ABSTRACT

Title of Document: **INVESTIGATING GENETIC AND HEALTH FACTORS RELATED TO AA AMYLOIDOSIS PREVALENCE IN CAPTIVE CHEETAHS (*ACINONYX JUBATUS*): IMPLICATIONS FOR POPULATION MANAGEMENT**

Ashley Danielle Franklin, PhD, 2014

Directed By: Professor Tom E. Porter
Animal and Avian Sciences

Adjunct Assistant Professor Adrienne E. Crosier
Smithsonian Conservation Biology Institute

Systemic amyloid A (AA) amyloidosis is an increasingly important cause of morbidity and mortality among captive cheetahs, yet wild cheetahs are virtually unaffected, suggesting the phenomenon is a result of the captive condition. The self-aggregating AA protein responsible for this disease, is a byproduct of serum amyloid A (SAA) protein degradation, an acute phase protein highly upregulated during inflammation. The objective of this study was to identify the relationship between genetics, stress, and inflammation with serum concentrations of the SAA protein and the incidence of AA amyloidosis in captive cheetahs. Fecal and serum samples collected from cheetahs held at the Smithsonian (NZP-SCBI) and Cheetah

Conservation Fund (CCF) facilities, as well as wild, free-ranging cheetahs, were examined. Enzyme-linked immunosorbent assays were used to measure SAA protein and proinflammatory cytokine concentrations in serum samples and cortisol concentrations in feces. Additionally, cheetahs were genotyped for the $SAA1A^{-97delG}$ single nucleotide polymorphism (SNP) in the promoter region of the SAA1 gene. This study was the first to demonstrate that serum concentrations of the SAA protein in cheetahs are affected by the $SAA1A^{-97delG}$ SNP (P=0.0453). However, the high prevalence of AA amyloidosis observed among captive cheetahs is not attributable to genetic differences at this locus, but rather appears to be related to stress and/or inflammation, as captive cheetahs at NZP-SCBI have significantly higher SAA protein concentrations in serum compared to captive cheetahs at CCF, regardless of genotype (P=0.0003). Captive cheetahs at NZP-SCBI show levels of stress (fecal cortisol concentrations) greater than their captive counterparts at CCF in Namibia. Interestingly, wild cheetahs and captive cheetahs at CCF in Namibia had significantly higher proinflammatory cytokine concentrations (TNF-α and IL-1β) in serum compared to cheetahs at NZP-SCBI (P<0.0001). It is possible that chronic stress may be suppressing the production of proinflammatory cytokines in the NZP-SCBI cheetah population. Controlling the currently high SAA protein concentrations associated with AA amyloidosis is the best strategy to decreasing the diseases prevalence among captive cheetahs. Promoting management practices that reduce stress could help re-establish proper immune system homeostasis and mitigate the overproduction of SAA protein, decreasing the probability of developing AA amyloidosis.

INVESTIGATING GENETIC AND HEALTH FACTORS RELATED TO AA AMYLOIDOSIS PREVALENCE IN CAPTIVE CHEETAHS (*ACINONYX JUBATUS*): IMPLICATIONS FOR POPULATION MANAGEMENT

By

Ashley Danielle Franklin

Dissertation submitted to the Faculty of the Graduate School of the
University of Maryland, College Park, in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
2014

Advisory Committee:
Professor Tom E. Porter, Chair
Adjunct Assistant Professor Adrienne E. Crosier
Associate Professor Carol Keefer
Assistant Professor Zhengguo Xiao
Associate Professor Carlos A. Machado

© Copyright by
Ashley Danielle Franklin
2014

Dedication

In loving memory of

David Rosaire Choinière

Acknowledgements

Firstly, I would like to thank my committee members, **Dr. Tom Porter, Dr. Adrienne Crosier, Dr. Carol Keefer, Dr. Zhengguo Xiao** and **Dr. Carlos Machado** for embarking on this journey with me. In the beginning, there was zero funding for this project, but my committee gave me the time to write grant proposals to fund and complete this research project that I was so passionate about doing. Ultimately, I was successful, and for their guidance and their patience, I am grateful. I would also like to recognize **Dr. Frank Siewerdt**, who initially advised me at the beginning of my PhD program before leaving the University in 2012. He was my biggest cheerleader and initially introduced me to Adrienne, whom without, this project would never have happened.

I would also like to recognize several people from the Cheetah Conservation Fund in Namibia who played an intimate role, either intellectually or physically, in my project. First, I would like to thank **Dr. Laurie Marker** who has gone from being an idol of mine, to a mentor, collaborator, and friend. Her passion for saving cheetahs is intoxicating and what she is doing for cheetahs in Namibia is remarkable. The fact that she welcomed me into their CCF family has been priceless. Second, I would like to recognize the folks who work in the genetics lab at CCF, **Dr. Anne Schmidt-Küntzel, Natalie Giesen**, and **Lusia Mhuulu**. Without Anne and Natalie, I would have been lost running the ABI sequencer and getting my permits completed to export the serum and fecal samples from Namibia into the USA. Lusia generously donated her time to genetically identify the unknown wild cheetah fecal samples for me, even after I had returned to the USA.

Almost every day I was at CCF, I was working with the husbandry team to prepare marked meals, feed cheetahs, and collect scat. My primary partners in crime were **Juliette Erdtsieck** (Head Cheetah Keeper) and **Ryan Sucaet** (Assistant Cheetah Keeper). These extraordinary souls were not forewarned of my arrival and my research demands, so you can imagine their surprise when I showed up and made their jobs quite a bit more difficult. Lucky for me, these are two of the most fantastic folks I have ever met and had the pleasure of working with. We managed to make picking up poop an exciting game of "Who's poop is this?"; you can never underestimate the excitement of pulling open a fresh scat sample to find it glitters gold or finding green lentils and oats that have swollen up to the size of M&M's. I would also like to recognize some of the interns and working guests that were enlisted to work hours of scat collecting including, but not limited to, **Margaret and David Wilkie, Justine Solesbee, Olivia Spagnuolo, Kelsey Fogle, Kelly Kapsar, Rachel Rowland, CJ and Stephanie Mantilla, Dr. Anna Ferreira** and **Laurie Schubert**.

I would like to thank **Dr. Karen Terio**, the cheetah SSP® pathologist, for both providing the voucher tissue samples with known AA amyloidosis histology as well as our thoughtful and insightful discussions about the project.

A large portion of my lab work was carried out in the endocrinology labs at the Smithsonian Conservation Biology Institute in Front Royal, VA. I'd like to thank **Dr. Diana Koester, Sarah Putnam, Nicole Presley** and **Morgan Maly** for their help and support in the lab. I'd also like to thank **Elliot Mattson** for his help with the PBMC stimulation experiment carried out in Dr. Xiao's lab at the University of Maryland College Park.

It is important to me to also thank **Dr. Bahram Momen**, who has served as both a teaching and statistics mentor. It was my pleasure to be his teaching assistant for several years in BIOM602 and I would not have the depth of understanding and appreciation of experimental design and statistics that I have if it were not for him. For this, I am eternally grateful, because he has made me a better scientist.

I would like to recognize the many sources of funding that I was able to acquire to piece this research project together, one step at a time, which includes the **Animal Sciences Graduate Students Association (ASGSA)**, the **Cosmos Club Foundation** (Washington, D.C.), the **Grants-in-Aid for Research** program from the National Academy of Sciences, administered by **Sigma Xi**, The Scientific Research Society, **Emanuel J. Friedman Philanthropies,** and the **William H. Donner Foundation**.

Being in the Animal and Avian Sciences Department at the University of Maryland for both my MSc and PhD programs, I have met and made a lot of really great friends along the way, who have helped me maintain my sanity. There are far too many people to list them all by name, but they know who they are. Thank you.

My family has also been incredibly supportive of me on this journey. My Mom, **Coralee Choinière**, who likely does not understand the majority of what I do, is always proud (no matter how long this has all taken) and always manages to find the opportunity to brag about me, even getting my work featured in our hometown newspaper. You would think she'd have stopped making me blush when I got well into adulthood, but she has still got those magical Mom powers. If I asked her to be at my defense seminar, I know she would hop on a plane (well, perhaps the train

because she hates flying) to be there. My siblings, **April and Andrew Choinière**, like to listen to me talk about scientific things. April has already declared that I should be the one to explain reproduction to her future children, because apparently the way I say things is nerdy and funny: that should be exciting! Unfortunately, my Dad, **David Choinière**, never got to hear me talk much about my PhD research, as I know he would have been a really good listener. I owe my natural mathematical abilities and nerdy science brain to him, but a few years ago, God felt it was his time to become an angel, and though I know he is watching over me from above, I wish he could be here to celebrate this with me.

Lastly, I have to thank my husband, **Keyan Franklin**, who has made many personal sacrifices for me to achieve my educational and professional goals. He is the one who has had to listen to me vent about any and all of my frustrations, as well as take care of my cats when I went gallivanting off to Africa, but he is also the one I enjoy celebrating all of my successes with. I love him so much and look forward to the new beginning our lives will take with a new job, in a new city, and finally starting a family.

Table of Contents

Dedication .... **ii**
Acknowledgements .... **iii**
Table of Contents .... **vii**
List of Tables .... **x**
List of Figures .... **xi**
List of Abbreviations .... **xiii**

Chapter 1: Review of literature, research objective and hypotheses . 1
Current status of the cheetah 1
Diseases among captive cheetahs 2
Stress 3
Stress among captive cheetahs 5
The acute phase response 6
Serum amyloid A: a major acute phase protein in domestic cats 7
Transcriptional regulation of the SAA gene 9
Amyloid A amyloidosis 13
Structure and expression of the cheetah SAA genes 14
Structure of SAA and AA proteins in cheetah 17
Amyloid A amyloidosis prevalence in cheetahs 18
Amyloid A amyloidosis in other species 20
Research objective and hypotheses 22
References 23

Chapter 2: Linking genetic differences to amyloid A amyloidosis prevalence in the cheetah (*Acinonyx jubatus*) 30
Abstract 30
Introduction 31
Methods 35
Sample collection 35
DNA isolation 36
Amplification and genotyping of the NF-κB binding site in the SAA1A promoter region 36
Quantification of serum amyloid A protein concentration in serum 41
Statistical analysis 41
Results 42
Serum amyloid A concentration in captive cheetahs is associated with genotype 42
Differences in $SAA1A^{-97delG}$ allele frequencies between populations 44
Discussion 47
Conclusion 51
Acknowledgements 51
References 52

Chapter 3: Induction of cytokine production in cheetah (*Acinonyx jubatus*) peripheral blood mononuclear cells by LPS and validation of feline-specific cytokine assays for analysis of cheetah serum 57
Abstract 57
Introduction 57
Methods 58
Sample Collection 58
Stimulation of cheetah PBMCs with LPS 59
Quantification of cytokine production in cell culture supernatants 59
Parallelism and cytokine recovery in serum 61
Statistical analysis 61
Results 62
LPS stimulation of cheetah PBMCs produces a measurable, cell concentration dependent increase in production of TNF-α, IL-1β, and IL-6 62
Percent recovery of cytokine standard from cheetah serum is dependent on the dilution factor 62
Parallelism observed between standards and spiked serum samples 65
Discussion 65
Conclusion 70
Acknowledgements 70
References 71

Chapter 4: Evaluation of the effects of chronic stress and inflammation on serum amyloid A concentrations in serum associated with increasing AA amyloidosis prevalence in captive cheetah populations 73
Summary 73
Introduction 75
Methods 80
Study Populations 80
Sample Collection 82
Hormone extraction from fecal samples 83
Quantification of cortisol in serum and fecal hormone extracts 83
Protein extraction from fecal samples 84
Quantification of total protein in fecal protein extracts 86
Quantification of serum amyloid A protein in serum and fecal protein extracts 86
Quantification of proinflammatory cytokines (TNF-α, IL-1β, IL-6) in serum 87
Univariate statistical analyses 87
Multivariate statistical analyses 91
Results 92
Fecal cortisol and associated metabolite concentrations and variability between populations 92
Population differences in fecal protein concentrations 94
Cortisol and serum amyloid A protein concentrations in serum 94

Population differences in proinflammatory cytokine (TNF-α, IL-1β, IL-6) concentrations in serum ... 98
Environmental associations with cortisol, inflammation, and SAA protein within captive populations ... 98
Variable correlations across populations ... 101
Variable correlations within populations ... 105
Descriptive Discriminant Analysis ... 105
Predictive Discriminant Analysis ... 111
Discussion ... 113
Conclusion ... 123
Acknowledgements ... 123
References ... 124

Chapter 5: Summary of study results and the implications for population management of captive cheetahs (*Acinonyx jubatus*).... 129
Genetics ... 129
Marker-assisted selection ... 129
Relevance of the SAA1A$^{-97delG}$ SNP in the NF-κB binding site in SAA1A promoter in captive cheetah population management ... 133
Inflammation ... 135
The role of proinflammatory cytokines (TNF-α, IL-1β, IL-6) in AA amyloidosis pathogenesis ... 135
Implications of inflammation for captive cheetah population management ... 138
Stress ... 139
The role of stress in AA amyloidosis pathogenesis ... 139
Implications of stress for captive cheetah population management ... 140
Other possible management considerations ... 141
Dietary requirements ... 141
Medical treatments ... 141
Conclusion ... 142
References ... 144

Appendix I. Confirmation of *SAA1A* promoter specificity of F3A primer via nested vs. non-nested PCR ... 147
Appendix II. Complete list of fecal and serum samples collected from cheetahs ... 150
Bibliography ... 152

List of Tables

Table 1.1. Previous literature investigating serum amyloid A (SAA) concentrations in healthy and sick domestic cats. 8

Table 2.1. Estimated allele frequencies of the *SAA1A*$^{-97delG}$ SNP for wild cheetahs in Namibia and captive cheetahs in North America. 45

Table 2.2. Estimated allele frequencies of the *SAA1A*$^{-97delG}$ SNP for captive cheetahs confirmed positive or negative for AA amyloidosis at time of necropsy. 46

Table 3.1. Cytokine concentrations in cell culture supernatants from cheetah PBMCs 24, 48 and 72 hours post-LPS stimulation. 64

Table 4.1. Cortisol variable correlation coefficients (r) across all cheetah populations. 103

Table 4.2. Serum variable correlation coefficients (r) across all cheetah populations. 104

Table 4.3. Cortisol variable correlation coefficients (r) within captive cheetah populations. 106

Table 4.4. Serum variable correlation coefficients (r) within captive cheetah populations. 107

Table 4.5. Correlation coefficients (r) of cortisol variables measured in feces with serum variables within the CCF captive cheetah populations. 108

Table 4.6. Correlation coefficients (r) of cortisol variables measured in feces with serum variables within the NZP-SCBI captive cheetah population. 109

Table 4.7. Total canonical structure indicating correlation coefficients (r) between original variables and CDF scores. 112

Table 4.8. Minimum and maximum proinflammatory cytokine concentrations observed within cheetah populations. 118

List of Figures

Figure 1.1. SAA protein production regulation in the liver. 12

Figure 2.1. Nucleotide sequence of the putative promoter region -262bp upstream of the cheetah SAA1A gene. 38

Figure 2.2. Representative chromatograms for the three SAA1A promoter genotypes after Sanger sequencing. 39

Figure 2.3. Representative output for the three SAA1A promoter genotypes from the ABI PRISM® 310 Genetic Analyzer. 40

Figure 2.4. Serum concentrations of serum amyloid A (SAA) protein in captive cheetahs at NZP-SCBI and CCF. 43

Figure 3.1. 24-well plate set-up for LPS stimulation of cheetah monocytes in cell culture. 60

Figure 3.2. Cytokine concentrations in cell culture supernatants from cheetah PBMCs (undiluted and 5X) at 24, 48 and 72 hours post-LPS stimulation. 63

Figure 3.3. Effect of increasing serum dilution on percent recovery of cytokine standards from spiked cheetah serum samples. 66

Figure 3.4. Parallelism between cytokine standards and cheetah serum (1:2 dilution) spiked with known concentrations of standard. 67

Figure 4.1. Parallelism between cheetah serum samples and the standard for an established EIA. 85

Figure 4.2 Representative profile for cortisol across a three month collection period for cheetah 114382. 89

Figure 4.3. Fecal cortisol and associated metabolite concentrations by population. Means ± SEM are presented. 93

Figure 4.4. Total protein and serum amyloid A protein concentrations in feces by population. Means ± SEM are presented. 95

Figure 4.5. Differences in serum cortisol concentrations (lsmeans ± SEM) across cheetah populations. 96

Figure 4.6. Comparison of serum log_{10}SAA concentrations (lsmeans ± SEM) across populations. 97

Figure 4.7. TNF-α and IL-1β cytokine concentrations in cheetah serum (lsmeans ± SEM) across populations. 99

Figure 4.8. Effects of environmental factors on baseline and average cortisol concentrations in feces among captive cheetahs at Cheetah Conservation Fund. 100

Figure 4.9. Population comparison of baseline and average cortisol in feces (A) and log_{10}SAA concentration in serum (B) across captive cheetah populations and environments. 102

Figure 4.10. The three populations of cheetahs included in the canonical discriminant analysis (NZP-SCBI, CCF, and Wild) can be discriminated with 76.8% accuracy based on the two canonical discriminant functions calculated (Can1 and Can2). 110

Figure 4.11. Fecal cortisol concentrations in wild cheetahs (N=7) by month. 114

Figure 5.1. Proinflammatory cytokine concentrations in captive cheetahs at CCF by age. 137

List of Abbreviations

AA	amyloid A
AAFCO	Association of American Feed Control Officials
ACTH	adrenocorticotropic hormone
AGP	alpha-1-acid glycoprotein
ANCOVA	analysis of covariance
ANOVA	analysis of variance
APP	acute phase protein
APR	acute phase response
ASO	antisense oligonucleotides
AVP	arginine vasopressin
AZA	Association of Zoos and Aquariums
BLUP	best linear unbiased prediction
C/EBP	CCAAT-enhancer-binding protein
CCF	Cheetah Conservation Fund
CD4	cluster of differentiation 4
CDA	canonical discriminant analysis
CDF	canonical discriminant function
CRH	corticotropin releasing hormone
CRP	C-reactive protein
DDA	descriptive discriminant analysis
DEX	dexamethasone
DSH	domestic short hair
E	epinephrine
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
GC	glucocorticoid
GR	glucocorticoid receptor
GRE	glucocorticoid response element
HPA	hypothalamic-pituitary-adrenal
IFN-γ	interferon gamma
IL-1β	interleukin-1 beta
IL-6	interleukin-6
IUCN	International Union for Conservation of Nature
IκB	I-kappa B
LPS	lipopolysaccharide
MAS	marker-assisted selection
NE	norepinephrine
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NZP	National Zoological Park

PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDA	predictive discriminant analysis
QTL	quantitative trait loci
SAA	serum amyloid A
SCBI	Smithsonian Conservation Biology Institute
SNP	single nucleotide polymorphism
SSP	Species Survival Plan
TH1	T helper cells (type 1)
TH2	T helper cells (type 2)
TLR2	toll-like receptor 2
TLR4	toll-like receptor 4
TNF-α	tumor necrosis factor alpha

Chapter 1: Review of literature, research objective and hypotheses

Current status of the cheetah

The cheetah, *Acinonyx jubatus*, is a member of the family *Felidae*, and ranges historically from Africa into Asia (Durant et al. 2008). Unfortunately, wild populations of cheetah are declining due to habitat loss and fragmentation, a depleted prey base, conflict with farmers and ranchers, exportation into the pet trade, as well as interspecific competition with large predators such as lions and hyenas (Durant et al. 2008). The wild population was recently estimated to be between 7,500 and 10,000 individuals (Durant et al. 2008). With the population in decline, cheetahs are listed as "vulnerable" on the IUCN Red List of Threatened Species™. At the end of 2012, the captive cheetah population was comprised of 1,661 individuals, among 250 facilities, in 44 countries (Marker 2013). Twenty-one percent of these captive animals are held and managed in North American facilities (Marker 2013).

Captive cheetahs in North America are currently managed under a Species Survival Plan (SSP®). A major goal of the SSP® for captive cheetahs in North America is to create a self-sustaining population that no longer requires the importation of cheetahs from Africa. Currently, this goal is not being met. However there are multiple ways to increase captive population growth including: improving reproduction (increasing mating and pregnancy success) and decreasing mortality (improving juvenile mortality and litter survival rates, improvements to diet and nutrition, decreasing disease prevalence). Ultimately, achievement of the SSP®'s goal

requires rigorous captive population management and the implementation of a new approach to the captive breeding program. Consequently, representatives from North American facilities meet each year to make decisions on breeding recommendations and movement of animals within and between facilities based on genetics, fertility, behavior, age and health (Grisham et al. 2013).

Aside from increasing mortality, diseases are a cause for concern to captive population managers because they affect behavior, inhibit the movement of animals between facilities, and impact the ability to anesthetize an individual for any required or special medical procedures, including assisted reproductive technologies.

Diseases among captive cheetahs

Diseases among cheetahs in captivity are exceedingly common, limiting their lifespan (Munson 1993), yet despite the high prevalence of many diseases observed in captive cheetahs in both North America and South Africa, wild cheetahs are virtually unaffected (Munson et al. 2005). This disparity suggests the phenomenon is not simply due to the low genetic diversity observed among both wild and captive cheetahs (Castro-Prieto et al. 2011, Drake et al. 2004, O'Brien et al. 1983, O'Brien et al. 1985, Yuhki and O'Brien 1990), but rather a result of the captive condition. Adrenal cortical hyperplasia is a condition that has been commonly documented among captive cheetahs (Bolton and Munson 1999, Munson et al. 1999, Munson et al. 2005, Terio et al. 2004), thus it has been speculated that stress due to the captive condition is the driving force behind many of these diseases.

Stress

Stress can be defined as a physiological or psychological state in which homeostasis is, or perceived to be, threatened (Chrousos 2009, Dhabhar 2009, Elenkov and Chrousos 1999). The biological reaction (or purpose) of acute stress is to divert energy resources to muscles while inhibiting energy storage, to stimulate immune function through the mobilization of immune cells from storage to peripheral tissues, to inhibit reproduction, to decrease feeding and appetite, to sharpen cognition, and to increase water retention (in cases of blood loss), all in an effort to increase the probability of short-term survival (Sapolski et al. 2000). The major effectors mediating homeostasis produced in the central nervous system are arginine vasopressin (AVP) and corticotropin-releasing hormone (CRH) (Chrousos 2009, Elenkov and Chrousos 1999); peripheral effectors also mediating homeostasis include catecholamines and glucocorticoids (GCs) (Chrousos 2009, Dhabhar 2009, Elenkov and Chrousos 1999).

Stress activates the sympathetic nervous system which stimulates the release of catecholamines (epinephrine and norepinephrine; E and NE) from the adrenal medulla. Stress also results in activation of the hypothalamic-pituitary-adrenal (HPA) axis by innervating neurons in the paraventricular nucleus of the hypothalamus to secrete CRH, which travels through the portal vein system to activate corticotropic cells in the anterior pituitary, which in turn release adrenocorticotropic hormone (ACTH) into circulation. The ACTH signal is received by cells of the adrenal cortex, stimulating the production and release of GCs, such as cortisol and corticosterone. Thus in short, acute stress is characterized by the enhanced secretion of E and NE,

later followed by GCs released from the adrenal gland (Chrousos 1995, Chrousos 2009, Dhabhar 2009, Elenkov and Chrousos 1999, Sapolski et al. 2000).

Psychological stressors can trigger the release of proinflammatory cytokines before any rise in GCs is detected (Dhabhar 2009, Sapolski et al. 2000). Specifically, catecholamines have been shown to stimulate the release of the proinflammatory cytokine IL-6 from immune cells (Chrousos 1995, Chrousos 2009). Conversely, proinflammatory cytokines are known to stimulate the stress system (HPA axis activation), causing the release of CRH (Baumann and Gauldie 1994, Chrousos 2009, Elenkov and Chrousos 1999, Sapolski et al. 2000). Peripheral CRH has been known to have proinflammatory actions (Elenkov and Chrousos 1999). Studies in mice have shown that the proinflammatory cytokine IL-6 is essential for activation of the HPA axis during immune challenges in the absence of CRH (Bethin et al. 2000); supporting evidence includes the presence of IL-6 receptors on the surface of pituitary corticotrophs and adrenocortical cells (Bethin et al. 2000). The same study also found the relative contribution of IL-6 for modulation of the adrenal response to stress greater in female mice, suggesting differences between sexes when it comes to the induction of the HPA axis in response to inflammation (Bethin et al. 2000).

Activation of the HPA axis by proinflammatory cytokines plays an important role in the negative feedback mechanism that controls systemic proinflammatory cytokine concentrations from becoming detrimental to the organism: both catecholamines and GCs suppress the secretion of most proinflammatory cytokines (Chrousos 1995, Chrousos 2009, Elenkov and Chrousos 1999, Sapolski et al. 2000).

Additionally, production of the anti-inflammatory cytokine IL-10 is upregulated by GCs (Elenkov and Chrousos 1999).

Stress among captive cheetahs

It has been widely accepted that captivity can impose psychological stress on many animal species. Measuring glucocorticoids in fecal samples across time can be a valuable, non-invasive tool for measuring stress in many animals, including cheetahs (Keay et al. 2006). Cheetahs defecate approximately once per day, therefore any diurnal fluctuations in cortisol due to circadian secretory patterns are attenuated in feces (Keay et al. 2006).

There is both functional (hypercortisolemia) and morphological evidence (adrenal hyperplasia/hypertrophy) for chronic stress among captive cheetahs, at levels much greater than their wild counterparts (Bolton and Munson 1999, Köster et al. 2007, Munson et al. 1999, Munson et al. 2005, Terio et al. 2004). Similarly, in captivity, when cheetahs are moved or housed on-exhibit, they tend to show greater levels of stress (higher GC concentrations in feces) than when moved or housed off-exhibit (Terio et al. 2004, Wells et al. 2004), and the increases observed after movement can persist for months (Wells et al. 2004). There is also greater daily variation in the GC concentrations of feces observed when cheetahs are moved on-exhibit (Wells et al. 2004). Evidence of stress (elevated cortisol and/or behavior) being linked to anestrous in captive cheetahs (downregulation of the reproductive system) has also been demonstrated (Jurke et al. 1997, Wielebnowski et al. 2002). Hence, it appears there is a clear association between the captive environment and stress in cheetahs.

Stress-related hypersecretion of the proinflammatory cytokine IL-6, in addition to hypercortisolism, may result in the chronic upregulation of the acute phase response (Chrousos 2009), contributing to the increase in disease and mortality observed in the captive cheetah population.

The acute phase response

The acute phase response (APR) is a systemic reaction that occurs early during inflammation, initiated by cells of the innate immune system in response to invading pathogens, tissue damage, and associated activation signals. Macrophages resident in affected tissues and monocytes in the blood are the immune cells primarily responsible for the initiation of the APR (Baumann and Gauldie 1994, Paltrinieri 2008). The liver is one of the primary targets for systemic inflammatory mediators (Baumann and Gauldie 1994, Jensen and Whitehead 1998, Paltrinieri 2008); it is the location of acute phase protein (APP) production (Baumann and Gauldie 1994, Cerón et al. 2005, Jensen and Whitehead 1998, Paltrinieri 2008). APPs, by definition, are proteins whose concentration in serum significantly increases or decreases during the APR. There is a high degree of similarity in the response patterns of several APPs to systemic inflammatory mediators during the APR across species (Baumann and Gauldie 1994, Cerón et al. 2005, Paltrinieri 2008), including C-reactive protein (CRP), serum amyloid A protein (SAA), alpha-1-acid glycoprotein (AGP), ceruloplasmin, haptoglobin, and albumin. The systemic inflammatory mediators generally responsible for the upregulation of CRP, SAA, and AGP production during the APR are the IL-1 type cytokines, such as IL-1β and TNF-α (Baumann and Gauldie 1994, Jensen and Whitehead 1998, Marhaug and Dowton 1994, Paltrinieri

2008). Additionally, the actions of the IL-1 type cytokines on the production of APPs are often synergistically enhanced in the presence of IL-6 (Baumann and Gauldie 1994, Cerón et al. 2005, Jensen and Whitehead 1998).

Serum amyloid A: a major acute phase protein in domestic cats

The major APPs that have been identified to date in the domestic cat are SAA and AGP (Cerón et al. 2005, Kajikawa et al. 1999, Paltrinieri 2008); CRP is not a highly reactive APR in domestic cats (Kajikawa et al. 1999). In humans, the concentration of SAA found in circulation bound to high-density lipoproteins increases as much as 1000-fold within 24-48 hours of the initiation of inflammation (Marhaug and Dowton 1994). The increase in SAA observed in domestic cats during the APR, approximately a 10-100 fold increase, is lower in magnitude compared to humans and other species, but still represents a major APP in this species (Cerón et al. 2005, Giordano et al. 2004, Kajikawa et al. 1999), and multiple studies have shown that the SAA concentrations in sick cats is significantly higher than the SAA concentrations in healthy cats (Table 1.1). A pattern of increased SAA concentrations among older domestic cats has also been reported (Campbell et al. 2004, Kann et al. 2012). The increase in SAA concentration with age appears to be associated with a decrease in white blood cells, peripheral blood lymphocytes and circulating eosinophils, and fewer $CD4^+$ T cells among older domestic cats (Campbell et al 2004).

The SAA protein is well conserved throughout evolution, indicating an important biological function (Marhaug and Dowton 1994). Possible roles of SAA during the APR include the recruitment of inflammatory cells to the primary site of

Table 1.1. Previous literature investigating serum amyloid A (SAA) concentrations in healthy and sick domestic cats.

Condition	[SAA] Mean ± SEM	Method	Author
Healthy (n = 26)	0.14 ± 0.05 μg/ml	ELISA* TIA^	*Tamamoto et al. 2008*
Diseased (n = 263)	7.52 ± UR μg/ml	ELISA* TIA^	*Tamamoto et al. 2008*
Healthy (n = 20)	16.6 ± 2.55 μg/ml	ELISA#	*Kajikawa et al. 1999*
Hospitalized (n = 20)	150.1 ± 16.3 μg/ml	ELISA#	*Kajikawa et al. 1999*
Healthy (n = 34)	1.8 ± 2.3 μg/ml	ELISA§	*Kann et al. 2012*
Sick (n = 32)	16.0 ± 32.1 μg/ml	ELISA§	*Kann et al. 2012*
Healthy (n = 24)	10.21 ± 1.70 μg/ml	ELISA§	*Giordano et al. 2004*
FCoV exposed (n = 7)	7.92 ± 2.69 μg/ml	ELISA§	*Giordano et al. 2004*
FIP infected (n = 32)	82.88 ± 8.88 μg/ml	ELISA§	*Giordano et al. 2004*
Healthy (n = 45)	0.60 ± 0.16 μg/ml	ELISA	*Sasaki et al. 2003*
Diseased (n = 312)	33.65 ± 3.83 μg/ml	ELISA	*Sasaki et al. 2003*

*BioSource Multispecies SAA ELISA kit, BioSource International, Inc., Camarillo, CA, U.S.A.
^Turbidimetric immunoassay for human SAA, LZ-SAA, Eiken Chemical Co., Ltd.
#Kajikawa, T., Furuta, A., Onishi, T., Sugii, S., 1996. Enzyme-linked immunosorbent assay for detection of feline serum amyloid A protein by use of immunological cross-reactivity of polyclonal anti-canine serum amyloid A protein antibody. J. Vet. Med. Sci. 58, 1141-1143.
§PHASE™ RANGE Multispecies SAA ELISA kit, Tridelta Development Limited, Wicklow, Ireland.
UR: Unreported.

inflammation, involvement in lipid metabolism and transport, increasing cytokine production in monocytes and macrophages, and stimulating nitric oxide production in macrophages (Cerón et al. 2005, Jensen and Whitehead 1998, Tamamoto et al. 2012). In domestic cats, SAA appears to also play a protective role in inflamed tissue by scavenging for oxidized cholesterol, thereby preventing prolonged tissue damage (Paltrinieri 2008).

Transcriptional regulation of the SAA gene

The CCAAT-enhancer-binding proteins (C/EBPs) are primarily responsible for the regulation of SAA transcription (Ray and Ray 1994). Both C/EBPβ and C/EBPδ are newly synthesized under acute phase conditions, activated by phosphorylation, and induce transcription of the SAA gene in a dose-dependent manner (Ray and Ray 1994). The C/EBP binding site in the promoter of the human SAA gene is located within the 265bp region upstream of the transcription start site (Marhaug and Dowton 1994). Also located within this region is a binding site for the transcription factor NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) (Marhaug and Dowton 1994). The active form of NF-κB is a heterodimer, usually comprised of two proteins, p65 and p50 (Barnes and Karin 1997, Beg et al. 1993).

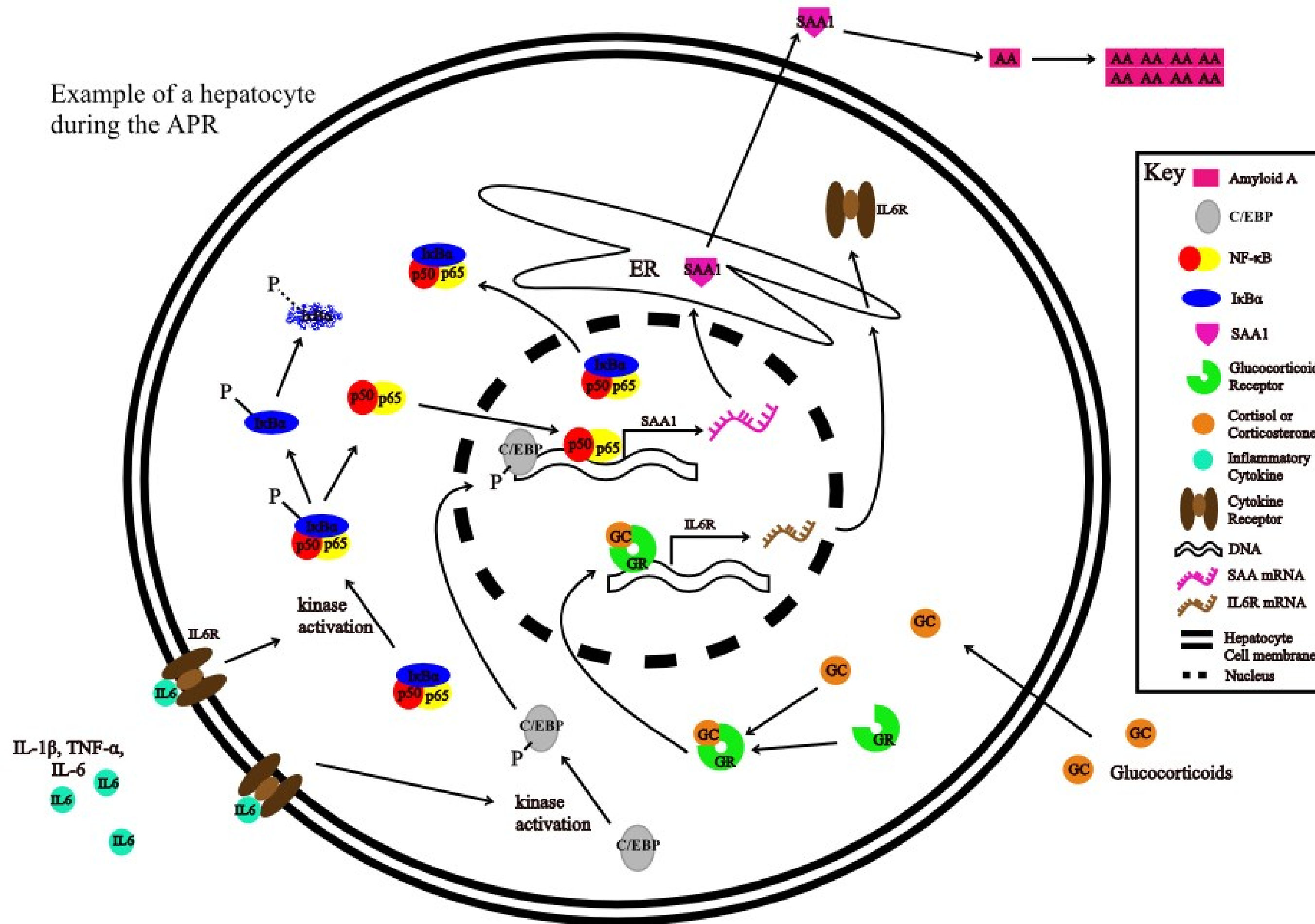
Without stimulation signaling, NF-κB is bound to IκBα or IκBβ in the cytoplasm of the cell and is prevented from entering the nucleus (Barnes and Karin 1997, Beg et al. 1993). However, in the presence of stimulation from proinflammatory cytokines, such as TNF-α (Beg et al. 1993, Scheinman et al. 1995) and IL-1β (Barnes and Karin 1997), IκBs are phosphorylated, disassociate from NF-

κB, and get degraded (Barnes and Karin 1997, Beg et al. 1993). The unbound NF-κB enters the nucleus and binds to the promoter region of target genes associated with inflammatory and immune responses, such as *SAA* (Barnes and Karin 1997, Beg et al. 1993). IL-1β, TNF-α, and IL-6 proinflammatory cytokines have all been shown to stimulate hepatic SAA production during the APR (Baumann and Gauldie 1994, Cerón et al. 2005, Jensen and Whitehead 1998, Marhaug and Dowton 1994, Paltrinieri 2008).

In addition to proinflammatory target genes, NF-κB induces synthesis of IκBα, therefore the activity of NF-κB is self-regulated (Barnes and Karin 1997). Glucocorticoids also induce transcription of IκBα (Auphan et al. 1995, Barnes and Karin 1997, Scheinman et al. 1995). Multiple studies have shown that IκBα synthesis is increased in the presence of dexamethasone (DEX: a synthetic glucocorticoid), causing fast reassociation of NF-κB with IκBα, thus reducing the translocation of NF-κB to the nucleus, even under cytokine stimulation (Auphan et al. 1995, Scheinman et al. 1995); this inhibitory effect of DEX is mediated through cytoplasmic glucocorticoid receptors (Auphan et al. 1995), with possible direct protein-protein interaction between the glucocorticoid receptor (GR) and NF-κB (Barnes and Karin 1997). Therefore, activated GRs are involved in blocking transcription factors required for the expression of proinflammatory target genes (Chrousos 1995). It is widely accepted that the expression of many cytokines (including TNF-α, IL-1β, and IL-6) is inhibited by GCs (Baumann and Gauldie 1994, Chrousos 1995, Jensen and Whitehead 1998, Paltrinieri 2008, Wiegers and Reul 1998).

Contrastingly, there is a positive, synergistic effect of GCs with cytokines in the induction of SAA synthesis during the APR (Chrousos 1995, Dhabhar 2009, Marhaug and Dowton 1994). This effect is the result of the upregulation of cytokine receptors on the surface of hepatic cells by GCs (Baumann and Gauldie 1994, Wiegers and Reul 1998), increasing the sensitivity of the hepatic cells to cytokine stimulation (Baumann and Gauldie 1994, Paltrinieri 2008, Thorn and Whitehead 2002, Wiegers and Reul 1998). During the APR, IL-1β and IL-6 can act on the pituitary-adrenal axis to stimulate production of ACTH, inducing the production of cortisol (Baumann and Gauldie 1994, Chrousos 2009, Elenkov and Chrousos 1999, Sapolski et al. 2000). Therefore, it appears GCs play a role in the systemic inflammatory response by increasing production of APPs during the APR. These effects, combined with the increased post-transcriptional stability of SAA mRNA due to the production of longer poly-A tails (Jensen and Whitehead 1998), contribute to the major increases in SAA concentration observed in serum during the APR. Higher SAA concentrations in serum were found to be positively correlated with prednisone treatment (a synthetic GC) in systemic lupus erythematosus patients compared to patients without treatment (Esmat et al. 2005). Unfortunately, this could mean that administering glucocorticoids as a therapy for diseases characterized with chronic inflammation may have an undesirable or even harmful effect resulting in higher serum SAA concentrations. Factors regulating SAA protein production in the liver are summarized in Figure 1.1.

Figure 1.1. SAA protein production regulation in the liver.



Amyloid A amyloidosis

The SAA protein normally has a short half-life, approximately 90 minutes in normal serum (Marhaug and Dowton 1994). Therefore, the concentration of SAA in serum returns to normal following the termination of inflammation. In multiple species, including domestic cats, monocytes contribute to SAA degradation (Migita et al. 2001, Tamamoto et al. 2012); however, this is inhibited in the presence of the cytokines IL-1β and IFN-γ (Migita et al. 2001), and glucocorticoids (Tamamoto et al. 2012). SAA itself can induce the expression and secretion of TNF-α and IL-1β from human macrophages through interactions with the TLR2 and TLR4 receptors (Niemi et al. 2011), contributing to its own persistence. Thus, chronic systemic inflammation can result in the persistent upregulation of SAA production, a decrease in SAA degradation, and an accumulation of SAA protein in the serum, providing evidence that it plays a role in amyloid A (AA) amyloidosis pathogenesis.

The N-terminal region of the SAA protein is the precursor for the AA protein, the first 10-15 amino acid residues of which are amyloidogenic (Marhaug and Dowton 1994). In abundance, the AA protein polymerizes to form fibrils which are insoluble and deposit into the liver, kidney, spleen and other organs. As stated previously, in the presence of inflammation, SAA may be slowly or improperly degraded, leading to an accumulation of AA protein (Marhaug and Dowton 1994). It has been shown that SAA can also induce the secretion of cathepsin B from macrophages through direct activation of the $P2X_7$ receptor (Niemi et al. 2011). This effect, considered together with evidence that cathepsin B is capable of degrading SAA into AA-like protein fragments (Röcken et al. 2005, Yamada et al. 1995), means

chronic inflammation may lead to the extracellular proteolytic processing of SAA into AA amyloidogenic fragments. Thus, AA amyloidosis is a disease induced by the over-accumulation and incomplete degradation of SAA and polymerization of AA protein into fibrils (Marhaug and Dowton 1994). Therefore, it is unsurprising that elevated SAA concentrations in serum have been found to correlate with AA amyloidosis, cancer and other inflammatory diseases in humans (Biran et al. 1986, Lachmann et al. 2007, Marhaug and Dowton 1994).

Structure and expression of the cheetah SAA genes

Four serum amyloid A protein genes (*SAA1A*, *SAA1B*, *SAA3A*, *SAA3B*) have been identified in the cheetah genome, yet these SAA proteins are monomorphic: all polymorphisms are observed in non-coding regions of the genes (Chen et al. 2012). This represents a decrease in polymorphisms in the cheetah SAA1 and SAA3 genes in comparison to human and mouse orthologs (Chen et al. 2012). Similar to other species, the SAA genes appear to be linked on the same chromosome due to the cosegregation of allelic haplotypes (Chen et al. 2012).

The structure of the cheetah SAA1 genes show high similarity to the human SAA1 and mouse Saa1 genes (Zhang et al. 2008b). Transcriptional induction of the SAA1 gene in cheetahs by IL-1β and IL-6 is dependent on the presence of both C/EBPβ and NF-κB elements (Zhang et al. 2008b). Nucleotide sequences of C/EBPβ and NF-κB target sites are highly conserved between cheetah and human (Zhang et al. 2008b). The NF-κB element in the promoter of the SAA1 gene in cheetahs cannot confer promoter activity alone, yet the C/EBPβ element retains partial promoter activity in the absence of the NF-κB element (Zhang et al. 2008b). Zhang et al.

(2008b) identified two alleles for a polymorphism consisting of a single guanine nucleotide deletion in the putative NF-κB binding site of the *SAA1* promoter. More recently four alleles have been identified for the SAA1A gene, only one of which ("allele 1") has 3 guanine residues at the -97 to -99 position (Chen et al. 2012); the three other alleles have the single guanine nucleotide deletion. The allele frequency of "allele 1" in the Chen et al. (2012) study was 29.41%, thus for all other $SAA1A^{-97delG}$ alleles combined, the allele frequency was 70.59%. These $SAA1A^{-97delG}$ alleles are associated with reduced transcriptional activity of the SAA1 gene in vitro (Zhang et al. 2008b).

Chen et al. (2012) found the expression pattern of the SAA genes differed depending on tissue type. SAA1A gene expression was found in the liver tissue of most cheetahs, however, no expression was found in the stomach or intestine. The SAA1B gene was not expressed in the liver, even in the presence of inflammation, suggesting the presence of a negative regulatory element in the SAA1B promoter upstream of the -667 position (Chen et al. 2012). In contrast, SAA3 gene expression was found in the liver and stomach, but not in the intestine (Chen et al. 2012). Both SAA1 and SAA3 gene expression is higher in the liver tissue of cheetahs with amyloidosis (Chen et al. 2012), suggesting that an increase in SAA protein production by the liver is important in the etiology of systemic amyloidosis.

Humans also have four SAA protein genes (*SAA1*, *SAA2*, *SAA3*, and *SAA4*): the SAA1 and SAA2 genes are responsible for the increased production of SAA in the liver during the APR, whereas the SAA3 gene is a pseudogene and the SAA4 gene is constitutively expressed in other tissue types (Jensen and Whitehead 1998).

Despite the upregulation of both SAA1 and SAA2 during the APR, it should also be noted that SAA1 is the protein primarily found in amyloid fibril deposits of human patients with secondary amyloidosis (Liepnieks et al. 1995). In humans, the addition of glucocorticoids enhances the induction of the SAA1 promoter by cytokines in a dose-dependent manner, however it does not enhance the induction of the SAA2 promoter by cytokines at any concentration (Thorn and Whitehead 2002), which suggests elevated GCs may cause a predisposition to developing amyloid deposits. The observed enhancement of SAA1 expression by GCs is dependent on the cytoplasmic GR (Thorn and Whitehead 2002). It was determined this effect was the result of a GRE (glucocorticoid response element) that is uninterrupted in the SAA1 promoter in humans, conferring glucocorticoid responsiveness of SAA under cytokine stimulation; the GRE in the promoter of the SAA2 gene is disrupted, resulting in the non-responsiveness to GCs (Thorn and Whitehead 2002). In the cheetah SAA1 gene promoter, the GRE is similarly disrupted, but the position of which is shifted and non-homologous to human, based on the sequence presented in Zhang et al. (2008b), thus it seems unlikely that glucocorticoids are directly increasing SAA1 transcription in cheetahs.

In human populations, there is evidence that a SNP could explain population differences in secondary AA amyloidosis prevalence in rheumatoid arthritis patients: a thymine (T) nucleotide at the -13 position in the promoter region of the SAA1 gene is primarily associated with amyloidosis risk in these human populations (Moriguchi et al. 2001). Interestingly, cheetahs have a T at the -13 position in the SAA1 promoter (Zhang et al. 2008b) and this nucleotide position is not polymorphic (Chen et al.

2012), therefore all cheetahs may be at an increased risk of developing secondary AA amyloidosis.

Structure of SAA and AA proteins in cheetah

The SAA1 protein encoded by the SAA1 gene is the precursor to the amyloid A protein identified as the major component of amyloid fibrils deposited into tissues during systemic amyloidosis in cheetahs (Bergstrom et al. 2006, Johnson et al. 1997, Ofri et al. 1997). The cheetah SAA protein is 129 amino acid residues in length (Zhang et al. 2008b), compared to the 122 in the human SAA protein (Woo et al. 1987). Initially it was reported that the cheetah AA protein had 90 amino acid residues (Johnson et al. 1997), however, more recently it has been reported to have 93 amino acid residues (Zhang et al. 2008b); the shorter length of the AA protein compared to the SAA1 protein is due to the loss of an 18 amino acid N-terminal signal peptide (presumably for secretion from hepatocytes), and C-terminal amino acids from the SAA1 protein (Zhang et al. 2008b). The amyloid A protein isolated from cheetah liver tissue is predominantly 10kDa in size, but 8kDa size proteins have also been detected (Johnson et al. 1997). In comparison to human AA protein, cheetah AA protein is one amino acid residue shorter at the N terminus and contains an eight amino acid insertion between human residues 69-70 (Johnson et al. 1997). The molecular mass of AA protein in feces is approximately 7.0kDa, smaller than that found in the liver due to deletions from both N and C terminal regions (Zhang et al. 2008a).

Amyloid fibrils isolated from liver tissue of cheetahs with amyloidosis only contain SAA1 protein derivatives (Zhang et al. 2008b), despite the expression of both

SAA1 and SAA3 genes in hepatocytes in cheetahs with amyloidosis. There is also evidence that undigested SAA protein is incorporated into amyloid fibrils in cheetah (Bergstrom et al. 2006).

Amyloid A amyloidosis prevalence in cheetahs

Systemic AA amyloidosis is an increasingly important cause of morbidity and mortality in captive cheetahs (Munson et al. 2005, Ofri et al. 1997, Papendick et al. 1997). The number of cases of systemic amyloidosis in captive cheetahs has increased over time from 20% of individuals in pre-1990 necropsies to 70% of individuals in 1995 (Papendick et al. 1997). Amyloid deposition can be observed in many different tissues including the kidney, liver, adrenal gland, parathyroid gland, spleen, stomach, intestine, lymph node, heart, pancreas and esophagus (Chen et al. 2012, Munson et al. 1999, Papendick et al. 1997). The majority of cases of AA amyloidosis can be considered "severe": 77% of cases involving the kidney and 71% of cases involving the liver (Papendick et al. 1997). Most commonly, amyloid deposition is observed in the kidneys, and among the cheetahs with renal amyloid, 52% of individuals also have hepatic amyloid, thus systemic amyloidosis (Papendick et al. 1997). Hepatic amyloid has not been observed in cheetahs with an absence of renal amyloid (Papendick et al. 1997). Due to these factors, renal failure is the sole or partial cause of death in 74% of cheetahs with amyloidosis, compared to only 20% of cheetahs without amyloidosis (Papendick et al. 1997). There is no association between the occurrence of amyloidosis and sex (male/female) (Papendick et al. 1997).

Papendick et al. (1997) found that all cheetahs diagnosed with amyloidosis also suffered from other significant chronic inflammatory diseases in organs other than kidney, most commonly chronic lymphoblastic gastritis. In the Papendick et al. (1997) study, the prevalence of severe gastritis in captive cheetahs increased from 16% of individuals in pre-1990 necropsies to 43% of individuals in 1995, an increase almost parallel to the increase in AA amyloidosis prevalence observed. Seventy-six percent of cheetahs with amyloidosis also had moderate to severe gastritis (Papendick et al. 1997), while close to 100% of cheetahs in captivity have some form of gastritis (Munson 1993, Munson et al. 1999, Munson et al. 2005). Both catecholamines and GCs induce a shift from T_H1 (cellular immunity) to T_H2 (humoral immunity) immune responses (Chrousos 2009, Dhabhar 2009, Elenkov and Chrousos 1999, Sapolski et al. 2000), therefore chronic stress-induced immune dysfunction might contribute to the persistence of *Helicobacter* infections in the stomach (generally defended against by the cellular immune response) (Chrousos 2009, Elenkov and Chrousos 1999), resulting in the high prevalence of gastritis in captive cheetahs compared to other species infected with the same stains of *Helicobacter* (Terio et al. 2011). Non-inflammatory conditions associated with gastritis in captive cheetahs include multifocal perivascular interstitial cardiac fibrosis and adrenocortical hypertrophy (Munson et al. 1999, Munson et al. 2005, Papendick et al. 1997).

Unrelated to stress or inflammation, there is some evidence presented that AA amyloidosis among captive cheetahs may be so highly prevalent because of husbandry practices, as the disease may be transmissible through feces among

cheetahs in a prion-like fashion (Caughey and Baron 2008, Zhang et al. 2008a) when housed as groups, however support of this hypothesis is limited.

Amyloid A amyloidosis in other species

In addition to cheetahs, AA amyloidosis has been identified in many other species. In some cases, susceptibility appears to be genetic (familial amyloidosis), such as observed in: Shar-Pei dogs (Rivas et al. 1993), Abyssinian (Boyce et al. 1984) and Siamese (van der Linde-Sipman et al. 1997) breeds of domestic cats, and black-footed cats (Terio et al. 2008). Domestic cats are an interesting species to examine because the traditional domestic short hair (DSH) cat is not often affected by amyloidosis, while the Siamese and Abyssinian breeds have inherited forms of the disease, though the genetic factors implicated in the heritable disease are different between these two breeds. There are 10 amino acid positions of variability identified among DSH, Siamese and Abyssinian cats. The cheetah AA amino acid sequence is more similar to that of Abyssinian cats than DSH cats: there is homology at 5 positions of variability with Abyssinian cats but only 2 with DSH cats (Johnson et al. 1997). This suggests the possibility that cheetah AA protein may be structured in a way that makes it more amyloidogenic compared to the domestic cat AA protein. When isolating amyloid A protein from liver tissues, two unique amino acid substitutions are found in the Siamese cat AA sequence when compared to Abyssinian cats (Niewold et al. 1999, van der Linde-Sipman et al. 1997). The 52A-V substitution found in Siamese cats is common among many other animal species, however the 46Q-R substitution discovered is not found in other mammals, but has been found in birds (Niewold et al. 1999). This 46Q-R substitution in Siamese cats is

not homologous and thus could influence the AA protein structure (Niewold et al. 1999). It is hypothesized that the AA sequence differences found among the breeds of domestic cats could explain the differences observed in the organ deposition patterns of amyloid during amyloidosis (Niewold et al. 1999).

Among other species, AA amyloidosis is considered "reactive" and does not have a genetic basis, but rather appears secondary to other inflammatory conditions. Secondary amyloidosis has been noted in several species including (but not limited to): bighorn sheep (Hadlow and Jellison 1962), bat (Gruber and Linke 1996), Dorcas gazelle (Rideout et al. 1989) and humans (De Beer et al. 1982). In humans, persistently elevated SAA concentrations lead to increased amyloid load and organ deterioration (Gillmore et al. 2001); mortality, amyloid burden, and renal prognosis are all significantly correlated with serum SAA concentration (De Beer et al. 1982, Lachmann et al. 2007). Similar to cheetahs, the main manifestation of secondary amyloidosis is renal dysfunction and failure (Lachmann et al. 2007). However, despite the negative effects of elevated SAA concentrations due to persistent systemic inflammation, it has been shown that amyloid deposits can regress and organ function can recover if SAA concentrations are controlled (kept low) (Gillmore et al. 2001). A promising therapy using SAA-specific antisense oligonucleotides (ASO) has been shown to reduce SAA concentration in mice, particularly by facilitating a more rapid return to baseline after episodic bursts in SAA concentration (Kluve-Beckerman et al. 2011). SAA-specific ASOs were also shown to reduce amyloid deposition in treated mice in which amyloidosis was previously induced (Kluve-Beckerman et al. 2011).

Whether ASOs could be a therapy to treat systemic amyloidosis in either humans or cheetahs has yet to be investigated.

Research objective and hypotheses

The overall objective of the following studies was to identify the relationship between genetics, inflammation, and stress with serum concentrations of the SAA protein and the incidence of amyloid A amyloidosis in captive cheetah populations. The specific hypotheses investigated include: (1) the single nucleotide deletion ($SAA1A^{-97delG}$) in the putative NF-κB binding site within the promoter region of the SAA1A gene locus is associated with lower serum SAA concentrations in cheetahs and with decreased prevalence of AA amyloidosis in deceased cheetahs; (2) higher fecal cortisol concentrations (as an indication of stress) are associated with higher serum SAA protein concentrations in cheetahs; (3) higher proinflammatory cytokine concentrations are associated with higher serum SAA protein concentrations in cheetahs; (4) elevated serum SAA concentrations are associated with greater amyloid deposition in the feces of cheetahs; and (5) the captive environment is associated with higher fecal cortisol concentrations, higher proinflammatory cytokine concentrations and higher serum SAA protein concentrations in cheetahs.

References

1. Auphan N, DiDonato JA, Rosette C, Helmberg A, Karin M. 1995. Immunosuppression by glucocorticoids: inhibition of NF-κB activity through induction of IκB synthesis. Science 270:286-290.

2. Biran H, Friedman N, Neumann L, Pras M, Shainkin-Kestenbaum R. 1986. Serum amyloid A (SAA) variations in patients with cancer: correlation with disease activity, stage, primary site, and prognosis. Journal of Clinical Pathology 39:794-797.

3. Barnes PJ, Karin M. 1997. Nuclear factor-κB - a pivotal transcription factor in chronic inflammatory diseases. New England Journal of Medicine 336:1066-1071.

4. Baumann H, Gauldie J. 1994. The acute phase response. Immunology Today 15:74-80.

5. Beg AA, Finco TS, Nantermet PV, Baldwin AS. 1993. Tumor necrosis factor and interleukin-1 lead to phosphorylation and loss of IκBα: a mechanism for NF-κB activation. Molecular and Cellular Biology 13:3301-3310.

6. Bergstrom J, Ueda M, Une Y, Sun X, Misumi S, Shoji S, Ando Y. 2006. Analysis of amyloid fibrils in the cheetah (*Acinonyx jubatus*). Amyloid 13(2): 93-98.

7. Bethin KE, Vogt SK, Muglia L. 2000. Interleukin-6 is an essential, corticotropin-releasing hormone independent stimulator of the adrenal axis during immune system activation. Proceedings of the National Academy of Sciences of the United States of America 97(16):9317-9322.

8. Bolton LA, Munson L. 1999. Glomerulosclerosis in captive cheetahs (*Acinonyx jubatus*). Veterinary Pathology 36:14-22.

9. Boyce JT, DiBartola SP, Chew DJ, Gasper PW. 1984. Familial renal amyloidosis in Abyssinian cats. Veterinary Pathology 21: 33-38.

10. Campbell DJ, Rawlings JM, Koelsch S, Wallace J, Strain JJ, Hannigan BM. 2004. Age-related differences in parameters of feline immune status. Veterinary Immunology and Immunopathology 100:73–80.

11. Castro-Prieto A, Wachter B, Sommer S. 2011. Cheetah paradigm revisited: MHC diversity in the world's largest free-ranging population. Molecular Biology and Evolution 28:1455-1468.

12. Caughey B, Baron G. 2008. Are cheetahs on the run from prion-like amyloidosis? Proceedings of the National Academy of Sciences of the United States of America 105(20):7113-7114.

13. Cerón JJ, Eckersall PD, Martinez-Subiela S. 2005. Acute phase proteins in dogs and cats: current knowledge and future perspectives. Veterinary Clinical Pathology 34:84-99.

14. Chen L, Une Y, Higuchi K, Mori M. 2012. Cheetahs have 4 serum amyloid A genes evolved through repeated duplication events. Journal of Heredity 103(1):115-129.

15. Chrousos GP. 1995. The hypothalamic-pituitary-adrenal axis and immune-mediated inflammation. The New England Journal of Medicine 332:1351-1362.

16. Chrousos GP. 2009. Stress and disorders of the stress system. Nature Reviews Endocrinology 5(7):374-81.

17. De Beer FC, Fagan EA, Hughes GRV, Mallya RK, Lanham JG, Pepys MB. 1982. Serum amyloid-A protein concentration in inflammatory disease and its relationship to the incidence of reactive systemic amyloidosis. The Lancet 320:231–234.

18. Dhabhar FS. 2009. Enhancing versus suppressive effects of stress on immune function: implications for immunoprotection and immunopathology. Neuroimmunomodulation 16:300-317.

19. Drake GJC, Kennedy LJ, Auty K, Ryvar R, Ollier WER, Kitchener AC, Freeman AR, Radford AD. 2004. The use of reference strand-mediated conformational analysis for the study of cheetah (*Acinonyx jubatus*) feline leucocyte antigen class II DRB polymorphisms. Molecular Ecology 13:221-229.

20. Durant S, Marker L, Purchase N, Belbachir F, Hunter L, Packer C, Breitenmoser-Wursten C, Sogbohossou E, Bauer H. 2008. *Acinonyx jubatus*. In: IUCN 2014. IUCN Red List of Threatened Species. Version 2014.1. <www.iucnredlist.org>. Downloaded on 17 June 2014.

21. Elenkov IJ, Chrousos GP. 1999. Stress hormone, Th1/Th2 patterns, pro/anti-inflammatory cytokines and susceptibility to disease. Trends in Endocrinology and Metabolism 10(9):359-368.

22. Esmat SM, EL-Sherif HE, Anwar S, Abdel-Atty S, Abdel-Reheim HA. 2005. Serum amyloid A protein level, and its significance in systemic lupus erythematosus patients. Egyptian Dermatology Online Journal 1(2):2.

23. Gillmore JD, Lovat LB, Persey MR, Pepys MB, Hawkins PN. 2001. Amyloid load and clinical outcome in AA amyloidosis in relation to circulating concentration of serum amyloid A protein. The Lancet 358:244-29.

24. Giordano A, Spagnolo V, Colombo A, Paltrinieri S. 2004. Changes in some acute phase protein and immunoglobulin concentrations in cats affected by feline infectious peritonitis or exposed to feline coronavirus infection. The Veterinary Journal 167:38–44.

25. Grisham J, Lackey LB, Spevak E. 2013. Population Analysis and Breeding and Transfer Plan: Cheetah (*Acinonyx jubatus*) AZA Species Survival Plan® Yellow Program. Population Management Center, Lincoln Park Zoo.

26. Gruber AD, Linke RP. 1996. Generalized AA-amyloidosis in a bat (*Pipistrellus pipistrellus*). Veterinary Pathology 33:428-430.

27. Hadlow WJ, Jellison WL. 1962. Amyloidosis in rocky mountain bighorn sheep. Journal of the American Veterinary Medical Association 141:243–247.

28. Jensen LE, Whitehead AS. 1998. Regulation of serum amyloid A protein expression during the acute-phase response. Biochemical Journal 334:489-503.

29. Johnson KH, Sletten K, Munson L, O'Brien TD, Papendick R, Westermark P. 1997. Amino acid sequence analysis of amyloid protein A (AA) from cats (captive cheetahs: *Acinonyx jubatus*) with a high prevalence of AA amyloidosis. Amyloid: International Journal of Experimental and Clinical Investigation 4:171-177.

30. Jurke MH, Czekala NM, Lindburg DG, Millard SE. 1997. Fecal corticoid metabolite measurements in the cheetah (*Acinonyx jubatus*). Zoo Biology 16:133-147.

31. Kajikawa T, Furuta A, Onishi T, Tajima T, Sugii S. 1999. Changes in concentrations of serum amyloid A protein, α-1-acid glycoprotein, haptoglobin, and C-reactive protein in feline sera due to induced inflammation and surgery. Veterinary Immunology and Immunopathology 68:91-98.

32. Kann RKC, Seddon JM, Henning J, Meers J. 2012. Acute phase proteins in healthy and sick cats. Research in Veterinary Science 93:649-654.

33. Keay JM, Singh J, Gaunt MC, Kaur T. 2006. Fecal glucocorticoids and their metabolites as indicators of stress in various mammalian species: a literature review. Journal of Zoo and Wildlife Medicine 37(3):234-244.

34. Kluve-Beckerman B, Hardwick J, Du L, Benson MD, Monia BP, Watt A, Crooke RM, Mullick A. 2011. Antisense oligonucleotide suppression of serum amyloid A reduces amyloid deposition in mice with AA amyloidosis. Amyloid: International Journal of Experimental and Clinical Investigation 18:136-146.

35. Köster LS, Schoeman JP, Meltzer DGA. 2007. ACTH stimulation test in the captive cheetah (*Acinonyx jubatus*). Journal of the South African Veterinary Association 78(3):133-136.

36. Lachmann HJ, Goodman HJB, Gilbertson JA, Gallimore JR, Sabin CA, Gillmore JD, Hawkins PN. 2007. Natural history and outcome in systemic AA amyloidosis. New England Journal of Medicine 356:2361-2371.

37. Liepnieks JJ, Kluve-Beckerman B, Benson MD. 1995. Characterization of amyloid A protein in human secondary amyloidosis: the predominant deposition of serum amyloid A1. Biochimica et Biophysica Acta 1270:81-86.

38. Marhaug G, Dowton SB. 1994. Serum amyloid A: an acute phase apolipoprotein and precursor of AA amyloid. Baillière's Clinical Rheumatology 8:553-573.

39. Marker L. 2013. 2012 International Cheetah (*Acinonyx jubatus*) Studbook. Namibia: Cheetah Conservation Fund.

40. Migita K, Yamasaki S, Shibatomi K, Ida H, Kita M, Kawakami A, Eguchi K. 2001. Impaired degradation of serum amyloid A (SAA) protein by cytokine-stimulated monocytes. Clinical and Experimental Immunology 123:408-411.

41. Moriguchi M, Terai C, Kaneko H, Koseki Y, Kajiyama H, Uesato M, Inada S, Kamatani N. 2001. A novel single-nucleotide polymorphism at the 5'-flanking region of SAA1 associated with risk of type AA amyloidosis secondary to rheumatoid arthritis. Arthritis and Rheumatism 44:1266-1272.

42. Munson L. 1993. Diseases of Captive Cheetahs (*Acinonyx jubatus*): Results of the Cheetah Research Council Pathology Survey, 1989-1992. Zoo Biology 12:105-124.

43. Munson L, Nesbit JW, Meltzer DGA, Colly LP, Bolton L, Kreik LPJ. 1999. Diseases of captive cheetahs (*Acinonyx jubatus jubatus*) in South Africa: a 20-year retrospective survey. Journal of Zoo and Wildlife Medicine 30(3):342-347.

44. Munson L, Terio K, Worley M, Jago M, Bagot-Smith A, Marker L. 2005. Extrinsic factors significantly affect patterns of disease in free-ranging and

captive cheetah (*Acinonyx jubatus*) populations. Journal of Wildlife Diseases 41(3):542-548.

45. Niemi K, Teirila L, Lappalainen J, Rajamaki K, Baumann MH, Oorni K, Wolff H, Kovanen PT, Matikainen S, Eklund KK. 2011. Serum amyloid A activates the NLRP3 inflammmasome via $P2X_7$ receptor and a cathepsin B-sensitive pathway. Journal of Immunology 186:6119-6128.

46. Niewold TA, van der Linde-Sipman JS, Murphy C, Tooten PCJ, Gruys E. 1999. Familial amyloidosis in cats: Siamese and Abyssinian AA proteins differ in primary sequence and pattern of deposition. Amyloid: International Journal of Experimental and Clinical Investigation 6:205-209.

47. O'Brien SJ, Roelke ME, Marker L, Newman A, Winkler CA, Meltzer D, Colly L, Evermann JF, Bush M, Wildt DE. 1985. Genetic basis for species vulnerability in the cheetah. Science 227:1428-1434.

48. O'Brien SJ, Wildt DE, Goldman D, Merril CR, Bush M. 1983. The cheetah is depauperate of genetic variation. Science 221:459-462.

49. Ofri R, Nyska A, Linke RP, Shtrasburg S, Livneh A, Gal R. 1997. Systemic amyloidosis in a cheetah (*Acinonyx jubatus*). Amyloid: International Journal of Experimental and Clinical Investigation 4(2):98-103.

50. Paltrinieri S. 2008. The feline acute phase reaction. The Veterinary Journal 177:26-35.

51. Papendick RE, Munson L, O'Brien TD, Johnson KH. 1997. Systemic AA amyloidosis in captive cheetahs (*Acinonyx jubatus*). Veterinary Pathology 34:549-556.

52. Ray A, Ray BK. 1994. Serum amyloid A gene expression under acute-phase conditions involves participation of inducible C/EBP-β and C/EBP-δ and their activation by phosphorylation. Molecular and Cellular Biology 14:4324-4332.

53. Rideout BA, Montali RJ, Wallace RS, Bush M, Phillips Jr. LG, Antonovych TT, Sabnis SG. 1989. Renal medullary amyloidosis in Dorcas gazelles. Veterinary Pathology 26:129-135.

54. Rivas AL, Tintle L, Meyerswallen V, Scarlett JM, Vantassell CP, Quimby FW. 1993. Inheritance of renal amyloidosis in Chinese Shar-pei dogs. Journal of Heredity 84:438-442.

55. Röcken C, Menard R, Bühling F, Vöckler S, Raynes J, Stix B, Krüger S, Roessner A, Kähne T. 2005. Proteolysis of serum amyloid A and AA amyloid proteins by cysteine proteases: cathepsin B generates AA amyloid proteins

and cathepsin L may prevent their formation. Annals of the Rheumatic Diseases 64:808–815.

56. Sapolsky RM, Romero LM, Munck AU. 2000. How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory and preparative actions. Endocrine Reviews 21(1):55-89.

57. Sasaki K, Ma Z, Khatlani TS, Okuda M, Inokuma H, Onishi T. 2003. Evaluation of feline serum amyloid A (SAA) as an inflammatory marker. Journal of Veterinary Medical Science 65:545–548.

58. Scheinman RI, Cogswell PC, Lofquist AK, Baldwin AS. 1995. Role of transcriptional activation of IκBα in mediation of immunosuppression by glucocorticoids. Science 270:283-286.

59. Tamamoto T, Ohno K, Goto-Koshino Y, Fujino Y, Tsujimoto H. 2012. Serum amyloid A uptake by feline peripheral macrophages. Veterinary Immunology and Immunopathology 150:47-52.

60. Tamamoto T, Ohno K, Ohmi A, Goto-Koshino Y, Tsujimoto H. 2008. Verification of measurement of the feline serum amyloid A (SAA) concentration by human SAA turbidimetric immunoassay and its clinical application. Journal of Veterinary Medical Science 70:1247-1252.

61. Terio KA, Marker L, Munson L. 2004. Evidence for chronic stress in captive but not free-ranging cheetahs (*Acinonyx jubatus*) based on adrenal morphology and function. Journal of Wildlife Diseases 40(2):259-266.

62. Terio KA, Munson L, Moore PF. 2011. Characterization of the gastric immune response in cheetahs (*Acinonyx jubatus*) with Helicobacter-associated gastritis. Veterinary Pathology 49(5):824-833.

63. Terio KA, O'Brien T, Lamberski N, Famula TR, Munsin L. 2008. Amyloidosis in black-footed cats (*Felis nigripes*). Veterinary Pathology 45:393-400.

64. Thorn CF, Whitehead AS. 2002. Differential glucocorticoid enhancement of the cytokine-driven transcriptional activation of the human acute phase serum amyloid A genes, SAA1 and SAA2. Journal of Immunology 169:399-406.

65. van der Linde-Sipman JS, Niewold TA, Tooten PCJ, de Neijs-Backer M, Gruys E. 1997. Generalized AA-amyloidosis in Siamese and Oriental cats. Veterinary Immunology and Immunopathology 56:1-10.

66. Wells A, Terio KA, Ziccardi MH, Munson L. 2004. The stress response to environmental change in captive cheetahs (*Acinonyx jubatus*). Journal of Zoo and Wildlife Medicine 35(1):8-14.

67. Wiegers GJ, Reul JMHM. 1998. Induction of cytokine receptors by glucocorticoids: functional and pathological significance. Trends in Pharmacological Sciences 19:317-321.

68. Wielebnowski NC, Ziegler K, Wildt DE, Lukas J, Brown JL. 2002. Impact of social management on reproductive, adrenal and behavioural activity in the cheetah (*Acinonyx jubatus*). Animal Conservation 5:291-301.

69. Woo P, Sipe J, Dinarello CA, Colton HR. 1987. Structure of a human serum amyloid A gene and modulation of its expression in transfected L cells. Biochemistry 262(32):15790-15795.

70. Yamada T, Liepnieks JJ, Kluve-Beckerman B, Benson MD. 1995. Cathepsin B generates the most common form of amyloid A (76 residues) as a degradation product of serum amyloid A. Scandinavian Journal of Immunology 41:94-97.

71. Yuhki N, O'Brien SJ. 1990. DNA variation of the mammalian major histocompatibility complex reflects genomic diversity and population history. Proceedings of the National Academy of Sciences of the United States of America 87:836-840.

72. Zhang B, Une Y, Fu X, Yan J, Ge F, Yao J, Sawashita J, Masayuki M, Tomozawa H, Kametani F, Higuchi K. 2008a. Fecal transmission of AA amyloidosis in the cheetah contributes to high incidence of disease. Proceedings of the National Academy of Sciences of the United States of America 105(20):7263-7268.

73. Zhang B, Une Y, Ge F, Fu X, Qian J, Zhang J, Sawashita J, Higuchi K, Mori M. 2008b. Characterization of the cheetah serum amyloid A1 gene: critical role and functional polymorphism of a cis-acting element. Journal of Heredity 99(4):355-363.

Chapter 2: Linking genetic differences to amyloid A amyloidosis prevalence in the cheetah (*Acinonyx jubatus*)

Abstract

Systemic amyloid A (AA) amyloidosis is a major cause of morbidity and mortality among captive cheetahs in North America. The self-aggregating AA protein responsible for this disease is a byproduct of serum amyloid A (SAA) protein degradation, an acute phase protein highly upregulated during inflammation. Transcriptional induction of the cheetah SAA1 gene is dependent on both C/EBPβ and NF-κB cis-acting elements in the promoter region. Two alleles exist for a single nucleotide polymorphism (SNP) consisting of a single guanine nucleotide deletion in the putative NF-κB binding site of the SAA1 promoter. This study showed that the $SAA1A^{-97delG}$ allele was associated with decreased SAA protein concentrations in the serum of captive cheetahs, suggesting that genetic differences between the populations at this locus may be affecting AA amyloidosis prevalence. The allele frequency of the SNP, $SAA1A^{-97delG}$, was significantly different between captive and wild cheetah populations: it was found more commonly among cheetahs in captivity. This disparity could be the result of past selection pressure in captivity favoring the $SAA1A^{-97delG}$ allele if the production of more SAA protein is linked to the development of AA amyloidosis; however, there was no significant difference in the frequency of the $SAA1A^{-97delG}$ allele between individuals who were confirmed AA amyloidosis positive and AA amyloidosis negative at the time of necropsy. Thus, even though there is evidence that having more copies of the $SAA1A^{-97delG}$ SNP results

in a decrease in the serum concentration of SAA protein in captive cheetahs, genotype is not associated with this disease within the North American population, suggesting that other factors are playing a role in the pathogenesis of AA amyloidosis among captive cheetahs.

Introduction

The acute phase response (APR) is a systemic reaction that occurs early during inflammation, initiated by cells of the innate immune system such as macrophages (Baumann and Gauldie 1994, Paltrinieri 2008). The liver is one of the primary targets for systemic inflammatory mediators such as the IL-1 type cytokines, IL-1β and TNF-α, as it is the primary location of acute phase protein (APP) production (Baumann and Gauldie 1994, Cerón et al. 2005, Jensen and Whitehead 1998, Marhaug and Dowton 1994, Paltrinieri 2008). APPs, by definition, are proteins whose concentration in serum significantly increase or decrease during the APR and there is a high degree of similarity in the response patterns of several APPs to systemic inflammatory mediators during the APR across species (Baumann and Gauldie 1994, Cerón et al. 2005, Paltrinieri 2008).

The major APPs that have been identified to date in the domestic cat are the serum amyloid A (SAA) protein and alpha-1-acid glycoprotein (AGP) (Cerón et al. 2005, Kajikawa et al. 1999, Paltrinieri 2008). In humans, the concentration of SAA found in circulation bound to high-density lipoproteins increases as much as 1000-fold within 24-48 hours of the initiation of inflammation (Marhaug and Dowton 1994). The increase in SAA observed in domestic cats during the APR, approximately a 10-100 fold increase, is lower in magnitude compared to humans and

other species, but still represents a major APP (Cerón et al. 2005, Giordano et al. 2004, Kajikawa et al. 1999) and multiple studies have shown that SAA concentrations in sick cats are significantly higher than SAA concentrations in healthy cats (Giordano et al. 2004, Kajikawa et al. 1999, Kann et al 2012, Sasaki et al. 2003, Tamamoto et al. 2008).

The SAA protein is well conserved throughout evolution, indicating an important biological function (Marhaug and Dowton 1994). Though its function is not completely understood, possible roles of SAA during the APR include the recruitment of inflammatory cells to the primary site of inflammation, involvement in lipid metabolism and transport, increasing cytokine production in monocytes/macrophages, stimulating nitric oxide production in macrophages, and scavenging for oxidized cholesterol, preventing prolonged tissue damage (Cerón et al. 2005, Jensen and Whitehead 1998, Paltrinieri 2008, Tamamoto et al. 2012). The SAA protein normally has a short half-life: approximately 90 minutes in normal serum (Marhaug and Dowton 1994), and the concentration of SAA in serum returns to normal following the termination of inflammation.

In multiple species, including domestic cats, monocytes contribute to SAA degradation (Migita et al. 2001, Tamamoto et al. 2012); however, this process is inhibited in the presence of the cytokines IL-1β and IFN-γ (Migita et al. 2001) and glucocorticoids (Tamamoto et al. 2012). SAA itself can induce the expression and secretion of TNF-α and IL-1β from human macrophages through interactions with the TLR2 and TLR4 receptors (Niemi et al. 2011), thus contributing to its own persistence. These effects, combined with the increased post-transcriptional stability

of SAA mRNA during the APR due to the production of longer poly-A tails (Jensen and Whitehead 1998), contribute to the major increases in SAA concentration observed in serum during the APR.

The N-terminal region of the SAA protein is the precursor for the amyloid A (AA) protein, the first 10-15 amino acid residues of which are amyloidogenic (Marhaug and Dowton 1994). In abundance, the AA protein polymerizes to form insoluble fibrils, which are deposited into the liver, kidney, spleen and other tissues (Marhaug and Dowton 1994). AA amyloidosis is a disease induced by the over-accumulation and incomplete degradation of SAA, polymerization of AA protein into fibrils and subsequent tissue deposition, resulting in a disruption in organ functionality (Marhaug and Dowton 1994).

It has been shown that SAA can also induce the secretion of cathepsin B from macrophages through direct activation of the $P2X_7$ receptor (Niemi et al. 2011). This effect, considered together with evidence that cathepsin B is capable of degrading SAA into AA-like protein fragments (Röcken et al. 2005, Yamada et al. 1995), means chronic inflammation may lead to the extracellular proteolytic processing of SAA into AA amyloidogenic fragments. Thus, it is unsurprising that elevated SAA concentrations in serum have been found to correlate with AA amyloidosis and other inflammatory diseases in humans (Lachmann et al. 2007, Marhaug and Dowton 1994).

The CCAAT-enhancer-binding proteins (C/EBPs) are primarily responsible for the upregulation of SAA transcription during the APR (Ray and Ray 1994). The C/EBPβ binding site in the promoter of the human SAA gene is located within the

265bp region upstream of the transcription start site (Marhaug and Dowton 1994). Also located within this region, is a binding site for the transcription factor NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) (Marhaug and Dowton 1994). In the presence of stimulation from pro-inflammatory cytokines, such as TNF-α (Beg et al. 1993, Scheinman et al. 1995) and IL-1β (Barnes and Karin 1997), the activated forms of C/EBPβ and NF-κB enter the nucleus and bind to the promoter region of target genes associated with inflammatory and immune responses, such as *SAA* (Barnes and Karin 1997, Beg et al. 1993, Ray and Ray 1994). IL-1β, TNF-α, and IL-6 proinflammatory cytokines have all been shown to stimulate hepatic SAA production during the APR (Baumann and Gauldie 1994, Cerón et al. 2005, Jensen and Whitehead 1998, Marhaug and Dowton 1994, Paltrinieri 2008).

Four serum amyloid A protein genes (*SAA1A*, *SAA1B*, *SAA3A*, *SAA3B*) have been identified in the cheetah genome (Chen et al. 2012). Chen et al. (2012) found that both SAA1 and SAA3 gene expression is higher in the liver tissue of cheetahs with amyloidosis (Chen et al. 2012), suggesting that an increase in SAA protein production by the liver is important in the etiology of systemic amyloidosis. The SAA1 protein encoded by the SAA1 gene is the precursor to the AA protein identified as the major component of amyloid fibrils deposited into tissues during systemic amyloidosis in cheetahs (Bergstrom et al. 2006, Johnson et al. 1997, Ofri et al. 1997). The structure of the cheetah SAA1 genes show high similarity to the human SAA1 and mouse Saa1 genes (Zhang et al. 2008).

Transcriptional induction of the SAA1 gene in cheetahs by IL-1β and IL-6 is dependent on the presence of both C/EBPβ and NF-κB elements (Zhang et al. 2008).

Nucleotide sequences of NF-κB and C/EBPβ target sites are highly conserved between cheetah and human (Zhang et al. 2008), however Zhang et al. (2008) identified two alleles for a polymorphism consisting of a single guanine nucleotide deletion (SNP) in the putative NF-κB binding site of the *SAA1A* promoter. More recently, four alleles have been identified for the SAA1A gene, only one of which ("allele 1") has 3 guanine residues at the -97 to -99 position (Chen et al. 2012); the three other alleles have the single guanine nucleotide deletion. These *SAA1A*$^{-97delG}$ alleles are associated with reduced transcriptional activity of the SAA1 gene in vitro (Zhang et al. 2008), though the effect *in vivo* has yet to be evaluated.

Systemic amyloid A amyloidosis is an increasingly important cause of morbidity and mortality in captive cheetahs, affecting up to 70% of individuals at necropsy (Munson et al. 2005, Ofri et al. 1997, Papendick et al. 1997) and despite the high prevalence of AA amyloidosis observed in captive cheetahs in both North America and South Africa, wild cheetahs are virtually unaffected (Munson et al. 2005). The aim of this study was to determine if the *SAA1A*$^{-97delG}$ SNP in the putative NF-κB binding site of the *SAA1A* promoter is associated with the production of SAA protein *in vivo* and if so, if the difference in AA amyloidosis prevalence between the wild and captive cheetah populations could be attributable to genetic differences at this locus.

Methods

Sample collection

Banked fecal and serum samples were obtained from cheetahs housed at the Smithsonian Conservation Biology Institute (SCBI) in Front Royal, VA, and National

Zoological Park (NZP) in Washington, DC (fecals: n=38 cheetahs from multiple institutions, serum: n=20 cheetahs at NZP-SCBI only). Previously extracted DNA or white blood cell samples and banked serum samples were obtained from the Cheetah Conservation Fund (CCF) in Namibia from wild-born cheetahs (DNA or WBC: n=47 cheetahs, serum: n=34 cheetahs). Fresh and formalin fixed tissue samples from deceased cheetahs with known AA amyloidosis histology were provided upon request by the cheetah Species Survival Plan® pathologist (n=48 cheetahs).

DNA isolation

DNA was extracted from fecal samples using the QIAamp® DNA Stool Mini Kit (QIAGEN®) following the protocol for the isolation of DNA from stool for human DNA analysis. DNA was extracted from white blood cell samples using the QIAamp® DNA Blood Mini Kit (QIAGEN®) following the protocol for DNA purification from blood or body fluids (spin protocol). DNA was extracted from fresh and formalin fixed tissues using the PUREGENE® Genomic DNA Purification Kit (Gentra Systems) following the solid tissue protocol with an extended incubation time in the Cell Lysis Buffer provided.

Amplification and genotyping of the NF-κB binding site in the SAA1A promoter region

The putative NF-κB binding site within the *SAA1A* promoter region was amplified using a semi-nested polymerase chain reaction (PCR). Initially, a larger region of the promoter was amplified using the F3A forward primer specific to the *SAA1A* gene (Chen et al. 2012). The reverse primer used was designed from the *SAA1*

promoter sequence reported in Zhang et al. (2008) and named "SAA1R" (5'-ACTGTGCCCTCCCCGTTGGG-3'). The second round of PCR used the SAA1F forward primer (5'-GACCGGCCAAGCTGGCTTCC-3') with the SAA1R reverse primer to produce a smaller PCR product for sequencing containing the putative NF-κB binding site (Figure 2.1). The first PCR product was either purified using the QIAquick® Gel Extraction Kit (QIAGEN®) or was treated with ExoSAP-IT® (Affymetrix) before being used as template for the second reaction. Both polymerase chain reactions were carried out using GoTaq® Green Master Mix (Promega) and entailed an initial denaturing step of 94^0C for 3 mins, followed by 35 cycles of 94^0C for 30sec (denature), 60^0C for 30sec (annealing) and 72^0C for 30sec (extension), followed by a final extension step of 72^0C for 10min. PCR products were genotyped by either standard Sanger sequencing via GENEWIZ® or by microsatellite analysis through the use of a fluorescently labelled SAA1F primer (SAA1F-FAM). To prepare samples for sequencing, the PCR products were purified using the QIAquick® Gel Extraction Kit (QIAGEN®) and premixed with primer before shipping to GENEWIZ®. Chromatograms were interpreted for determination of genotype. $SAA1A^{+/+}$ and $SAA1A^{-97delG/-97delG}$ genotypes contained clear distinguishable peaks on the chromatogram. $SAA1A^{+/-97delG}$ heterozygotes generated a chromatogram with overlapping peaks beginning at the -97 guanine deletion site in the *SAA1A* promoter (Figure 2.2). When running the fluorescently labelled PCR products on the ABI PRISM® 310 Genetic Analyzer, homozygotes produced a single peak, and heterozygotes produced two peaks. Homozygote genotypes could be distinguished based on PCR product length (Figure 2.3).

Figure 2.1. Nucleotide sequence of the putative promoter region -262bp upstream of the cheetah SAA1A gene.

```
-262  CCGACTCGGA  CAGCAGAGCC  CTCCTCCTGA  GCCCCAGCTT  CCAGGCAGAC
-212  CGGCCAAGCT  GGCTTCCTCA  CAGTTGCACA  ACTGGAAGTT  GACCTGTGGA
-162  TGAAGAAAAC  CACGGGTGTC  CGGTAAGTTT  TCCTTTGCAG  GTCTCTCGGG
-112  GCCATGACCT  GGAGGGACCT  TTCTGGCCAC  CAGGTCTGGC  TTTTCTTGCG
-62   TCCCCATGGG  GCGCCCAACG  GGGAGGGCAC  AGTATAAATC  CCGGCCACCT
-12   CTCCCAGCAG  GCAGGGACCG  GCAGCTCTGC  TCCA
```

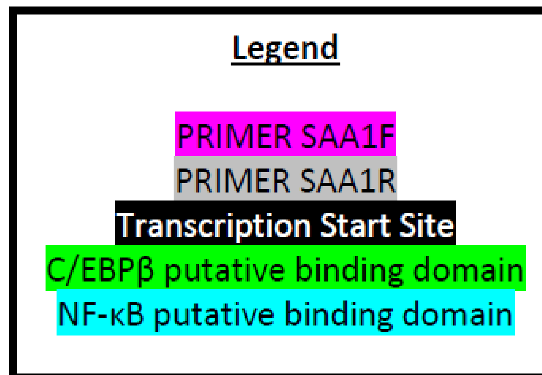


Figure 2.2. Representative chromatograms for the three SAA1A promoter genotypes after Sanger sequencing. Top: homozygous *SAA1A*$^{+}$; Middle: heterozygous *SAA1A*$^{+/-97delG}$; Bottom: homozygous *SAA1A*$^{-97delG}$.

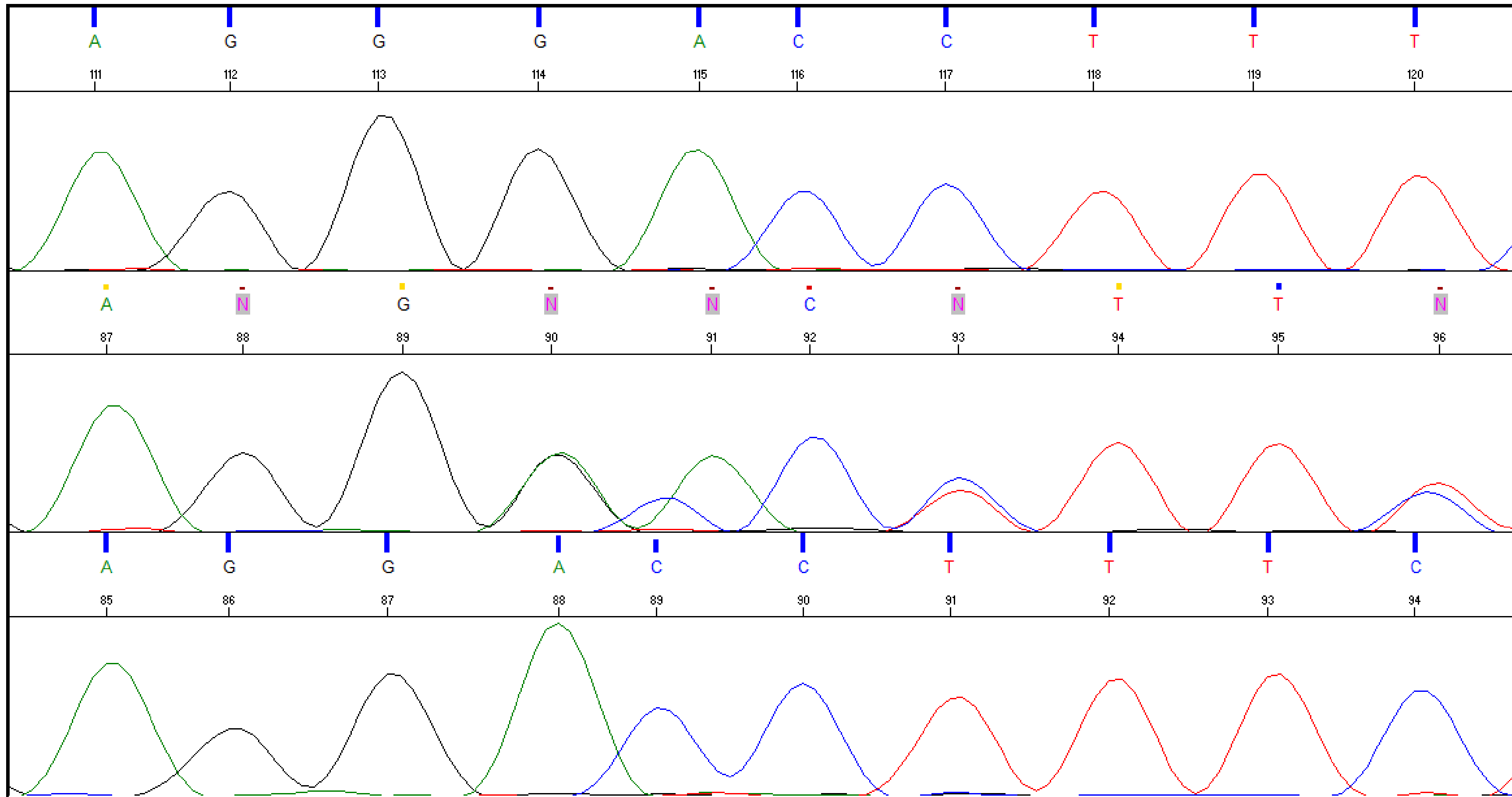
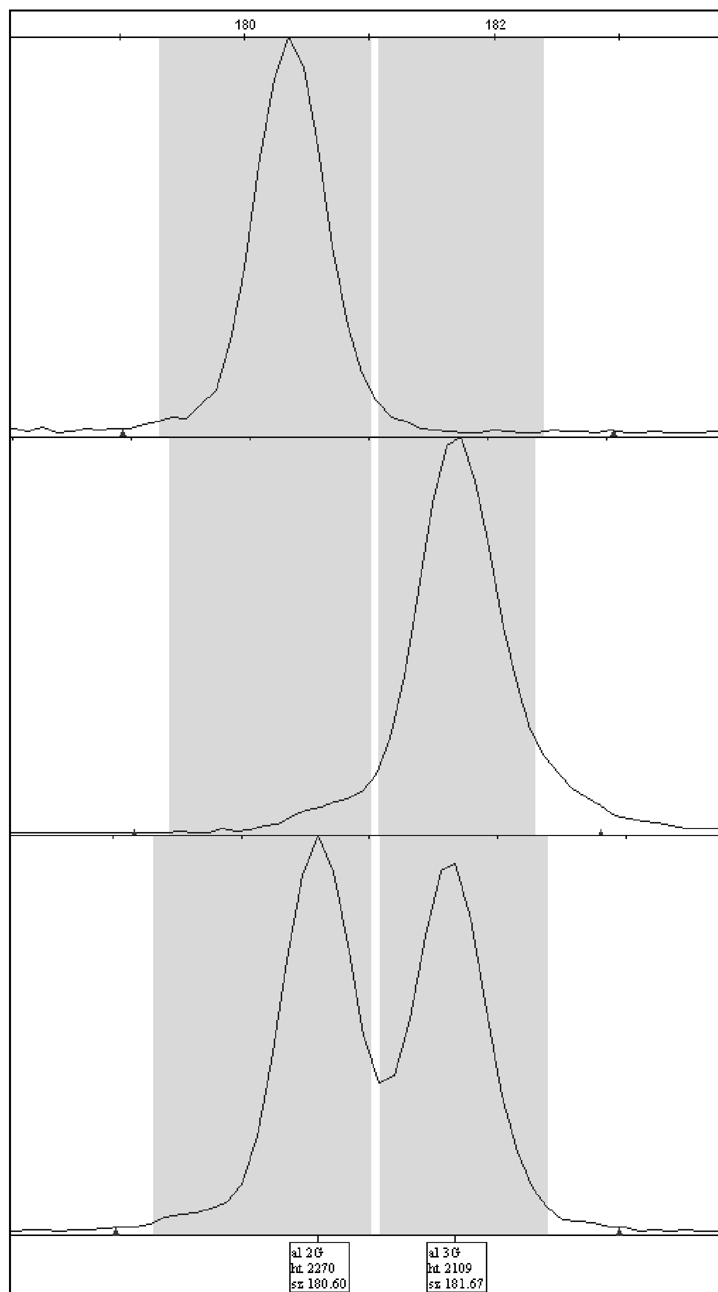


Figure 2.3. Representative output for the three SAA1A promoter genotypes from the ABI PRISM® 310 Genetic Analyzer. Top: homozygous $SAA1A^{-97delG}$; Middle: homozygous $SAA1A^{+}$; Bottom: heterozygous $SAA1A^{+/-97delG}$.



To confirm specific amplification of the SAA1A gene only, second round PCR primers (SAA1F/R; non-specific: amplifies both SAA1A and SAA1B promoters) were used to amplify the SAA1 promoter region for six cheetahs (2 with each genotype) and compared to the nested PCR results. The SAA1B gene lacks the $SAA1A^{-97delG}$ SNP (Chen et al. 2012), thus amplification of both SAA1A and SAA1B promoters simultaneously should produce 4 amplified products: 2 should always be $SAA1A^{+}$ and the other 2 will be dictated based on SAA1A genotype. See Appendix I for representative results.

Quantification of serum amyloid A protein concentration in serum

Serum amyloid A concentration was measured in serum samples using the Phase^{TM} Range Multispecies SAA ELISA kit (Tridelta Development Ltd: Cat No. TP 802). Serum samples were diluted 1:100 in the provided sample diluent before use. Anti-SAA/HRP conjugate (50µl) and sample/control/standard (50µl, in duplicate) were added to each pre-coated well and incubated for 1.5 hours on a shaker at room temperature. After washing, 100µl of TMB substrate solution was added to each well, and the plate was incubated for 15 minutes at room temperature. Stop solution was added (100µl) and absorbance for each well was read at 450nm using 620nm as a reference.

Statistical analysis

Serum amyloid A concentration was log (base 10) transformed before data analysis due to a significant departure from normality. Log_{10}[SAA] was compared between the three genotypes of the *SAA1A* gene using an ANCOVA with the number

of SNP copies (of the *SAA1A*$^{-97delG}$ SNP) as a quantitative factor and captive population (NZP-SCBI vs. CCF) as a fixed categorical factor, assuming heterogeneous variances for captive populations, using PROC MIXED in SAS Enterprise Guide version 6.1 with the Kenward and Roger (DDFM=KR) option for adjustment of denominator degrees of freedom. Initially, the interaction term between SNP copies and captive population was included in the statistical model, however, it was removed due to being highly insignificant.

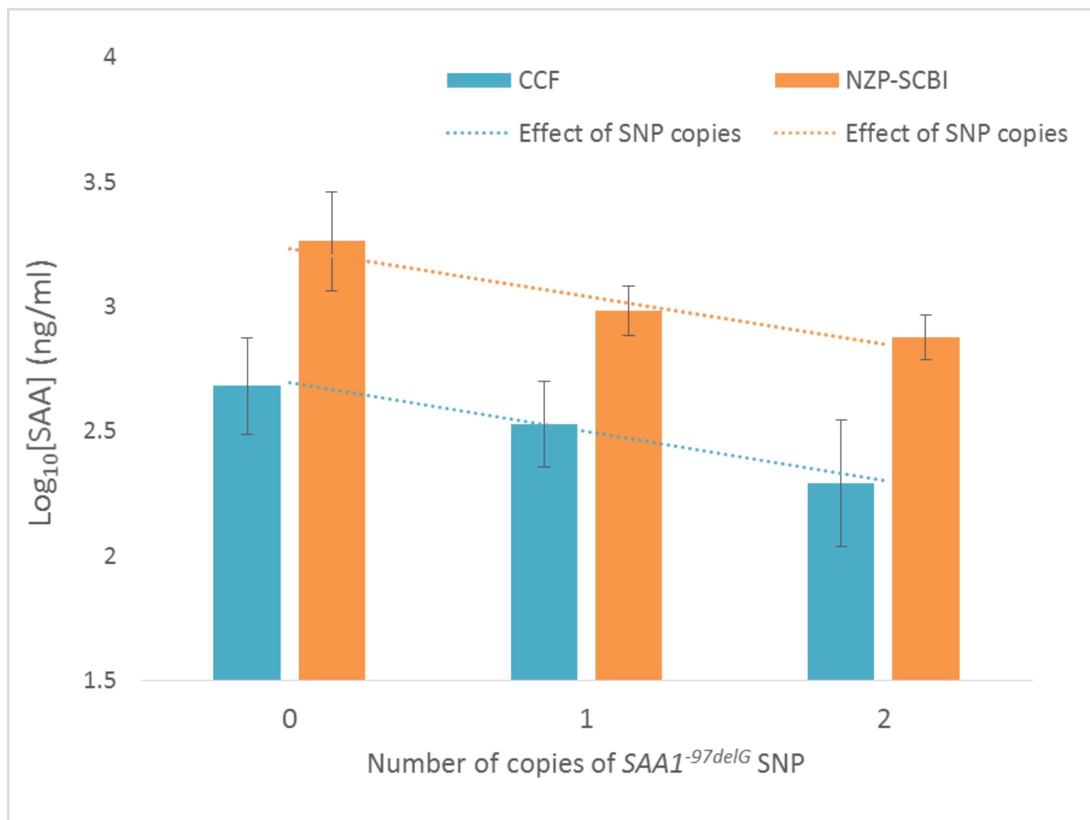
Allele frequencies for the captive North American and wild Namibian cheetah populations were estimated using the software MENDEL (Lange et al. 2013) utilizing known genotypes and complete pedigree information available for all individuals. Genotype and allele frequencies were compared between populations using a Chi-square Test of Homogeneity.

Results

Serum amyloid A concentration in captive cheetahs is associated with genotype

Based on the ANCOVA, a significant decrease in the $\log_{10}$SAA concentration in serum was found as the number of copies of the *SAA1A*$^{-97delG}$ SNP increased (P=0.0453; Figure 2.4). There was also a significant effect of captive population: captive cheetahs at NZP-SCBI have significantly higher $\log_{10}$SAA concentrations in serum compared to captive cheetahs at CCF (P=0.0003; Figure 2.4), regardless of the number of SNP copies. Interestingly, even captive cheetahs at NZP-SCBI who were homozygous for the *SAA1A*$^{-97delG}$ allele had higher $\log_{10}$SAA concentrations, on average, than CCF cheetahs who were homozygous *SAA1A*$^{+}$ (Figure 2.4). The differences observed between the two captive populations explained approximately

Figure 2.4. Serum concentrations of serum amyloid A (SAA) protein in captive cheetahs at NZP-SCBI and CCF. Unrestricted least-square means of the log (base 10) SAA concentrations ± SE by genotype are presented.



four times the amount of variation in $\log_{10}$SAA concentrations as was explained by genotype.

Differences in $SAA1A^{-97delG}$ allele frequencies between populations

When examining the genotype frequencies of the SNP in the NF-κB binding site in the *SAA1A* promoter region of the captive North American and wild Namibian cheetah populations, there was no evidence to reject Hardy-Weinberg equilibrium in either population, thus allele frequencies between populations were compared. The allele frequency of the single base pair deletion in the promoter region of the *SAA1A* gene, $SAA1A^{-97delG}$, was significantly different between populations (P=0.0414): the $SAA1A^{-97delG}$ allele is more common in the captive North American cheetah population when compared to wild Namibian cheetahs (Table 2.1).

Allele frequencies were also compared among deceased cheetahs from the captive North American population between individuals who were confirmed to be AA amyloidosis positive and those confirmed AA amyloidosis negative at the time of necropsy. There was no significant difference in the frequency of the $SAA1A^{-97delG}$ allele between these two groups of captive cheetahs (Table 2.2). A power analysis was performed using PROC POWER in SAS Enterprise Guide version 6.1 to determine the number of cheetahs that would need to be genotyped to declare the difference in allele frequencies observed significantly different and it was concluded that 52 cheetahs per group (positive/negative) would be required to ensure 90% power. Additional voucher samples may be genotyped in the future to increase the power of the analysis, but based on the current available data, AA amyloidosis diagnosis appears to be independent of genotype.

Population	**Allele**	**Estimated[1] Frequency ± Standard Error**
Namibia (Wild, n=47)	*SAA1A*$^{-97delG}$	0.5032[a] ±0.0600
	SAA1A^{+}	0.4968 ±0.0600
North America (Captive, n=38)	*SAA1A*$^{-97delG}$	0.7158[b] ±0.0796
	SAA1A^{+}	0.2842 ±0.0796

Table 2.1. Estimated allele frequencies of the *SAA1A*$^{-97delG}$ SNP for wild cheetahs in Namibia and captive cheetahs in North America. [a,b] Indicates a significant difference in allele frequency based on the Chi-square Test of Homogeneity, P=0.0414. [1]Estimated using the software MENDEL (Lange et al. 2013) utilizing complete pedigree information available for all individuals.

AA Amyloidosis Diagnosis	Allele	Estimated[1] Frequency ± Standard Error
Positive (n=27)	*SAA1A*$^{-97delG}$	0.8750 ±0.1170
	SAA1A^{+}	0.1250 ±0.1170
Negative (n=21)	*SAA1A*$^{-97delG}$	0.5980 ±0.0781
	SAA1A^{+}	0.4020 ±0.0781

Table 2.2. Estimated allele frequencies of the *SAA1A*$^{-97delG}$ SNP for captive cheetahs confirmed positive or negative for AA amyloidosis at time of necropsy. [1]Estimated using the software MENDEL (Lange et al. 2013)) utilizing complete pedigree information available for all individuals.

Discussion

Amyloid A amyloidosis has been identified in many species. In some cases, susceptibility appears to be genetic (familial amyloidosis), as observed in: Shar-Pei dogs (Rivas et al. 1993), Abyssinian (Boyce et al. 1984) and Siamese (van der Linde-Sipman et al. 1997) breeds of domestic cats, and black-footed cats (Terio et al. 2008). Among other species, AA amyloidosis is considered "reactive" and does not have a genetic basis, but rather appears secondary to other inflammatory conditions, as observed in: bighorn sheep (Hadlow and Jellison 1962), bat (Gruber and Linke 1996), Dorcas gazelle (Rideout et al. 1989) and humans (De Beer et al. 1982). Domestic cats are an interesting species to examine because the traditional domestic short hair (DSH) cat is not often affected by amyloidosis, while the Siamese and Abyssinian breeds have inherited forms of the disease, though the genetic differences implicated in the heritable disease are different between these two breeds. The cheetah AA protein amino acid sequence is more similar to that of Abyssinian cats than DSH cats (Johnson et al. 1997), which suggests the possibility that cheetah AA protein may be structured in a way that makes it more amyloidogenic compared to the domestic cat AA protein. However, AA amyloidosis is uncommon among wild cheetahs (Munson et al. 2005), suggesting that the AA protein structure is not the cause of AA amyloidosis observed in cheetahs, therefore not of a true "familial" type.

The identification of the SNP in the promoter region of the SAA1A gene in cheetahs by Zhang et al. (2008), suggested that AA amyloidosis in captive cheetahs may still be linked to genetic differences, as the $SAA1A^{-97delG}$ SNP is associated with a decrease in transcription of the SAA1A gene *in vitro* (Zhang et al. 2008). This study

shows that the $SAA1A^{-97delG}$ SNP is similarly associated with a decrease in the SAA concentration in serum of captive cheetahs. However, more striking were the differences in SAA concentrations that were observed between the two captive populations examined, NZP-SCBI and CCF. NZP-SCBI cheetahs had significantly higher SAA concentrations compared to CCF cheetahs. Though both populations are captive, it seems that major environmental differences between the captive environments at NZP-SCBI and CCF play a bigger role in the differences in SAA concentrations observed. This supports previous theories that suggests the high prevalence of AA amyloidosis observed among captive cheetahs is the result of differences imposed by the captive environment.

It has been widely accepted that captivity imposes psychological stress on several species of animals (e.g. camels (Padalino et al. 2014), house sparrows (Martin et al. 2012), orangutans (Weingrill et al. 2011), chukar partridge (Dickens et al. 2009), and Bengal tigers (Sajjad et al 2011)). Psychological stress activates the hypothalamic-pituitary-adrenal (HPA) axis leading to the production and release of glucocorticoids (GCs), such as cortisol and corticosterone (Chrousos 1995). There is a positive, synergistic effect of GCs with cytokines in the induction of SAA synthesis during the APR (Chrousos 1995, Marhaug and Dowton 1994). This effect is the result of the upregulation of cytokine receptors on the surface of hepatic cells by GCs (Baumann and Gauldie 1994, Wiegers and Reul 1998), increasing the sensitivity of the hepatic cells to cytokine stimulation (Baumann and Gauldie 1994, Paltrinieri 2008, Thorn and Whitehead 2002, Wiegers and Reul 1998). During the APR, IL-1β and IL-6 can also act on the adrenal-pituitary axis to stimulate production of ACTH,

inducing the production of cortisol (Baumann and Gauldie 1994). Therefore, it appears GCs play a role in the systemic inflammatory response by increasing production of APPs during the APR. There is both functional (hypercortisolemia) and morphological (adrenal hyperplasia) evidence for chronic stress (persistently elevated glucocorticoids) among captive cheetahs, at levels much greater than their wild counterparts (Terio et al. 2004). Therefore, it is possible that chronic stress among captive cheetahs, imposed by the environment, potentiates the development of AA amyloidosis during inflammation, resulting in the observed increase in the incidence of AA amyloidosis among captive cheetahs compared to animals in the wild (Munson et al. 2005).

Papendick et al. (1997) found that all cheetahs diagnosed with amyloidosis also suffered from other significant chronic inflammatory diseases in organs other than kidney, most commonly chronic lymphoblastic gastritis. The prevalence of severe gastritis in captive cheetahs increased from 16% of individuals in pre-1990 necropsies to 43% of individuals in 1995, an increase almost parallel to the increase in AA amyloidosis prevalence observed in the same study (Papendick et al. 1997). Seventy-six percent of cheetahs with amyloidosis have moderate to severe gastritis (Papendick et al. 1997), while close to 100% of captive cheetahs have some form of gastritis (Munson 1993, Munson et al. 1999, Munson et al. 1995). Therefore, the high prevalence of AA amyloidosis in captive cheetahs is more likely a result of chronic inflammation inducing the overproduction of SAA in general, particularly in animals that are chronically stressed.

In human populations, there is evidence that a SNP could explain population differences in secondary AA amyloidosis prevalence in rheumatoid arthritis patients: a thymine (T) nucleotide at the -13 position in the promoter region of the SAA1 gene is primarily associated with amyloidosis risk in these human populations (Moriguchi et al. 2001). Interestingly, cheetahs have a T at the -13 position in the SAA1 promoter (Zhang et al. 2008) and this nucleotide position is not polymorphic (Chen et al. 2012), therefore all cheetahs may be at an increased risk of developing secondary AA amyloidosis.

It has been shown that persistently elevated SAA concentration leads to increased amyloid load and organ deterioration in humans (Gillmore et al. 2001): mortality, amyloid burden, and renal prognosis are all significantly correlated with serum SAA concentration (De Beer et al. 1982, Lachmann et al. 2007). Similar to cheetahs, the main manifestation of secondary amyloidosis in human patients is renal dysfunction and failure (Lachmann et al. 2007).

This study has also showed that the $SAA1A^{-97delG}$ allele is significantly more common among the captive North American population compared to the wild Namibian cheetah population. It is possible that captivity-induced chronic stress and/or inflammation has imposed natural selection pressure within the captive population to favor the $SAA1A^{-97delG}$ allele (associated with less SAA production). However, the lack of a relationship between the SNP and AA amyloidosis diagnosis found in this study and the Chen et al. (2012) study suggests that the population differences observed in allele frequencies are most likely due to an unrepresentative

founder population or genetic drift within the captive North American, as opposed to selection.

Conclusion

This study was the first to demonstrate that the concentration of SAA protein in serum in captive cheetahs is associated with the $SAA1A^{-97delG}$ SNP in the promoter region of the SAA1A gene. However, based on the significant difference observed between captive populations and the lack of association between the SNP and AA amyloidosis diagnosis in captive North American cheetahs, the high prevalence of AA amyloidosis observed among captive cheetahs is not primarily attributable to genetic differences at this locus, but rather appears to be related to stress and/or chronic inflammation within captivity.

Acknowledgements

Funding for this project was provided by the Animal Sciences Graduate Student Association (ASGSA) at the University of Maryland College Park, the Cosmos Club Foundation (Washington D.C.), and Emmanuel J. Friedman Philanthropies.

References

1. Barnes PJ, Karin M. 1997. Nuclear factor-κB - a pivotal transcription factor in chronic inflammatory diseases. New England Journal of Medicine 336:1066-1071.

2. Baumann H, Gauldie J. 1994. The acute phase response. Immunology Today 15:74-80.

3. Beg AA, Finco TS, Nantermet PV, Baldwin AS. 1993. Tumor necrosis factor and interleukin-1 lead to phosphorylation and loss of IκBα: a mechanism for NF-κB activation. Molecular and Cellular Biology 13:3301-3310.

4. Bergstrom J, Ueda M, Une Y, Sun X, Misumi S, Shoji S, Ando Y. 2006. Analysis of amyloid fibrils in the cheetah (*Acinonyx jubatus*). Amyloid 13(2): 93-98.

5. Boyce JT, DiBartola SP, Chew DJ, Gasper PW. 1984. Familial renal amyloidosis in Abyssinian cats. Veterinary Pathology 21: 33-38.

6. Cerón JJ, Eckersall PD, Martinez-Subiela S. 2005. Acute phase proteins in dogs and cats: current knowledge and future perspectives. Veterinary Clinical Pathology 34:84-99.

7. Chen L, Une Y, Higuchi K, Mori M. 2012. Cheetahs have 4 serum amyloid A genes evolved through repeated duplication events. Journal of Heredity 103(1):115-129.

8. Chrousos, GP. 1995. The hypothalamic-pituitary-adrenal axis and immune-mediated inflammation. The New England Journal of Medicine 332:1351-1362.

9. De Beer FC, Fagan EA, Hughes GRV, Mallya RK, Lanham JG, Pepys MB. 1982. Serum amyloid-A protein concentration in inflammatory disease and its relationship to the incidence of reactive systemic amyloidosis. The Lancet 320:231–234.

10. Dickens MJ, Earle KA, Romero LM. 2009. Initial transference of wild birds to captivity alters stress physiology. General and Comparative Endocrinology 160:76–83.

11. Gillmore JD, Lovat LB, Persey MR, Pepys MB, Hawkins PN. 2001. Amyloid load and clinical outcome in AA amyloidosis in relation to circulating concentration of serum amyloid A protein. The Lancet 358:244-29.

12. Giordano A, Spagnolo V, Colombo A, Paltrinieri S. 2004. Changes in some acute phase protein and immunoglobulin concentrations in cats affected by feline infectious peritonitis or exposed to feline coronavirus infection. The Veterinary Journal 167:38–44.

13. Gruber AD, Linke RP. 1996. Generalized AA-amyloidosis in a bat (*Pipistrellus pipistrellus*). Veterinary Pathology 33:428-430.

14. Hadlow WJ, Jellison WL. 1962. Amyloidosis in rocky mountain bighorn sheep. Journal of the American Veterinary Medical Association 141:243–247.

15. Jensen LE, Whitehead AS. 1998. Regulation of serum amyloid A protein expression during the acute-phase response. Biochemical Journal 334:489-503.

16. Johnson KH, Sletten K, Munson L, O'Brien TD, Papendick R, Westermark P. 1997. Amino acid sequence analysis of amyloid protein A (AA) from cats (captive cheetahs: *Acinonyx jubatus*) with a high prevalence of AA amyloidosis. Amyloid: International Journal of Experimental and Clinical Investigation 4:171-177.

17. Kajikawa T, Furuta A, Onishi T, Tajima T, Sugii S. 1999. Changes in concentrations of serum amyloid A protein, α-1-acid glycoprotein, haptoglobin, and C-reactive protein in feline sera due to induced inflammation and surgery. Veterinary Immunology and Immunopathology 68:91-98.

18. Kann RKC, Seddon JM, Henning J, Meers J. 2012. Acute phase proteins in healthy and sick cats. Research in Veterinary Science 93:649-654.

19. Lachmann HJ, Goodman HJB, Gilbertson JA, Gallimore JR, Sabin CA, Gillmore JD, Hawkins PN. 2007. Natural history and outcome in systemic AA amyloidosis. New England Journal of Medicine 356:2361-2371.

20. Lange K, Papp JC, Sinsheimer JS, Sripracha R, Zhou H, Sobel EM. 2013. Mendel: The Swiss army knife of genetic analysis programs. Bioinformatics 29:1568-1570.

21. Marhaug G, Dowton SB. 1994. Serum amyloid A: an acute phase apolipoprotein and precursor of AA amyloid. Baillière's Clinical Rheumatology 8:553-573.

22. Martin LB, Brace AJ, Urban A, Coon C, Liebl AL. 2012. Does immune suppression during stress occur to promote physical performance? Journal of Experimental Biology 215:4097-4103.

23. Migita K, Yamasaki S, Shibatomi K, Ida H, Kita M, Kawakami A, Eguchi K. 2001. Impaired degradation of serum amyloid A (SAA) protein by cytokine-stimulated monocytes. Clinical and Experimental Immunology 123:408-411.

24. Moriguchi M, Terai C, Kaneko H, Koseki Y, Kajiyama H, Uesato M, Inada S, Kamatani N. 2001. A novel single-nucleotide polymorphism at the 5'-flanking region of SAA1 associated with risk of type AA amyloidosis secondary to rheumatoid arthritis. Arthritis and Rheumatism 44:1266-1272.

25. Munson L. 1993. Diseases of captive cheetahs (*Acinonyx jubatus*): results of the Cheetah Research Council pathology survey, 1989-1992. Zoo Biology 12:105-124.

26. Munson L, Nesbit JW, Meltzer DGA, Colly LP, Bolton L, Kreik LPJ. 1999. Diseases of captive cheetahs (*Acinonyx jubatus jubatus*) in South Africa: a 20-year retrospective survey. Journal of Zoo and Wildlife Medicine 30(3):342-347.

27. Munson L, Terio K, Worley M, Jago M, Bagot-Smith A, Marker L. 2005. Extrinsic factors significantly affect patterns of disease in free-ranging and captive cheetah (*Acinonyx jubatus*) populations. Journal of Wildlife Diseases 41(3):542-548.

28. Niemi K, Teirila L, Lappalainen J, Rajamaki K, Baumann MH, Oorni K, Wolff H, Kovanen PT, Matikainen S, Eklund KK. 2011. Serum amyloid A activates the NLRP3 inflammmasome via $P2X_7$ Receptor and a cathepsin B-sensitive pathway. Journal of Immunology 186:6119-6128.

29. Ofri R, Nyska A, Linke RP, Shtrasburg S, Livneh A, Gal R. 1997. Systemic amyloidosis in a cheetah (*Acinonyx jubatus*). Amyloid: International Journal of Experimental and Clinical Investigation 4(2): 98-103.

30. Padalino B, Aube L, Fatnassi M, Monaco D, Khorchani T, Hammadi M, Lacalandra GM. 2014. Could dromedary camels develop stereotypy? The first description of stereotypical behaviour in housed male dromedary camels and how it is affected by different management systems. PLOS One 9(2):e89093.

31. Paltrinieri S. 2008. The feline acute phase reaction. The Veterinary Journal 177:26-35.

32. Papendick RE, Munson L, O'Brien TD, Johnson KH. 1997. Systemic AA amyloidosis in captive cheetahs (*Acinonyx jubatus*). Veterinary Patholology 34:549-556.

33. Ray A, Ray BK. 1994. Serum amyloid A gene expression under acute-phase conditions involves participation of inducible C/EBP-β and C/EBP-δ and their activation by phosphorylation. Molecular and Cellular Biology 14:4324-4332.

34. Rideout BA, Montali RJ, Wallace RS, Bush M, Phillips Jr. LG, Antonovych TT, Sabnis SG. 1989. Renal medullary amyloidosis in Dorcas gazelles. Veterinary Pathology 26:129-135.

35. Rivas AL, Tintle L, Meyerswallen V, Scarlett JM, Vantassell CP, Quimby FW. 1993. Inheritance of renal amyloidosis in Chinese Shar-pei dogs. Journal of Heredity 84:438-442.

36. Röcken C, Menard R, Bühling F, Vöckler S, Raynes J, Stix B, Krüger S, Roessner A, Kähne T. 2005. Proteolysis of serum amyloid A and AA amyloid proteins by cysteine proteases: cathepsin B generates AA amyloid proteins and cathepsin L may prevent their formation. Annals of the Rheumatic Diseases 64:808–815.

37. Sajjad S, Farooq U, Anwar M, Khurshid A, Bukhari SA. 2011. Effect of captive environment on plasma cortisol level and behavioral pattern of Bengal tigers (*Panthera tigris tigris*). Pakistan Veterinary Journal 31(3):195-198.

38. Sasaki K, Ma Z, Khatlani TS, Okuda M, Inokuma H, Onishi T. 2003. Evaluation of feline serum amyloid A (SAA) as an inflammatory marker. Journal of Veterinary Medical Science 65:545–548.

39. Scheinman RI, Cogswell PC, Lofquist AK, Baldwin AS. 1995. Role of transcriptional activation of IκBα in mediation of immunosuppression by glucocorticoids. Science 270:283-286.

40. Tamamoto T, Ohno K, Goto-Koshino Y, Fujino Y, Tsujimoto H. 2012. Serum amyloid A uptake by feline peripheral macrophages. Veterinary Immunology and Immunopathology 150:47-52.

41. Tamamoto T, Ohno K, Ohmi A, Goto-Koshino Y, Tsujimoto H. 2008. Verification of Measurement of the feline serum amyloid A (SAA) concentration by human SAA turbidimetric immunoassay and its clinical application. Journal of Veterinary Medical Science 70:1247-1252.

42. Terio KA, Marker L, Munson L. 2004. Evidence for chronic stress in captive but not free-ranging cheetahs (*Acinonyx jubatus*) based on adrenal morphology and function. Journal of Wildlife Diseases 40: 259-266.

43. Terio KA, O'Brien T, Lamberski N, Famula TR, Munsin L. 2008. Amyloidosis in black-footed cats (*Felis nigripes*). Veterinary Pathology 45:393-400.

44. Thorn CF, Whitehead AS. 2002. Differential glucocorticoid enhancement of the cytokine-driven transcriptional activation of the human acute phase serum amyloid A genes, SAA1 and SAA2. Journal of Immunology 169:399-406.

45. van der Linde-Sipman JS, Niewold TA, Tooten PCJ, de Neijs-Backer M, Gruys E. 1997. Generalized AA-amyloidosis in Siamese and Oriental cats. Veterinary Immunology and Immunopathology 56:1-10.

46. Weingrill T, Willems EP, Zimmermann N, Steinmetz H, Heistermann M. 2001. Species-specific patterns in fecal glucocorticoid and androgen levels in zoo-living orangutans (*Pongo* spp.). General and Comparative Endocrinology 172:446–457.

47. Wiegers GJ, Reul JMHM. 1998. Induction of cytokine receptors by glucocorticoids: functional and pathological significance. Trends in Pharmacological Sciences 19:317-321.

48. Yamada T, Liepnieks JJ, Kluve-Beckerman B, Benson MD. 1995. Cathepsin B generates the most common form of amyloid A (76 residues) as a degradation product of serum amyloid A. Scandinavian Journal of Immunology 41:94-97.

49. Zhang B, Une Y, Ge F, Fu X, Qian J, Zhang J, Sawashita J, Higuchi K, Mori M. 2008b. Characterization of the cheetah serum amyloid A1 gene: critical role and functional polymorphism of a cis-acting element. Journal of Heredity 99(4):355-363.

Chapter 3: Induction of cytokine production in cheetah (*Acinonyx jubatus*) peripheral blood mononuclear cells by LPS and validation of feline-specific cytokine assays for analysis of cheetah serum

Abstract

Peripheral blood mononuclear cells were isolated from the whole blood of cheetahs and stimulated with lipopolysaccharide (LPS) to establish cross-reactivity between cheetah proinflammatory cytokines TNF-α, IL-1β, and IL-6 and feline-specific cytokine antibodies provided in commercially available ELISA kits (R&D Systems). To enable the use of these kits (designed for cell culture supernatants) for analyzing cytokine concentrations in cheetah serum, percent recovery and parallelism of feline cytokine standards in serum were also evaluated. This study validates the use of commercially available feline DuoSet® ELISA kits (R&D Systems) for measuring the concentration of proinflammatory cytokines TNF-α, IL-1β, and IL-6 in serum samples collected from cheetahs based on positive cross-reactivity of cheetah proteins with feline-specific antibodies, percent recovery and parallelism.

Introduction

Proinflammatory cytokines are produced and released from lymphocytes in response to external stimuli that signal the presence of foreign pathogens or tissue damage. Lipopolysaccharide (LPS), an endotoxin found on the surface of gram-negative bacteria, binds to the Toll-like receptor 4 (TLR4) on lymphocytes eliciting a strong proinflammatory immune response, thus it is used very often and effectively in

inducing the production of proinflammatory cytokines from lymphocytes in cell culture. A dose of 5µg/ml of LPS has been shown to be sufficient to increase TNF-α production in feline peritoneal exudate cells in culture (Otto and Rawlings 1995). When injected into domestic cats and dogs, LPS stimulates an increase in rectal temperature (DeClue et al. 2009, LeMay et al. 1990), as well as increases in plasma concentrations of TNF-α and IL-6 (DeClue et al. 2009, LeMay et al. 1990, Otto and Rawlings 1995). In both domestic cats and dogs, IL-6 activity persists longer than TNF activity after LPS injection (DeClue et al. 2009, LeMay et al. 1990). In domestic cats, there is a delayed response of plasma IL-1β concentrations to LPS stimulation (compared to TNF-α and IL-6): a small increase in IL-1β is observed 6hr post-LPS stimulation (DeClue et al. 2009). IL-1β responses in dogs are unknown.

The goal of this study was to produce cheetah proinflammatory cytokines *in vitro* through the isolation and stimulation of peripheral blood mononuclear cells (PBMCs) isolated from whole blood using LPS to establish cross-reactivity between cheetah cytokines and feline-specific cytokine antibodies provided in commercially available ELISA kits (R&D Systems). To enable the use of these kits (designed for cell culture supernatants) for analyzing cytokine concentrations in cheetah serum, percent recovery and parallelism of feline cytokine standards in serum was also evaluated.

Methods

Sample Collection

Fresh whole blood samples (n=3 cheetahs) were collected in Vacutainer® tubes containing EDTA by staff veterinarians at the Smithsonian Conservation

Biology Institute (SCBI) in Front Royal, VA. Whole blood samples were transported on ice to the University of Maryland for PBMC isolation and cell culture. Samples were diluted 1:2 with PBS and slowly layered on lymphocyte separation medium (10ml) and centrifuged for 30 min at 400 × g. Cells at the interface of the media were collected, washed in PBS, and resuspended in 11ml cell culture media (RPMI-1640 media, pH 7.4 supplemented with 10% fetal bovine serum, 2mM glutamine, 100U/ml penicillin, 100µg/ml streptomycin). Final concentration of cells was approximately 10 million cells/ml media for each sample.

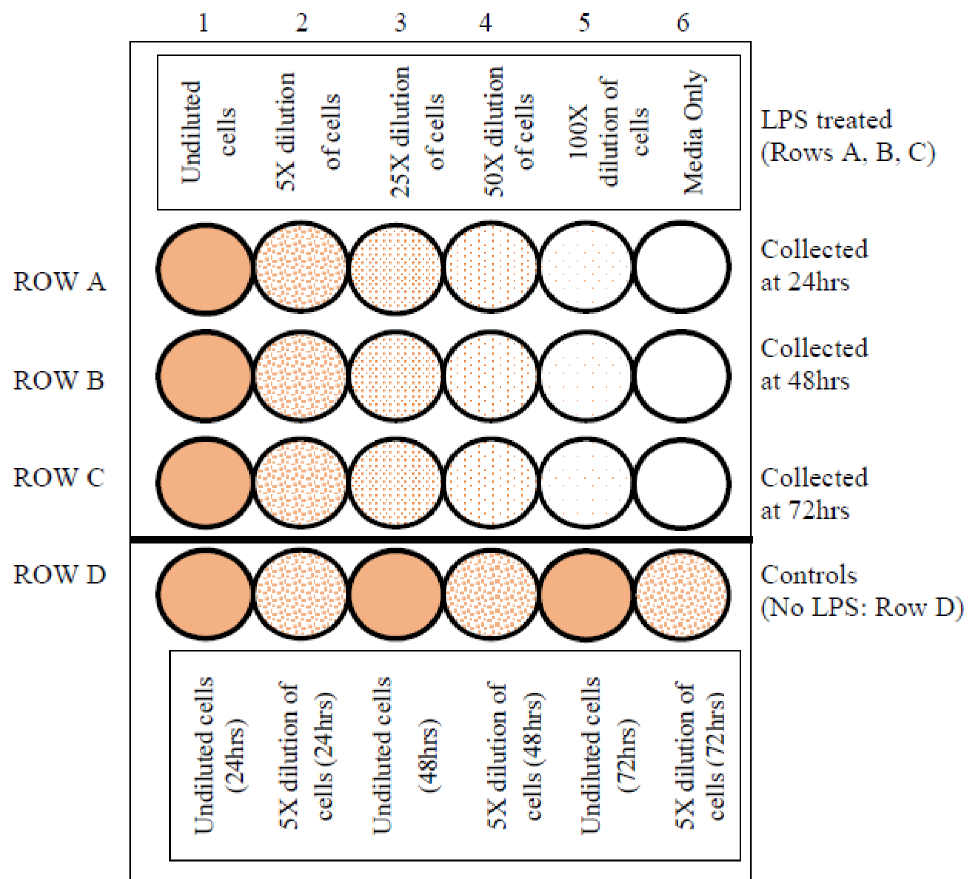
Stimulation of cheetah PBMCs with LPS

Isolated lymphocytes were added to a 24-well plate in 1.5ml of media, either undiluted or as a 5x, 25x, 50x or 100x dilution in cell culture media (Figure 3.1; 3 replicates, 1 plate per cheetah). Plates were incubated for 2 hours at 5% CO_2 at 37ºC to allow monocytes to adhere to the plate. Wells were gently washed with media via careful aspiration to remove any unbound lymphocytes. Media was replaced (1.5ml) either with or without 5µg/ml LPS (Figure 3.1). Cell culture supernatants were removed at 24, 48 or 72 hours post-LPS stimulation (Figure 3.1) and stored at -20ºC until assayed.

Quantification of cytokine production in cell culture supernatants

Feline DuoSet® ELISA kits (R&D Systems) for TNF-α, IL-1β, and IL-6 were used following the manufacturer's instructions to quantify the concentration of cytokines produced in the cheetah PBMC cell culture supernatants after LPS stimulation.

Figure 3.1. 24-well plate set-up for LPS stimulation of cheetah monocytes in cell culture.



Parallelism and cytokine recovery in serum

Feline DuoSet® ELISA kits (R&D Systems) for TNF-α, IL-1β, and IL-6 were used following the manufacturer's instructions (substituting 10% FBS in PBS for the blocking buffer) to quantify the concentration of cytokines in different dilutions of cheetah serum (undiluted serum, 2x, 3x, 4x, 5x, 10x, 20x, 50x) spiked with a constant quantity of standard towards the lower end of the range of detection (25pg/ml for TNF-α, 50pg/ml for IL-1β and IL-6) to investigate if the dilution of serum affected the percent recovery of the standard.

Feline DuoSet® ELISA kits (R&D Systems) for TNF-α, IL-1β, and IL-6 were used following the manufacturer's instructions (substituting 10% FBS in PBS for the blocking buffer) to quantify the concentration of cytokines in a constant dilution of cheetah serum (1:2) spiked with multiple concentrations of standard (0, 10, 25, 50, 100, 356.25, 712.5, and 1425 pg/ml TNF-α and 0, 20, 50, 100, 200, 787.5, 1575, 3150 pg/ml IL-1β and IL-6) to check for parallelism between the spiked samples and the standard curve.

Statistical analysis

To investigate differences in cytokine production by PMBCs in cell culture, a repeated-measures ANOVA in PROC MIXED of SAS Enterprise Guide (version 6.1) was conducted with dilution factor, LPS stimulation (Yes or No), time and all associated interaction terms as fixed effects and cheetah as a random effect (block), with time as the repeated variable using an unstructured covariance matrix. A one-sided contrast was used to detect increases in cytokine production in response to LPS

stimulation (at undiluted and 5x concentrations of cells only) due to the incomplete factorial treatment structure. The effects of dilution factor and time were examined for LPS stimulated cell culture supernatants only. Treatment least square means were compared using a Bonferroni correction to control for an experimentwise error rate at $\alpha=0.05$.

Results

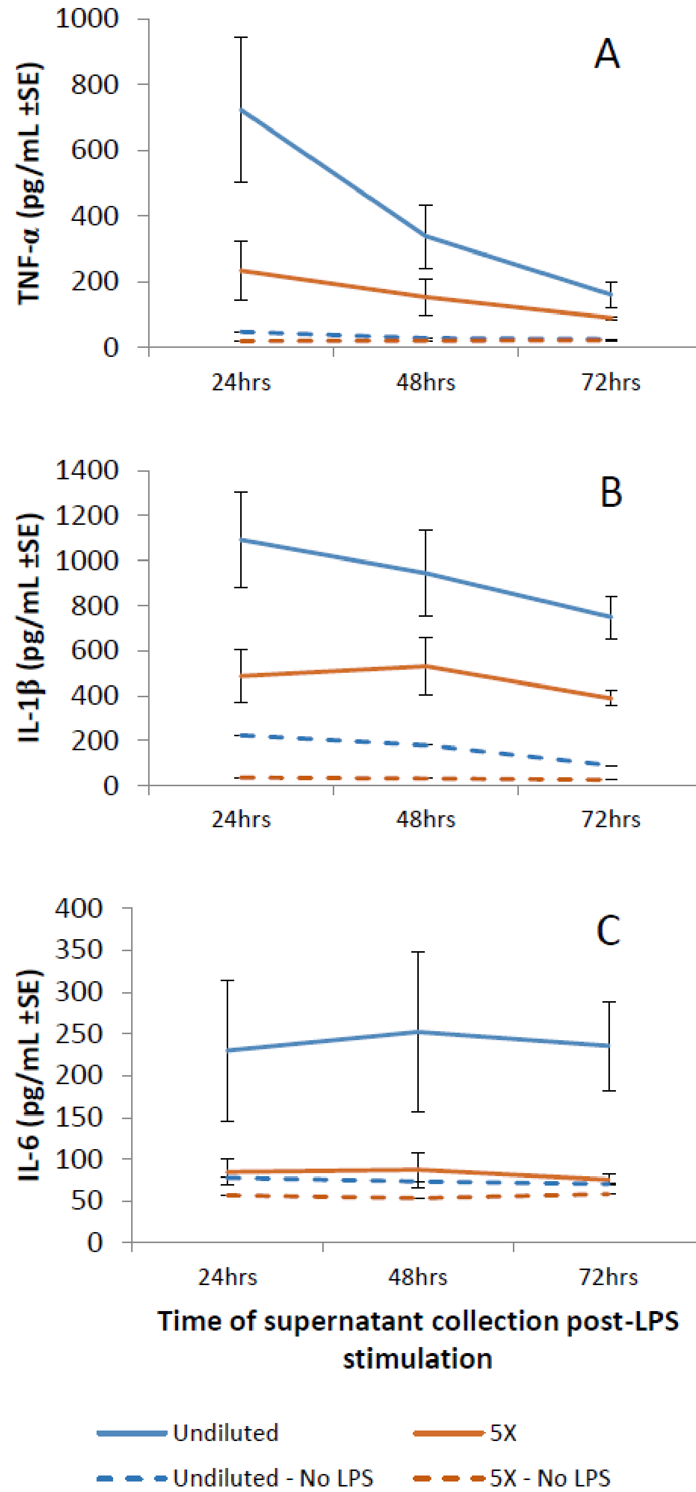
LPS stimulation of cheetah PBMCs produces a measurable, cell concentration dependent increase in production of TNF-α, IL-1β, and IL-6

There was a significant positive induction of proinflammatory cytokine production by cheetah PBMCs in culture following stimulation with LPS for all three cytokines measured compared to unstimulated cells (Figure 3.2): TNF-α ($P=0.0007$), IL1-β ($P<0.0001$), and IL-6 ($P=0.0196$). The amount of cytokines produced was also dependent on the concentration of PBMCs present (Table 3.1). A statistically significant effect of time was only observed in supernatants from undiluted cells. The concentrations of TNF-α and IL-1β both decreased over time, though the concentration of TNF-α appeared to decrease more rapidly (Figure 3.2, Table 3.1). There was no significant difference observed in IL-6 concentration across time points (Figure 3.2, Table 3.1).

Percent recovery of cytokine standard from cheetah serum is dependent on the dilution factor

The dilution of serum used when spiking samples with a constant, known amount of cytokine standard affected percent recovery for all three cytokines

Figure 3.2. Cytokine concentrations in cell culture supernatants from cheetah PBMCs (undiluted and 5X) at 24, 48 and 72 hours post-LPS stimulation. (A) TNF-α; (B) IL-1β; (C) IL-6.



		Cytokine Concentration LSmeans ± SE (pg/ml)		
	Dilution Factor	**24hrs**	**48hrs**	**72hrs**
TNF-α	*Undiluted*	^{A}723.09 ± 220.39^{a}	^{B}338.69 ± 96.44^{a}	^{C}161.24 ± 39.68^{a}
	5x	235.06 ± 90.39^{ab}	154.24 ± 55.20^{ab}	91.06 ± 3.97^{ab}
	25x	71.47 ± 14.15^{b}	49.59 ± 8.91^{b}	40.16 ± 2.64^{b}
	50x	38.13 ± 4.89^{b}	33.52 ± 2.76^{b}	30.16 ± 1.97^{b}
	100x	27.09 ± 2.21^{b}	27.12 ± 2.84^{b}	27.36 ± 0.51^{b}
	Media	***	***	18.47 ± 1.60^{b}
IL-1β	*Undiluted*	^{A}1091.86 ± 209.14^{a}	^{AB}943.11 ± 190.54^{a}	^{B}747.42 ± 95.56^{a}
	5x	486.70 ± 116.09^{b}	530.05 ± 125.80^{ab}	387.18 ± 33.70^{b}
	25x	244.48 ± 38.81^{b}	225.31 ± 41.41^{b}	173.74 ± 28.02^{bc}
	50x	117.74 ± 8.84^{b}	132.93 ± 5.99^{b}	102.53 ± 23.64^{c}
	100x	77.49 ± 11.04^{b}	84.56 ± 13.04^{b}	79.11 ± 17.32^{c}
	Media	***	***	***
IL-6	*Undiluted*	^{A}229.59 ± 84.29^{a}	^{A}251.93 ± 96.33^{a}	^{A}235.36 ± 53.64^{a}
	5x	84.41 ± 15.64^{a}	87.22 ± 20.74^{a}	75.32 ± 6.19^{b}
	25x	***	***	***
	50x	***	***	***
	100x	***	***	***
	Media	***	***	***

Table 3.1. Cytokine concentrations in cell culture supernatants from cheetah PBMCs 24, 48 and 72 hours post-LPS stimulation. ^{A,B,C}Designate significant differences within the undiluted cell culture supernatants across time. ^{a,b,c}Designate significant differences across dilution factors at the given time point. Mean comparisons were declared significant at where P<0.05 after Bonferroni correction. ***indicates concentration below the detection limit of the assay.

measured (TNF-α, IL-1β, and IL-6; Figure 3.3). In all cases, percent recovery increased as the serum sample dilution increased (Figure 3.3). There was a large amount of variation between the three cytokine assays in percent recovery at a given dilution factor. A 1:2 dilution of serum resulted in approximately 45%, 82%, and 7% recovery of TNF-α, IL-1β, and IL-6 standards, respectively (Figure 3.3).

Parallelism observed between standards and spiked serum samples

Adequate parallelism between spiked serum (1:2 dilution) samples and the standard curve was observed across a large range of cytokine concentrations for all three cytokines measured (TNF-α: 31.25-1000 pg/ml, $r=0.99690$, $P<0.0001$; IL-1β: 31.25-2000 pg/ml, $r=0.99634$, $P<0.0001$; and IL-6: 62.5-2000pg/ml, $r=0.99635$, $P<0.0001$). Larger departures from parallelism as the concentration of the spike increased was observed (Figure 3.4).

Discussion

The ability to measure inflammatory markers, such as serum concentrations of cheetah proinflammatory cytokines, has become progressively more desirable due to the high prevalence of several diseases observed within captive cheetah populations in North America and South Africa (Munson et al. 2005). A disease that has become of increasing concern is systemic amyloid A (AA) amyloidosis (Munson et al. 2005, Ofri et al. 1997, Papendick et al. 1997), which has been shown to be associated with other chronic inflammatory diseases, most commonly chronic lymphoblastic gastritis (Papendick et al. 1997). Close to 100% of cheetahs in captivity have some form of gastritis (Munson 1993, Munson et al. 1999, Munson et al. 2005), which may lead to

Figure 3.3. Effect of increasing serum dilution on percent recovery of cytokine standards from spiked cheetah serum samples.

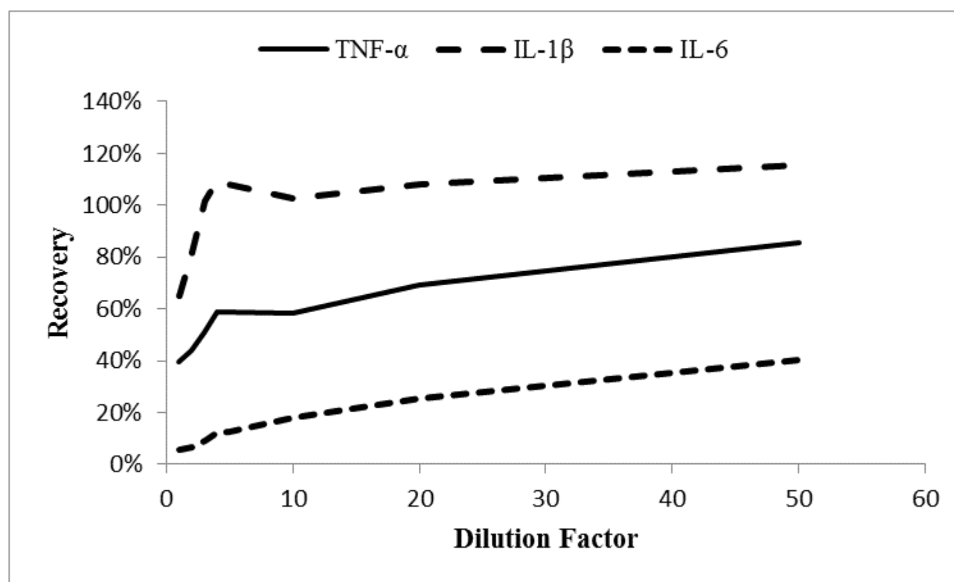
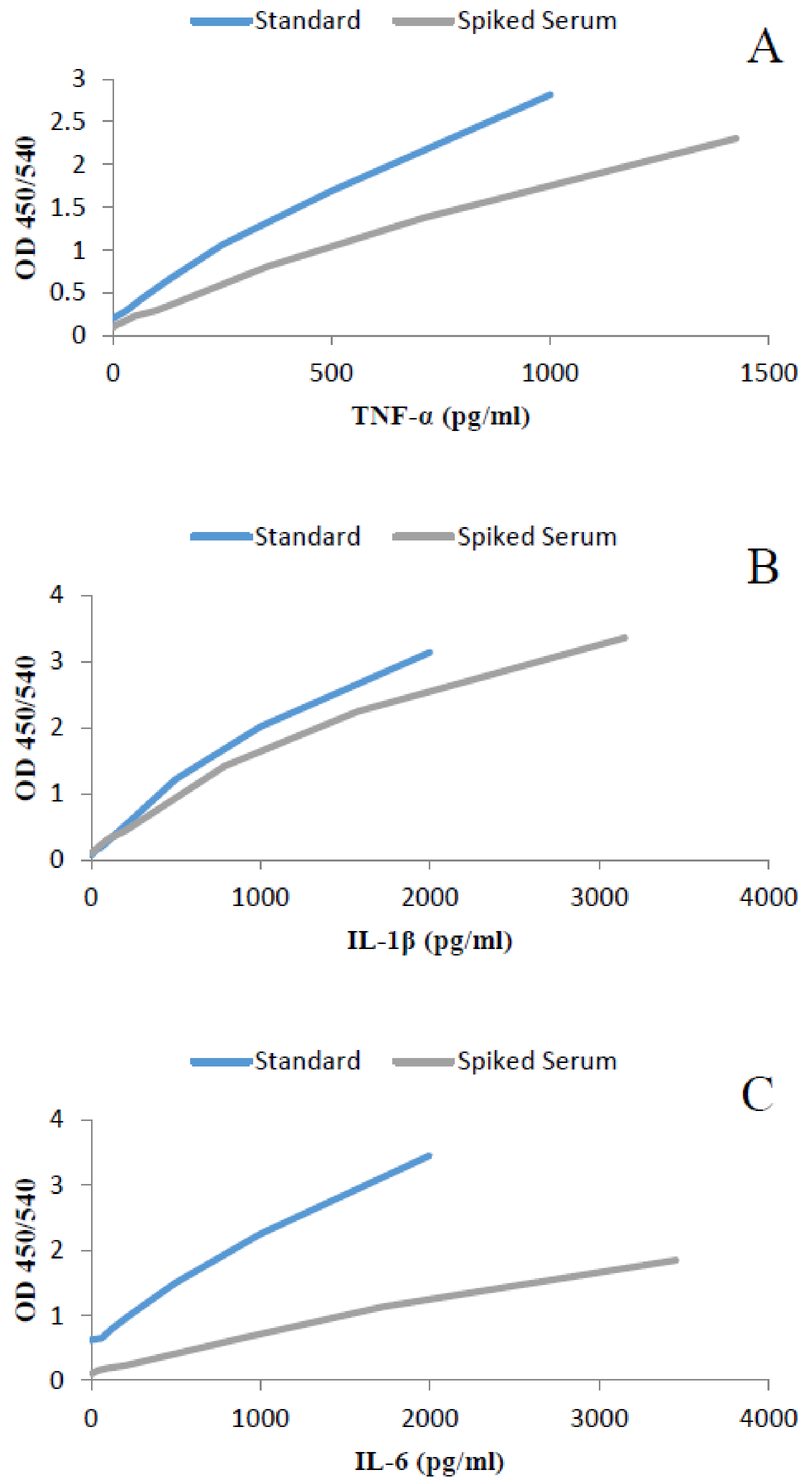


Figure 3.4. Parallelism between cytokine standards and cheetah serum (1:2 dilution) spiked with known concentrations of standard. (A) TNF-α; (B) IL-1β; (C) IL-6.



chronic or persistent episodes of inflammation. Chronic systemic inflammation leads to the persistent upregulation of production of the amyloid A precursor protein, serum amyloid A (SAA), as well as a decrease in SAA degradation and an accumulation of SAA protein in the serum, providing evidence that it plays a role in AA amyloidosis pathogenesis.

The systemic inflammatory mediators generally responsible for the upregulation of SAA production are the IL-1 type cytokines, such as IL-1β and TNF-α (Baumann and Gauldie 1994, Jensen and Whitehead 1998, Marhaug and Dowton 1994, Paltrinieri 2008). The actions of the IL-1 type cytokines on the production of acute phase proteins, such as SAA, are also often synergistically enhanced in the presence of IL-6 (Baumann and Gauldie 1994, Cerón et al. 2005, Jensen and Whitehead 1998). Therefore, there is a need to be able to measure TNF-α, IL-1β, and IL-6 in cheetah serum samples to investigate how proinflammatory cytokines may be related to the diseases observed in captive cheetahs, such as AA amyloidosis.

Previous work has shown the amino acid sequence of the cheetah IFN-γ cytokine to be 99% homologous to the domestic cat sequence (Maas et al. 2010), thus it would be expected that there is conservation across most protein binding sites. Cheetah proinflammatory cytokines TNF-α, IL-1β, and IL-6 have not been sequenced, but it was expected that there may be similarly high levels of homology between the cheetah and domestic cat proteins such that ELISA kits designed to bind to domestic cat proteins would cross-react with cheetah proteins. This study has shown that the feline-specific antibodies provided in the commercially available ELISA kits for measuring TNF-α, IL-1β, and IL-6 in cell culture supernatants (R&D

Systems) are cross-reactive with cheetah proinflammatory cytokines as they were able to detect increases in cytokine concentrations in PMBC cell culture supernatants post-LPS stimulation (vs. control) as well as show cell concentration dependent responses.

There was substantial variability in the ability of the ELISA kits to detect TNF-α, IL-1β, and IL-6 in serum samples. The IL-1β ELISA worked quite well with cheetah serum, even at lower dilution factors. The TNF-α ELISA worked fairly well with cheetah serum, though the percent recovery was less than ideal. The percent recovery was very poor for the IL-6 ELISA. Therefore, even though parallelism was acceptable for all ELISA kits, it is likely that TNF-α and most certainly IL-6 concentrations will be underestimated when measuring cheetah serum samples. Percent recovery was improved by increasing the dilution factor. However, assuming that physiological concentrations of these cytokines in serum is going to be fairly low in healthy individuals, it is not possible to use a very high dilution factor. A previous study in domestic cats used a 1:2 dilution of plasma demonstrating a peak in IL-6 at 1200pg/ml, and IL-1β concentrations were measured around 86pg/ml following a low-dose endotoxin infusion (DeClue et al. 2009). The limit of detection for the IL-6 assay on cheetah serum was 62.5pg/ml. Therefore, if peaks in the concentrations of IL-6 in cheetah serum are in the same range as domestic cats, increasing the dilution factor up to a 1:10 may be possible to help improve percent recovery and parallelism, whilst a 1:2 dilution factor is likely sufficient for TNF-α and IL-1β assays.

Conclusion

This study has validated the use of commercially available ELISA kits (R&D Systems) for measuring the concentration of proinflammatory cytokines TNF-α, IL-1β, and possibly IL-6 in serum samples collected from cheetahs based on positive cross-reactivity of cheetah proteins with feline-specific antibodies, percent recovery and parallelism.

Acknowledgements

Funding for this project was provided by Emmanuel J. Friedman Philanthropies.

References

1. Baumann H, Gauldie J. 1994. The acute phase response. Immunology Today 15:74-80.

2. Cerón JJ, Eckersall PD, Martinez-Subiela S. 2005. Acute phase proteins in dogs and cats: current knowledge and future perspectives. Veterinary Clinical Pathology 34:84-99.

3. DeClue AE, Williams KJ, Sharp C, Haak C, Lechner E, Reinero CR. 2009. Systemic response to low-dose endotoxin infusion in cats. Veterinary Immunology and Immunopathology 132:167-174.

4. Jensen LE, Whitehead AS. 1998. Regulation of serum amyloid A protein expression during the acute-phase response. Biochemical Journal 334:489-503.

5. LeMay DR, LeMay LG, Kluger MJ, D'Alecy LG. 1990. Plasma profiles of IL-6 and TNF with fever-inducing doses of lipopolysaccharide in dogs. American Journal of Physiology 259:R126-132.

6. Maas M, Rhijn IV, Allsopp M, Rutten V. 2010. Lion (*Panthera leo*) and cheetah (*Acinonyx jubatus*) IFN-γ sequences. Veterinary Immunology and Immunopathology 134:296–298.

7. Marhaug G, Dowton SB. 1994. Serum amyloid A: an acute phase apolipoprotein and precursor of AA amyloid. Baillière's Clinical Rheumatology 8:553-573.

8. Munson L. 1993. Diseases of Captive Cheetahs (*Acinonyx jubatus*): Results of the Cheetah Research Council Pathology Survey, 1989-1992. Zoo Biology 12:105-124.

9. Munson L, Nesbit JW, Meltzer DGA, Colly LP, Bolton L, Kreik LPJ. 1999. Diseases of captive cheetahs (*Acinonyx jubatus jubatus*) in South Africa: a 20-year retrospective survey. Journal of Zoo and Wildlife Medicine 30(3):342-347.

10. Munson L, Terio K, Worley M, Jago M, Bagot-Smith A, Marker L. 2005. Extrinsic factors significantly affect patterns of disease in free-ranging and captive cheetah (*Acinonyx jubatus*) populations. Journal of Wildlife Diseases 41(3):542-548.

11. Ofri R, Nyska A, Linke RP, Shtrasburg S, Livneh A, Gal R. 1997. Systemic amyloidosis in a cheetah (*Acinonyx jubatus*). Amyloid: International Journal of Experimental and Clinical Investigation 4(2):98-103.

12. Otto CM, Rawlings CA. 1995. Tumor necrosis factor production in cats in response to lipopolysaccharide: an in vivo and in vitro study. Veterinary Immunology and Immunopathology 49:183-188.

13. Paltrinieri S. 2008. The feline acute phase reaction. The Veterinary Journal 177:26-35.

14. Papendick RE, Munson L, O'Brien TD, Johnson KH. 1997. Systemic AA amyloidosis in captive cheetahs (*Acinonyx jubatus*). Veterinary Pathology 34:549-556.

Chapter 4: Evaluation of the effects of chronic stress and inflammation on serum amyloid A concentrations in serum associated with increasing AA amyloidosis prevalence in captive cheetah populations

Summary

Systemic amyloid A (AA) amyloidosis is a major cause of morbidity and mortality among captive cheetahs in North America, however wild cheetahs are virtually unaffected. The AA protein, a self-aggregating protein responsible for this disease, is a byproduct from the degradation of serum amyloid A (SAA) protein, an acute phase protein highly upregulated during inflammation. IL-1β, TNF-α, and IL-6 proinflammatory cytokines have all been shown to stimulate hepatic SAA production during the acute phase response (APR). There is a positive, synergistic effect of glucocorticoids with cytokines in the induction of SAA synthesis during the APR. The aim of this study was to investigate the potential effect of stress and inflammation on SAA production in order to gain a better understanding of the differences observed in AA amyloidosis prevalence between captive and wild populations.

A multivariate statistical approach (canonical discriminant analysis) was taken to investigate how population differences across multiple correlated variables measured can distinguish the populations from each other. Two significant canonical discriminant functions, Can1 and Can2, were produced that defined the differences between three populations (captive populations NZP-SCBI and CCF, and wild

cheetahs). Can1 best defined the difference between NZP-SCBI cheetahs and Namibian cheetahs (CCF and wild), and Can2 best defined the separation between Namibian cheetahs, CCF vs. wild.

Log_{10}SAA concentration was significantly positively correlated with Can1 (r=0.54, P<0.0001), which had similar significant positive correlations with baseline cortisol, average cortisol, and baseline standard deviation, and was negatively correlated with IL-1β and TNF-α. The low cytokine concentrations found among NZP-SCBI cheetahs compared to CCF and wild cheetahs is possibly the result of proinflammatory cytokine production getting suppressed in immune cells via glucocorticoids. Log_{10}SAA concentration was not associated with Can2, as log_{10}[SAA] was not significantly different between CCF and wild cheetah populations. Can2 was positively correlated with baseline cortisol, average cortisol, baseline standard deviation, total standard deviation, as well as IL-1β and TNF-α concentrations.

Closer examination of the effects of environmental factors including exercise, enclosure size and exposure to humans on serum SAA protein concentrations within the CCF population revealed that cheetahs in smaller enclosures (<10 acres) had significantly higher log_{10}[SAA] than cheetahs kept in larger enclosures (P=0.0190). Similarly, baseline cortisol and average cortisol were marginally higher in cheetahs kept in smaller enclosures (P=0.0793 and P=0.0534, respectively). In humans, elevated SAA concentrations have been found to correlate with AA amyloidosis incidence. Thus, it appears the best strategy for decreasing AA amyloidosis prevalence among captive cheetahs is through management practices that reduce

stress, which could help re-establish proper immune system homeostasis and mitigate the overproduction of SAA protein.

Introduction

Stress can be defined as a physiological or psychological state in which homeostasis is, or perceived to be, threatened (Chrousos 2009, Dhabhar 2009, Elenkov and Chrousos 1999). Stress activates the sympathetic nervous system which stimulates the release of catecholamines (epinephrine and norepinephrine; E and NE) from the adrenal medulla. Stress also results in activation of the hypothalamic-pituitary-adrenal (HPA) axis, signaling the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary into circulation. The ACTH signal is received by cells of the adrenal cortex, stimulating the production and release of glucocorticoids (GCs), such as cortisol and corticosterone. In short, acute stress is characterized by the enhanced secretion of E and NE, later followed by GCs released from the adrenal gland (Chrousos 1995, Chrousos 2009, Dhabhar 2009, Elenkov and Chrousos 1999, Sapolski et al. 2000).

Psychological stressors can trigger the release of proinflammatory cytokines before any rise in GCs is detected (Dhabhar 2009, Sapolski et al. 2000). Proinflammatory cytokines are known to stimulate the stress system (HPA axis activation), causing the release of CRH (Baumann and Gauldie 1994, Chrousos 2009, Elenkov and Chrousos 1999, Sapolski et al. 2000), and peripheral CRH has been known to have proinflammatory actions (Elenkov and Chrousos 1999). Activation of the HPA axis by proinflammatory cytokines plays an important role in the negative feedback mechanism that controls systemic proinflammatory cytokine concentrations

from becoming detrimental to the organism: both catecholamines and GCs suppress the secretion of most proinflammatory cytokines (Chrousos 1995, Chrousos 2009, Elenkov and Chrousos 1999, Sapolski et al. 2000). It has been widely accepted that captivity can impose psychological stress on many animal species. Measuring glucocorticoids in fecal samples across time can be a valuable, non-invasive tool for measuring stress in many animals, including cheetahs (Keay et al. 2006).

There is both functional (hypercortisolemia) and morphological (adrenal hyperplasia/hypertrophy) evidence for chronic stress among captive cheetahs, at levels much greater than their wild counterparts (Bolton and Munson 1999, Köster et al. 2007, Munson et al. 1999, Munson et al. 2005, Terio et al. 2004). In captivity, when cheetahs are moved or housed on-exhibit, they tend to show higher concentrations of glucocorticoids in feces than when moved or housed off-exhibit (Terio et al. 2004, Wells et al. 2004), and the increases observed after movement can persist for months (Wells et al. 2004). There is also greater daily variation in the GC concentrations of feces observed when cheetahs are moved on-exhibit (Wells et al. 2004). Evidence of stress (elevated cortisol and/or behavior) being linked to anestrous in captive cheetahs (downregulation of the reproductive system) has also been demonstrated (Jurke et al. 1997, Wielebnowski et al. 2002). Hence, it appears there is a clear association between the captive environment and stress in cheetahs. Stress-related hypersecretion of the proinflammatory cytokine IL-6, in addition to hypercortisolism, may result in the chronic upregulation of the acute phase response (Chrousos 2009) in cheetahs, contributing to the increase in disease and mortality observed in the captive population.

The acute phase response (APR) is a systemic reaction that occurs early during inflammation, initiated by cells of the innate immune system in response to invading pathogens, tissue damage and associated activation signals. Macrophages resident in affected tissues and monocytes in the blood are the immune cells primarily responsible for the initiation of the APR (Baumann and Gauldie 1994, Paltrinieri 2008). The liver is one of the primary targets for systemic inflammatory mediators (Baumann and Gauldie 1994, Jensen and Whitehead 1998, Paltrinieri 2008); it is the location of acute phase protein (APP) production (Baumann and Gauldie 1994, Cerón et al. 2005, Jensen and Whitehead 1998, Paltrinieri 2008). APPs, by definition, are proteins whose concentration in serum significantly increase or decrease during the APR, including C-reactive protein (CRP), serum amyloid A protein (SAA), alpha-1-acid glycoprotein (AGP), ceruloplasmin, haptoglobin and albumin. The systemic inflammatory mediators generally responsible for the upregulation of CRP, SAA and AGP production during the APR are the IL-1 type cytokines, such as IL-1β and TNF-α (Baumann and Gauldie 1994, Jensen and Whitehead 1998, Marhaug and Dowton 1994, Paltrinieri 2008). Additionally, the actions of the IL-1 type cytokines on the production of APPs are often synergistically enhanced in the presence of IL-6 (Baumann and Gauldie 1994, Cerón et al. 2005, Jensen and Whitehead 1998).

The major APPs that have been identified to date in the domestic cat are SAA and AGP (Cerón et al. 2005, Kajikawa et al. 1999, Paltrinieri 2008). The increase in SAA observed in domestic cats during the APR, approximately a 10-100 fold increase, is lower in magnitude compared to humans and other species, but still represents a major APP in this species (Cerón et al. 2005, Giordano et al. 2004,

Kajikawa et al. 1999). Multiple studies have shown that the SAA concentrations in sick domestic cats are significantly higher than the SAA concentrations in healthy domestic cats (Giordano et al. 2004, Kajikawa et al. 1999, Kann et al. 2012, Sasaki et al. 2003, Tamamoto et al. 2008).

Transcriptional induction of the cheetah SAA1 gene is dependent on both the CCAAT-enhancer-binding protein beta (C/EBPβ) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) cis-acting elements located in the promoter region (Marhaug and Dowton 1994). In the presence of stimulation from proinflammatory cytokines, C/EBPβ and NF-κB enter the nucleus and bind to the promoter region of the SAA1 gene inducing transcription (Barnes and Karin 1997, Beg et al. 1993). IL-1β, TNF-α, and IL-6 proinflammatory cytokines have all been shown to stimulate hepatic SAA production during the APR (Baumann and Gauldie 1994, Cerón et al. 2005, Jensen and Whitehead 1998, Marhaug and Dowton 1994, Paltrinieri 2008).

There is a positive, synergistic effect of GCs with cytokines in the induction of SAA synthesis during the APR (Chrousos 1995, Dhabhar 2009, Marhaug and Dowton 1994). This effect is the result of the upregulation of cytokine receptors on the surface of hepatic cells by GCs (Baumann and Gauldie 1994, Wiegers and Reul 1998), increasing the sensitivity of the hepatic cells to cytokine stimulation (Baumann and Gauldie 1994, Paltrinieri 2008, Thorn and Whitehead 2002, Wiegers and Reul 1998). Contrastingly, it is widely accepted that the production of many proinflammatory cytokines (including TNF-α, IL-1β and IL-6) in leukocytes is

inhibited by GCs (Baumann and Gauldie 1994, Chrousos 1995, Jensen and Whitehead 1998, Paltrinieri 2008, Wiegers and Reul 1998).

The SAA protein normally has a short half-life, approximately 90 minutes in normal serum (Marhaug and Dowton 1994). Therefore the concentration of SAA in serum returns to normal following the termination of inflammation. In multiple species, including domestic cats, monocytes contribute to SAA degradation (Migita et al. 2001, Tamamoto et al. 2012). However, this is inhibited in the presence of the cytokines IL-1β and IFN-γ (Migita et al. 2001), and glucocorticoids (Tamamoto et al. 2012). The amyloid A (AA) protein responsible for causing AA amyloidosis, is a byproduct of SAA protein degradation. AA amyloidosis is a disease induced by the over-accumulation and incomplete degradation of SAA and polymerization of the AA protein into fibrils (Marhaug and Dowton 1994), which are insoluble and get deposited into organs, ultimately leading to organ dysfunction and organ failure. Therefore, chronic systemic inflammation can result in the persistent upregulation of SAA production, a decrease in SAA degradation, and an accumulation of SAA protein in the serum, providing evidence that it plays a role in amyloid A (AA) amyloidosis pathogenesis.

Systemic AA amyloidosis is an increasingly important cause of morbidity and mortality in captive cheetahs (Munson et al. 2005, Ofri et al. 1997, Papendick et al. 1997). The number of cases of systemic amyloidosis in captive cheetahs has increased over time from 20% of individuals in pre-1990 necropsies to 70% of individuals in 1995 (Papendick et al. 1997). Amyloid deposition can be observed in many different tissues: the kidney, liver, adrenal gland, parathyroid gland, spleen,

stomach, intestine, lymph node, heart, pancreas and esophagus (Chen et al. 2012, Munson et al. 1999, Papendick et al. 1997). Most commonly, amyloid deposition is observed in the kidneys, and renal failure is the sole or partial cause of death in 74% of cheetahs with amyloidosis, compared to only 20% of cheetahs without amyloidosis (Papendick et al. 1997).

The aim of this study was to investigate the potential effects of stress (due to captivity) and inflammation on serum concentrations of SAA protein that may be linked to AA amyloidosis pathogenesis, in order to gain a better understanding of the differences observed in AA amyloidosis prevalence between captive and wild cheetah populations.

Methods

Study Populations

Cheetahs from two captive populations and one wild population are represented in this study. The captive Smithsonian population (NZP-SCBI) is comprised of cheetahs housed at either the National Zoological Park (NZP) in Washington, DC, or the Smithsonian Conservation Biology Institute (SCBI) in Front Royal, VA. Cheetahs at the NZP are on-exhibit to the public approximately 10-12 hours per day, 7 days a week. The SCBI is not open to the public, and thus these cheetahs are off-exhibit. Enclosure sizes at both facilities are <0.5 acres. All of these cheetahs were born in captivity.

The captive Cheetah Conservation Fund (CCF) population is located near Otjiwarongo, Namibia. These cheetahs were all born in the wild and housed in captivity after being found orphaned, or often are removed from farmland as

“problem” animals trapped by farmers. Some of these cheetahs will remain captive for the remainder of their life, while others may be released back into the wild, depending on individual circumstances. CCF cheetahs are kept in a variety of different types of enclosures. Cheetahs with no chance of being released are held in smaller enclosures (approx. 2 acres) at the center of CCFs main buildings, including an office, education center, gift shop and café, etc. These cheetahs are on-exhibit to the public, including being exercised on a lure system for visitors and fed in front of the public daily. Some of these cheetahs have been raised as ambassador animals and have frequent human contact with their handlers and can be walked on leashes outside of their enclosures. Other cheetahs at CCF are housed in large (>10 acres) “drive-thru safari” type enclosures. These cheetahs are “retired” from the most public enclosures, therefore previously habituated to humans. They are not exercised or fed in front of the public, but are exposed to multiple “drive-thrus” per day, where safari trucks seek out the animals for tourists to take photographs.

CCF also houses several cheetahs away from the public, many of which have a high probability of being released to the wild. Some of these animals are exercised regularly by chasing the feeding truck, but some are not due to physical constraints. One of these enclosures is quite large (>100acres), which houses the wildest females to be released.

CCF also has a “soft-release camp”. This is a former game ranch (approx. 10,000 acres) that includes many game species (oryx, kudu, hartebeest, eland, etc.) as well as a low density of predators (hyena, leopard). Cheetahs to be released to the wild are first put into the soft-release camp to see if they are able to find water

sources and hunt successfully, without too much pressure from predators. If a cheetah proves to be successful, CCF finds an appropriate location for them to be reintroduced to the wild.

The third population studied included wild cheetahs in Namibia whose home range was close to, or included, CCF central property and surrounding farmland. All of these cheetahs were male and made up three coalitions. These individuals were chosen because multiple fecal samples per individual were able to be collected and identified, and several (two coalitions) were previously captured and medically examined by CCF staff, providing an opportunity for blood sampling.

Sample Collection

Fecal samples were obtained from cheetahs at the Smithsonian Conservation Biology Institute (SCBI) in Front Royal, VA (N=17), and National Zoological Park (NZP) in Washington, DC (N=4). Samples were collected approximately every 2 or 3 days over three consecutive months resulting in 36-50 samples per cheetah. Serum samples were also obtained from 20 of the 21 cheetahs. Similarly, fecal samples were also obtained from cheetahs at the CCF in Namibia. Samples from wild-born, captive cheetahs (N=34) were collected every 1 or 2 days over three consecutive weeks, resulting in 7-15 samples per cheetah. Cheetahs housed together were fed insoluble markers such as uncooked corn, lentils, oats, rice or colored glitter in order to identify ownership of fecal samples.

Fecal samples were also obtained from wild cheetahs (N=7: 1-15 samples per cheetah) and cheetahs in "soft-release" (N=14: 1-9 samples per cheetah) as available. Genetic markers were used by CCF staff to identify ownership of fecal samples.

Serum samples were obtained for all of the captive cheetahs, 4 of 7 wild cheetahs, and 6 of 14 cheetahs in "soft-release". For a complete list of sample numbers per individual, see Appendix II.

Hormone extraction from fecal samples

Fecal cortisol and associated metabolites were obtained from samples using an ethanol/methanol extraction procedure. Samples were first lyophilized, pulverized and approximately 0.2g of powdered feces was shaken for 30 minutes in 5ml of ethanol (90%). Samples were centrifuged at 1500 x g for 20 min, then supernatant was recovered and the pellet resuspended in 5ml of ethanol (90%), shaken for 1 min and re-centrifuged. Ethanol supernatants were combined and dried down completely under air. Extracts were reconstituted in 3ml ethanol (100%), vortexed briefly, sonicated for 15 min, and dried down completely under air. Extracts were next reconstituted in 1ml methanol (100%), vortexed briefly, sonicated for 15 min, and dried down completely under air. Extracted samples were finally reconstituted in 1ml PBS ("neat" sample). Recovery of ^{3}H-cortisol added to fecal samples before extraction was calculated, and the extraction procedure was repeated for any samples in which recovery did not exceed 60%. Samples were stored at -20 ºC until analyzed.

Quantification of cortisol in serum and fecal hormone extracts

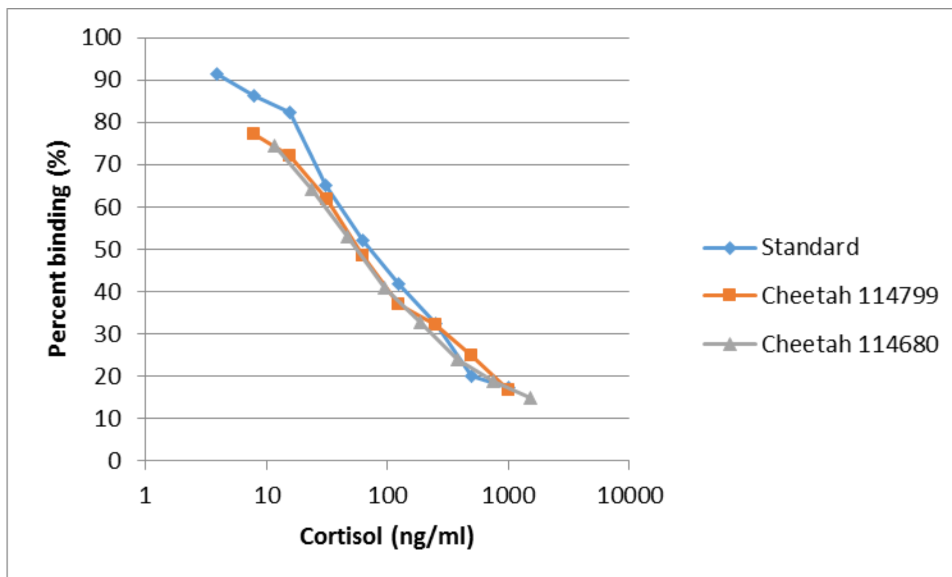
Serum samples and fecal hormone extracts were diluted 1:25 and 1:20, respectively, in PBS before analysis. Samples were analyzed in duplicate using a competitive enzyme-linked immunosorbent assay (ELISA) previously validated for use in cheetah for fecal hormone extracts (Young et al. 2004), featuring a polyclonal

antibody and associated horseradish-peroxidase (HRP) ligand. The EIA was validated for use with cheetah serum samples based on parallelism (Figure 4.1; 3.90–1000 pg/well; $r=0.99532$, $P<0.0001$ and $r=0.99356$, $P<0.0001$, for cheetahs 114799 and 114680 with standard, respectively). Fecal hormone extract samples with percent binding of Cort-HRP above 80% were re-analyzed without dilution ("neat" sample). Samples with percent binding of Cort-HRP below 20% were re-diluted an additional 10-fold and re-analyzed. The inter-assay coefficients of variation (CV) for two internal controls (n=69 assays) were 8.9% and 12.2%, and the intra-assay CV was <10%.

Protein extraction from fecal samples

To maximize the amount of serum amyloid A protein and its byproducts extracted from fecal samples while eliminating other proteins, a method adapted from Pras' method (1968) was used. Fifty milligrams of dried, pulverized feces was measured into a 2ml microcentrifuge tube. A salt solution, 0.15M NaCl, was used to eliminate saline soluble proteins from the sample by adding 1ml to the microcentrifuge tube, mixing the sample for 5 mins on a vortexer, centrifuging the tube for 10 mins at 15,000 x g, and then decanting the supernatant. This was repeated six additional times. Saline supernatants were discarded. Samples were then washed by the addition of 1ml diH_2O to the sample, mixing the sample for 5 mins on a vortexer, centrifuging the tube for 10 mins at 15,000 x g, then pipetting a fixed amount of supernatant (800µl) from the sample. This was repeated two times with 1ml diH_2O being added and removed, next a single step with the addition of 600µl

Figure 4.1. Parallelism between cheetah serum samples and the standard for an established EIA.



diH_2O and removal of 500µl, then a single step with the addition of 500µl diH_2O and removal of 700µl. The final centrifuge spin was done at full speed for the table top centrifuge (approximately 21,000 x g). Because amyloid proteins are water soluble, diH_2O supernatants were collected and pooled for a total of 4 ml collected.

Quantification of total protein in fecal protein extracts

Total concentration of protein in fecal protein extracts was quantified using the Bio-Rad Protein Assay kit, utilizing the protocol for the microassay procedure. Concentrations of β-amyloid (Aβ) [1-40] human protein (Novex®; Invitrogen Cat No. 03-136) ranging from 1.875 – 8.75 µg/ml was used as a standard for the assay.

Quantification of serum amyloid A protein in serum and fecal protein extracts

Serum amyloid A concentration was measured in serum and fecal protein extract samples using the Phase™ Range Multispecies SAA ELISA kit (Tridelta Development Ltd: Cat No. TP 802). Serum samples were diluted 1:100 in the provided sample diluent before use. Anti-SAA/HRP conjugate (50µl) and sample/control/standard (50µl, in duplicate) were added to each pre-coated well and incubated for 1.5 hours on a shaker at room temperature. After washing, 100µl of TMB substrate solution was added to each well and the plate was incubated for 15 minutes at room temperature. Stop solution was added (100µl) and absorbance for each well was read at 450nm using 620nm as a reference. A subset of the data collected from serum samples was previously reported in Chapter 2. The inter-assay coefficients of variation (CV) for two internal controls (n=5 assays) were 8.5% and 27.2%.

Quantification of proinflammatory cytokines (TNF-α, IL-1β, IL-6) in serum

Feline DuoSet® ELISA kits (R&D Systems) for TNF-α, IL-1β, and IL-6 were used following the manufacturer's instructions (validated in Chapter 3) to quantify the concentration of the cytokines in the cheetah serum samples. Samples were diluted 1:2 in PBS (with 1% BSA) before TNF-α and IL-1β assays. Samples were diluted 1:10 in PBS (with 1% BSA) before IL-6 assays. The inter-assay CV for two internal controls (n=5 assays) for TNF-α were 12.9% and 15.3%. The inter-assay CV for two internal controls (n=5 assays) for IL-1β were 3.3% and 4.5%. The inter-assay CV for two internal controls (n=5 assays) for IL-6 were 7.3% and 9.5%.

Univariate statistical analyses

Five variables were calculated from the cortisol concentrations quantified from fecal extracts: average cortisol (average), baseline cortisol (baseline), total cortisol variation (cortSTD), cortisol variation around baseline only (cortSTDbase), and frequency of cortisol peaks per sample collected (cortPEAKS). Average cortisol is the arithmetic mean of cortisol across all samples collected for an individual. Baseline was calculated using an iterative process that eliminates samples with high or low values if they fall outside the 95% confidence interval for the mean. After samples are removed, iterations continue until no more samples fall outside of the 95% confidence interval. Due to low sample numbers, particularly for captive cheetahs at CCF and wild cheetahs, an additional step in which samples were eliminated to create a balance of 50% of the remaining samples falling above and 50% of samples falling below baseline was employed. CortSTD is the standard deviation of the average (includes all samples). CortSTDbase is the standard

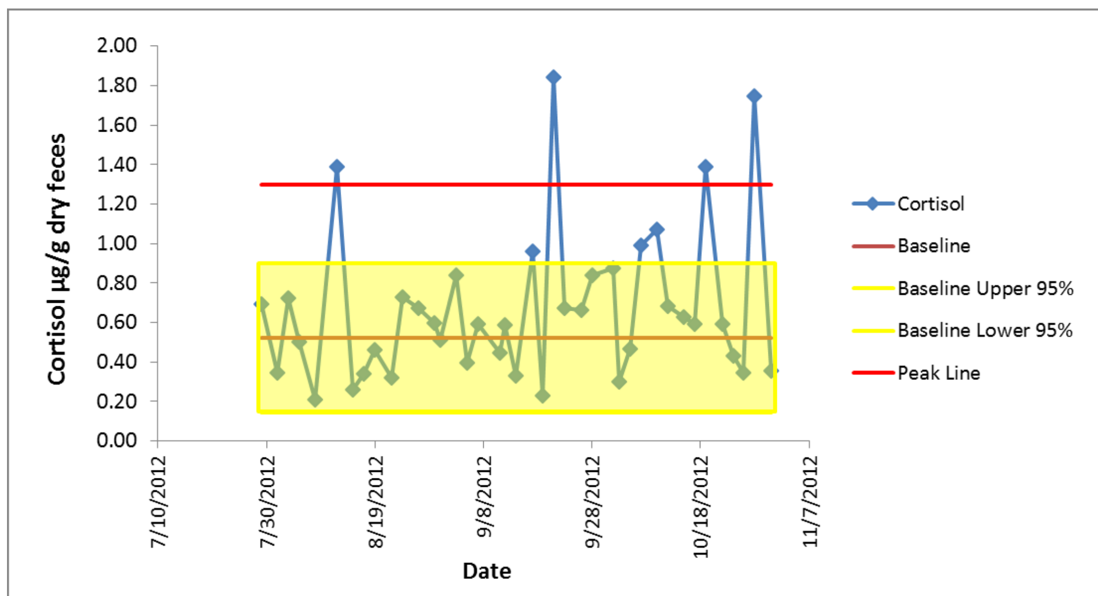
deviation of only the samples used in calculation of baseline cortisol. Cortisol peaks were determined as samples that were outside of the 95% confidence interval based on the baseline cortisol and cortSTD. Peaks were standardized per the number of fecal samples collected for an individual such that cortPEAKS = number of peaks / total number of samples. See Figure 4.2 for a representative cortisol profile from individual 114382. Cheetah 1340 was determined to be an outlier (extreme high values) and excluded from the analysis for baseline cortisol, average cortisol, cortSTD and cortSTDbase.

The five cortisol variables were analyzed using PROC MIXED of SAS Enterprise Guide (version 6.1) with the Kenward and Roger (DDFM=KR) option for adjustment of denominator degrees of freedom, including age as a covariate, status (captive, wild or soft-release), population (Namibia or North America), and sex (male/female) as fixed categorical factors and assuming heterogeneous variances for the status*population*sex groupings. Effects of status, population and sex were determined using a set of non-orthogonal contrasts due to the incomplete factorial nature of the status*population*sex groupings.

Total protein content of fecals and SAA concentration in fecals were analyzed using the same statistical model and contrasts as the previous cortisol variables, except the covariate age was excluded due to the lack of a significant relationship with the response variables. Total protein was included as a covariate in the analysis for SAA concentration.

SAA concentration in serum was log (base 10) transformed before analysis due to a high departure from normality. Log_{10}SAA, serum cortisol, TNF-α, and IL-1β

Figure 4.2 Representative profile for cortisol across a three month collection period for cheetah 114382.



concentrations were also analyzed using the same statistical model and contrasts as the fecal variables excluding the covariate age, again due to a high lack of a significant relationship with the response variables. IL-6 concentrations were similarly analyzed assuming homogeneous variances. Cheetah 114681 was determined to be an outlier (extreme high values) and excluded from the analysis for TNF-α and IL-1β.

The effect of environmental factors on average cortisol, baseline cortisol, cortSTD, cortSTDbase, log_{10}[SAA], [TNF-α], and [IL-1β] within the captive CCF population was also examined. Environmental factors included exposure to humans (High: "on-exhibit" or frequent exposure; Low: "off-exhibit" or sees few individuals, few times a day), regular exercise (Yes: 1^+ times per week; No: <1 time per week), and enclosure size (Large: >10 acres; Small: <10 acres). Based on these criteria, cheetahs were separated into 6 groups. The fecal variables were analyzed using PROC MIXED of SAS Enterprise Guide (version 6.1) including age as a covariate, and human exposure, exercise, and enclosure size as fixed categorical factors. Effects of human exposure, exercise, and enclosure size were determined using a set of orthogonal contrasts due to the incomplete factorial nature of the groupings. Statistical significance between groups was declared at α=0.10.

The effect of environment on average cortisol, baseline cortisol, cortSTD, cortSTDbase, [log_{10}SAA], [TNF-α], and [IL-1β] within the captive Smithsonian population was also examined. Using the above classifications, cheetahs made only two groups determined by location, thus cheetahs held at NZP were compared versus cheetahs held at SCBI.

Correlation analyses were used to determine the relationships between all variables measured in the fecal and serum samples, both across populations and within each captive population (NZP-SCBI and CCF). Within population correlations for the wild and soft-release cheetahs were not performed due to insufficient sample size.

Multivariate statistical analyses

AA amyloidosis status of the cheetahs measured in this study is unknown. Therefore, a multivariate statistical approach (canonical discriminant analysis: CDA) was taken to investigate how population differences across the multiple correlated variables measured can distinguish the populations from each other. CDA is a process that defines independent linear combinations of variables (canonical discriminant functions: CDFs) that maximize the differences between the groups. The DISCRIM procedure in SAS Enterprise Guide (version 6.1) with parametric, linear classification rules, and prior probabilities proportional to sample sizes was used to perform both descriptive and predictive canonical discriminant analyses (DDA and PDA, respectively). "Soft-release" cheetahs were excluded from this analysis due to missing values for too many individuals.

DDA was used to separate the populations of cheetahs (CLASS variables: NZP-SCBI, CCF, and Wild). Independent variables included in the analysis were average cortisol, baseline cortisol, cortSTD, cortSTDbase, $\log_{10}$[SAA], [TNF-α], and [IL-1β]. Within-group covariance matrices were used as the basis of the measure of the squared distance.

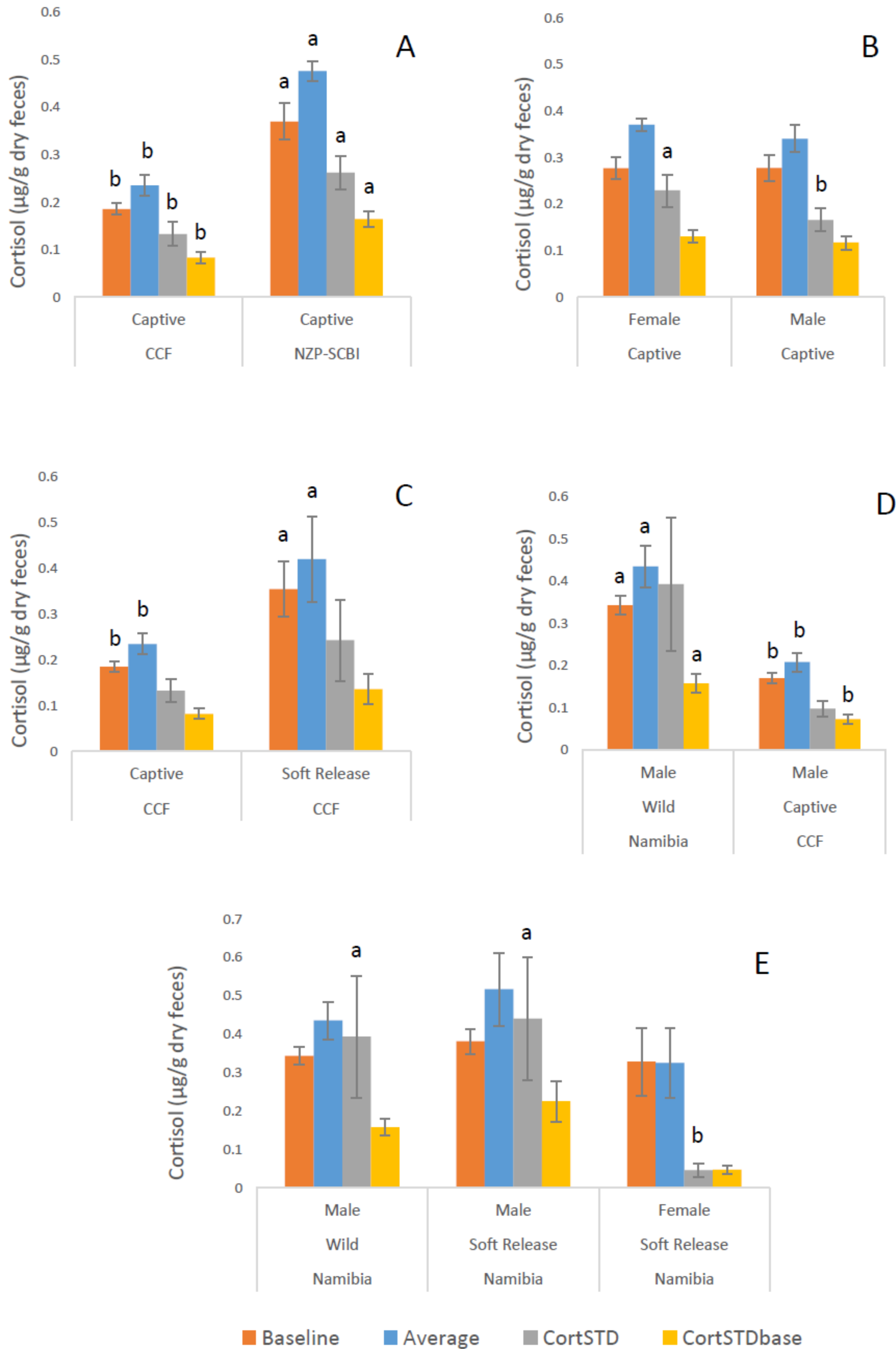
For PDA, a model was constructed to predict population membership based on the CDFs. Resubstitution and cross-validation (also known as jackknifing) methods were used to evaluate the performance of the discriminant model (Huberty 1994).

Results

Fecal cortisol and associated metabolite concentrations and variability between populations

Captive cheetahs at Smithsonian facilities have higher baseline cortisol, higher average cortisol and greater daily variation in cortisol (both cortSTD and cortSTDbase) when compared to captive cheetahs at CCF ($P<0.0005$; Figure 4.3A). Differences between male and female cheetahs in captivity was only detected for cortSTD: captive females have greater overall variation in cortisol compared to captive males ($P=0.0581$; Figure 4.3B). Cheetahs put into “soft-release” at CCF have significantly higher baseline and average cortisol compared to captive cheetahs at CCF ($P<0.05$; Figure 4.3C). The only difference detected between males and females in “soft-release” was in variation around baseline: females showed less variability around baseline compared to males ($P=0.0393$; Figure 4.3E). Due to the limitation of having only male wild cheetahs sampled, wild cheetahs were compared to male captive and “soft-release” cheetahs at CCF. Wild males have significantly higher baseline cortisol, average cortisol, and greater variation around baseline (cortSTDbase) than captive male cheetahs ($P<0.05$; Figure 4.3D). There were no significant differences between wild male cheetahs and male cheetahs in “soft-

Figure 4.3. Fecal cortisol and associated metabolite concentrations by population. Means ± SEM are presented. [a,b]Designate significant differences between groups based on non-orthogonal contrasts, P<0.06.



release" (Figure 4.3E). No differences between any populations were detected for cortPEAKS.

Population differences in fecal protein concentrations

Wild male cheetahs have significantly higher protein content in feces compared to captive male cheetahs at CCF (P=0.0536; Figure 4.4A). Similarly, cheetahs in "soft-release" have significantly higher protein content in feces compared to captive cheetahs (P=0.0051l, Figure 4.4A). The only significant difference observed in SAA content in feces was between captive cheetahs at CCF vs. NZP-SCBI: significantly more SAA was detected in the feces of CCF cheetahs (P=0.0002; Figure 4.4B).

Cortisol and serum amyloid A protein concentrations in serum

Though there was no difference in the serum concentrations of cortisol between captive cheetahs at CCF and captive cheetahs at NZP-SCBI (Figure 4.5A), female cheetahs in captivity have significantly higher serum cortisol concentrations compared to male cheetahs in captivity (P=0.0214; Figure 4.5B). Female cheetahs in "soft-release" had significantly lower cortisol concentrations in serum than captive CCF females (P=0.001: Figure 4.5C). There was no difference in serum cortisol concentrations between captive male cheetahs at CCF and wild males (Figure 4.5D).

The $\log_{10}$SAA concentration in serum was significantly different between captive cheetahs at CCF and captive cheetahs at NZP-SCBI. Captive cheetahs at both the NZP and SCBI have significantly higher $\log_{10}$SAA concentrations in serum (P=0.0008; Figure 4.6). Female cheetahs in "soft-release" had a similar significant

Figure 4.4. Total protein and serum amyloid A protein concentrations in feces by population. Means ± SEM are presented. *Denotes a significant difference between two populations, P=0.0536. **Denotes a signficant difference between populations (sexes pooled), P<0.01.

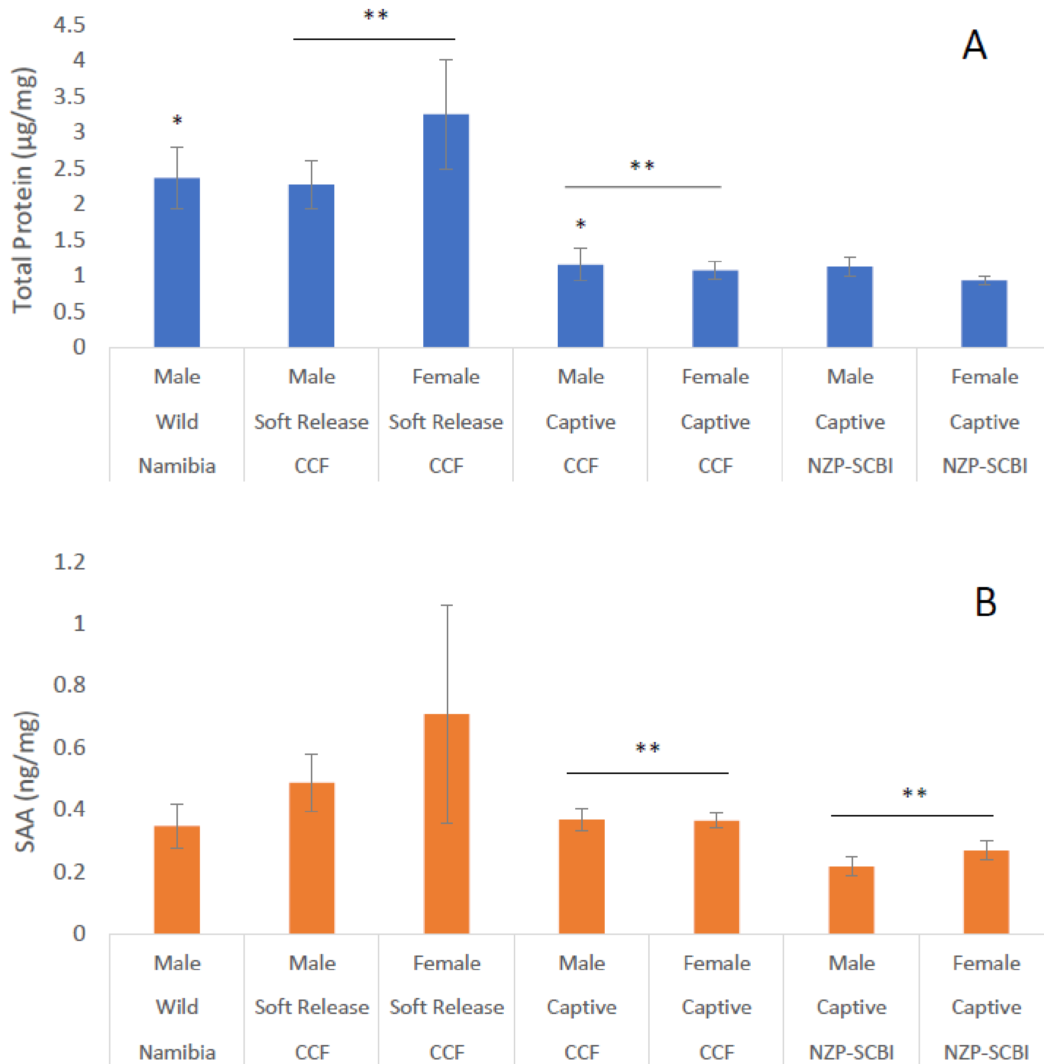


Figure 4.5. Differences in serum cortisol concentrations (lsmeans ± SEM) across cheetah populations. [a,b]Designate significant differences between groups based on non-orthogonal contrasts, P<0.05.

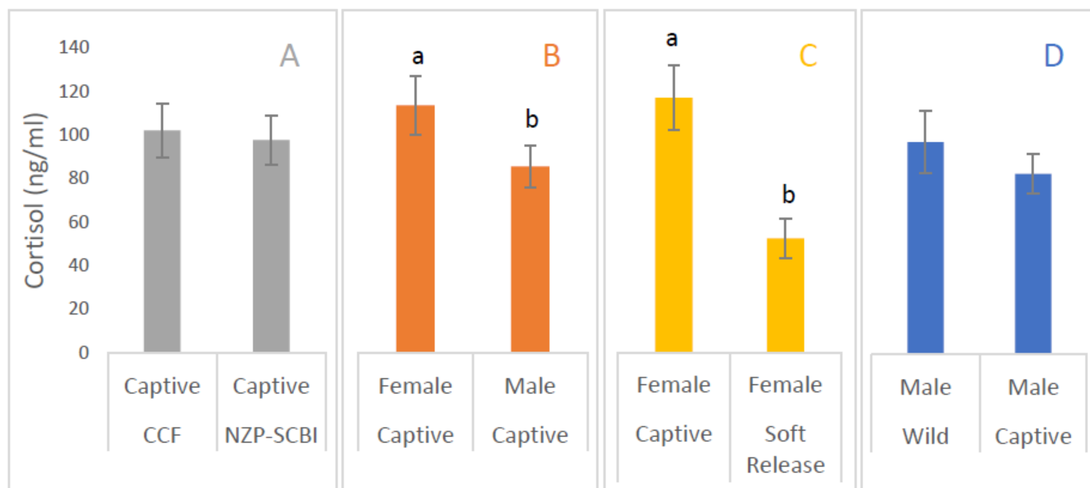
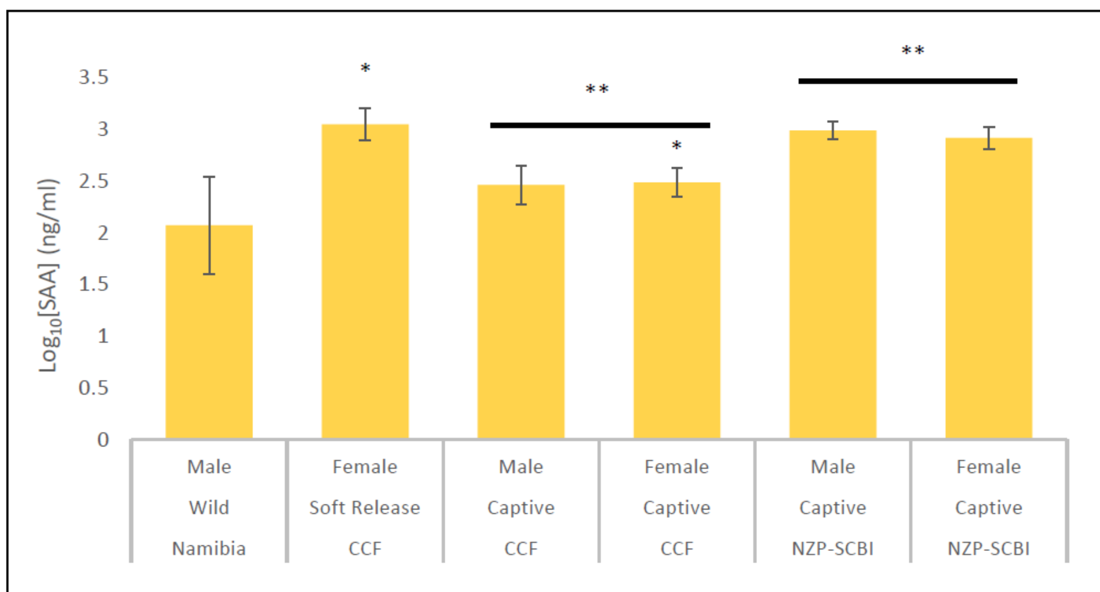


Figure 4.6. Comparison of serum log_{10}SAA concentrations (lsmeans ± SEM) across populations. *Denotes a significant difference between two populations, P=0.0156. **Denotes a signficant difference between populations (sexes pooled), P=0.0008.



increase in log_{10}SAA concentrations compared to captive females at CCF (P=0.0156; Figure 4.6). There was no significant difference between captive CCF cheetahs and wild cheetahs (Figure 4.6).

Population differences in proinflammatory cytokine (TNF-α, IL-1β, IL-6) concentrations in serum

For both TNF-α and IL-1β, wild cheetahs as well as "soft-release" and captive cheetahs at CCF had significantly higher cytokine concentrations in serum compared to captive cheetahs at NZP-SCBI (P<0.0001; Figure 4.7). Analyzing the results of the IL-6 assay is not appropriate, as all samples assayed fell between zero and the lowest standard. Thus all concentrations were determined based on extrapolation of the data. Additionally, it appears all concentrations are likely overestimated after they were multiplied by 10 for the dilution factor used considering the relative abundances of the three cytokines after LPS stimulation (Chapter 3). Nonetheless, there was a significant difference in IL-6 concentrations in serum between wild males and captive male cheetahs at CCF: wild males had significantly higher IL-6 concentrations (P<0.0001). There was no significant differences detected in IL-6 concentrations between "soft-release", captive CCF and captive NZP-SCBI populations.

Environmental associations with cortisol, inflammation, and SAA protein within captive populations

Among cheetahs at CCF, higher baseline and average cortisol concentrations were found to be associated with high exposure to humans and small enclosures (P<0.1; Figure 4.8). CortSTD, cortSTDbase, and cortPEAKS did not differ between

Figure 4.7. TNF-α and IL-1β cytokine concentrations in cheetah serum (lsmeans ± SEM) across populations. [a,b]Designate significant differences between groups based on non-orthogonal contrasts, $P<0.05$.

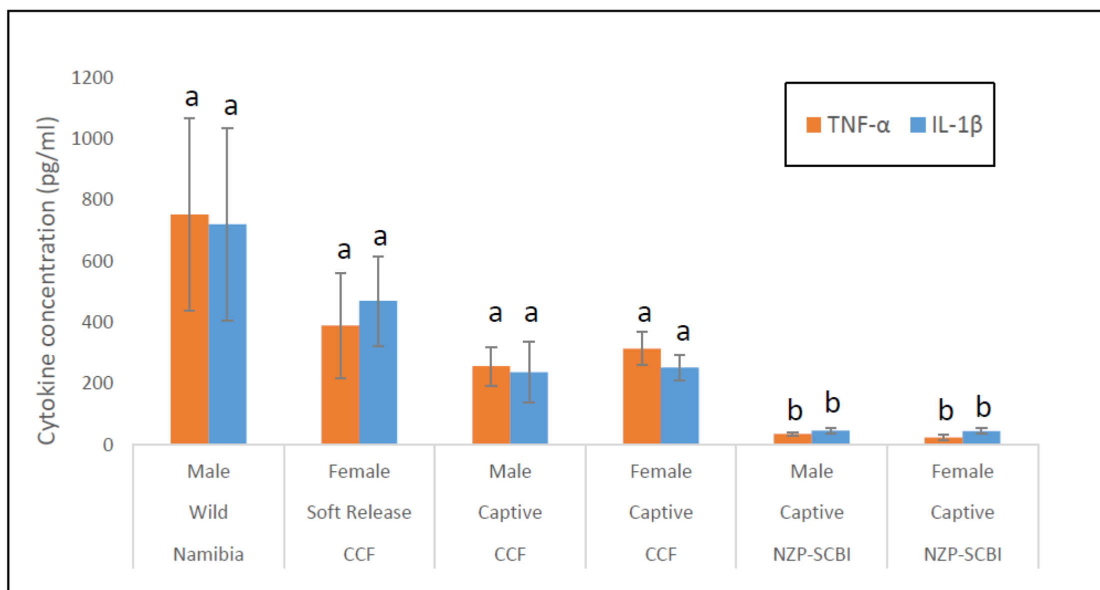
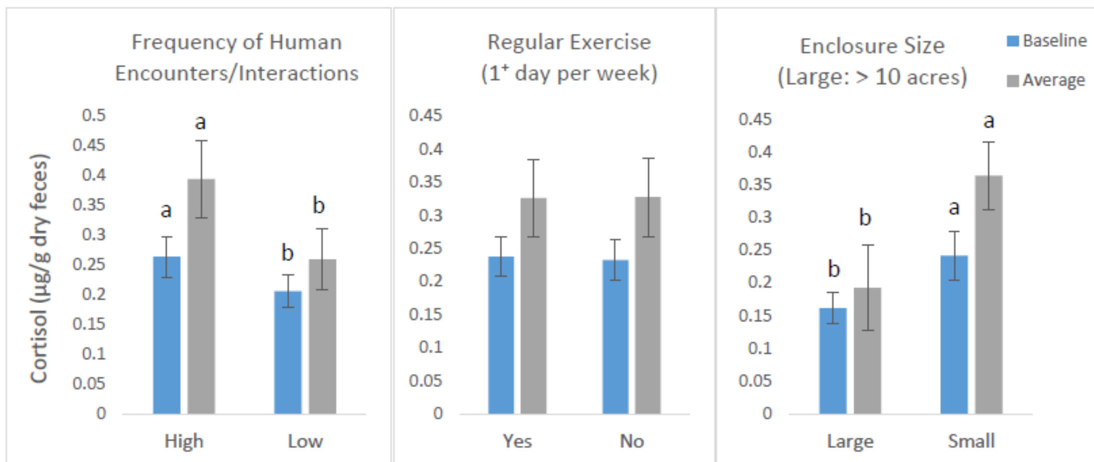


Figure 4.8. Effects of environmental factors on baseline and average cortisol concentrations in feces among captive cheetahs at Cheetah Conservation Fund. [a,b]Indicates a significant difference between environments at $P<0.1$.

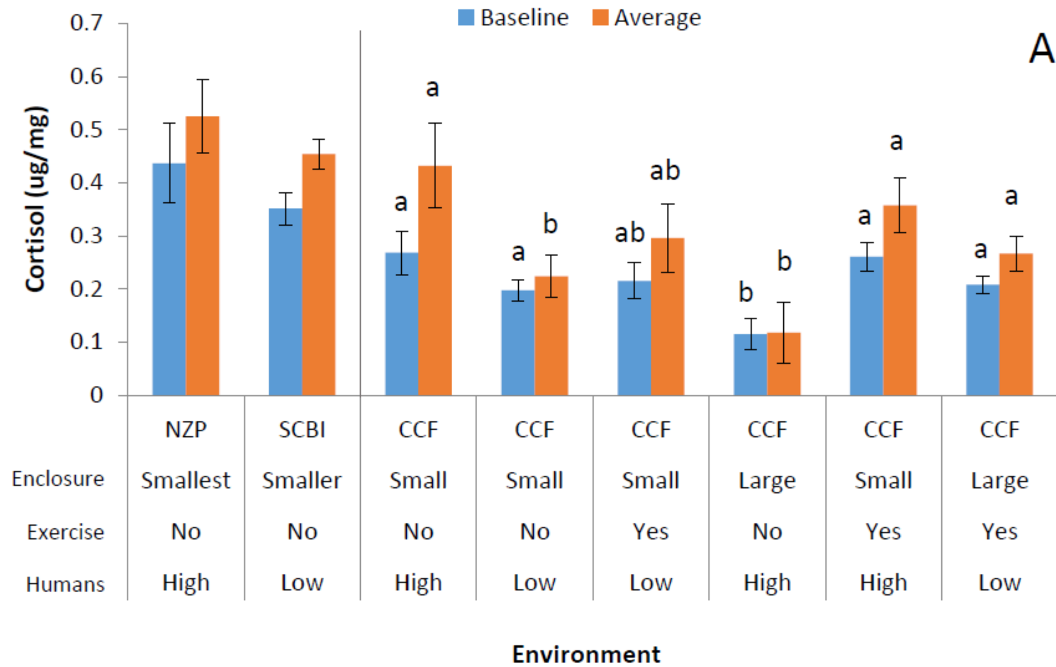


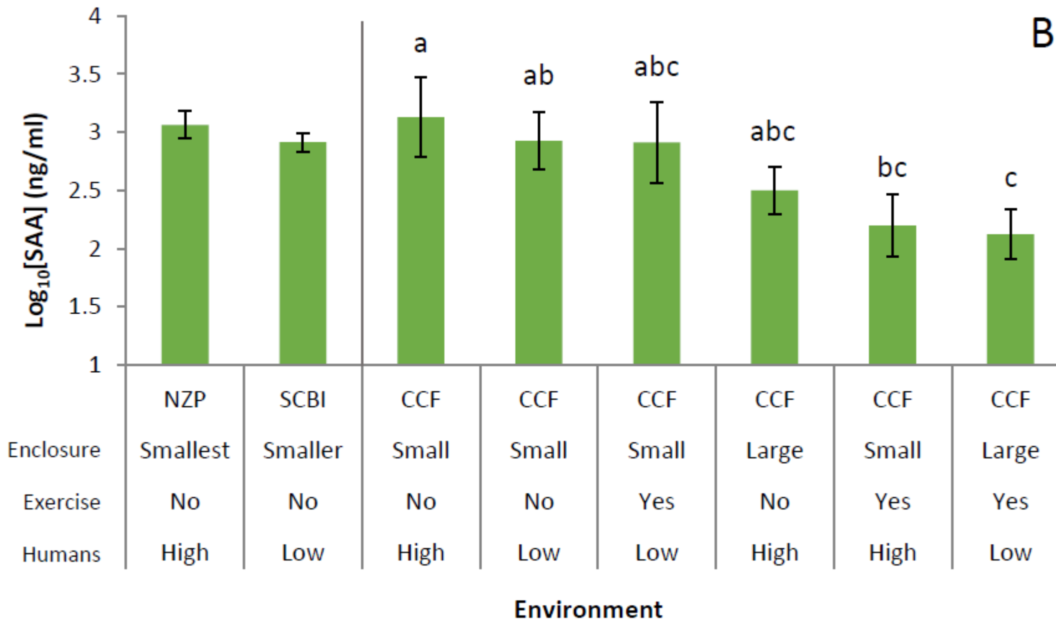
captive environments. Similarly, higher serum concentrations of $\log_{10}$SAA and IL-1β were associated with small enclosures ($P=0.019$ and $P=0.0413$, respectively). TNF-α concentrations did not differ between captive environments. There were no significant differences between cheetahs held at the two different Smithsonian locations (NZP and SCBI) for any of the variables examined, however proinflammatory cytokines TNF-α and IL-1β were marginally higher in cheetahs on-exhibit at NZP versus off-exhibit at SCBI ($P=0.0527$ and $P=0.1208$, respectively). In general, values for $\log_{10}$SAA and cortisol variables for the NZP-SCBI cheetahs most closely resembled those of CCF cheetahs in small enclosures, without regular exercise and high exposure to humans (Figure 4.9).

Variable correlations across populations

Baseline cortisol, average cortisol, cortSTD and cortSTDbase are significantly positively correlated with one another ($P \leq 0.0002$; Table 4.1). CortPEAKS was significantly positively correlated with average cortisol and cortSTD only ($P \leq 0.0003$; Table 4.1), which is logical since all cortisol peaks are included in the calculation of these variables, yet removed from the calculation of baseline cortisol and cortSTDbase. Proinflammatory cytokines TNF-α, IL-1β and IL-6 were all significantly positively correlated in serum ($P \leq 0.0035$; Table 4.2). There were no significant correlations between cortisol variables measured in fecals (baseline cortisol, average cortisol, cortSTD, cortSTDbase, and cortPEAKS) and variables measured in serum (cortisol, TNF-α, IL-1β, IL-6 and $\log_{10}$SAA concentrations).

Figure 4.9. Population comparison of baseline and average cortisol in feces (A) and $\log_{10}$SAA concentration in serum (B) across captive cheetah populations and environments. Means among CCF groups that do not share a letter are significantly different based on Fisher's LSD multiple mean comparison test at P<0.05.





Pearson Correlation Coefficients (r) **Prob >|r| under H_0: ρ=0**	**Age**	**Baseline**	**Average**	**CortSTD**	**CortSTDbase**	**CortPEAKS**
Age	1.0	0.12400 0.2892	0.14174 0.2251	0.15164 0.2171	0.17922 0.1467	-0.01751 0.8815
Baseline		1.0	0.81577 <0.0001	0.44011 0.0002	0.70619 <0.0001	-0.06409 0.5849
Average			1.0	0.81313 <0.0001	0.82438 <0.0001	0.40977 0.0003
CortSTD				1.0	0.63231 <0.0001	0.49858 <0.0001
CortSTDbase					1.0	0.03973 0.7496
CortPEAKS						1.0

Table 4.1. Cortisol variable correlation coefficients (r) across all cheetah populations**.** Significant correlation coeffients are in red (P<0.05).

Pearson Correlation Coefficients (r) Prob >|r| under H_0: ρ=0	Age	Cortisol	TNF-α	IL-1β	IL-6	$Log_{10}SAA$
Age	1.0	0.03601 0.7656	-0.1161 0.3350	-0.1079 0.3704	-0.1965 0.1005	0.11066 0.3583
Cortisol		1.0	0.10529 0.3822	0.05358 0.6572	-0.1237 0.3039	0.10271 0.3940
TNF-α			1.0	0.81265 <0.0001	0.49700 <0.0001	-0.19897 0.0962
IL-1β				1.0	0.34255 0.0035	-0.10793 0.3703
IL-6					1.0	-0.03160 0.7936
$Log_{10}SAA$						1.0

Table 4.2. Serum variable correlation coefficients (r) across all cheetah populations. Significant correlation coeffients are in red (P<0.05).

Variable correlations within populations

When variable correlations were examined independently by population, a significant positive relationship between age and cortisol in feces was detected for baseline cortisol, average cortisol, cortSTD, and cortSTDbase among CCF cheetahs, and for average cortisol and cortSTDbase among NZP-SCBI cheetahs ($P<0.05$; Table 4.3). In contrast, age was not associated with any variables measured in serum (Table 4.4). Log_{10}SAA concentration was positively correlated with IL-6 in CCF cheetahs only ($P=0.0468$; Table 4.4). Log_{10}SAA concentration was positively correlated with cortSTD and cortSTDbase in NZP-SCBI cheetahs only ($P<0.05$; Tables 4.5 and 4.6). A false significant association between IL-1β and baseline cortisol was detected in the NZP-SCBI cheetah population due to cheetah 114701 having both the highest baseline cortisol and IL-1β concentration in serum. When cheetah 114701 was removed from the analysis, the relationship between the two variables was no longer significant.

Descriptive Discriminant Analysis

Two significant canonical discriminant functions (Can1 and Can2) were produced that defined the underlying dimensions in the data that explained the differences between the three populations (NZP-SCBI, CCF and wild cheetahs). Can1 explained over 71% of the total variation among the three populations and best defined the difference between NZP-SCBI cheetahs and Namibian cheetahs (captive and wild). Can2 explained the remaining variation between the populations and best defined the separation between Namibian cheetahs, CCF vs. wild (Figure 4.10).

Pearson Correlation Coefficients (r) Prob >|r| under H_0: ρ=0	Age	Baseline	Average	CortSTD	CortSTDbase	CortPEAKS
Age	1.0	0.66442 <0.0001	0.56338 0.0006	0.46209 0.0068	0.49231 0.0036	0.13540 0.4525
Baseline	0.38907 0.0813	1.0	0.90837 <0.0001	0.75991 <0.0001	0.70152 <0.0001	0.11039 0.5408
Average	0.50276 0.0202	0.90540 <0.0001	1.0	0.92717 <0.0001	0.84193 <0.0001	0.30568 0.0836
CortSTD	0.36160 0.1073	0.22487 0.3271	0.58099 0.0057	1.0	0.83397 <0.0001	0.26934 0.1296
CortSTDbase	0.44998 0.0407	0.52963 0.0135	0.79557 <0.0001	0.90549 <0.0001	1.0	0.31249 0.0766
CortPEAKS	-0.2658 0.2442	-0.4830 0.0266	-0.3527 0.1168	-0.13083 0.5719	-0.36223 0.1066	1.0

Table 4.3. Cortisol variable correlation coefficients (r) within captive cheetah populations. CCF cheetahs are highlighted in orange. NZP-SCBI cheetahs are highlighted in green. Significant correlation coeffients are in red (P<0.05).

Pearson Correlation Coefficients (r) **Prob >|r| under H_0: ρ=0**	**Age**	**Cortisol**	**TNF-α**	**IL-1β**	**IL-6**	**Log_{10}SAA**
Age	1.0	0.11656 0.4623	-0.13812 0.3831	-0.1002 0.5277	-0.17937 0.2557	0.04726 0.7663
Cortisol	0.07720 0.7534	1.0	0.25174 0.1078	0.16268 0.3033	-0.14465 0.3607	0.24241 0.1219
TNF-α	0.11878 0.6282	0.01175 0.9619	1.0	0.66504 <0.0001	0.07813 0.6229	-0.03779 0.8122
IL-1β	0.00984 0.9681	-0.15748 0.5197	0.76372 0.0001	1.0	-0.21003 0.1819	0.04226 0.7904
IL-6	-0.34866 0.1435	-0.30414 0.2055	0.05533 0.8220	0.09179 0.7086	1.0	0.30852 0.0468
Log_{10}SAA	0.01622 0.9475	-0.152263 0.5328	-0.11013 0.6536	-0.29597 0.2186	0.00862 0.9720	1.0

Table 4.4. Serum variable correlation coefficients (r) within captive cheetah populations. CCF cheetahs are highlighted in orange. NZP-SCBI cheetahs are highlighted in green. Significant correlation coeffients are in red (P<0.05).

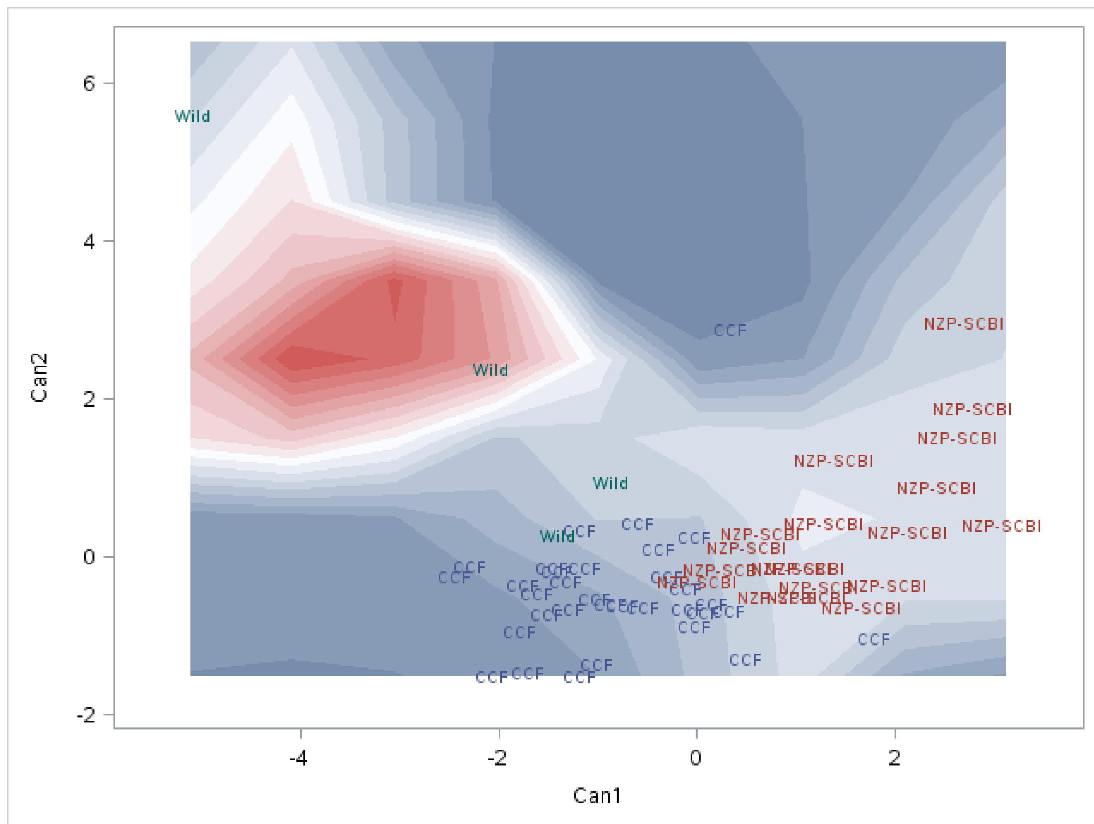
Pearson Correlation Coefficients (r) Prob >|r| under H_0: ρ=0	Baseline	Average	CortSTD	CortSTDbase	CortPEAKS
Cortisol	-0.00628 0.9723	-0.03141 0.8622	0.08647 0.6323	0.05950 0.7422	-0.08684 0.6309
TNF-α	-0.02754 0.8791	-0.01775 0.9219	0.10878 0.5468	0.07248 0.6885	-0.16409 0.3615
IL-1β	-0.05577 0.7579	-0.03003 0.8682	0.11183 0.5355	-0.01686 0.9258	-0.06248 0.7298
IL-6	-0.00034 0.9985	0.01678 0.9262	0.04545 0.8017	-0.03618 0.8415	-0.05029 0.7811
Log_{10}SAA	0.07003 0.6986	0.09255 0.6085	0.18384 0.3058	-0.13541 0.4525	-0.16252 0.3662

Table 4.5. Correlation coefficients (r) of cortisol variables measured in feces with serum variables within the CCF captive cheetah populations.

Pearson Correlation Coefficients (r) **Prob >|r| under H_0: ρ=0**	**Baseline**	**Average**	**CortSTD**	**CortSTDbase**	**CortPEAKS**
Cortisol	-0.05894 0.8106	-0.05324 0.8286	-0.16021 0.5124	-0.22025 0.3649	0.21657 0.3732
TNF-α	0.37783 0.1107	0.23236 0.3384	-0.23734 0.3279	0.01381 0.9553	-0.19361 0.4271
IL-1β	0.47671 0.0391	0.30208 0.2088	-0.22583 0.3526	0.02160 0.9301	-0.18643 0.4447
IL-6	-0.13648 0.5774	-0.12925 0.5979	0.14307 0.5590	0.14308 0.5590	-0.38510 0.1035
Log_{10}SAA	0.06439 0.7934	0.26946 0.2646	0.59236 0.0075	0.54940 0.0148	-0.27274 0.2586

Table 4.6. Correlation coefficients (r) of cortisol variables measured in feces with serum variables within the NZP-SCBI captive cheetah population. Significant correlation coeffients are in red ($P<0.05$).

Figure 4.10. The three populations of cheetahs included in the canonical discriminant analysis (NZP-SCBI, CCF, and Wild) can be discriminated with 76.8% accuracy based on the two canonical discriminant functions calculated (Can1 and Can2). Individual animals are plotted based on their canonical discriminant function scores and identified by population.



The total canonical structure (the correlation coefficients between the individual variables and the canonical scores) is presented in Table 4.7. $Log_{10}SAA$ concentration was significantly positively correlated with Can1 ($P<0.0001$), which had similar positive correlations with baseline cortisol, average cortisol, and cortSTDbase, and was negatively correlated with IL-1β and TNF-α. $Log_{10}SAA$ concentration was not associated with Can2. Can2 was positively correlated with baseline cortisol, average cortisol, cortSTDbase, cortSTD, as well as IL-1β and TNF-α concentrations.

Predictive Discriminant Analysis

When resubstitution was used to evaluate the performance of the discriminant model, 51 out of 56 cheetahs were classified correctly, resulting in a total error-count-estimate of 8.93%. The 5 misclassified cheetahs were all CCF cheetahs that were misclassified as NZP-SCBI cheetahs. When the cross-validation method was used, 43 out of the 56 cheetahs were classified correctly, resulting in a total error-count-estimate of 23.21%. All four wild cheetahs were misclassified as CCF cheetahs. Because only 3 wild cheetahs are used to generate the discriminant model created in each iteration, the amount of information about wild cheetahs is very limited and individual differences can make large changes to the model. Thus, it is unsurprising that the individual left out during any given iteration was always misclassified. Also when using the cross-validation method, 6 CCF cheetahs were misclassified as NZP-SCBI cheetahs and 3 NZP-SCBI cheetahs were misclassified as CCF cheetahs. There were no misclassifications from wild to NZP-SCBI cheetahs or vice versa.

Pearson Correlation Coefficients (r) **Prob >|r| under H_0: ρ=0**		
	Can1	**Can2**
Baseline	0.63419 <0.0001	0.71595 <0.0001
Average	0.58696 <0.0001	0.78028 <0.0001
CortSTD	0.00991 0.9422	0.82604 <0.0001
CortSTDbase	0.51181 <0.0001	0.58292 <0.0001
TNF-α	-0.77241 <0.0001	0.36460 0.0057
IL-1β	-0.58093 <0.0001	0.35873 0.0066
Log_{10}SAA	0.53937 <0.0001	-0.07967 0.5595

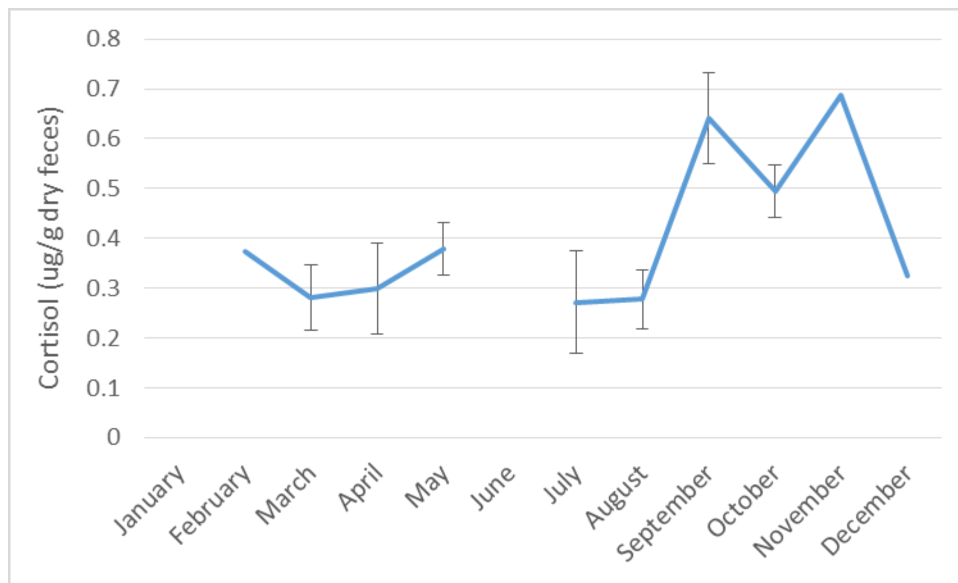
Table 4.7. Total canonical structure indicating correlation coefficients (r) between original variables and CDF scores. Significant correlations are in red.

Discussion

Previous work done by Terio et al. (2004) and Munson et al. (2005) has suggested that the high prevalence of gastritis and AA amyloidosis observed in captive cheetahs in North America is likely related to the stress associated with being in captivity. In this study it was shown that the captive Smithsonian population of cheetahs has significantly higher levels of cortisol compared to the captive CCF population. In contrast to the previous studies, wild cheetahs demonstrated equally high levels of cortisol as the captive Smithsonian population. It is not entirely unexpected that wild cheetahs have higher baseline cortisol and cortSTDbase compared to CCF cheetahs, because there was less statistical power to declare samples as acute stress peaks and eliminate them from the calculations. Also, one may expect the wild environment to be more unpredictable in terms of food availability, hunting success and the presence of predators, which may cause more frequent acute stress peaks and thus higher average cortisol and cortSTD. Cheetahs defecate approximately once per day, therefore any diurnal fluctuations in cortisol due to circadian secretory patterns are attenuated in feces (Keay et al. 2006).

It is also possible that seasonal changes in cortisol are affecting the data. When wild cheetah samples are averaged by month, it seems clear that there is an increase in cortisol from September to November, which is the end of the winter season when food and water is most scarce (Figure 4.11). This corresponds to periods of anestrous observed in captive Namibian females between August and December in a previous study during a particularly dry winter (Terio et al. 2003). Studies among other species have also shown that a high abundance of predators as well as starvation

Figure 4.11. Fecal cortisol concentrations in wild cheetahs (N=7) by month. LSMeans ± SE are presented where data was available.



are positively correlated with glucocorticoid concentrations (Busch and Hayward 2009) and that moderately elevated glucocorticoids can actually be related to increased survival in the wild for some species (Busch and Hayward 2009, Dhabhar 2009). Also, cheetahs that were previously held captive at CCF and subsequently put into “soft-release” had similarly high levels of cortisol. Considering these factors, it is likely that even though wild cheetahs were not found to have statistically significant differences in measures of stress, that their stress is more acute or seasonal in nature and does not affect these animals the same as the chronic levels of stress seen in the North American captive cheetah population in this and previous studies (Bolton and Munson 1999, Munson et al. 1999, Munson et al. 2005, Terio et al. 2004).

The ELISA assay for SAA protein concentrations in fecal protein extracts was tested as a non-invasive tool for monitoring amyloid fibril deposition in feces, as seen in cheetahs with moderate to severe amyloidosis (Zhang et al. 2008). The idea for its use was based on the assumption that the antibody used in the assay binds the most conserved region of the SAA protein across species, which is retained as part of the AA protein (Johnson et al. 1997), thus it may be an indirect measurement of amyloid fibril accumulation in feces. The only statistical difference found in fecal SAA concentrations was between captive cheetahs at CCF and captive NZP-SCBI cheetahs, and though significant, it does not appear to be biologically relevant as the difference between the populations was small and it is uncorrelated with $\log_{10}$SAA concentrations in serum. Therefore, it was concluded that fecal SAA protein concentrations would not be a good way to assess possible amyloid fibril deposition in feces or amyloidosis status in cheetahs.

Overall protein concentrations in feces were significantly higher in wild and "soft-release" cheetahs compared to captive cheetahs. This difference seems likely due to differences in diet, as captive cheetahs are fed supplemented muscle meat either on or off the bone (CCF and NZP-SCBI, respectively), whereas wild and "soft-release" cheetahs are eating whole carcasses, including bits of hair and hide that provide additional fiber in the diet and likely increase diet digestibility, as well as internal organs, such as heart and liver, that increase the concentration of protein in the diet. Whether dietary differences have an effect on amyloidosis pathogenesis has not been investigated.

Log_{10}SAA concentrations in serum were significantly higher in the NZP-SCBI captive population compared to captive CCF and wild cheetahs. This is expected given previous research showing that AA amyloidosis among cheetahs in captivity is exceedingly common in North America, yet exceptionally rare in wild cheetahs (Munson et al. 2005). Furthermore, studies in humans have found elevated SAA concentrations in serum to correlate with AA amyloidosis (Biran et al. 1986, Lachmann et al. 2007, Marhaug and Dowton 1994). Wild cheetahs appeared to have lower log_{10}SAA concentrations compared to captive CCF. However, this difference could not be declared statistically significant, likely due to the small sample size for wild cheetahs. Therefore, it appears that SAA concentrations in serum of cheetahs is similarly correlated with the probability of developing AA amyloidosis.

Previous work in humans has shown that amyloid deposits can regress and organ function can recover if SAA concentrations are controlled (kept low) (Gillmore et al. 2001). A promising therapy using SAA-specific antisense oligonucleotides

(ASO) has been shown to reduce SAA concentration in mice, particularly by facilitating a more rapid return to baseline after episodic bursts in SAA concentration (Kluve-Beckerman et al. 2011). SAA-specific ASOs were also shown to reduce amyloid deposition in treated mice in which amyloidosis was previously induced (Kluve-Beckerman et al. 2011). Whether ASOs could be a therapy to treat systemic amyloidosis in either humans or cheetahs has yet to be investigated.

Papendick et al. (1997) found that all cheetahs diagnosed with amyloidosis also suffered from other significant chronic inflammatory diseases in organs other than kidney, most commonly chronic lymphoblastic gastritis. Thus, it appears the high prevalence of AA amyloidosis among captive cheetahs is related to chronic inflammation. Knowing that almost 100% of captive cheetahs in North America have gastritis (a chronic inflammatory condition) to some degree (Munson 1993, Munson et al. 1999, Munson et al. 2005), it was expected that proinflammatory cytokine concentrations would be higher in the captive NZP-SCBI population compared to the other cheetah populations. In fact, the opposite was observed in this study. TNF-α and IL-1β were significantly lower in the NZP-SCBI cheetah population compared to the other cheetah populations. Aside from the mean concentrations of proinflammatory cytokines, worth noting is the differences in the range of concentrations observed for each population. The range of concentrations within the captive NZP-SCBI population was much smaller compared to CCF and wild cheetah populations (Table 4.8). It is possible that captive NZP-SCBI cheetahs are not often exposed to new pathogens or are less likely to incur injuries such that they have low proinflammatory cytokine concentrations. However, one serum sample was knowingly taken 4 days

Cytokine	Population	Minimum (pg/ml)	Maximum (pg/ml)
TNF-α	NZP-SCBI	9.67	90.42
	CCF (captive)	14.63	956.27
	CCF (soft-release)	26.89	934.02
	Wild	247.22	1661.04
IL-1β	NZP-SCBI	15.61	121.10
	CCF (captive)	25.76	1822.81
	CCF (soft-release)	33.05	856.20
	Wild	321.05	1657.62

Table 4.8. Minimum and maximum proinflammatory cytokine concentrations observed within cheetah populations.

post-surgery and even this individual did not show elevated cytokines. Therefore, these results may be indicative of dysregulation of the immune system and suppression of the production of proinflammatory cytokines in captive NZP-SCBI cheetahs due to a chronic elevation of glucocorticoids.

Glucocorticoids induce the transcription of IκBα protein (Auphan et al. 1995, Barnes and Karin 1997, Scheinman et al. 1995). Without stimulation signaling, NF-κB is bound to IκBα or IκBβ in the cytoplasm of the cell and is prevented from entering the nucleus (Barnes and Karin 1997, Beg et al. 1993). However, in the presence of stimulation from proinflammatory cytokines, such as TNF-α (Beg et al. 1993, Scheinman et al. 1995) and IL-1β (Barnes and Karin 1997), IκBs are phosphorylated, disassociate from NF-κB, and get degraded (Barnes and Karin 1997, Beg et al. 1993). The unbound NF-κB enters the nucleus and binds to the promoter region of target genes associated with inflammatory and immune responses, including many proinflammatory cytokines (Barnes and Karin 1997, Beg et al. 1993).

Multiple studies have shown that IκBα synthesis is increased in the presence of dexamethasone (DEX: a synthetic glucocorticoid), causing fast reassociation of NF-κB with IκBα, thus reducing the translocation of NF-κB to the nucleus, even under cytokine stimulation (Auphan et al. 1995, Scheinman et al. 1995). This inhibitory effect of DEX is mediated through cytoplasmic glucocorticoid receptors (Auphan et al. 1995), with possible direct protein-protein interaction between the glucocorticoid receptor (GR) and NF-κB (Barnes and Karin 1997). Therefore, activated GRs are involved in blocking transcription factors required for the expression of proinflammatory target genes (Chrousos 1995). It is widely accepted

that the expression of many cytokines (including TNF-α, IL-1β and IL-6) is inhibited by GCs (Baumann and Gauldie 1994, Chrousos 1995, Jensen and Whitehead 1998, Paltrinieri 2008, Wiegers and Reul 1998). Therefore, determining the role of inflammation in AA amyloidosis pathogenesis in the captive North American cheetah population, in the presence of high GC concentrations, will require a more complete look at systemic immune regulation and the permissive vs. suppressive effects of GCs on immune function. It is interesting to note that among the captive CCF population, which had significantly lower cortisol, that IL-6 was positively correlated with $\log_{10}$SAA concentrations, suggesting that in the absence of chronically elevated GCs, proinflammatory cytokines do play an important role in the amount of SAA produced, as expected.

Using a canonical discriminant analysis allowed the investigation of the relationship between the stress variables, proinflammatory cytokine concentrations and SAA protein concentrations simultaneously. This analysis revealed that the stress variables best discriminate the NZP-SCBI captive population from the captive CCF population along the same axis significantly associated with $\log_{10}$SAA concentrations. Therefore, based on this analysis it can be predicted that captive individuals with greater levels of stress will have an increased risk of developing AA amyloidosis. This is supported by the significant positive correlation between cortSTD and $\log_{10}$[SAA] in the NZP-SCBI population. Whether this association is a direct relationship between the role of GCs in the induction of SAA production during the APR or indirectly through other associated mechanisms is unknown. Interestingly, in this study captive female cheetahs had significantly higher cortSTD

compared to male captive cheetahs across both captive populations, which suggests that females may have an increased probability of developing AA amyloidosis, though a previous study declared that there is no association between the occurrence of amyloidosis and sex (Papendick et al. 1997).

Worth noting is the lack of correlation observed between serum cortisol concentrations and cortisol concentrations in feces. The lack of correlation was likely due to the timing of sample collection as well as the effect of sample collection itself. The majority of serum samples used in this study were not collected during the same time period as fecal sample collection. This is the result of using only opportunistic serum sample collection methods, such that an individual had to be under anesthesia for a purpose unrelated to this study for a sample to be collected. An effort was made to minimize the time between serum and fecal sample collection to minimize the effect on the data. For several of the male cheetahs, serum was collected during an anesthesia for electroejaculation procedures. Depending on the time during the anesthesia the serum sample was collected, cortisol concentrations may have been affected by the procedure itself, as described in Wildt et al. (1984). Also, it is possible that the method of capture and/or drugs used for anesthesia can affect serum cortisol concentrations. Therefore, any differences in these factors between animals or facilities can add a lot of variability to the serum cortisol concentration data. These effects may also explain the lack of correlation observed between serum cortisol concentrations and the other variables measured in serum: proinflammatory cytokine and SAA protein concentrations.

The captive CCF cheetah population provides the opportunity to begin to investigate the possible relationships between environmental factors such as exposure to humans, exercise or enclosure size with stress, inflammation and SAA protein concentrations, due to the size of the property and the various enclosures the cheetahs are kept in. The stress level of cheetahs included in this study appeared to be affected by both enclosure size and the frequency of human exposure, such that animals in small enclosures and animals exposed to a lot of people (on-exhibit) had higher cortisol concentrations in feces. This agrees with previous work demonstrating that when cheetahs are moved or housed on-exhibit, they tend to show greater levels of stress (higher GC concentrations) than when moved or housed off-exhibit (Terio et al. 2004, Wells et al. 2004) and the increases observed after movement can persist for months (Wells et al. 2004).

Similarly, cheetahs in smaller enclosures had significantly higher $\log_{10}$SAA concentrations than cheetahs kept in larger enclosures. When enclosure size is considered continuous (in lieu of small vs. large), $\log_{10}$[SAA] decreases by 0.008ng/ml for every additional acre of enclosure space. Therefore, to get effective decreases in $\log_{10}$[SAA] will require significant increases in enclosure size, which are likely unattainable at North American institutions.

Exercise also decreased $\log_{10}$[SAA], however this effect was dependent upon the other environmental factors, such that it only occurred among cheetahs kept in small enclosures with high human exposure. Regardless, considering the limitations in increasing enclosure size and reducing exposure to humans, regular exercise can likely be beneficial for the health of captive North American cheetahs.

Conclusion

Captive cheetahs at Smithsonian facilities show levels of stress greater than their captive counterparts at CCF in Namibia. These indicators of stress are associated with higher serum concentrations of the SAA protein, increasing the probability that they will develop AA amyloidosis during their lifetime. Until appropriate treatments become available that may control serum SAA protein concentrations, the best strategy to decreasing AA amyloidosis prevalence will be promoting captive population management practices that reduce stress, such as increasing enclosure size, or increasing opportunities for exercise where cheetahs are housed in small enclosures with high human exposure. Such practices could help re-establish proper immune system homeostasis and mitigate the overproduction of SAA protein, decreasing the probability of developing AA amyloidosis.

Acknowledgements

Funding for this project was provided by Emmanuel J. Friedman Philanthropies, the Grants-in-Aid for Research program from the National Academy of Sciences, administered by Sigma Xi, The Scientific Research Society and the William H. Donner Foundation

References

1. Auphan N, DiDonato JA, Rosette C, Helmberg A, Karin M. 1995. Immunosuppression by glucocorticoids: Inhibition of NF-κB activity through induction of IκB synthesis. Science 270:286-290.

2. Barnes PJ, Karin M. 1997. Nuclear factor-κB - a pivotal transcription factor in chronic inflammatory diseases. New England Journal of Medicine 336:1066-1071.

3. Baumann H, Gauldie J. 1994. The acute phase response. Immunology Today 15:74-80.

4. Beg AA, Finco TS, Nantermet PV, Baldwin AS. 1993. Tumor necrosis factor and interleukin-1 lead to phosphorylation and loss of IκBα: a mechanism for NF-κB activation. Molecular and Cellular Biology 13:3301-3310.

5. Biran H, Friedman N, Neumann L, Pras M, Shainkin-Kestenbaum R. 1986. Serum amyloid A (SAA) variations in patients with cancer: correlation with disease activity, stage, primary site, and prognosis. Journal of Clinical Pathology 39:794-797.

6. Bolton LA, Munson L. 1999. Glomerulosclerosis in Captive Cheetahs (*Acinonyx jubatus*). Veterinary Pathology 36:14-22.

7. Busch DS, Hayward LS. 2009. Stress in a conservation context: A discussion of glucocorticoid actions and how levels change with conservation-relevant variables. Biological Conservation 142:2844-2853.

8. Cerón JJ, Eckersall PD, Martinez-Subiela S. 2005. Acute phase proteins in dogs and cats: current knowledge and future perspectives. Veterinary Clinical Pathology 34:84-99.

9. Chen L, Une Y, Higuchi K, Mori M. 2012. Cheetahs have 4 serum amyloid A genes evolved through repeated duplication events. Journal of Heredity. 103(1):115-129.

10. Chrousos GP. 1995. The hypothalamic-pituitary-adrenal axis and immune-mediated inflammation. The New England Journal of Medicine 332:1351-1362.

11. Chrousos GP. 2009. Stress and disorders of the stress system. Nature Reviews Endocrinology 5(7):374-81.

12. Dhabhar FS. 2009. Enhancing versus suppressive effects of stress on immune function: implications for immunoprotection and immunopathology. Neuroimmunomodulation 16:300-317.

13. Elenkov IJ, Chrousos GP. 1999. Stress hormone, Th1/Th2 patterns, pro/anti-inflammatory cytokines and susceptibility to disease. Trends in Endocrinology and Metabolism 10(9):359-368.

14. Huberty CJ. 1994. Applied discriminant analysis. John Wiley and Sons, New York, New York, USA.

15. Gillmore JD, Lovat LB, Persey MR, Pepys MB, Hawkins PN. 2001. Amyloid load and clinical outcome in AA amyloidosis in relation to circulating concentration of serum amyloid A protein. The Lancet 358:244-29.

16. Giordano A, Spagnolo V, Colombo A, Paltrinieri S. 2004. Changes in some acute phase protein and immunoglobulin concentrations in cats affected by feline infectious peritonitis or exposed to feline coronavirus infection. The Veterinary Journal 167:38–44.

17. Jensen LE, Whitehead AS. 1998. Regulation of serum amyloid A protein expression during the acute-phase response. Biochemical Journal 334:489-503.

18. Johnson KH, Sletten K, Munson L, O'Brien TD, Papendick R, Westermark P. 1997. Amino acid sequence analysis of amyloid protein A (AA) from cats (captive cheetahs: *Acinonyx jubatus*) with a high prevalence of AA amyloidosis. Amyloid: International Journal of Experimental and Clinical Investigation 4:171-177.

19. Jurke MH, Czekala NM, Lindburg DG, Millard SE. 1997. Fecal corticoid metabolite measurements in the cheetah (*Acinonyx jubatus*). Zoo Biology 16:133-147.

20. Kajikawa T, Furuta A, Onishi T, Tajima T, Sugii S. 1999. Changes in concentrations of serum amyloid A protein, α-1-acid glycoprotein, haptoglobin, and C-reactive protein in feline sera due to induced inflammation and surgery. Veterinary Immunology and Immunopathology 68:91-98.

21. Kann RKC, Seddon JM, Henning J, Meers J. 2012. Acute phase proteins in healthy and sick cats. Research in Veterinary Science 93:649-654.

22. Keay JM, Singh J, Gaunt MC, Kaur T. 2006. Fecal glucocorticoids and their metabolites as indicators of stress in various mammalian species: a literature review. Journal of Zoo and Wildlife Medicine 37(3):234-244.

23. Kluve-Beckerman B, Hardwick J, Du L, Benson MD, Monia BP, Watt A, Crooke RM, Mullick A. 2011. Antisense oligonucleotide suppression of serum amyloid A reduces amyloid deposition in mice with AA amyloidosis. Amyloid: International Journal of Experimental and Clinical Investigation 18:136-146.

24. Köster LS, Schoeman JP, Meltzer DGA. 2007. ACTH stimulation test in the captive cheetah (*Acinonyx jubatus*). Journal of the South African Veterinary Association 78(3):133-136.

25. Lachmann HJ, Goodman HJB, Gilbertson JA, Gallimore JR, Sabin CA, Gillmore JD, Hawkins PN. 2007. Natural history and outcome in systemic AA amyloidosis. New England Journal of Medicine 356:2361-2371.

26. Marhaug G, Dowton SB. 1994. Serum amyloid A: an acute phase apolipoprotein and precursor of AA amyloid. Baillière's Clinical Rheumatology 8:553-573.

27. Migita K, Yamasaki S, Shibatomi K, Ida H, Kita M, Kawakami A, Eguchi K. 2001. Impaired degradation of serum amyloid A (SAA) protein by cytokine-stimulated monocytes. Clinical and Experimental Immunology 123:408-411.

28. Munson L. 1993. Diseases of Captive Cheetahs (*Acinonyx jubatus*): Results of the Cheetah Research Council Pathology Survey, 1989-1992. Zoo Biology 12:105-124.

29. Munson L, Nesbit JW, Meltzer DGA, Colly LP, Bolton L, Kreik LPJ. 1999. Diseases of captive cheetahs (*Acinonyx jubatus jubatus*) in South Africa: a 20-year retrospective survey. Journal of Zoo and Wildlife Medicine 30(3):342-347.

30. Munson L, Terio K, Worley M, Jago M, Bagot-Smith A, Marker L. 2005. Extrinsic factors significantly affect patterns of disease in free-ranging and captive cheetah (*Acinonyx jubatus*) populations. Journal of Wildlife Diseases 41(3):542-548.

31. Ofri R, Nyska A, Linke RP, Shtrasburg S, Livneh A, Gal R. 1997. Systemic amyloidosis in a cheetah (*Acinonyx jubatus*). Amyloid: International Journal of Experimental and Clinical Investigation 4(2):98-103.

32. Paltrinieri S. 2008. The feline acute phase reaction. The Veterinary Journal 177:26-35.

33. Papendick RE, Munson L, O'Brien TD, Johnson KH. 1997. Systemic AA amyloidosis in captive cheetahs (*Acinonyx jubatus*). Veterinary Pathology 34:549-556.

34. Pras M, Schubert M, Zucker-Franklin D, Rimon A, Franklin EC. 1968. The characterization of soluble amyloid prepared in water. The Journal of Clinical Investigation 47:924-933.

35. Sapolsky RM, Romero LM, Munck AU. 2000. How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory and preparative actions. Endocrine Reviews 21(1):55-89.

36. Sasaki K, Ma Z, Khatlani TS, Okuda M, Inokuma H, Onishi T. 2003. Evaluation of feline serum amyloid A (SAA) as an inflammatory marker. Journal of Veterinary Medical Science 65:545–548.

37. Scheinman RI, Cogswell PC, Lofquist AK, Baldwin AS. 1995. Role of transcriptional activation of IκBα in mediation of immunosuppression by glucocorticoids. Science 270:283-286.

38. Tamamoto T, Ohno K, Goto-Koshino Y, Fujino Y, Tsujimoto H. 2012. Serum amyloid A uptake by feline peripheral macrophages. Veterinary Immunology and Immunopathology 150:47-52.

39. Tamamoto T, Ohno K, Ohmi A, Goto-Koshino Y, Tsujimoto H. 2008. Verification of measurement of the feline serum amyloid A (SAA) concentration by human SAA turbidimetric immunoassay and its clinical application. Journal of Veterinary Medical Science 70:1247-1252.

40. Terio KA, Marker L, Munson L. 2004. Evidence for chronic stress in captive but not free-ranging cheetahs (*Acinonyx jubatus*) based on adrenal morphology and function. Journal of Wildlife Diseases 40(2):259-266.

41. Terio KA, Marker L, Overstrom EW, Brown JL. 2003. Analysis of ovarian and adrenal activity in Namibian cheetahs. South African Journal of Wildlife Research 33(2):71-78.

42. Thorn CF, Whitehead AS. 2002. Differential glucocorticoid enhancement of the cytokine-driven transcriptional activation of the human acute phase serum amyloid A genes, SAA1 and SAA2. Journal of Immunology 169:399-406.

43. Wells A, Terio KA, Ziccardi MH, Munson L. 2004. The stress response to environmental change in captive cheetahs (*Acinonyx jubatus*). Journal of Zoo and Wildlife Medicine 35(1):8-14.

44. Wiegers GJ, Reul JMHM. 1998. Induction of cytokine receptors by glucocorticoids: functional and pathological significance. Trends in Pharmacological Sciences 19:317-321.

45. Wielebnowski NC, Ziegler K, Wildt DE, Lukas J, Brown JL. 2002. Impact of social management on reproductive, adrenal and behavioural activity in the cheetah (*Acinonyx jubatus*). Animal Conservation 5:291-301.

46. Wildt DE, Meltzer D, Chakraborta PK, Bush M. 1984. Adrenal-testicular-pituitary relationships in the cheetah subjected to anesthesia/electroejaculation. Biology of Reproduction 30:665-672.

47. Young KM, Walker SL, Lanthier C, Waddell WT, Monfort SL, Brown JL. 2004. Noninvasive monitoring of adrenocortical activity in carnivores by fecal glucocorticoid analyses. General and Comparative Endocrinology 137(2):148-165.

48. Zhang B, Une Y, Fu X, Yan J, Ge F, Yao J, Sawashita J, Masayuki M, Tomozawa H, Kametani F, Higuchi K. 2008. Fecal transmission of AA amyloidosis in the cheetah contributes to high incidence of disease. Proceedings of the National Academy of Sciences of the United States of America 105(20):7263-7268.

Chapter 5: Summary of study results and the implications for population management of captive cheetahs (*Acinonyx jubatus*)

Genetics

Marker-assisted selection

Among the commercial animal industry, marker-assisted selection (MAS) is a movement towards using genetic data to make selection and breeding decisions that have been traditionally made using phenotypic records based on best linear unbiased prediction (BLUP; Henderson 1984). The use of MAS has historically been constrained by the limited availability of genomic information. With costs for whole genome sequencing and resequencing decreasing and the invention of novel SNP (single nucleotide polymorphism) discovering technologies, the genomic information required to apply marker-assisted selection in management practices is becoming much more feasible to acquire. Fernando and Grossman (1989) developed a model for BLUP that could incorporate the marker information into the genetic evaluation of individuals still accounting for fixed effects and phenotypic data. This model is even able to accommodate individuals who have missing or incomplete marker information.

Traits that are generally targeted by commercial breeding programs have economic value, are quantitative in nature, and are affected by small gene effects from many loci, otherwise termed quantitative trait loci (QTL), and the influence of the environment. Statistical associations are measured between phenotypic

observations and the QTL. Variants (marker alleles) of the QTL that are significantly associated with favorable phenotypic values (and likely associated with a gene that controls the trait of interest) are targeted for selection. Individuals who carry these alleles are thus preferential breeders.

MAS is variable in its success, mainly due to the unknown relationship between the marker alleles and the genes of interest. A marker located within the gene of interest yields the greatest success, particularly when the gene has a large effect on the trait, because association between the marker and the gene is perfect. Markers in linkage disequilibrium with the gene of interest show some success, but are limited by the quality of the data used to determine the statistical associations and the distance between the marker and the gene. Genetic improvement from MAS is also limited to the variance in the trait that is explained by the QTL. Important to note as well, is that individual markers or genes cannot be selected. Whole individuals are ultimately the unit for selection and positive effect alleles at one locus may be counter-balanced by negative effect alleles at another. The breeding value for an individual is thus determined by a sum of all the allele effects across all loci (Fernando and Grossman 1989).

MAS can be used to increase the rate of genetic gain in commercially bred animal populations when compared to traditional selection methods (Meuwissen et al. 2001). However, long term predictions of gain are dependent upon the number of alleles, the initial allele frequencies and the distribution of allele effects (Meuwissen and Goddard 1996), and response rates to MAS decrease rapidly after many

generations of selection due to the breakdown of linkage-disequilibrium (Habier et al. 2009, Meuwissen et al. 2001).

When to use marker-assisted selection

Marker-assisted selection is most effectively used when accuracy of selection is low using traditional methods. This occurs most frequently when heritability of a trait is low or the number of data points (phenotypic records obtainable) is low due to expense or availability. Relative efficiency of MAS is greatest when the heritability of the selected trait is low (Lande and Thompson 1990) because phenotypic information from the individual is less informative alone for genetic merit. Marker-assisted selection increases selection accuracy in young animals, because selection decisions can be made before there is a record for the trait of interest (Calus et al. 2008, Lande and Thompson 1990, Meuwissen and Goddard 1996). Earlier selection of individuals reduces the generation interval in a population, which further increases the rate of genetic gain obtained. Accuracy of selection can also be low for disease resistance-related traits (unobservable in most cases) and sex-limited traits (because records only exist for one sex). MAS provides the ability to select opposite sex individuals in the event of sex-limited traits.

Role of MAS in captive breeding programs

Marker-assisted selection in conservation breeding programs can play a similar role as it does in commercial breeding, but with a different purpose. MAS could be beneficial to conservation programs by increasing the effective size of small populations (Wang 2001) and decreasing inbreeding accumulation (Pedersen et al. 2009) in excess of current breeding practices. It could also be used to select

individuals who have underrepresented genotypes (or haplotypes) to maintain genetic diversity in captivity (Ballou and Lacy 1995), particularly in populations where previous breeding has been highly skewed towards individuals who perform well in captivity (Ballou and Lacy 1995). Identification of marker alleles that are unique in an individual would make it a strong selection candidate to ensure representation of that genotype in the next generation.

There are multiple strategies that exist today for breeders from zoos and other wildlife centers to utilize when making breeding decisions. The two most common strategies to date are maximum avoidance of inbreeding (MAI; Kimura and Crow 1963) and minimizing mean kinship, defined as the average of the kinship coefficients between an individual and all other living individuals (MK; Ballou and Lacy 1995). MAI works by minimizing any further inbreeding accumulation in the population, without utilizing any historical pedigree information. MK works by minimizing the overall level of relationship among individuals in a population. MK does use historical pedigree information and is advantageous over MAI when there are unequal contributions of the initial founders to the current population (Montgomery et al. 1997). Minimizing mean kinship, while considering demographic constraints, as a breeding strategy appears to have been the most effective to date for retaining the greatest amount of founder genetic diversity (Ballou and Lacy 1995, Montgomery et al. 1997, Rodriguez-Clark 1999). However, whether this provides any fitness advantages or improves the probability of persistence for populations under natural selective pressures has yet to be determined (Montgomery et al. 1997, Rodriguez-Clark 1999).

The use of genomic information can also give population managers a more accurate estimate of kinship between individuals and inbreeding accumulation, making both MAI and MK more effective strategies for conserving genetic diversity through captive breeding programs. One of the greatest benefits MAS can likely provide in conservation breeding is the ability to make selection decisions within families (among full siblings) whose genetic merit would otherwise appear equal.

General criteria for the use of genotyping in non-model species includes of the ability to obtain high quality genotypes from small amounts or low quality DNA samples (Morin et al. 2004) and low cost and rapid ascertainment of sequence information per genome (Morin et al. 2004). If a species is threatened or endangered, there may be very few individuals to sample. Screening fewer individuals is going to find more markers with intermediate allele frequencies (ascertainment bias), which would not be selection target loci of interest when the goal is to identify and preserve rare or unique haplotypes. To detect SNPs of greater interest, candidate genes with known functions that influence fitness or fitness-related traits could be sequenced and analyzed for SNPs (Morin et al. 2004). SNPs closely linked to such genes are likely better candidates for genetic improvement through marker-assisted selection. The SNP in the putative NF-κB binding site of the *SAA1A* promoter in cheetahs is an example of a possible candidate.

Relevance of the $SAA1A^{-97delG}$ SNP in the NF-κB binding site in SAA1A promoter in captive cheetah population management

Before beginning this study, it was hypothesized that animals with the $SAA1A^{+}$ allele should have greater transcriptional induction of the SAA1A gene

during inflammation and that this allele would be associated with a higher incidence of AA amyloidosis. If this relationship was true, the SNP in the putative NF-κB binding site within the promoter region of the SAA1A gene locus would be a potential target for MAS. MAS could be a beneficial component of the captive breeding program to decrease the prevalence of AA amyloidosis among captive cheetahs and promote a self-sustaining population.

Based on the results of this study (Chapter 2), even though there was a significant decrease in the $\log_{10}$SAA protein concentration in serum as the number of copies of the $SAA1A^{-97delG}$ SNP increased, thus appearing to reduce the risk of developing AA amyloidosis in the presence of chronic or persistent inflammation, I cannot recommend this locus to be targeted for MAS in the captive North American cheetah population for the following reasons: (1) the $SAA1A^{-97delG}$ allele is already the more common allele in the captive North American cheetah population, thus selecting individuals with this allele would actually decrease the genetic diversity at this locus and this opposes the overall goals of the captive breeding program; and (2) there was no association found between the SNP and AA amyloidosis diagnosis in the captive North American cheetah population, therefore the differences in the $\log_{10}$SAA protein concentration observed between genotypes may not be relevant biologically in terms of producing a predisposition to AA amyloidosis.

Inflammation

The role of proinflammatory cytokines (TNF-α, IL-1β, IL-6) in AA amyloidosis pathogenesis

Almost 100% of captive cheetahs in North America have gastritis (a chronic inflammatory condition) to some degree (Munson 1993, Munson et al. 1999, Munson et al. 2005). However, the present study found proinflammatory cytokine concentrations to be highest and most variable among wild and "soft-release" cheetahs and lowest among the captive Smithsonian cheetahs. If one were to examine the Smithsonian population only, they may suggest that cheetahs are immune deficient, citing evidence for the lack of genetic variation in cheetahs, including a study that showed a lack of skin graft rejection among unrelated cheetahs (O'Brien et al. 1985). However, more recent studies have shown that the MHC class I α1 and α2, as well as MHC class II β1 domains are not monomorphic, with some amino acid differences located in sites important for antigen binding (Castro-Prieto et al. 2011). Therefore, cheetahs are not entirely immune-incompetent.

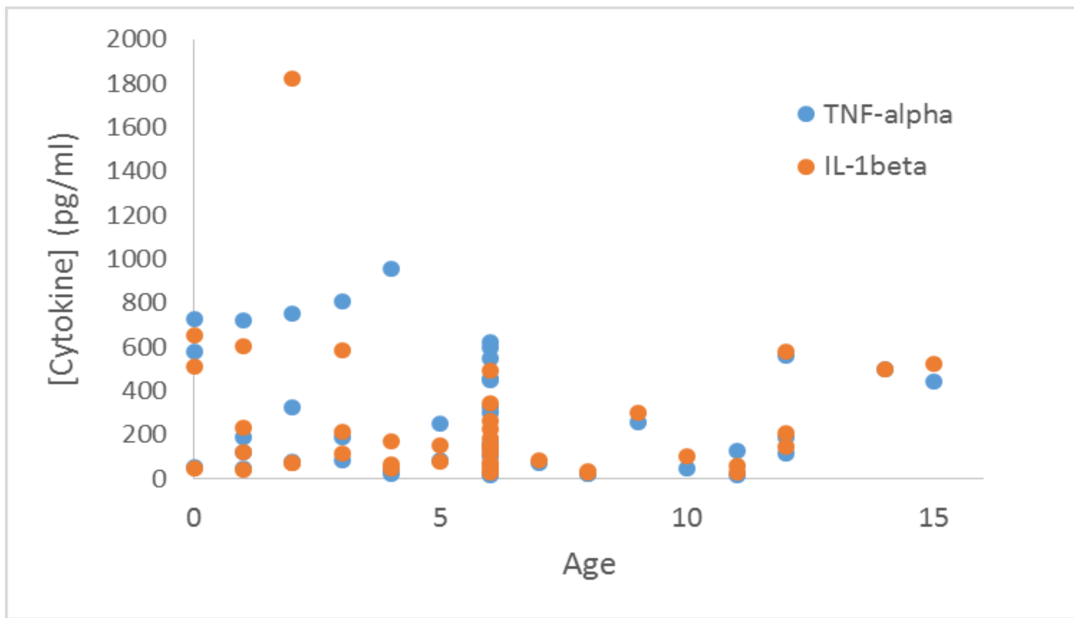
One SCBI cheetah, 114681, did have high proinflammatory cytokine concentrations in the range of wild cheetahs, but was excluded from the analysis for being a significant outlier relative to the NZP-SCBI population. In addition, the PBMC stimulation experiment (Chapter 3) was successfully able to induce activation of and proinflammatory cytokine production from freshly isolated cheetah monocytes. Both of these results suggest that cheetahs are not immune-incompetent. Interesting to note is that the amount of cytokines produced in response to LPS stimulation was widely varied between the three different cheetahs' cells, which may

suggest some individuals' cells are more capable of responding to stimulus, though this effect may not be genetic because the three male cheetahs used in the experiment were full-sibling brothers. It could be possible that "pre-treatment" *in vivo* of the monocytes with glucocorticoids could have made them less responsive, however the individual with the least responsive cells actually had the lowest concentration of cortisol in a serum sample drawn at the same time as the whole blood collection, so that does not appear to be the explanation. It is also possible that the number of cells remaining in culture after "washing" were not equal between animals. A cell-concentration dependent effect was observed during this experiment within animals, so it would be logical to think the differences observed between individuals could, to some degree, be attributable to the same effect.

In domestic cats, the production of cytokines decreases from youth to middle age then increases again in elderly animals (Paltrinieri 2008). There is not enough variability among the NZP-SCBI captive cheetahs to look for this type of effect; however, we do see a similar pattern among the captive cheetahs at CCF (Figure 5.1).

Though not intuitive, the way that captive cheetahs are fed may be related to the amounts of circulating proinflammatory cytokines. Insulin is inhibitive to IL-1 and IL-6 type cytokine responses (Baumann and Gauldie 1994). Most of the captive cheetahs at SCBI are fed once a day, but for those individuals being fed more than once a day, it is possible that more frequent releases of insulin may contribute to the lower concentrations of proinflammatory cytokines observed in these animals. Captive cheetahs at CCF are fed only once a day, 6 days per week and wild or "soft-release" cheetahs likely feed only once every few days, depending on the frequency

Figure 5.1. Proinflammatory cytokine concentrations in captive cheetahs at CCF by age.



and success of hunting efforts. It can be argued that if the captive cheetahs have elevated epinephrine due to stress that insulin secretion is inhibited (Norris 2007). However, elevated epinephrine also inhibits proinflammatory cytokine production, thus it is difficult to separate these effects and their influences on the differences in proinflammatory cytokines observed between populations.

There are numerous studies that show various ways in which proinflammatory cytokine gene expression and protein production is inhibited by glucocorticoids, thus I believe the most likely explanation for this study's results is dysregulation of the immune system and suppression of the production of proinflammatory cytokines in captive NZP-SCBI cheetahs due to chronic stress (discussed in more detail in Chapter 4).

Implications of inflammation for captive cheetah population management

Previous studies have shown that all cheetahs diagnosed with amyloidosis also suffer from other significant chronic inflammatory diseases (Papendick et al. 1997) such that the high prevalence of AA amyloidosis among captive cheetahs is likely related to chronic inflammation. Unfortunately, this study was unable to get a clear picture of chronic inflammation among captive North American cheetahs based on a single blood sample from each individual and the measurement of just three cytokines, particularly when these cheetahs showed evidence of stress (elevated cortisol) that may be suppressing the production of proinflammatory cytokines. Further studies are required to understand the role of proinflammatory cytokines in AA amyloidosis pathogenesis specifically within the North American captive cheetah population.

Stress

The role of stress in AA amyloidosis pathogenesis

Based on this study, the most significant factor related to SAA protein concentration in serum and the probability of developing AA amyloidosis was stress: stress is measured as the baseline and/or average cortisol concentration in feces over time. The physiological changes that occur in response to acute stress are intended to increase the short-term survival of an organism, but when stress becomes chronic, these same changes can become detrimental. There is evidence for seasonal changes in glucocorticoids (GCs) due to changes in the allostatic load, or the animal's requirements to survive (Busch and Hayward 2009). This, combined with an increase in acute stress events (hunting, injury, intra- or inter-specific conflicts), is the most likely explanation for the higher cortisol concentrations observed in wild cheetahs compared to captive CCF cheetahs in the same environment.

Hunting is one of the most fundamental behaviors observed in felid species, and when felids are put in captivity, they are deprived of the ability to hunt. Though this may protect them from the physical stress of hunting or getting injured, any physiological benefits associated with hunting (e.g. mental stimulation and exercise) are also lost. In this study, exercise was associated with a decrease in SAA protein concentrations in cheetahs kept in small enclosures with frequent human exposure. This suggests a physiological benefit of exercise among cheetahs in a zoological setting, perhaps simply by reducing stress, or a more complex scenario such as the activation of the sympathetic nervous system decreasing proinflammatory cytokines via its anti-inflammatory actions. Many of the captive cheetahs at CCF are exercised

regularly, either on a lure system or by chasing the feeding truck. Therefore, exercise may partially explain the population differences observed in stress and/or SAA protein concentrations. Larger enclosures and low frequency of exposure to humans were also associated with lower SAA protein concentrations in serum.

Implications of stress for captive cheetah population management

Regardless of the mechanism behind the association of stress with higher SAA protein concentrations, it seems that measures should be taken to decrease stress whenever possible. This may mean limiting the number of times an individual is moved between facilities, since this is known to be associated with long-term increases in stress in cheetahs (Wells et al. 2004). For individuals that are identified as being particularly stressed, it may mean considering moving them to a facility where they will no longer be on exhibit to the public (decreasing exposure to humans), particularly if the animal has a breeding recommendation. Limiting the number of keepers that are taking care of the cheetahs on a daily basis may be another effective way to decrease stress associated with human interactions.

Since cheetahs are known as the fastest land mammal, increasing the amount of exercise individuals are getting could be a really unique management strategy employed that may even improve the visitor experience at a zoo if exercising is done during business hours and the public has access to watch. A good example of this is the Cheetah Encounter Show at the Cincinnati Zoo. Cheetahs used in this program have lower fecal GCs compared to other cheetahs (Bill Swanson, personal communication).

Other possible management considerations

Dietary requirements

A recently published study by Liu et al. (2014) found that mice hepatocytes cultured in zinc deficient media stimulated with IL-6 and IL-1β produced significantly more SAA1 protein than cells cultured in zinc-sufficient media. The study illustrated that zinc deficiency results in more phosphorylation of IκB proteins, leading to their disassociation from the NF-κB transcription factor, subsequently allowing NF-κB to enter the nucleus and promote transcription of the SAA1 gene (Liu et al. 2014). Therefore, it is possible that captive cheetahs eating a zinc deficient diet may be predisposed to developing AA amyloidosis.

Species specific dietary requirements for exotic feline species are not actually known. Diets fed to captive felids in Association of Zoos and Aquariums (AZA) accredited institutions meet the Association of American Feed Control Officials (AAFCO) criteria determined for domestic cats, as they are the closest model species for larger captive felids. It is possible that cheetahs are unique and have higher zinc requirements than domestic cats. Even so, captive diets seem to be over supplemented with zinc (Cheryl Morris and Michael Maslanka, personal communication), so this is likely not the driving force behind the high incidence of AA amyloidosis in captivity. Future studies may consider measuring serum concentrations of zinc to see if it is linked to the increases in SAA protein concentrations observed.

Medical treatments

Due to the positive, synergistic effect of GCs with cytokines in the induction of SAA synthesis during the APR (Chrousos 1995, Dhabhar 2009, Marhaug and

Dowton 1994), administering glucocorticoids as a therapy for diseases characterized with chronic inflammation may have an undesirable or even harmful effect resulting in higher serum SAA concentrations. Therefore, it may not be appropriate for use in cheetahs. The benefit of GC treatment vs. the possibility of developing AA amyloidosis should be considered before treatment is given.

A promising therapy using SAA-specific antisense oligonucleotides (ASO) has been shown to reduce SAA concentration in mice, particularly by facilitating a more rapid return to baseline after episodic bursts in SAA concentration (Kluve-Beckerman et al. 2011). SAA-specific ASOs were also shown to reduce amyloid deposition in treated mice in which amyloidosis was previously induced (Kluve-Beckerman et al. 2011). Whether ASOs could be a therapy to treat systemic amyloidosis in either humans or cheetahs has yet to be investigated.

The simplest answer to decreasing AA amyloidosis prevalence among captive cheetahs may simply be finding a way to control gastritis since AA amyloidosis is almost always associated with gastritis (Papendick et al. 1997). However, gastritis also appears to be linked to chronic stress in captive cheetahs, so controlling stress should always be emphasized.

Conclusion

Controlling the amount of psychological stress imposed on cheetahs needs to be emphasized foremost in the management of captive cheetah populations because chronic stress is so intimately linked to decreased health in this species. In cases where stress is unavoidable, possible dietary considerations, exercise or medical

treatments may be found to be beneficial in decreasing SAA protein concentrations and decreasing AA amyloidosis prevalence.

References

1. Ballou JD, Lacy RC. 1995. Identifying genetically important individuals for management of genetic variation in pedigreed populations. In Ballou, JD, Gilpin M, Foose TJ (eds.), Population Management for Survival and Recovery: Analytical Methods and Strategies in Small Population Conservation, 76-111, Columbia University Press, New York.

2. Baumann H, Gauldie J. 1994. The acute phase response. Immunology Today 15(2):74-80.

3. Busch DS, Hayward LS. 2009. Stress in a conservation context: A discussion of glucocorticoid actions and how levels change with conservation-relevant variables. Biological Conservation 142:2844-2853.

4. Calus MPL, Meuwissen THE, de Roos APW, Veerkamp RF. 2008. Accuracy of genomic selection using different methods to define haplotypes. Genetics 178:553-561.

5. Castro-Prieto A, Wachter B, Sommer S. 2011. Cheetah paradigm revisited: MHC diversity in the world's largest free-ranging population. Molecular Biology and Evolution 28:1455-1468.

6. Chrousos GP. 1995. The hypothalamic-pituitary-adrenal axis and immune-mediated inflammation. The New England Journal of Medicine 332:1351-1362.

7. Dhabhar FS. 2009. Enhancing versus suppressive effects of stress on immune function: Implications for immunoprotection and immunopathology. Neuroimmunomodulation 16:300-317.

8. Fernando RL, Grossman M. 1989. Marker assisted selection using best linear unbiased prediction. Genetics Selection Evolution 21:467-477.

9. Habier D, Fernando RL, Dekkers JCM. 2009. Genetic selection using low density marker panels. Genetics 182(1):343–353.

10. Henderson CR. 1984. Applications of linear models in animal breeding. University of Guelph, Guelph, Ontario.

11. Kimura M, Crow JF. 1963. On the maximum avoidance of inbreeding. Genetical Research 4:399-415.

12. Kluve-Beckerman B, Hardwick J, Du L, Benson MD, Monia BP, Watt A, Crooke RM, Mullick A. 2011. Antisense oligonucleotide suppression of

serum amyloid A reduces amyloid deposition in mice with AA amyloidosis. Amyloid: International Journal of Experimental and Clinical Investigation 18:136-146.

13. Lande R, Thompson R. 1990. Efficiency of marker-assisted selection in the improvement of quantitative traits. Genetics 124:743-756.

14. Liu M-J, Bao S, Napolitano JR, Burris DL, Yu L, Tridandapani S, Knoell DL. 2014. Zinc regulates the acute phase response and serum amyloid A production in response to sepsis through JAK-STAT3 signaling. PLOS One 9(4):e94934. doi:10.1371/journal.pone.0094934.

15. Marhaug G, Dowton SB. 1994. Serum amyloid A: an acute phase apolipoprotein and precursor of AA amyloid. Baillière's Clinical Rheumatology 8:553-573.

16. Meuwissen THE, Goddard ME. 1996. The use of marker haplotypes in animal breeding schemes. Genetics Selection Evolution 28:161-176.

17. Meuwissen THE, Hayes BJ, Goddard ME. 2001. Prediction of total genetic value using genome-wide dense marker maps. Genetics 157:1819-1829.

18. Montgomery ME, Ballou JD, Nurthen RK, England PR, Briscoe DA, Frankham R. 1997. Minimizing kinship in captive breeding programs. Zoo Biology 16:377-389.

19. Morin PA, Luikart G, Wayne RK, and the SNP workshop group. 2004. SNPs in ecology, evolution and conservation. Trends in Ecology and Evolution 19:209-216.

20. Norris DO. 2007. Vertebrate Endocrinology (4th ed.). Elsevier Academic Press, Burlington, MA.

21. O'Brien SJ, Roelke ME, Marker L, Newman A, Winkler CA, Meltzer D, Colly L, Evermann JF, Bush M, Wildt DE. 1985. Genetic Basis for Species Vulnerability in the Cheetah. Science 227:1428-1434.

22. Paltrinieri S. 2008. The feline acute phase reaction. The Veterinary Journal 177:26-35.

23. Papendick RE, Munson L, O'Brien TD, Johnson KH. 1997. Systemic AA amyloidosis in captive cheetahs (*Acinonyx jubatus*). Veterinary Pathology 34:549-556.

24. Pedersen LD, Sorensen AC, Berg P. 2009. Marker-assisted selection can reduce true as well as pedigree-estimated inbreeding. Journal of Dairy Science 92:2214-2223.

25. Rodriguez-Clark KM. 1999. Genetic theory and evidence supporting current practices in captive breeding for conservation. In Landweber LF, Dobson AP. (eds.), Genetics and the Extinction of Species, 47-65, Princeton University Press, Princeton.

26. Wang J. 2001. Optimal marker-assisted selection to increase the effective size of small populations. Genetics 157:867-874.

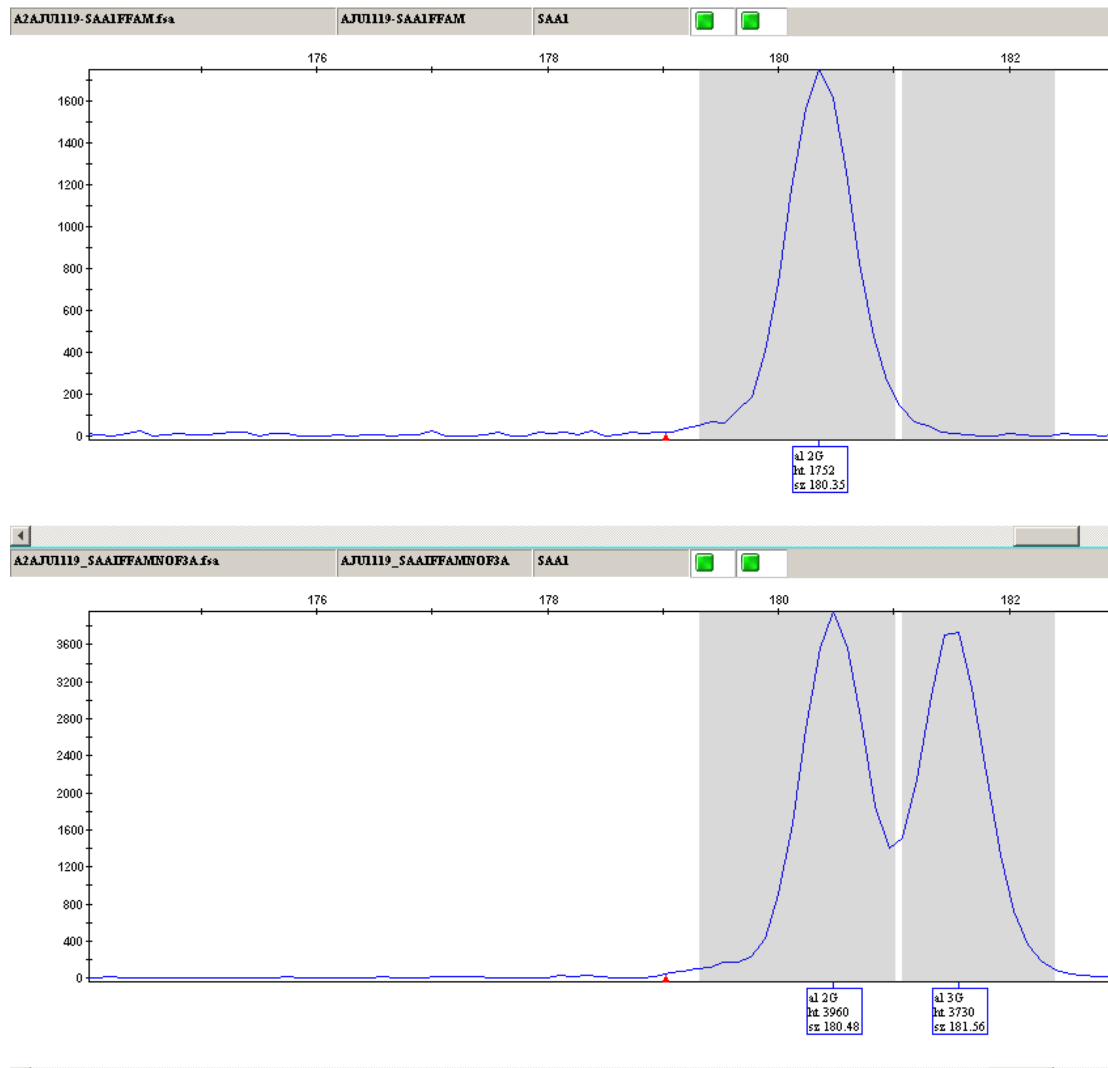
27. Wells A, Terio KA, Ziccardi MH, Munson L. 2004. The stress response to environmental change in captive cheetahs (*Acinonyx jubatus*). Journal of Zoo and Wildlife Medicine 35(1):8-14.

Appendix I. Confirmation of *SAA1A* promoter specificity of F3A primer via nested vs. non-nested PCR.

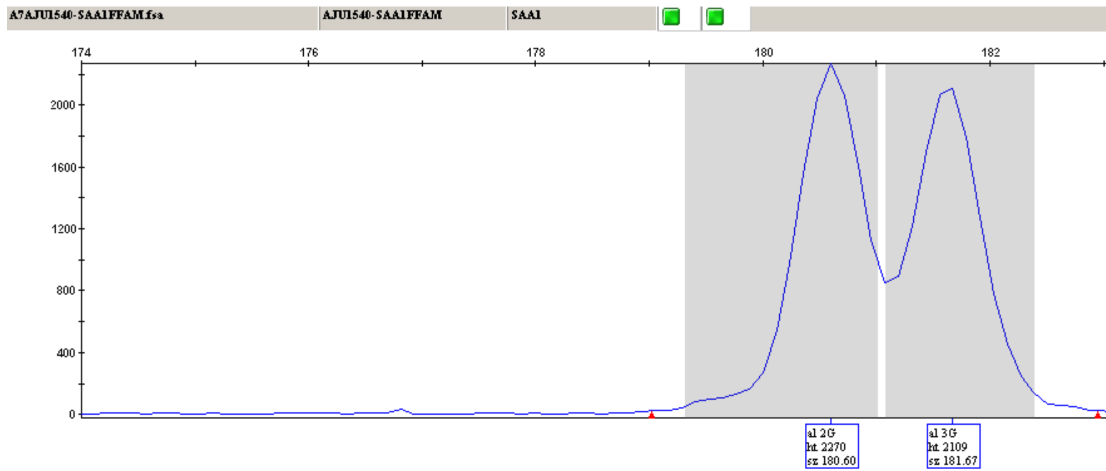
SAA1FFAM = 2nd round PCR product from nested PCR

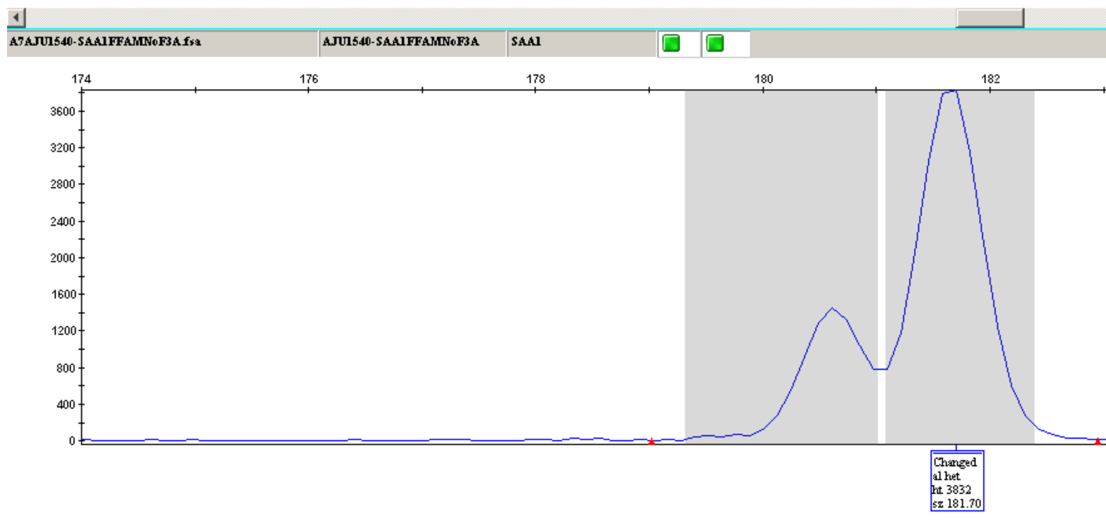
SAA1FFAMNOF3A = PCR product from SAA1F/SAA1R primers only. No 1st round of PCR performed (F3A primer).

Representative *SAA1A*$^{-97delG/-97delG}$

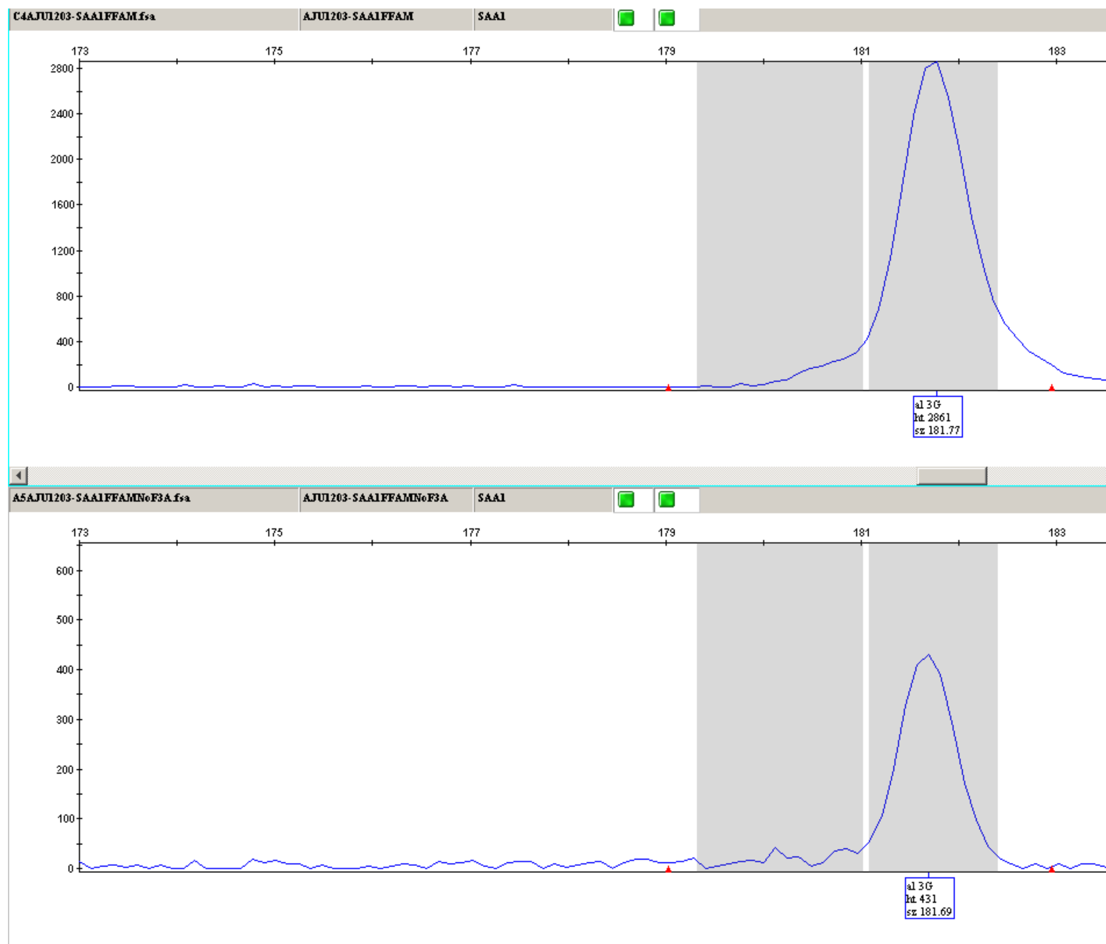


Representative $SAA1A^{+/-97delG}$





Representative *SAA1A*$^{+/+}$



Appendix II. Complete list of fecal and serum samples collected from cheetahs.

Status	Population	Location	ID	Sex	Age	Fecal samples (#)	Serum sample (Y/N)
Captive	North America	NZP	113904	Female	11	43	Yes
Captive	North America	SCBI	113906	Female	10	44	Yes
Captive	North America	NZP	114193	Male	6	39	Yes
Captive	North America	NZP	114194	Male	6	39	Yes
Captive	North America	NZP	114195	Male	6	41	Yes
Captive	North America	SCBI	114277	Female	7	39	Yes
Captive	North America	SCBI	114382	Female	5	40	Yes
Captive	North America	SCBI	114486	Female	8	50	Yes
Captive	North America	SCBI	114539	Male	3	39	Yes
Captive	North America	SCBI	114540	Male	3	38	Yes
Captive	North America	SCBI	114541	Male	3	39	Yes
Captive	North America	SCBI	114645	Male	1	43	Yes
Captive	North America	SCBI	114680	Male	1	40	Yes
Captive	North America	SCBI	114681	Female	1	40	Yes
Captive	North America	SCBI	114682	Female	2	37	Yes
Captive	North America	SCBI	114701	Male	8	37	Yes
Captive	North America	SCBI	114702	Male	8	42	Yes
Captive	North America	SCBI	114735	Male	1	36	Yes
Captive	North America	SCBI	114736	Female	2	41	No
Captive	North America	SCBI	114748	Female	8	50	Yes
Captive	North America	SCBI	114799	Male	9	39	Yes
Captive	Namibia	CCF	1119	Female	15	14	Yes
Captive	Namibia	CCF	1203	Female	13	16	Yes
Captive	Namibia	CCF	1204	Female	13	14	Yes
Captive	Namibia	CCF	1206	Female	13	13	Yes
Captive	Namibia	CCF	1233	Female	13	14	Yes
Captive	Namibia	CCF	1237	Female	13	13	Yes
Captive	Namibia	CCF	1340	Female	15	10	Yes
Captive	Namibia	CCF	1355	Female	11	14	Yes
Captive	Namibia	CCF	1380	Female	10	13	Yes
Captive	Namibia	CCF	1444	Female	9	7	Yes
Captive	Namibia	CCF	1493	Female	9	14	Yes
Captive	Namibia	CCF	1510	Female	7	14	Yes
Captive	Namibia	CCF	1511	Female	7	14	Yes
Captive	Namibia	CCF	1512	Female	7	14	Yes
Captive	Namibia	CCF	1513	Male	7	14	Yes
Captive	Namibia	CCF	1514	Female	7	14	Yes

Captive	Namibia	CCF	1515	Male	7	14	Yes
Captive	Namibia	CCF	1516	Male	7	14	Yes
Captive	Namibia	CCF	1517	Female	7	14	Yes
Captive	Namibia	CCF	1518	Male	7	15	Yes
Captive	Namibia	CCF	1560	Female	5	14	Yes
Captive	Namibia	CCF	1565	Male	5	14	Yes
Captive	Namibia	CCF	1578	Female	5	14	Yes
Captive	Namibia	CCF	1600	Female	3	15	Yes
Captive	Namibia	CCF	1601	Male	3	14	Yes
Captive	Namibia	CCF	1602	Male	3	14	Yes
Captive	Namibia	CCF	1603	Female	3	14	Yes
Captive	Namibia	CCF	1607	Male	3	14	Yes
Captive	Namibia	CCF	1616	Male	3	14	Yes
Captive	Namibia	CCF	1627	Male	2	14	Yes
Captive	Namibia	CCF	1632	Female	5	9	Yes
Captive	Namibia	CCF	1640	Female	0	14	Yes
Captive	Namibia	CCF	1641	Female	0	14	Yes
Captive	Namibia	CCF	1642	Female	1	14	Yes
Soft-Release	Namibia	Bellebenno	1243	Female	10	3	Yes
Soft-Release	Namibia	Bellebenno	1348	Female	8	2	Yes
Soft-Release	Namibia	Bellebenno	1349	Female	8	4	Yes
Soft-Release	Namibia	Bellebenno	1351	Female	8	1	Yes
Soft-Release	Namibia	Bellebenno	1513	Male	6	2	No
Soft-Release	Namibia	Bellebenno	1515	Male	6	1	No
Soft-Release	Namibia	Bellebenno	1516	Male	6	1	No
Soft-Release	Namibia	Bellebenno	1518	Male	6	1	No
Soft-Release	Namibia	Bellebenno	1539	Male	6	6	No
Soft-Release	Namibia	Bellebenno	1540	Male	4	5	No
Soft-Release	Namibia	Bellebenno	1545	Male	3	5	No
Soft-Release	Namibia	Bellebenno	1560	Female	4	1	Yes
Soft-Release	Namibia	Bellebenno	1561	Male	3	9	No
Soft-Release	Namibia	Bellebenno	1578	Female	4	1	Yes
Wild	Namibia	Wild	1537	Male	6	9	Yes
Wild	Namibia	Wild	1538	Male	6	1	Yes
Wild	Namibia	Wild	1542	Male	6	15	Yes
Wild	Namibia	Wild	1543	Male	6	15	Yes
Wild	Namibia	Wild	pop169	Male	unk	7	No
Wild	Namibia	Wild	pop170	Male	unk	7	No
Wild	Namibia	Wild	Scat1161	Male	unk	10	No

NZP = Smithsonian National Zoological Park, SCBI = Smithsonian Conservation Biology Institute, CCF = Cheetah Conservation Fund, Bellebenno = Soft-release camp at CCF, unk = unknown.

Bibliography

1. Auphan N, DiDonato JA, Rosette C, Helmberg A, Karin M. 1995. Immunosuppression by glucocorticoids: Inhibition of NF-κB activity through induction of IκB synthesis. Science 270:286-290.

2. Ballou JD, Lacy RC. 1995. Identifying genetically important individuals for management of genetic variation in pedigreed populations. In Ballou, JD, Gilpin M, Foose TJ (eds.), Population Management for Survival and Recovery: Analytical Methods and Strategies in Small Population Conservation, 76-111, Columbia University Press, New York, USA.

3. Barnes PJ, Karin M. 1997. Nuclear factor-κB - a pivotal transcription factor in chronic inflammatory diseases. New England Journal of Medicine 336:1066-1071.

4. Baumann H, Gauldie J. 1994. The acute phase response. Immunology Today 15:74-80.

5. Beg AA, Finco TS, Nantermet PV, Baldwin AS. 1993. Tumor necrosis factor and interleukin-1 lead to phosphorylation and loss of IκBα: a mechanism for NF-κB activation. Molecular and Cellular Biology 13:3301-3310.

6. Bergstrom J, Ueda M, Une Y, Sun X, Misumi S, Shoji S, Ando Y. 2006. Analysis of amyloid fibrils in the cheetah (*Acinonyx jubatus*). Amyloid 13(2): 93-98.

7. Bethin KE, Vogt SK, Muglia L. 2000. Interleukin-6 is an essential, corticotropin-releasing hormone independent stimulator of the adrenal axis during immune system activation. Proceedings of the National Academy of Sciences of the United States of America 97(16):9317-9322.

8. Biran H, Friedman N, Neumann L, Pras M, Shainkin-Kestenbaum R. 1986. Serum amyloid A (SAA) variations in patients with cancer: correlation with disease activity, stage, primary site, and prognosis. Journal of Clinical Pathology 39:794-797.

9. Bolton LA, Munson L. 1999. Glomerulosclerosis in captive cheetahs (*Acinonyx jubatus*). Veterinary Pathology 36:14-22.

10. Boyce JT, DiBartola SP, Chew DJ, Gasper PW. 1984. Familial renal amyloidosis in Abyssinian cats. Veterinary Pathology 21: 33-38.

11. Busch DS, Hayward LS. 2009. Stress in a conservation context: A discussion of glucocorticoid actions and how levels change with conservation-relevant variables. Biological Conservation 142:2844-2853.

12. Calus MPL, Meuwissen THE, de Roos APW, Veerkamp RF. 2008. Accuracy of genomic selection using different methods to define haplotypes. Genetics 178:553-561.

13. Campbell DJ, Rawlings JM, Koelsch S, Wallace J, Strain JJ, Hannigan BM. 2004. Age-related differences in parameters of feline immune status. Veterinary Immunology and Immunopathology 100:73–80.

14. Castro-Prieto A, Wachter B, Sommer S. 2011. Cheetah paradigm revisited: MHC diversity in the world's largest free-ranging population. Molecular Biology and Evolution 28:1455-1468.

15. Caughey B, Baron G. 2008. Are cheetahs on the run from prion-like amyloidosis? Proceedings of the National Academy of Sciences of the United States of America 105(20):7113-7114.

16. Cerón JJ, Eckersall PD, Martinez-Subiela S. 2005. Acute phase proteins in dogs and cats: current knowledge and future perspectives. Veterinary Clinical Pathology 34:84-99.

17. Chen L, Une Y, Higuchi K, Mori M. 2012. Cheetahs have 4 serum amyloid A genes evolved through repeated duplication events. Journal of Heredity 103(1):115-129.

18. Chrousos GP. 1995. The hypothalamic-pituitary-adrenal axis and immune-mediated inflammation. The New England Journal of Medicine 332:1351-1362.

19. Chrousos GP. 2009. Stress and disorders of the stress system. Nature Reviews Endocrinology 5(7):374-81.

20. De Beer FC, Fagan EA, Hughes GRV, Mallya RK, Lanham JG, Pepys MB. 1982. Serum amyloid-A protein concentration in inflammatory disease and its relationship to the incidence of reactive systemic amyloidosis. The Lancet 320:231–234.

21. DeClue AE, Williams KJ, Sharp C, Haak C, Lechner E, Reinero CR. 2009. Systemic response to low-dose endotoxin infusion in cats. Veterinary Immunology and Immunopathology 132:167-174.

22. Dhabhar FS. 2009. Enhancing versus suppressive effects of stress on immune function: Implications for immunoprotection and immunopathology. Neuroimmunomodulation 16:300-317.

23. Dickens MJ, Earle KA, Romero LM. 2009. Initial transference of wild birds to captivity alters stress physiology. General and Comparative Endocrinology 160:76–83.

24. GJC, Kennedy LJ, Auty K, Ryvar R, Ollier WER, Kitchener AC, Freeman AR, Radford AD. 2004. The use of reference strand-mediated conformational analysis for the study of cheetah (*Acinonyx jubatus*) feline leucocyte antigen class II DRB polymorphisms. Molecular Ecology 13:221-229.

25. Durant S, Marker L, Purchase N, Belbachir F, Hunter L, Packer C, Breitenmoser-Wursten C, Sogbohossou E, Bauer H. 2008. *Acinonyx jubatus*. In: IUCN 2014. IUCN Red List of Threatened Species. Version 2014.1. <www.iucnredlist.org>. Downloaded on 17 June 2014.

26. Elenkov IJ, Chrousos GP. 1999. Stress hormone, Th1/Th2 patterns, pro/anti-inflammatory cytokines and susceptibility to disease. Trends in Endocrinology and Metabolism 10(9):359-368.

27. Esmat SM, EL-Sherif HE, Anwar S, Abdel-Atty S, Abdel-Reheim HA. 2005. Serum amyloid A protein level, and its significance in systemic lupus erythematosus patients. Egyptian Dermatology Online Journal 1(2):2.

28. Fernando RL, Grossman M. 1989. Marker assisted selection using best linear unbiased prediction. Genetics Selection Evolution 21:467-477.

29. Gillmore JD, Lovat LB, Persey MR, Pepys MB, Hawkins PN. 2001. Amyloid load and clinical outcome in AA amyloidosis in relation to circulating concentration of serum amyloid A protein. The Lancet 358:244-29.

30. Giordano A, Spagnolo V, Colombo A, Paltrinieri S. 2004. Changes in some acute phase protein and immunoglobulin concentrations in cats affected by feline infectious peritonitis or exposed to feline coronavirus infection. The Veterinary Journal 167:38–44.

31. Grisham J, Lackey LB, Spevak E. 2013. Population Analysis and Breeding and Transfer Plan: Cheetah (*Acinonyx jubatus*) AZA Species Survival Plan® Yellow Program. Population Management Center, Lincoln Park Zoo.

32. Gruber AD, Linke RP. 1996. Generalized AA-amyloidosis in a bat (*Pipistrellus pipistrellus*). Veterinary Pathology 33:428-430.

33. Habier D, Fernando RL, Dekkers JCM. 2009. Genetic selection using low density marker panels. Genetics 182(1):343–353.

34. Hadlow WJ, Jellison WL. 1962. Amyloidosis in rocky mountain bighorn sheep. Journal of the American Veterinary Medical Association 141:243–247.

35. Henderson CR. 1984. Applications of linear models in animal breeding. University of Guelph, Guelph, Ontario.

36. Huberty CJ. 1994. Applied discriminant analysis. John Wiley and Sons, New York, USA.

37. Jensen LE, Whitehead AS. 1998. Regulation of serum amyloid A protein expression during the acute-phase response. Biochemical Journal 334:489-503.

38. Johnson KH, Sletten K, Munson L, O'Brien TD, Papendick R, Westermark P. 1997. Amino acid sequence analysis of amyloid protein A (AA) from cats (captive cheetahs: *Acinonyx jubatus*) with a high prevalence of AA amyloidosis. Amyloid: International Journal of Experimental and Clinical Investigation 4:171-177.

39. Jurke MH, Czekala NM, Lindburg DG, Millard SE. 1997. Fecal corticoid metabolite measurements in the cheetah (*Acinonyx jubatus*). Zoo Biology 16:133-147.

40. Kajikawa, T., Furuta, A., Onishi, T., Sugii, S., 1996. Enzyme-linked immunosorbent assay for detection of feline serum amyloid A protein by use of immunological cross-reactivity of polyclonal anti-canine serum amyloid A protein antibody. Journal of Veterinary Medical Science 58:1141-1143.

41. Kajikawa T, Furuta A, Onishi T, Tajima T, Sugii S. 1999. Changes in concentrations of serum amyloid A protein, α-1-acid glycoprotein, haptoglobin, and C-reactive protein in feline sera due to induced inflammation and surgery. Veterinary Immunology and Immunopathology 68:91-98.

42. Kann RKC, Seddon JM, Henning J, Meers J. 2012. Acute phase proteins in healthy and sick cats. Research in Veterinary Science 93:649-654.

43. Keay JM, Singh J, Gaunt MC, Kaur T. 2006. Fecal glucocorticoids and their metabolites as indicators of stress in various mammalian species: a literature review. Journal of Zoo and Wildlife Medicine 37(3):234-244.

44. Kimura M, Crow JF. 1963. On the maximum avoidance of inbreeding. Genetical Research 4:399-415.

45. Kluve-Beckerman B, Hardwick J, Du L, Benson MD, Monia BP, Watt A, Crooke RM, Mullick A. 2011. Antisense oligonucleotide suppression of serum amyloid A reduces amyloid deposition in mice with AA amyloidosis. Amyloid: International Journal of Experimental and Clinical Investigation 18:136-146.

46. Köster LS, Schoeman JP, Meltzer DGA. 2007. ACTH stimulation test in the captive cheetah (*Acinonyx jubatus*). Journal of the South African Veterinary Association 78(3):133-136.

47. Lachmann HJ, Goodman HJB, Gilbertson JA, Gallimore JR, Sabin CA, Gillmore JD, Hawkins PN. 2007. Natural history and outcome in systemic AA amyloidosis. New England Journal of Medicine 356:2361-2371.

48. Lande R, Thompson R. 1990. Efficiency of marker-assisted selection in the improvement of quantitative traits. Genetics 124:743-756.

49. Lange K, Papp JC, Sinsheimer JS, Sripracha R, Zhou H, Sobel EM. 2013. Mendel: The Swiss army knife of genetic analysis programs. Bioinformatics 29:1568-1570.

50. LeMay DR, LeMay LG, Kluger MJ, D'Alecy LG. 1990. Plasma profiles of IL-6 and TNF with fever-inducing doses of lipopolysaccharide in dogs. American Journal of Physiology 259:R126-132.

51. Liepnieks JJ, Kluve-Beckerman B, Benson MD. 1995. Characterization of amyloid A protein in human secondary amyloidosis: the predominant deposition of serum amyloid A1. Biochimica et Biophysica Acta 1270:81-86.

52. Liu M-J, Bao S, Napolitano JR, Burris DL, Yu L, Tridandapani S, Knoell DL. 2014. Zinc regulates the acute phase response and serum amyloid A production in response to sepsis through JAK-STAT3 signaling. PLOS One 9(4):e94934. doi:10.1371/journal.pone.0094934.

53. Maas M, Rhijn IV, Allsopp M, Rutten V. 2010. Lion (*Panthera leo*) and cheetah (*Acinonyx jubatus*) IFN-γ sequences. Veterinary Immunology and Immunopathology 134:296–298.

54. Marhaug G, Dowton SB. 1994. Serum amyloid A: an acute phase apolipoprotein and precursor of AA amyloid. Baillière's Clinical Rheumatology 8:553-573.

55. Marker L. 2013. 2012 International Cheetah (*Acinonyx jubatus*) Studbook. Namibia: Cheetah Conservation Fund.

56. Martin LB, Brace AJ, Urban A, Coon C, Liebl AL. 2012. Does immune suppression during stress occur to promote physical performance? Journal of Experimental Biology 215:4097-4103.

57. Meuwissen THE, Goddard ME. 1996. The use of marker haplotypes in animal breeding schemes. Genetics Selection Evolution 28:161-176.

58. Meuwissen THE, Hayes BJ, Goddard ME. 2001. Prediction of total genetic value using genome-wide dense marker maps. Genetics 157:1819-1829.

59. Migita K, Yamasaki S, Shibatomi K, Ida H, Kita M, Kawakami A, Eguchi K. 2001. Impaired degradation of serum amyloid A (SAA) protein by cytokine-stimulated monocytes. Clinical and Experimental Immunology 123:408-411.

60. Montgomery ME, Ballou JD, Nurthen RK, England PR, Briscoe DA, Frankham R. 1997. Minimizing kinship in captive breeding programs. Zoo Biology 16:377-389.

61. Moriguchi M, Terai C, Kaneko H, Koseki Y, Kajiyama H, Uesato M, Inada S, Kamatani N. 2001. A novel single-nucleotide polymorphism at the 5'-flanking region of SAA1 associated with risk of type AA amyloidosis secondary to rheumatoid arthritis. Arthritis & Rheumatism 44:1266-1272.

62. Morin PA, Luikart G, Wayne RK, and the SNP workshop group. 2004. SNPs in ecology, evolution and conservation. Trends in Ecology and Evolution 19:209-216.

63. Munson L. 1993. Diseases of Captive Cheetahs (*Acinonyx jubatus*): Results of the Cheetah Research Council Pathology Survey, 1989-1992. Zoo Biology 12:105-124.

64. Munson L, Nesbit JW, Meltzer DGA, Colly LP, Bolton L, Kreik LPJ. 1999. Diseases of captive cheetahs (*Acinonyx jubatus jubatus*) in South Africa: a 20-year retrospective survey. Journal of Zoo and Wildlife Medicine 30(3):342-347.

65. Munson L, Terio K, Worley M, Jago M, Bagot-Smith A, Marker L. 2005. Extrinsic factors significantly affect patterns of disease in free-ranging and captive cheetah (*Acinonyx jubatus*) populations. Journal of Wildlife Diseases 41(3):542-548.

66. Niemi K, Teirila L, Lappalainen J, Rajamaki K, Baumann MH, Oorni K, Wolff H, Kovanen PT, Matikainen S, Eklund KK. 2011. Serum amyloid A activates the NLRP3 inflammmasome via P2X7 Receptor and a cathepsin B-sensitive pathway. Journal of Immunology 186:6119-6128.

67. Niewold TA, van der Linde-Sipman JS, Murphy C, Tooten PCJ, Gruys E. 1999. Familial amyloidosis in cats: Siamese and Abyssinian AA proteins differ in primary sequence and pattern of deposition. Amyloid: International Journal of Experimental and Clinical Investigation 6:205-209.

68. Norris DO. 2007. Vertebrate Endocrinology (4th ed.). Elsevier Academic Press, Burlington, MA.

69. O'Brien SJ, Roelke ME, Marker L, Newman A, Winkler CA, Meltzer D, Colly L, Evermann JF, Bush M, Wildt DE. 1985. Genetic basis for species vulnerability in the cheetah. Science 227:1428-1434.

70. O'Brien SJ, Wildt DE, Goldman D, Merril CR, Bush M. 1983. The cheetah is depauperate of genetic variation. Science 221:459-462.

71. Ofri R, Nyska A, Linke RP, Shtrasburg S, Livneh A, Gal R. 1997. Systemic amyloidosis in a cheetah (*Acinonyx jubatus*). Amyloid: International Journal of Experimental and Clinical Investigation 4(2): 98-103.

72. Otto CM, Rawlings CA. 1995. Tumor necrosis factor production in cats in response to lipopolysaccharide: an in vivo and in vitro study. Veterinary Immunology and Immunopathology 49:183-188.

73. Padalino B, Aube L, Fatnassi M, Monaco D, Khorchani T, Hammadi M, Lacalandra GM. 2014. Could dromedary camels develop stereotypy? The first description of stereotypical behaviour in housed male dromedary camels and how it is affected by different management systems. PLOS One 9(2):e89093.

74. Paltrinieri S. 2008. The feline acute phase reaction. The Veterinary Journal 177:26-35.

75. Papendick RE, Munson L, O'Brien TD, Johnson KH. 1997. Systemic AA amyloidosis in captive cheetahs (*Acinonyx jubatus*). Veterinary Pathology 34:549-556.

76. Pedersen LD, Sorensen AC, Berg P. 2009. Marker-assisted selection can reduce true as well as pedigree-estimated inbreeding. Journal of Dairy Science 92:2214-2223.

77. Pras M, Schubert M, Zucker-Franklin D, Rimon A, Franklin EC. 1968. The characterization of soluble amyloid prepared in water. The Journal of Clinical Investigation 47:924-933.

78. Ray A, Ray BK. 1994. Serum amyloid A gene expression under acute-phase conditions involves participation of inducible C/EBP-β and C/EBP-δ and their activation by phosphorylation. Molecular and Cellular Biology 14:4324-4332.

79. Rideout BA, Montali RJ, Wallace RS, Bush M, Phillips Jr. LG, Antonovych TT, Sabnis SG. 1989. Renal medullary amyloidosis in Dorcas gazelles. Veterinary Pathology 26:129-135.

80. Rivas AL, Tintle L, Meyerswallen V, Scarlett JM, Vantassell CP, Quimby FW. 1993. Inheritance of renal amyloidosis in Chinese Shar-pei dogs. Journal of Heredity 84:438-442.

81. Röcken C, Menard R, Bühling F, Vöckler S, Raynes J, Stix B, Krüger S, Roessner A, Kähne T. 2005. Proteolysis of serum amyloid A and AA amyloid proteins by cysteine proteases: cathepsin B generates AA amyloid proteins and cathepsin L may prevent their formation. Annals of the Rheumatic Diseases 64:808–815.

82. Rodriguez-Clark KM. 1999. Genetic theory and evidence supporting current practices in captive breeding for conservation. In Landweber LF, Dobson AP. (eds.), Genetics and the Extinction of Species, 47-65, Princeton University Press, Princeton.

83. Sajjad S, Farooq U, Anwar M, Khurshid A, Bukhari SA. 2011. Effect of captive environment on plasma cortisol level and behavioral pattern of Bengal tigers (*Panthera tigris tigris*). Pakistan Veterinary Journal 31(3):195-198.

84. Sapolsky RM, Romero LM, Munck AU. 2000. How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory and preparative actions. Endocrine Reviews 21(1):55-89.

85. Sasaki K, Ma Z, Khatlani TS, Okuda M, Inokuma H, Onishi T. 2003. Evaluation of feline serum amyloid A (SAA) as an inflammatory marker. Journal of Veterinary Medical Science 65:545–548.

86. Scheinman RI, Cogswell PC, Lofquist AK, Baldwin AS. 1995. Role of transcriptional activation of IκBα in mediation of immunosuppression by glucocorticoids. Science 270:283-286.

87. Tamamoto T, Ohno K, Goto-Koshino Y, Fujino Y, Tsujimoto H. 2012. Serum amyloid A uptake by feline peripheral macrophages. Veterinary Immunology and Immunopathology 150:47-52.

88. Tamamoto T, Ohno K, Ohmi A, Goto-Koshino Y, Tsujimoto H. 2008. Verification of measurement of the feline serum amyloid A (SAA) concentration by human SAA turbidimetric immunoassay and its clinical application. Journal of Veterinary Medical Science 70:1247-1252.

89. Terio KA, Marker L, Munson L. 2004. Evidence for chronic stress in captive but not free-ranging cheetahs (*Acinonyx jubatus*) based on adrenal morphology and function. Journal of Wildlife Diseases 40(2):259-266.

90. Terio KA, Munson L, Moore PF. 2011. Characterization of the gastric immune response in cheetahs (*Acinonyx jubatus*) with Helicobacter-associated gastritis. Veterinary Pathology 49(5):824-833.

91. Terio KA, Marker L, Overstrom EW, Brown JL. 2003. Analysis of ovarian and adrenal activity in Namibian cheetahs. South African Journal of Wildlife Research 33(2):71-78.

92. Terio KA, O'Brien T, Lamberski N, Famula TR, Munsin L. 2008. Amyloidosis in black-footed cats (*Felis nigripes*). Veterinary Pathology 45:393-400.

93. Thorn CF, Whitehead AS. 2002. Differential glucocorticoid enhancement of the cytokine-driven transcriptional activation of the human acute phase serum amyloid A genes, SAA1 and SAA2. Journal of Immunology 169:399-406.

94. van der Linde-Sipman JS, Niewold TA, Tooten PCJ, de Neijs-Backer M, Gruys E. 1997. Generalized AA-amyloidosis in Siamese and Oriental cats. Veterinary Immunology and Immunopathology 56:1-10.

95. Wang J. 2001. Optimal marker-assisted selection to increase the effective size of small populations. Genetics 157:867-874.

96. Weingrill T, Willems EP, Zimmermann N, Steinmetz H, Heistermann M. 2001. Species-specific patterns in fecal glucocorticoid and androgen levels in zoo-living orangutans (*Pongo* spp.). General and Comparative Endocrinology 172:446–457.

97. Wells A, Terio KA, Ziccardi MH, Munson L. 2004. The stress response to environmental change in captive cheetahs (*Acinonyx jubatus*). Journal of Zoo and Wildlife Medicine 35(1):8-14.

98. Wiegers GJ, Reul JMHM. 1998. Induction of cytokine receptors by glucocorticoids: functional and pathological significance. Trends in Pharmacological Sciences 19:317-321.

99. Wielebnowski NC, Ziegler K, Wildt DE, Lukas J, Brown JL. 2002. Impact of social management on reproductive, adrenal and behavioural activity in the cheetah (*Acinonyx jubatus*). Animal Conservation 5:291-301.

100. Wildt DE, Meltzer D, Chakraborta PK, Bush M. 1984. Adrenal-testicular-pituitary relationships in the cheetah subjected to anesthesia/electroejaculation. Biology of Reproduction 30:665-672.

101. Woo P, Sipe J, Dinarello CA, Colton HR. 1987. Structure of a human serum amyloid A gene and modulation of its expression in transfected L cells. Biochemistry 262(32):15790-15795.

102. Yamada T, Liepnieks JJ, Kluve-Beckerman B, Benson MD. 1995. Cathepsin B generates the most common form of amyloid A (76 residues) as a degradation product of serum amyloid A. Scandinavian Journal of Immunology 41:94-97.

103. Young KM, Walker SL, Lanthier C, Waddell WT, Monfort SL, Brown JL. 2004. Noninvasive monitoring of adrenocortical activity in carnivores by fecal glucocorticoid analyses. General and Comparative Endocrinology 137(2):148-165.

104. Yuhki N, O'Brien SJ. 1990. DNA variation of the mammalian major histocompatibility complex reflects genomic diversity and population history. Proceedings of the National Academy of Sciences of the United States of America 87:836-840.

105. Zhang B, Une Y, Fu X, Yan J, Ge F, Yao J, Sawashita J, Masayuki M, Tomozawa H, Kametani F, Higuchi K. 2008. Fecal transmission of AA amyloidosis in the cheetah contributes to high incidence of disease. Proceedings of the National Academy of Sciences of the United States of America 105(20):7263-7268.

106. Zhang B, Une Y, Ge F, Fu X, Qian J, Zhang J, Sawashita J, Higuchi K, Mori M. 2008. Characterization of the cheetah serum amyloid A1 gene: critical role and functional polymorphism of a cis-acting element. Journal of Heredity 99(4):355-363.