Aus der Abteilung für Evolutionäre Ökologie
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Immunity of cheetahs (*Acinonyx jubatus*): an evolutionary, comparative and life history perspective

Inaugural-Dissertation
zur Erlangung des Grades
Doctor of Philosophy (PhD) in Biomedical Sciences
im Rahmen des Programmes der Dahlem Research School (DRS)
der Freien Universität Berlin

vorgelegt von
Sonja Kirsten Heinrich
Tierärztin aus Heidenheim

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CONTENT

CHAPTER 1- GENERAL INTRODUCTION

In this thesis I present the results of a series of studies on different components of the immune system of the cheetah (*Acinonyx jubatus*). I have chosen a comparative, evolutionary life history perspective to ask how features of the cheetah immune system may have evolved. This is both of fundamental and applied relevance because the cheetah is considered as a threatened species. A special focus is put on the comparison with other carnivore species, which are not considered as genetically impoverished as the cheetah. Another focus is put on immune investment strategies to maximise immune functions when resources are limited. In this introduction I sketch the scientific background to such an evolutionary approach and how my studies are relevant to conservation efforts of the cheetah.

1.1 The conservation of cheetahs

Cheetahs *(Acinonyx jubatus)* were once widely distributed throughout Africa and Asia but occur today only in fragmented populations. Their numbers in the wild have dramatically decreased in the last century from an estimated 100,000 in 1900 (Myers 1975) to approximately 15,000 in 1998 (Marker 1998) and approximately 7,100 in 2016 (Durant et al. 2017), with Southern Africa hosting the largest population of around 4,000 (Durant et al. 2017). Cheetahs are known for their low genetic diversity and have been characterised for many years as a classic example of a species with low genetic diversity and associated high disease vulnerability. Limited genetic diversity was demonstrated for allelic isozymes and soluble proteins (O'Brien et al. 1983), skin grafts (O'Brien et al. 1985), fluctuating skull asymmetry (Wayne et al. 1986, Modi et al. 1987, but see Kieser and Groeneveld 1991), mitochondrial DNA (Menotti-Raymond and O'Brien 1993), minisatellite DNA (Menotti-Raymond and O'Brien 1993) and microsatellite DNA (Menotti-Raymond and O'Brien 1995), although some of this evidence has been subsequently disputed (Caughley 1994, Merola 1994).

Most importantly, cheetahs also exhibit low diversity at the genes of the major histocompatibility complex (MHC) class I, which was first examined by restriction fragment length polymorphism analysis (Yuhki and O'Brien 1990). The MHC class I genes encode peptides that mediate the immune response to viral infections, and it was therefore proposed that the cheetah is particularly vulnerable to infectious diseases (Grisham and Killmar 1997, Marker 2000, Marker et al. 2003). Cheetahs kept in breeding facilities and zoos showed high

mortality and a frequent occurrence of diseases (Evermann et al. 1986, Junge et al. 1991, Munson 1993, Munson et al. 1999), the most prominent case being an outbreak of feline infectious peritonitis (FIP) in a zoological facility in North America in 1982-1983, when over 60% of the cheetah population died (Evermann et al. 1986).

However, a high occurrence of diseases has been recorded only in captive cheetahs. Free-ranging cheetahs exhibited good general health (Caro 1994, Munson et al. 2005, Thalwitzer et al. 2010), despite being seropositive for many viral diseases, which suggests that free-ranging cheetahs effectively respond to pathogenic challenges (Munson et al. 2004, Thalwitzer et al. 2010, Krengel et al. 2015). This raised the question whether MHC diversity was underestimated because early studies in cheetahs either used low resolution molecular methods (Yuhki and O'Brien 1990) or small sample sizes (Yuhki and O'Brien 1994). A recent study used high resolution molecular methods on samples from 194 cheetahs and reported slightly higher, but still overall low levels of MHC diversity, namely ten MHC class I alleles and four MHC class II DRB alleles (Castro-Prieto et al. 2011). This suggested that cheetahs might be an example of species which present low MHC diversity but have high disease resistance. This phenomenon has also been described in other species, for example in North American and European moose *(Alces alces* (Mikko and Andersson 1995)), Chillingham cattle *(Bos taurus* (Visscher et al. 2001)), Northern elephant seals (*Mirounga angustirostris* (Hoelzel et al. 1999)) and mountain goats *(Oreamnus americanus* (Mainguy et al. 2007)). However, comprehensive studies of the immune responses of these species are currently lacking. By assessing six different immune parameters in approximately 400 cheetahs, this study provides the first overall assessment of the immune responses of this species and investigates the question of whether the immune system of cheetahs is generally impaired. It first compares one part of the immune system of the cheetah with five sympatric carnivore species, which are expected to exhibit higher genetic diversity (chapter 2). It then compares more thoroughly three parts of the immune system of the cheetah to the sympatric leopard (*Panthera pardus*) (chapter 3) and between age classes, sexes and pathogen pressures in cheetahs (chapter 4).

1.2 Eco-immunology

During the last decade, examinations of immune responses of wildlife species have gained increasing attention, giving rise to the new field of eco-immunology. Eco-immunology aims to understand how immune responses vary in the natural environment with different

parameters, including life history traits, Darwinian fitness costs and trade-offs as potential explanatory variables for variation among immune components within and among species (Boughton et al. 2011).

Pathogens have the ability to reduce Darwinian fitness, which imposes a strong selective pressure on the hosts (Loye and Zuk 1991, Sheldon and Verhulst 1996). Hosts have developed various defence mechanisms to fend off pathogens and ensure survival, one of the most important being the immune system (Saino et al. 1997). In vertebrates, the immune system comprises an innate and an adaptive part (Janeway et al. 2001). Innate responses provide a rapid, first line, general unspecific defence, whereas adaptive responses take longer to develop but confer long-lasting, highly specific protection against a particular pathogen (Janeway et al. 2001). The innate immune system can be further divided into a constitutive and an induced part (Schmid-Hempel and Ebert 2003). The constitutive part is always present at low levels in the blood and responds immediately. Constitutive components of the innate immune system can induce local inflammation as well as the systemic inflammatory response (Lee 2006).

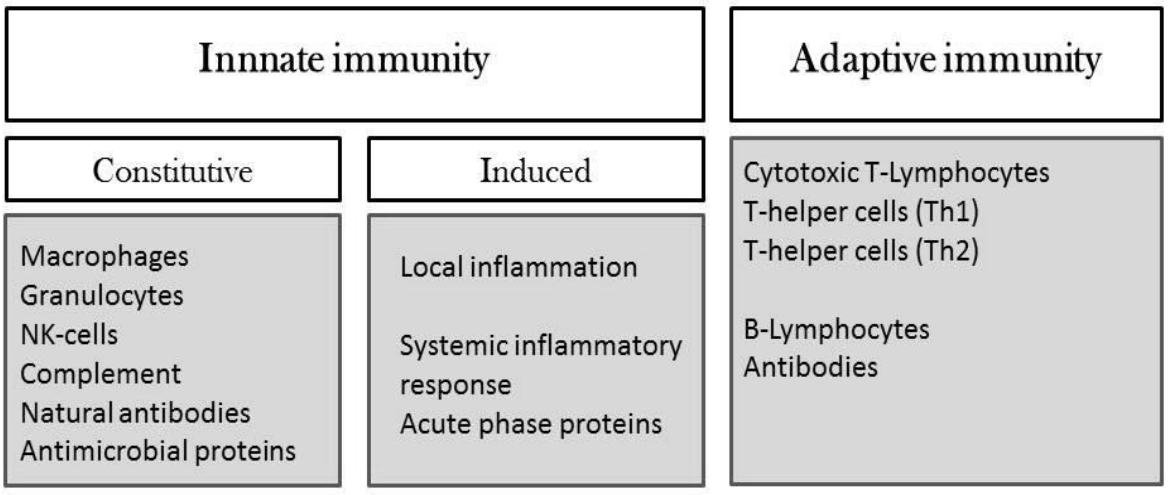

Figure 1: Structure of the immune system classified by function and the effectors involved when challenged by pathogens.

Different parts of the immune system do not work independently, but interact with each other in a complex arrangement. The type of immune response depends on the type of disease organism, i.e. bacterium, virus, single eukaryotic parasite, multi-cellular parasite or cancer cell, and other factors, such as virulence, entry route, prior exposure or dose (Matson et al. 2006), thus relating to traits of pathogens as well as their mode of transmission. Pathogen

transmission risk is influenced by ecological factors such as foraging behaviour (DeVault et al. 2003), social organisation (Côté and Poulin 1995, Wilson et al. 2003) or body mass (Kuris et al. 1980), which may influence the responses of the immune system (Vitone et al. 2004). I therefore asked whether the constitutive innate immunity of cheetahs and sympatric carnivore species has been shaped by these factors or was simply a function of their phylogenetic relatedness.

A range of immunological techniques has been developed and adapted to non-model organisms to gain a more integrative, comprehensive understanding of environment-immune interactions (Demas et al. 2011). However, the relationship between measures of immunity and disease resistance is complex, and therefore the interpretation of results is not straightforward (Adamo 2004). Immunological techniques that measure functional responses ("functional assays") often provide a good estimate of how effective immune responses are in protecting individuals from disease and should therefore be preferred when assessing immunocompetence over other methods (Viney et al. 2005). One functional assay is the bacterial killing assay (BKA) which provides an integrative measure of constitutive innate immunity (Tieleman et al. 2005, French and Neuman-Lee 2012). It determines the ability of an organism to remove a pathogen that could be encountered in the wild. Although the BKA has been in use since 1975 in studies on human immunocompetence (Keusch et al. 1975), its first application to eco-immunology of wildlife did not occur until 1994 (Barriga et al. 1994) and only received attention from 2005 onwards (Tieleman et al. 2005). Since then it has been mainly used in research on avian species (Tieleman et al. 2005, Matson et al. 2006, McGraw et al. 2006, Forsman et al. 2008, Rubenstein et al. 2008, Morrison et al. 2009, Ardia et al. 2010) and has been substantially improved, to be less time and material consuming. It has been adapted to different taxa (Stow et al. 2007, Garcia et al. 2010, French and Neuman-Lee 2012, Kuo et al. 2013) but prior to this study, it has been rarely used for mammals (Ezenwa et al. 2012, Schneeberger et al. 2013).

Single measures of immunity, as with a BKA, do not demonstrate complete resistance to infectious diseases (Adamo 2004). Rather, immunity should be assessed by using a range of techniques, because a low immune response from one part of the immune system may be compensated for by a high immune response by another part (Norris and Evans 2000, Boughton et al. 2011). I assessed the "immunocompetence" of cheetahs by simultaneously measuring six indices whilst controlling for differences in allostatic load, the wear and tear on the body and the energy required to cope with deviations from homeostasis caused by

stressful events (McEwen and Stellar 1993, Romero and Wingfield 2016) which might influence these immune measures.

1.3 Trade-offs in eco-immunology

Mounting and maintaining immune functions have evolved to increase survival. However, activation of components of the immune system can be energetically costly (Lochmiller and Deerenberg 2000). Thus, immune functions are unlikely to be maximized at all times. This leads to potential trade-offs with other life history traits, such as reproduction, growth and development, particularly when resources are limited (Zuk and Stoehr 2002, East et al. 2015). There may also be potential "competition" or synergisms between different parts of the immune system (Norris and Evans 2000, Matson et al. 2006). Therefore, much of the variability in pathogen susceptibility within and between individuals may be a consequence of differential investment in the immune system (Klasing 2004).

In an evolutionary ecology context it may be possible that a reduction in immunogenetic variability which impairs one part of the immune system increases selection pressure on other parts of the immune system to provide a compensatory response (Råberg et al. 2002, Zysling et al. 2009). Cheetahs have a relatively low genetic diversity which may impair their adaptive immune system (O'Brien et al. 1985, Charruau et al. 2011). In contrast, African leopards live sympatrically with cheetahs in Namibia and exhibit a relatively high genetic diversity (Spong et al. 2000, Uphyrkina et al. 2001). Thus I hypothesized that cheetahs may have a weaker adaptive immunity than leopards and that this weakness may be compensated by a higher competence of other parts of the immune system.

The costs of immunity are typically divided into three components, the costs of (1) development, (2) maintenance and (3) use of the immune system during an acute or chronic challenge (Klasing 2004). These costs vary substantially at different life stages for different parts of the immune system. The costs of development are primarily of an energetic nature to fuel the initial burst of leucopoiesis and the lengthy process of developing a diverse repertoire of lymphocytes expressing unique antigen receptors. The costs of maintenance are the costs of producing those substrates needed to replace leucocytes, antibodies and plasma proteins which are lost during normal cell and protein turnover. The costs of using the immune system are both energetic, for mobilising the responding cell types and fueling their effector functions, and immunopathologic for recovering from losses in tissue function and damage incurred by the effector mechanisms of immune cells (Klasing and Leshchinsky 1999).

Maintaining constitutive levels of protective cells and proteins at protective levels is likely to be the most rapid and effective defence, but implies high energetic costs (Klasing 2004). Adaptive immunity, on the other hand, is thought to have small energetic costs in terms of maintenance, but has the disadvantage of taking longer to come into action as it requires at least four days of activation before contributing significantly to the protection against novel pathogens (Lee 2006). Once adaptive immunity against a specific pathogen is established, repeated exposures to the same pathogen are quickly and effectively fended off through specific adaptive responses with the help of memory cells. Slow growth of individuals, long life span and repeated challenges by the same pathogen(s) favour an investment in adaptive immunity, because this part of the immune system requires an extensive period of developmental processes (Klasing and Leshchinsky 1999). I therefore expect cheetahs to invest resources into this part of the immune system and that adults should have a stronger adaptive immunity than younger animals. Adaptive immunity also requires a large energetic investment during ontogeny (Klasing and Leshchinsky 1999). Thus, young cheetahs are expected to rely on innate immunity and have equally strong innate immune responses as adults.

In species with slow growth, long life span and without helpers (Moehlman and Hofer 1997), females are likely to maximise fitness through an increase in longevity and the associated increase in reproductive output through a larger number of litters, whereas males are likely to maximise fitness through a high mating rate (Bateman 1948, Rolff 2002). Thus, mammalian males could in theory afford a weaker immune response than mammalian females. Indeed, some studies showed that males had lower antibody titres (Kacprzak-Bergman 1994, Olsen and Kovacs 1996), weaker antibody responses (Daniels and Belosevic 1994) and a higher parasite burden (Poulin 1996, Zuk and McKean 1996, Moore and Wilson 2002) than females. Such differences are probably mediated by sex steroid hormones, especially by testosterone (Folstad and Karter 1992, Muehlenbein and Bribiescas 2005). However, testosterone probably does not suppress immune function in general but only some parts of the immune system and enhances others (Hasselquist et al. 1999, Ezenwa et al. 2012). Previous studies demonstrated that testosterone had a suppressive effect on immunoglobulin concentration (Hirota et al. 1976, Grossman 1985, Saino et al. 1995, Duffy et al. 2000, Casto et al. 2001) which forms part of the adaptive immunity. Thus, I expect cheetah males to have a weaker adaptive immunity than females.

Reproductive effort has been identified as one factor responsible for variation in the investment in the immune system (Ellison 2003). Thus, immune functions may become reduced when individuals,

particularly females, invest in reproduction. A number of studies support this by demonstrating decreases in immune parameters (Deerenberg and Arpanius 1997, Nordling et al. 1998, Saino et al. 2002, Ardia 2005) or greater susceptibility to parasites during demanding times of reproduction (Møller 1993, Norris et al. 1994). For example, Ardia and colleagues manipulated brood size in tree swallows (*Tachineta bicolor*) and exposed breeding females to red blood cells of sheep. Females raising enlarged broods produced fewer secondary antibodies than females raising normal-sized control broods (Ardia et al. 2003). In female spotted hyenas (*Crocuta crocuta*), infection with *Ancylostoma* and *Ancylostoma* egg load was higher in lactating females than in non-lactating females and females nursing twins had higher egg loads than females nursing singletons (East et al. 2015). In most mammals, investment in offspring is unevenly divided between the sexes, with females bearing most of the energetic burden during pregnancy and lactation (Ellison 2003, Lee 2006). In female cheetahs, resources may be particularly limited, because their slim body is built for high speed chases and might lack large energy reserves in form of fat depots (Laurenson 1995). Cheetah cubs stay with their mothers for approximately 18 months and require the same amount of meat per day during the last months before independence as their mothers, while contributing little to or perhaps even impeding hunting success (Caro 1994), thereby increasing the factual work load for the female. Thus, I expect adult female cheetahs to invest less in the innate part of immunity because it represents the more expensive part in adults.

Such energetic limitations should be reduced in captive animals which have regular access to food. Captive cheetahs in Namibia live in large enclosures in their natural habitat. They are not permitted to reproduce, and are regularly examined and vaccinated by a veterinarian. These cheetahs live in close proximity to other cheetahs, people and their domestic dogs and cats, thus they are expected to be exposed to a higher pathogen pressure than free-ranging cheetahs.

1.4 Immunity and variation in allostatic load (“stress”)

Free-ranging animals are susceptible to changes in allostatic load (“stress”) when subjected to researcher interventions such as capture and handling (Romero et al. 2009, McEwen and Wingfield 2010). This in turn can significantly influence many immune measurements (Millet et al. 2007). The stress response is considered a general adaptive response crucial for survival as it is designed to endure, avoid or recover from averse stimuli (Martin 2009, Ottaviani and Malagoli 2009). Especially in domestic cats (*Felis catus*), leucocytosis is a common

consequence of stress (Cowell and Decker 2000, Paltrinieri 2008). Concentrations of glucocorticoids rise quickly after the capture of free-ranging animals, initiating characteristic changes in the leucocyte component of the vertebrate immune system. These changes are an increase in numbers of neutrophils and a decrease in the number of lymphocytes (Davis et al. 2008). Thus, whereas leucocyte profiles read from blood smears can be directly related to the concentration of glucocorticoids, such information does not predict the ability of that individual to mount an effective immune response (Davis et al. 2008).

Capture and handling are strong stressors for wildlife species (Hofer and East 1998) and elicit an acute stress response that is likely to be similar to the preparation of the immune system to a possible intruder (Dhabhar 2009). This acute stress response may influence those immune measurements which change quickly in response to elevated levels of catecholamines or glucocorticoids. This may be the case for acute phase proteins such as serum amyloid A, which increases in concentration in a study where laboratory rats were exposed to a short-term stressor (Deak et al. 1997). Other immune measurements such as the bacterial killing assay are not sensitive to catecholamines and glucocorticoids or respond much more slowly, as has been demonstrated in bats (Strobel et al. 2015) and birds, when sampling took place up to 30 min after capture (Buehler et al. 2008). Knowledge about which immune measurements are associated with stress is important for the correct interpretation of measurements of immunity. I assessed the stress of capture and handling by measuring the serum concentration of cortisol, the glucocorticoid found in mammals (Voigt et al. 2004), and the ratio of neutrophils to lymphocytes in the blood. I checked which immune measurements were associated with these measurements and was thus able to statistically control for this potentially confounding factor.

1.5 Objectives of this thesis

Loss of genetic diversity may render populations more vulnerable to pathogens if this is a consequence of inbreeding depression or depletion of variation in genes responsible for immunity against parasites. Owing to their low genetic diversity, cheetahs have been regarded as vulnerable to infectious diseases for decades, but the functionality of their immune system has not been investigated.

The first aim of this thesis was to assess whether the strength of the constitutive innate immunity, which represents the first line of defence against invading pathogens, was lower in cheetahs than in carnivore species with higher genetic diversity. In a first step, I adapted the

protocol of the bacterial killing assay (BKA) to several carnivore species and adjusted the method in such a way that the results of different species were comparable. I also investigated whether the bacterial killing capacity of serum dropped with increasing duration of storage in mammals, which had been shown to be the case for birds (Liebl and Martin 2009) but not for bats (Schneeberger et al. 2013). This paved the way for the first comparison of a functional immune response of the cheetah with other sympatric carnivore species. Ecological factors are of central importance in the emergence and transmission of diseases and thus may shape the immune system of a species. Therefore, I investigated whether the constitutive innate immunity of carnivore species was linked to their social organization, foraging behaviour or body mass.

The second purpose of this thesis was to investigate whether impaired immunity in one part of the immune system might be compensated for by other components of immunity. MHC molecules present pathogen-derived antigens to the effector cells of the immune system and thus trigger the adaptive immune response (Radwan et al. 2010). Different parts of immunity are important during different stages of infection, and various effectors of innate immunity can kill a broad spectrum of pathogens before the adaptive immunity needs to intervene (Acevedo-Whitehouse and Cunnigham 2006). Thus, individuals might prefer to invest in other immune components if adaptive immunity is impaired (Matson 2006) or too expensive. I investigated this idea by comparing the immune responses of cheetahs to those of leopards using six different immunological measurements. Because “stress” of capture and handling may significantly influence immune measurements, I also assessed the level of allostatic load as a possible confounding factor.

Finally I investigated other factors that may lead to differing immune responses in cheetahs such as age, sex or exposure to pathogens. Mounting and maintaining an immune function is energetically costly and thus might cause trade-offs with various life history traits. Females carry a strong energetic burden of reproduction, which might reduce their immune responses during reproductive efforts. Adaptive immune responses need time and energy to be developed and thus immune responses might change as animals grow older. Additionally, investment in immunity should be influenced by external factors, for example challenges by pathogens. Thus I investigated differences between environments of low and high exposure of pathogens, which corresponded to free-ranging animals and animals kept in large enclosures on Namibian farms. Here, I also assessed the level of allostatic load as a possible confounding factor and checked for differences between free-ranging and captive animals.

The results of my studies are presented in three manuscripts in chapters 2, 3 and 4.

Chapter 2: Compares the strength of the constitutive innate immune response with the BKA in six carnivore species, the caracal, the cheetah, the leopard, the lion (*Panthera leo*), the brown hyena (*Hyaena brunnea*) and the black-backed jackal (*Canis mesomelas*). Specifically, I investigated:

- whether immune responses reflect transmission risk of pathogens as assessed by ecological factors such as social organisation, foraging behaviour or body mass;
- whether immune responses can be predicted from the phylogenetic relatedness of the species;
- whether the duration of storage of serum reduces bacterial killing capacity in cheetah and leopard serum.

Chapter 3: Investigates immune investment strategies of two sympatric carnivore species with different genetic diversity: the cheetah and the leopard. Specifically, I investigated:

- whether humoral immunity differed between the two species in adaptive immunity, constitutive innate immunity and induced innate immunity;
- whether the two species differed in their response to trapping and handling by measuring allostatic load ('stress').

Chapter 4: Investigates possible trade-offs of immune functions with life history traits. It also evaluates handling associated changes in allostatic load and determines its possible influence on immune parameters. Specifically I compared in cheetahs:

- immune responses between males and females;
- immune responses between young and old animals;
- immune responses between environments of high and low pathogen exposure;
- levels of allostatic load between captive and free-ranging individuals.

Finally, in **chapter 5** I summarise the key findings of this thesis and discuss them in the broader context of the evolutionary ecology of the immune system.

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CHAPTER 2 – A COMPARISON WITH OTHER CARNIVORE SPECIES

First Manuscript

Feliform carnivores have a distinguished constitutive innate immune response

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RESEARCH ARTICLE

Feliform carnivores have a distinguished constitutive innate immune response

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ABSTRACT

Determining the immunological phenotype of endangered and threatened populations is important to identify those vulnerable to novel pathogens. Among mammals, members of the order Carnivora are particularly threatened by diseases. We therefore examined the constitutive innate immune system, the first line of protection against invading microbes, of six free-ranging carnivore species; the black-backed jackal (*Canis mesomelas*), the brown hyena (*Hyena brunnea*), the caracal (*Caracal caracal*), the cheetah (*Acinonyx jubatus*), the leopard (*Panthera pardus*) and the lion (*Panthera leo*) using a bacterial killing assay. The differences in immune responses amongst the six species were independent of their foraging behaviour, body mass or social organisation but reflected their phylogenetic relatedness. The bacterial killing capacity of black-backed jackals, a member of the suborder Caniformia, followed the pattern established for a wide variety of vertebrates. In contrast, the five representatives of the suborder Feliformia demonstrated a killing capacity at least an order of magnitude higher than any species reported previously, with a particularly high capacity in caracals and cheetahs. Our results suggest that the immunocompetence of threatened felids such as the cheetah has been underestimated and its assessment ought to consider both innate and adaptive components of the immune system.

KEY WORDS: Bacterial killing assay, Carnivores, Constitutive innate immunity, Canids, Felids

INTRODUCTION

One key factor threatening mammalian wildlife populations are pathogens and parasites (Smith et al., 2009). Members of the order Carnivora are particularly threatened by diseases; according to the IUCN red list, 25% of carnivores are considered as threatened and within those 27% of the 30 species in the family Canidae and 8% of the 36 species in the family Felidae are threatened by diseases (Pedersen et al., 2007; Smith et al., 2009). Knowing the immunocompetence of threatened species is therefore particularly important for disease management because it is a critical aspect of disease resistance and thus survival (Graham et al., 2011). In carnivores, species feeding on carrion are more likely to be infected than species feeding exclusively on freshly killed prey because of a higher abundance of pathogens colonizing carrion (DeVault et al., 2003). Larger species may also have a higher infection risk than smaller species because larger bodies need more food and may harbour more pathogens than smaller ones (Kuris et al., 1980; Morand and Poulin, 1998; Schneeberger et al., 2013; but see Vitone et al., 2004). Social species live under higher pathogen pressure than solitary ones because of higher intraspecific contact rates and closer proximity of individuals (Altizer et al., 2003). Additionally, higher rates of intraspecific horizontal transmission and multiple infections are associated with the evolution of increased virulence which is promoted by social species (Wilson et al., 2003).

Pathogen transmission risk and virulence should be reflected in species-specific patterns of immunocompetence because pathogens impose a strong selective pressure on their hosts (Schneeberger et al., 2013; Wilson et al., 2003). Thus, species with a high transmission risk should maintain a higher immunocompetence than species with a low transmission risk. Alternatively, closely related species might exhibit a similar immunocompetence because many immune system components are genetically encoded as many other traits and might be shared through common ancestors (Caroll and Prodeus, 1998; Lee, 2006; Ochsenbein and Zinkernagel, 2000).

Here we investigate the strength of the constitutive innate immune system in six free-ranging carnivore species from the same carnivore guild with different foraging behaviour, body size and social organisation (Table 1) using a bacterial killing assay (BKA). This assay is useful because it measures a functional response of the innate immune system of an animal (Tieleman et al., 2005). The BKA determines the ability to eliminate a pathogen encountered and has been demonstrated to be an excellent predictor of the susceptibility to a variety of bacterial infections in humans (Keusch et al., 1975). It is easily adaptable to different species without the need of species specific reagents, which makes it suitable for comparative studies (Millet et al., 2007; Schneeberger et al., 2013). Its interpretation is clear and straightforward and it has been successfully used to estimate constitutive innate immunity in many different species, e.g. in bats (Schneeberger et al., 2013), gazelles (Ezenwa et al., 2012), spotted hyena (Flies et al., 2015), skinks (Kuo et al., 2013) and many bird species (Matson et al., 2006; McGraw and Klasing, 2006; Millet et al., 2007). The constitutive part of the innate immune system is always present at low levels in the blood and provides a rapid, first line defences against parasites and pathogens (Janeway et al., 2001).

We used four felids; the caracal (*Caracal caracal*), cheetah (*Acinonyx jubatus*), leopard (*Panthera pardus*) and lion (*Panthera leo*), and the brown hyena (*Hyaena brunnea*, family Hyaenidae), all from suborder Feliformia, and one canid, the black-backed jackal (*Canis mesomelas*) from suborder Caniformia. If foraging behaviour is the best predictor for the constitutive innate immunocompetence, brown hyenas and black-backed jackals

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Table 1. Foraging behaviour, diet, body mass and social organisation of the six examined carnivore species*

	Caracal (*Caracal caracal*)	Cheetah (*Acinonyx jubatus*)	Leopard (*Panthera pardus*)	Lion (*Panthera leo*)	Brown hyena (*Hyaena brunnea*)	Black-backed jackal (*Canis mesomelas*)
Foraging behaviour	Mainly hunter	Hunter	Hunter and scavenger	Hunter and scavenger	Mainly scavenger	Mainly scavenger
Body mass	Small (<20 kg)	Medium (20-100 kg)	Medium (20-100 kg)	Large (>100 kg)	Medium (20-100 kg)	Small (<20 kg)
Social organisation	Solitary	Solitary, males form stable coalitions	Solitary	Large stable groups	Solitary or small fission-fusion groups	Socially monogamous in small family groups

*Characteristics following Kingdon et al., 2013

should have the highest immunocompetence and cheetahs the lowest, if body size is the best predictor, lions should have the highest immunocompetence and black-backed jackals and caracals the lowest, and if social organisation is the best predictor, lions should have the highest values and leopards and caracals the lowest. Alternatively, the immunocompetence follows the phylogenetic relationships of the species.

RESULTS

Samples were stored between six months and 11 years, and thawed for the first time. There was no difference in BKA results between (1) measurements of the 46 animals sampled repeatedly (Skillings Mack Statistic=4.55, 10.000 permutations, P=0.31), (2) cheetah samples stored for different numbers of years (Jonckheere–Terpstra test, JT=26,851, 10.000 permutations, P=0.48; Fig. 1A) and (3) leopard samples stored for different numbers of years (JT=203.5, 10.000 permutations, P=0.90; Fig. 1B). We therefore used samples from all study years and chose randomly one sample of individuals sampled repeatedly to avoid pseudo-replications in the data set. Two cheetah family groups were responsible for the drop in killing capacity of cheetah serum in 2005 (Fig. 1A). The three cubs of one mother and a group of three young males had particularly low results (ranks 0, 0, 2 and 0, 0, 0, respectively). Their immune system might have been affected by illness at the time of capture although they did not show any clinical signs.

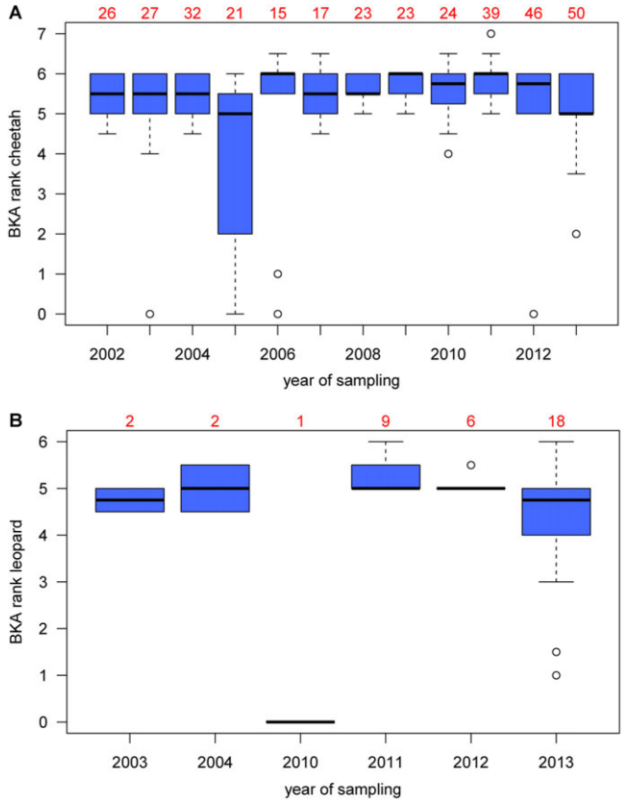

Fig. 1. BKA ranks of samples from different years. For cheetahs (A) and leopards (B). Numbers above boxplots represent sample sizes. Circles depict values more than 1.5 times the interquartile range below the first quartile. Changes between years were not significant.

Species differed in their BKA results (Kruskal–Wallis test, H=85.56, d.f.=5, P<0.001). Posthoc pairwise comparison revealed similar BKA ranks for caracals and cheetahs, for lions and leopards and for lions and brown hyenas (Table 2). The highest bacterial killing capacity was measured in caracals and cheetahs and the lowest in black-backed jackals (Fig. 2). There was a positive linear relationship between the BKA distance matrix and the genetic distances between species (Mantel test, r=0.773, P=0.023; Fig. 3).

Black-backed jackals had BKA values similar to those of coyotes (*Canis latrans*), house finches [*Haemorhous* (formerly *Carpodacus*) *mexicanus*], newts (*Taricha granulosa*) or garter snakes (*Thamnophis elegans*) (French and Neuman-Lee, 2012), whereas all feliform species had substantially higher BKA values (Fig. 4A).

The complete dataset of BKA results is available in the Supplementary information.

DISCUSSION

Within free-ranging mammals, comparative eco-immunological studies were previously available only from bats and rodents (Schneeberger et al., 2013; Tian et al., 2015), and for individual species from a variety of vertebrates (French and Neuman-Lee, 2012). Here we extend such studies by comparing the bacterial killing capacity of six sympatric carnivore species.

Storage duration had no effect on the bacterial killing capacity of carnivore serum against *Escherichia coli* (*E.coli*) when comparing

Table 2. *P*-values of posthoc pairwise comparison of BKA ranks of six carnivore species with Dwass–Steel–Critchlow–Fligner test based on 1000 Monte Carlo permutations

	Caracal	Cheetah	Leopard	Lion	Brown hyena
Cheetah	0.565	-	-	-	-
Leopard	<0.001	<0.001	-	-	-
Lion	0.002	<0.001	0.607	-	-
Brown hyena	<0.001	<0.001	0.003	0.622	-
Black-backed jackal	<0.001	<0.001	<0.001	<0.001	<0.001

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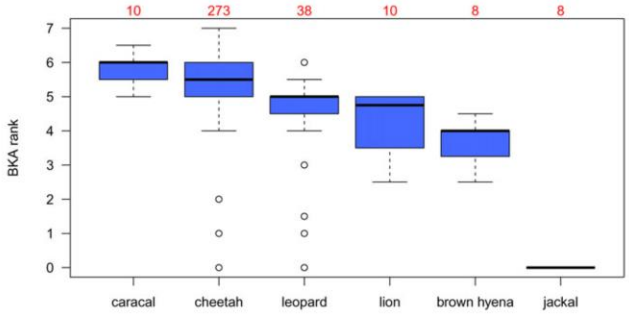

Fig. 2. BKA ranks of the six carnivore species. Numbers above boxplots represent sample sizes. Circles depict values more than 1.5 times the interquartile range below the first quartile.

samples frozen for 6 months up to 11 years. In bats, storing duration of plasma between 41 up to 81 days did not change the bacterial killing capacity (Schneeberger et al., 2013), similar to plasma of flycatchers (*Myiarchus cinerascens*) and bluebirds (*Sialia mexicana*) which retained bacterial killing capacity after 19-53 days (average 30 days) of storage at −80°C (Jacobs and Fair, 2016). Also, plasma of tree swallows (*Tachycineta bicolor*) stored for 6 months at −80°C revealed similar results as fresh plasma (Morrison et al., 2009). However, in some bird species bacterial killing capacity can also drop drastically within the first few weeks of storage. In house sparrows (*Passer domesticus*), bacterial killing capacity of plasma dropped from approximately 50% to 15% within the first three weeks when samples were stored at −40°C (Liebl and Martin, 2009), similar to the plasma of chickens (*Gallus gallus*) that dropped close to zero in most samples after 19-53 days stored at −80°C (Jacobs and Fair, 2016). These differences might be due to different storage temperatures, samples used (serum or plasma) or might reflect a difference in sensitivity of humoral effectors to storage length for different species. However, even if our samples lost bacterial killing capacity within the first few weeks, it cannot be much, because nearly all samples of the feliform carnivores had an initial bacterial killing capacity of ≥95%, irrespective of the storage time.

Foraging behaviour was not a good predictor of the innate immunocompetence of the six carnivore species. Brown hyenas and black-backed jackals, the main carrion feeders, showed the lowest BKA values instead of the predicted highest. Also, BKA values of large species were not higher than those of smaller species, because the two smallest species, caracal and black-backed jackal, showed the highest and lowest BKA values, respectively. Nor did social organization explain BKA values because lions, the most social species, showed an intermediate BKA result, whereas caracals and leopards, both solitary, had higher and similar values compared to lions, respectively.

The latter might be surprising because among mammals and other vertebrates social group size appears to be an important predictor of parasite risk (Côté and Poulin, 1995). The different branches of the immune system convey different costs and benefits and it is possible that different branches may compensate each other (Norris and Evans, 2000). Unique to adaptive immunity is the immunological memory that confers long-lasting immunity against pathogens and is advantageous in case of repeated infections with the same pathogen because its response is quick and specific (Janeway et al., 2001). Strong adaptive immunity may thus be more advantageous for social species, because social species often experience higher and repeated pathogen pressure from the same pathogen (Côté and Poulin, 1995). Consistent with this, T- and B-cell mediated adaptive immune responses were significantly stronger in colonial than non-colonial bird species (Møller et al., 2001). However, the bacterial killing assay used in this study estimates the strength of the constitutive innate immune response. Thus, sociality of a species might not influence the innate immune response but rather the adaptive immune response. Furthermore, immunity against parasites that are classified as generalists, i.e. infect many host species, and are transmitted by vectors or through contaminated soil, food or water might be less influenced by social organisation than immunity against parasites that are classified as specialists (Altizer et al., 2003). Because our BKA estimates the immune response against *E.coli*, an ubiquitous pathogen that infects many host species, sociality might not be very important for the pathogen transmission risk of this bacterium.

Of the six carnivore species we examined in this study, the cheetah is known to have a low genetic variability, including at the major histocompatibility complex (MHC), a multigene family crucial to the adaptive immune defense of vertebrates (Castro-Prieto et al., 2011b; O'Brien et al., 1983). It was suggested that the cheetah was highly susceptible to infectious diseases, but this was not confirmed for free-ranging cheetahs (Thalwitzer et al., 2010). This suggests that the different immune branches (adaptive and innate) can compensate each other and would explain the high BKA results in cheetahs. In line with this are the lower BKA results of leopards which have a higher MHC variability than cheetahs (Castro-Prieto et al., 2011a). Within the Hyaenidae, striped hyenas (*Hyaena hyaena*) and spotted hyenas (*Crocuta crocuta*) have a similar MHC variability and a higher one than cheetahs and leopards (Califf et al., 2013), but the MHC variability of brown hyenas is not described, nor the ones of caracals, African lions, and black-backed jackals. Thus, adaptive and innate immunity may compensate each other but more information for the carnivore species in this study is needed to confirm these first results. Although the bacterial killing capacity of free-ranging and captive spotted hyenas was studied, methodological differences make it difficult to compare the results of the study (Flies et al., 2015) to the ones obtained by us. Additional information on other aspects of the immune system for

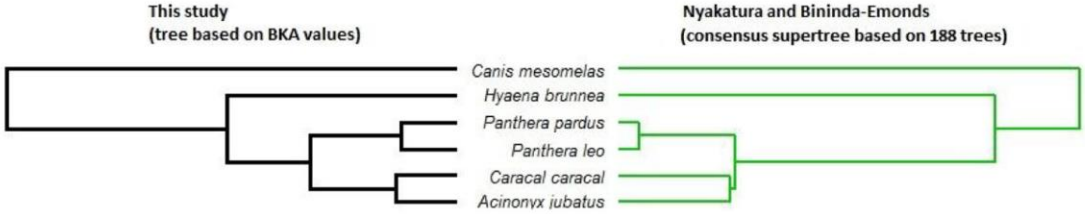

Fig. 3. Comparison of phylogenies derived from BKA values (this study) and the supertree reported by Nyakatura and Bininda-Emonds (2012). BKA results were hierarchically clustered with the centroid method (D'haeseleer, 2005).

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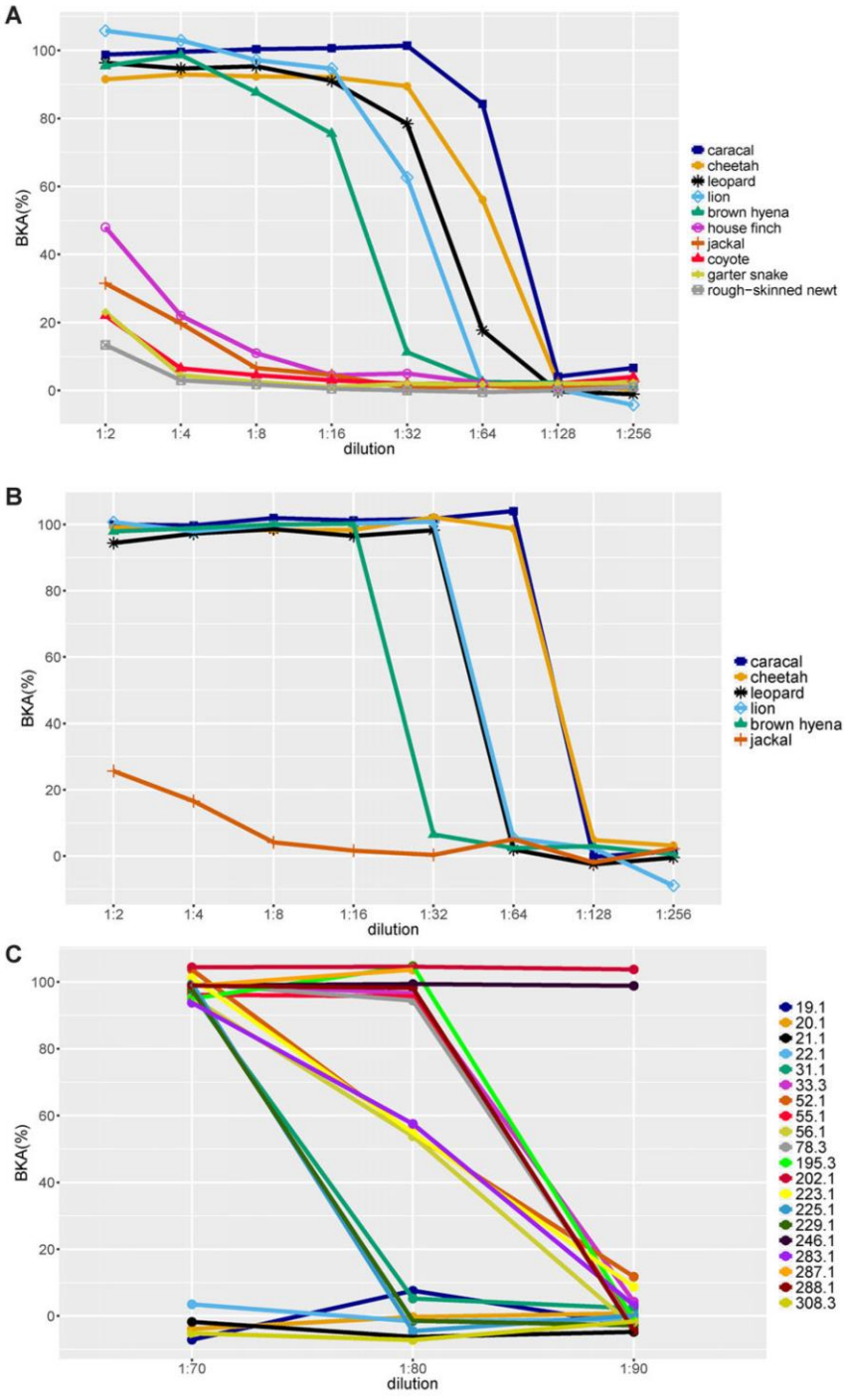

Fig. 4. BKA values for different species. (A) Mean BKA values for eight serial dilutions of the six carnivore species from this study and four other species (a carnivore, a bird, a reptile and an amphibian) previously published. Values for published species are from Fig. 2A from French and Neuman-Lee (2012). (B) BKA values for eight serial dilutions of six carnivore species. Lines represent one randomly chosen individual per species. (C) BKA values for dilutions 1:70, 1:80 and 1:90 for 20 randomly selected cheetahs.

these species is sparse. Studies on African lions have mostly focused on infectious diseases such as tuberculosis (Viljoen et al., 2015) and canine distemper virus (Roelke-Parker et al., 1996), but a functional characterisation of their immune system has not been attempted.

The only good predictor of the strength of the constitutive innate immune system of our species was the phylogeny, because the tree based on BKA results was similar to the one representing evolutionary relationships between the species (Nyakatura and Bininda-Emonds, 2012). These results are intriguing from at least two perspectives: (1) The strong selection pressure assumed to be exerted by different environments as represented by variation in foraging behaviour, body size or social organisation did not fine-tune the innate immune system, being – in comparison with the adaptive response – the evolutionarily older part of the immune system (Janeway et al., 2001). (2) The ancestors of feliform carnivores developed a superior form of constitutive innate immune response.

All feliform species had BKA values an order of magnitude higher than those previously reported from various vertebrates and those of black-backed jackals. It is therefore unlikely that the 8% of

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felids threatened by diseases suffer from a weakness of their constitutive immune system.

MATERIAL AND METHODS

Sample collection

Between 2002 and 2013 we blood-sampled 275 cheetahs (194 males, 81 females), 38 leopards (19 males, 19 females), ten lions (7 males, 3 females), ten caracals (5 males, 4 females, 1 unknown), eight black-backed jackals (6 males, 1 female, 1 unknown) and eight brown hyenas (4 males, 4 females). Animals were free-ranging on commercial farmland in east-central Namibia, except for six lions which were sampled in Etosha National Park in north-central Namibia and in Caprivi region in north-eastern Namibia. Forty-eight cheetahs, four lions, one leopard and one caracal were wild born animals kept in large enclosures on privately owned farms or at the AfriCat Foundation, a non-profit conservation facility for carnivores in central Namibia. All free-ranging animals, except lions, were captured in box traps, immobilized as previously described (Thalwitzer et al., 2010; Wachter et al., 2011) and released again at the site of capture. Free-ranging lions were darted from a vehicle on bait or on a kill. Captive animals were immobilized in their enclosures. Thirty, eight, four, two and one cheetahs were sampled twice, three, four, five and six times, respectively, and one caracal was sampled twice, resulting in 421 samples.

Blood was taken from the cephalic vein with sterile Vacutainer® serum tubes (Becton Dickinson), transported to the field laboratory in a cooler box and centrifuged within 24 h after sampling. Serum samples were sub-sampled, frozen and stored in liquid nitrogen until transported to Germany on dry ice or in liquid nitrogen, in full compliance with the Convention on International Trade and in Endangered Species (CITES), and then stored at −80°C.

The sex ratio of the species was similar (Fisher's exact test, P=0.090), thus we combined all samples for analyses. Animal immobilizations and sample collections were authorized by the Ministry of Environment and Tourism of Namibia and complied with the laws of the country.

Bacterial killing assay (BKA)

To measure the constitutive immune system, we used the bacterial killing assay (BKA) (Liebl and Martin, 2009; Tieleman et al., 2005), which measures the capacity of plasma to kill microorganisms and integrates many important humoral components of constitutive innate immunity. The bacterial killing assay was conducted with *E.coli* (ATCC No 8739), a ubiquitous gram-negative bacteria, with which all studied species regularly come into contact. We followed the method proposed by French and Neumann-Lee but used larger volumes (French and Neuman-Lee, 2012). Lyophilized pellets of *E.coli* were reconstituted in 10 ml sterile phosphate-buffered saline (PBS). The reconstituted bacteria were plated on a blood-agar plate and incubated at 37°C for 24 h. One colony was transferred into 5 ml of Tryptic Soy Broth (TSB) (Sigma Aldrich, Steinheim, Germany) 4-6 h before the assay and incubated at 37°C while placed on a shaker at 170 rpm (Stuart orbital incubator S1500, Bibby Scientific, Staffordshire, UK). Directly prior to the assay, we diluted the bacteria to a concentration of McFarland 0.5. This solution was then diluted by 10^3 with sterile PBS to obtain the bacterial working solution with an approximate concentration of 1.5×10^5 cells/ml.

Under a sterile hood we serially diluted 44 µl of each serum sample with PBS from 1:2 to 1:256 in a 96-well plate and added 10 µl of bacterial working solution to each dilution. We mixed 10 µl of bacterial working solution with 44 µl of PBS as positive control and used PBS only as negative control. The plate was covered and put on a plate shaker for 1 min at 150 rpm. It was incubated for 30 min at 37°C and again gently mixed on a plate shaker for 1 min at 150 rpm. Then, 250 µl of TSB, pre-warmed to 37°C, were added to all wells and the plate was again put on a plate shaker for 1 min at 100 rpm. Samples were measured in duplicates at 300 nm using a microplate reader (Biotek; µQuant Microplate Spectrophotometer, Winooski, USA) to determine the background absorbance at the starting point of bacterial growth. After an incubation of 12 h at 37°C the absorbance of the samples was measured again.

The absorbance readings of the negative controls at 0 h and 12 h of incubation should be similar. However, we noticed a slight discolouring in TSB during incubation when TSB was prepared 2 days or less before use, resulting in a drop of absorbance in negative controls by up to 14%. If the samples, the reagents or the plates were contaminated, a raise in absorbance would be expected. We therefore suggest using only TSB that has been prepared at least 2 days prior to the assay. To account for the affected plates, we subtracted the mean absorbance of negative controls at 0 h of incubation from the one at 12 h of incubation (=δC). We then subtracted absorbance of samples at 0 h of incubation from the ones at 12 h of incubation (=δA_i, δA_j, …) and added δC to each δA. The mean of the duplicates (=$(\delta A_i+\delta A_j)/2$ =$\emptyset D_{i,j}$) was used to calculate the bacterial killing capacity as $1-\emptyset D_{i,j}/\emptyset P\times100$, with øP being the mean absorbance of the positive controls.

We used serial serum dilutions from 1:2 to 1:256 to identify optimal working dilutions for each species (French and Neuman-Lee, 2012). At one of these dilutions, samples should kill on average approximately 50% of bacteria. We did not detect such a dilution for the carnivore species tested, except for black-backed jackals, because in preliminary tests killing capacity systematically dropped from ∼100% to 0% between two serial dilutions (Fig. 4B). The drop in bacterial killing capacity for cheetahs, the majority of our samples, mostly occurred between dilutions 1:64 and 1:128. We therefore investigated this phenomenon in more detail and diluted additional aliquots of 20 randomly selected cheetahs to 1:70, 1:80 and 1:90. Still, we could not identify an optimal dilution for cheetahs, because the bacterial killing capacity was for all dilutions in most cases either 100% or 0% (Fig. 4C). Thus, we concluded that the assay allows the bacteria to grow to saturation if the plasma is not able to kill 100% of bacteria. This means that there might be a fine-tuned threshold dilution for each individual at which it cannot kill the bacteria any more. This is similar to the findings of a recent study on BKA in spotted hyenas (Flies et al., 2015), but differs from other studies that identified optimal working dilutions for each species (French and Neuman-Lee, 2012; Liebl and Martin, 2009). Because we could not identify an optimal dilution for most of the species, we performed serial dilutions for all samples and all species and attributed a rank to the last dilution before the drop to 0% killing, i.e. dilution 1:2 corresponded to rank 1, dilution 1:4 to rank 2, etc. Because we measured all samples as duplicates, we sometimes measured at one particular dilution 100% bacterial killing for one aliquot and 0% for the other one, resulting in a mean value of 50% for this dilution. Such individuals were assigned the mean of the ranks of both dilutions with 100% and 50% bacterial killing respectively. If bacterial killing did not reach 100% even at dilution 1:2, the rank score was 0. Consequently, all black-backed jackals had ranks of 0.

Statistical analyses

All statistical analyses were performed using the open-source software R version 3.0.2 (R-Core-Team, 2015). To test whether phylogeny is a good predictor for bacterial killing capacity, we calculated mean BKA values for each species at each dilution. We then calculated a Euclidean distance matrix for all possible pairs of species. We used a Mantel test to compare this distance matrix with a published phylogenetic distance matrix (Nyakatura and Bininda-Emonds, 2012). The R package 'clinfun' (https://cran.r-project.org/web/packages/clinfun/) was used for the Jonckheere–Terpstra test, the R package 'Skillings.Mack' (https://cran.r-project.org/web/packages/Skillings.Mack/) for the Skillings–Mack test and the packages 'ape' (Paradis et al., 2004), 'picante' (Kembel et al., 2010) and 'geiger' (Harmon et al., 2008) for examination of whether phylogenetic relationships of species are a good predictor of the bacterial killing capacity of the species. Fig. 4 was created with the R package 'ggplot2' (Wickham, 2009).

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Competing interests

The authors declare no competing or financial interests.

Author contributions

S.K.H. collected many of the samples, carried out the laboratory work, analysed the data and drafted the manuscript. B.W. and G.Á.C. conceived and designed the

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study, helped with data analysis and writing of the manuscript. J.M., S.T. and O.H.K.A. contributed additional samples. H.H. guided data analysis and critically reviewed the manuscript. All authors gave final approval for publication.

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Supplementary information

Supplementary information available online at
http://bio.biologists.org/lookup/suppl/doi:10.1242/bio.014902/-/DC1

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Supplementary data

BKA results at different dilution of the six carnivore species from this study with information on BKA rank (see main text how ranks were assigned), sex, year of sampling and roaming status. Animal IDs consist of consecutive numbers in front of the period and the number of sampling events after the period, for example, the animal ID 33.4 represents animal number 33 that was sampled for the 4th time.

animal ID	BKA (%) at dilution 1:2	BKA (%) at dilution 1:4	BKA (%) at dilution 1:8	BKA (%) at dilution 1:16	BKA (%) at dilution 1:32	BKA (%) at dilution 1:64	BKA (%) at dilution 1:128	BKA (%) at dilution 1:256	BKA rank	sex	species	year of sampling	roaming status
1.1	96.24	100.29	100.08	96.76	98.16	3.10	1.96	-1.18	5	male	cheetah	2002	free-ranging
2.1	91.53	93.80	95.35	99.21	100.75	99.53	0.77	1.91	6	male	cheetah	2002	free-ranging
3.1	93.47	95.40	96.35	99.92	103.37	99.38	-0.68	1.37	6	male	cheetah	2002	free-ranging
4.1	94.07	97.28	98.32	102.56	103.50	104.11	2.27	3.07	6	male	cheetah	2002	free-ranging
5.1	89.48	92.65	92.86	96.03	99.36	100.62	5.34	1.61	6	male	cheetah	2002	free-ranging
6.1	84.93	90.82	92.78	91.23	98.87	93.71	9.61	3.39	6	male	cheetah	2002	free-ranging
7.1	97.25	96.00	99.02	99.02	105.03	103.54	2.85	8.15	6	male	cheetah	2002	free-ranging
8.1	97.76	100.01	100.84	101.83	100.92	100.57	-4.10	-5.62	6	male	cheetah	2002	free-ranging
9.1	97.05	96.47	94.34	97.82	98.16	-5.58	-0.54	1.25	5	male	cheetah	2003	free-ranging
10.1	92.81	94.03	94.54	96.22	48.86	2.54	2.96	0.22	4.5	female	cheetah	2003	free-ranging
11.1	88.70	93.26	94.00	95.72	99.46	3.46	1.00	0.05	5	female	cheetah	2003	free-ranging
12.1	87.70	93.33	94.59	95.80	51.98	5.69	-0.27	-6.35	4.5	male	cheetah	2003	free-ranging
13.1	95.59	97.21	98.69	100.32	2.50	-2.63	-5.83	-11.28	4	female	cheetah	2003	free-ranging
14.1	92.69	93.87	92.44	93.54	96.07	95.69	0.30	0.42	6	male	cheetah	2003	free-ranging
15.1	99.60	105.28	104.26	104.92	105.86	107.37	12.80	3.68	6	male	cheetah	2004	free-ranging
16.1	91.86	95.10	94.16	93.95	49.36	2.97	3.05	0.46	4.5	male	cheetah	2004	free-ranging
17.1	94.79	99.84	99.75	100.00	100.41	52.02	1.54	-5.93	5.5	male	cheetah	2004	free-ranging
18.1	99.30	99.15	100.82	99.07	100.31	99.11	4.11	3.75	6	male	cheetah	2004	free-ranging
19.1	99.18	98.58	98.29	98.38	102.21	98.78	4.80	3.11	6	female	cheetah	2005	free-ranging
20.1	55.10	49.62	2.88	1.08	0.98	1.13	-1.45	-5.88	0	female	cheetah	2005	free-ranging

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animal ID	BKA (%) at dilution 1:2	BKA (%) at dilution 1:4	BKA (%) at dilution 1:8	BKA (%) at dilution 1:16	BKA (%) at dilution 1:32	BKA (%) at dilution 1:64	BKA (%) at dilution 1:128	BKA (%) at dilution 1:256	BKA rank	sex	species	year of sampling	roaming status
21.1	14.69	12.74	3.30	-0.18	1.77	0.50	-7.36	0.76	0	female	cheetah	2005	free-ranging
22.1	26.64	8.54	7.82	1.93	-7.31	-15.21	-19.02	-17.51	0	male	cheetah	2005	free-ranging
23.1	94.07	86.61	92.50	97.59	98.39	49.71	-0.62	-3.48	5.5	male	cheetah	2005	free-ranging
24.1	94.95	98.54	6.35	0.65	3.46	0.30	-0.39	0.95	2	male	cheetah	2005	free-ranging
25.1	26.48	13.25	7.88	3.35	1.39	1.48	2.85	1.31	0	female	cheetah	2005	free-ranging
26.1	NA	29.19	17.15	5.36	-2.63	-3.27	1.71	-0.06	0	male	cheetah	2005	free-ranging
26.2	97.52	98.88	98.33	100.47	100.69	53.82	3.41	1.60	5.5	male	cheetah	2006	free-ranging
27.1	22.77	18.24	8.72	3.17	3.82	3.70	-0.18	-5.53	0	male	cheetah	2006	free-ranging
28.1	13.98	5.27	13.53	14.19	11.16	12.14	6.99	-0.86	0	male	cheetah	2006	free-ranging
29.1	93.08	5.85	-6.87	-7.18	0.40	-2.45	-2.95	-1.92	1	male	cheetah	2006	free-ranging
30.1	95.22	94.92	95.09	96.38	96.88	98.29	3.85	1.39	6	male	cheetah	2006	free-ranging
31.1	99.62	102.43	101.87	103.20	102.49	103.05	0.10	-0.26	6	male	cheetah	2006	free-ranging
32.1	101.20	100.75	104.18	109.62	109.13	106.96	12.76	1.92	6	male	cheetah	2007	free-ranging
33.1	97.24	97.58	97.58	98.02	99.23	99.37	2.80	5.89	6	male	cheetah	2007	free-ranging
33.2	91.60	94.05	96.38	94.88	96.26	52.07	3.52	1.98	5.5	male	cheetah	2008	free-ranging
33.3	101.48	98.63	101.16	101.48	98.15	53.26	-3.09	0.13	5.5	male	cheetah	2009	free-ranging
33.4	95.32	96.92	96.69	98.38	98.76	49.15	-1.51	0.85	5.5	male	cheetah	2010	free-ranging
34.1	99.64	101.20	97.56	99.68	102.72	100.74	44.84	-4.26	6.5	male	cheetah	2007	free-ranging
34.2	96.54	97.20	98.48	98.79	98.92	6.88	2.29	-2.78	5	male	cheetah	2007	free-ranging
35.1	96.73	97.75	98.04	100.41	100.33	11.50	1.65	5.37	5	male	cheetah	2007	free-ranging
36.1	96.80	100.29	97.46	97.82	54.02	10.19	3.18	-1.85	4.5	male	cheetah	2007	free-ranging
37.1	97.68	98.43	97.42	97.64	97.16	5.12	7.72	-0.35	5	male	cheetah	2007	free-ranging
38.1	98.17	100.73	102.33	101.63	102.63	53.27	-3.97	-3.02	5.5	female	cheetah	2008	free-ranging
39.1	96.52	96.83	95.63	85.85	98.44	100.40	2.90	-2.72	6	male	cheetah	2008	free-ranging
40.1	96.36	97.20	99.56	99.37	102.80	99.93	0.70	0.97	6	male	cheetah	2008	free-ranging
41.1	94.88	96.64	98.08	99.75	102.99	100.39	-0.51	0.60	6	male	cheetah	2008	free-ranging

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animal ID	BKA (%) at dilution 1:2	BKA (%) at dilution 1:4	BKA (%) at dilution 1:8	BKA (%) at dilution 1:16	BKA (%) at dilution 1:32	BKA (%) at dilution 1:64	BKA (%) at dilution 1:128	BKA (%) at dilution 1:256	BKA rank	sex	species	year of sampling	roaming status
41.2	92.60	95.45	95.36	97.66	98.56	99.41	2.03	4.29	6	male	cheetah	2008	free-ranging
42.1	102.38	101.97	101.89	106.63	100.38	102.46	3.80	1.19	6	male	cheetah	2008	free-ranging
43.1	95.96	97.79	99.66	99.45	100.16	100.20	0.41	4.07	6	male	cheetah	2008	free-ranging
44.1	97.42	98.39	99.14	99.05	98.57	12.79	0.53	-0.09	5	male	cheetah	2008	free-ranging
45.1	98.52	99.54	97.91	97.64	97.77	5.60	6.62	-2.03	5	female	cheetah	2008	free-ranging
46.1	97.07	99.97	101.26	99.88	101.87	4.41	0.43	2.20	5	male	cheetah	2008	free-ranging
47.1	97.16	101.00	101.22	101.05	101.09	50.22	0.52	0.43	5.5	male	cheetah	2008	free-ranging
48.1	96.77	100.36	99.67	100.18	100.01	101.91	-0.91	2.03	6	male	cheetah	2008	free-ranging
49.1	102.46	99.47	98.52	98.08	97.91	49.94	-3.19	-0.60	5.5	male	cheetah	2008	free-ranging
49.2	92.30	95.39	93.15	99.73	98.75	0.93	-0.55	0.12	5	male	cheetah	2008	free-ranging
50.1	96.59	103.21	100.00	101.43	100.79	3.89	5.37	-7.72	5	male	lion	2013	captive
51.1	93.69	97.00	93.15	99.15	99.10	99.87	1.20	2.63	6	male	cheetah	2008	free-ranging
52.1	95.80	94.56	99.98	101.17	98.78	100.17	16.43	4.45	6	female	cheetah	2008	free-ranging
53.1	95.25	96.55	94.00	97.63	98.39	51.02	-1.40	-2.29	5.5	female	cheetah	2008	free-ranging
54.1	99.94	103.36	99.74	97.64	101.26	5.26	2.58	0.57	5	male	cheetah	2009	free-ranging
54.2	98.10	98.27	99.61	99.45	56.26	6.04	2.57	0.11	4.5	male	cheetah	2010	free-ranging
55.1	102.70	105.26	103.93	107.01	106.44	105.88	0.53	1.76	6	female	cheetah	2009	captive
56.1	99.06	104.34	104.29	106.49	106.39	107.37	1.35	2.17	6	female	cheetah	2009	captive
57.1	115.58	110.55	113.99	106.46	111.15	64.62	2.87	8.93	5.5	female	cheetah	2009	free-ranging
57.2	96.78	97.47	97.24	98.29	98.84	3.86	-0.63	-0.68	5	female	cheetah	2012	free-ranging
58.1	95.82	101.55	102.33	102.33	101.96	53.50	-0.46	-4.15	5.5	male	cheetah	2011	free-ranging
58.2	93.93	95.36	96.82	97.61	98.28	52.38	3.22	-0.79	5.5	male	cheetah	2012	free-ranging
59.1	96.47	95.88	85.23	93.81	2.06	-6.13	-2.03	0.92	4	male	cheetah	2010	free-ranging
59.2	NA	104.63	106.70	108.39	104.20	97.63	3.72	7.39	6	male	cheetah	2010	free-ranging
59.3	96.75	90.42	95.16	94.10	94.46	95.94	0.09	-3.79	6	male	cheetah	2011	free-ranging
60.1	101.23	103.30	99.72	101.66	53.06	2.99	5.01	4.32	4.5	male	cheetah	2010	free-ranging

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61.1	95.73	97.41	98.67	98.22	98.35	98.76	8.91	0.07	6	male	cheetah	2010	free-ranging
62.1	93.15	95.24	93.45	97.96	98.17	99.49	-0.39	0.33	6	male	cheetah	2010	free-ranging
63.1	98.25	99.46	100.31	100.76	99.91	2.99	-3.59	3.66	5	male	cheetah	2010	free-ranging
64.1	100.84	103.64	101.14	102.70	103.99	52.63	5.96	7.47	5.5	male	cheetah	2010	free-ranging
65.1	98.65	99.79	100.41	101.11	102.70	103.22	3.88	1.46	6	male	cheetah	2012	free-ranging
66.1	95.36	97.27	98.40	99.57	102.89	100.64	0.63	4.00	6	female	cheetah	2011	free-ranging
67.1	26.28	56.56	42.47	98.77	99.66	98.30	0.68	2.50	6	male	cheetah	2011	free-ranging
68.1	96.45	101.80	100.42	104.43	103.22	102.49	5.73	5.00	6	male	cheetah	2011	free-ranging
69.1	103.26	107.58	105.42	105.42	105.16	3.46	1.22	-1.15	5	female	cheetah	2011	free-ranging
70.1	96.68	98.10	97.22	100.27	98.47	98.39	54.84	3.05	6.5	male	cheetah	2011	free-ranging
71.1	93.10	93.88	95.10	96.14	97.36	97.62	-1.09	-5.00	6	male	cheetah	2011	free-ranging
72.1	99.30	100.85	102.15	101.97	103.83	100.89	54.54	1.08	6.5	male	cheetah	2011	free-ranging
73.1	100.42	100.85	103.40	101.33	104.47	101.24	5.17	-2.20	6	female	cheetah	2011	free-ranging
74.1	99.99	102.88	102.53	101.07	106.89	101.28	0.91	-4.35	6	female	cheetah	2011	free-ranging
75.1	96.61	99.90	98.40	97.49	98.44	8.57	5.54	3.88	5	female	cheetah	2011	free-ranging
76.1	96.09	98.56	97.60	97.81	100.77	54.29	5.89	2.63	5.5	male	cheetah	2011	free-ranging
77.1	100.34	103.51	102.07	101.19	102.62	102.20	5.32	0.20	6	male	cheetah	2011	free-ranging
78.1	95.17	98.95	100.77	100.38	98.69	48.67	-0.92	-1.31	5.5	male	cheetah	2011	free-ranging
78.2	97.62	104.79	103.87	104.66	103.96	56.75	5.21	9.49	5.5	male	cheetah	2011	free-ranging
78.3	96.95	98.12	99.30	100.98	99.19	99.58	3.73	-1.65	6	male	cheetah	2011	free-ranging
78.4	97.65	98.94	97.93	100.86	98.11	53.02	3.63	4.82	5.5	male	cheetah	2012	free-ranging
78.5	101.25	104.09	103.94	104.82	106.73	2.49	-7.63	-0.35	5	male	cheetah	2013	free-ranging
78.6	96.97	96.48	96.97	97.83	100.63	9.30	-10.07	4.67	5	male	cheetah	2013	free-ranging
79.1	96.64	99.42	100.13	100.51	100.46	102.68	0.28	2.36	6	female	cheetah	2011	free-ranging
80.1	95.84	96.92	98.01	96.79	100.14	98.71	-1.52	0.26	6	female	cheetah	2011	free-ranging
81.1	93.89	96.48	97.82	97.45	98.95	98.83	6.06	2.97	6	female	cheetah	2012	free-ranging

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82.1	91.47	97.60	99.85	100.18	100.36	53.61	-2.50	1.61	5.5	male	cheetah	2012	free-ranging
83.1	95.21	98.16	99.89	98.26	101.53	101.77	-2.60	-1.05	6	male	cheetah	2012	free-ranging
84.1	97.11	99.30	100.01	100.43	100.47	100.81	3.18	-0.93	6	male	cheetah	2012	free-ranging
85.1	97.50	99.79	99.70	100.06	100.98	2.87	3.53	-1.22	5	male	cheetah	2012	free-ranging
86.1	100.98	107.15	105.14	104.92	106.63	105.88	3.72	10.28	6	male	cheetah	2012	free-ranging
87.1	98.65	99.01	98.51	100.83	98.51	51.69	0.87	2.19	5.5	male	cheetah	2012	free-ranging
88.1	94.81	97.95	99.30	100.05	99.93	100.56	1.50	-1.90	6	male	cheetah	2012	free-ranging
88.2	99.94	100.91	100.86	101.42	100.80	3.40	5.80	1.76	5	male	cheetah	2013	free-ranging
88.3	99.35	100.87	99.65	104.84	104.44	105.76	3.30	0.50	6	male	cheetah	2013	free-ranging
89.1	94.62	97.23	100.25	100.15	86.32	5.81	4.13	-8.36	5	male	lion	2013	captive
90.1	97.28	98.17	98.77	99.60	101.23	6.90	10.41	-2.09	5	male	lion	2013	captive
91.1	89.52	94.61	95.65	99.26	95.40	99.06	4.78	3.89	6	male	cheetah	2013	free-ranging
92.1	94.61	96.48	98.73	99.05	97.09	3.34	-2.22	-10.36	5	female	cheetah	2013	free-ranging
93.1	100.86	79.48	107.97	104.25	104.58	4.55	4.39	5.97	5	female	cheetah	2013	free-ranging
94.1	100.85	98.92	100.85	101.00	98.19	-7.28	3.95	4.42	5	male	cheetah	2013	free-ranging
95.1	97.93	99.91	100.53	101.31	100.59	20.70	0.62	1.25	5	female	cheetah	2013	free-ranging
96.1	93.83	100.48	100.34	98.55	103.08	98.84	-12.81	5.35	6	male	cheetah	2013	free-ranging
97.1	97.16	102.04	101.54	-4.39	-0.32	3.50	-1.44	-2.81	3	female	cheetah	2013	free-ranging
98.1	93.66	95.42	96.91	53.31	-0.52	-2.60	4.76	3.53	3.5	male	cheetah	2013	free-ranging
99.1	90.15	95.34	96.80	98.69	61.67	16.72	-11.25	-14.13	4.5	male	cheetah	2013	free-ranging
100.1	96.77	100.35	100.58	103.74	104.67	103.00	2.90	-0.91	6	male	cheetah	2013	free-ranging
101.1	92.60	93.30	96.39	96.39	59.92	37.62	3.02	-10.12	4.5	male	cheetah	2014	free-ranging
102.1	95.97	97.97	98.30	99.98	98.30	100.16	5.18	-1.01	6	female	caracal	2003	free-ranging
103.1	100.03	99.76	101.98	101.29	101.77	104.08	-0.25	1.41	6	male	caracal	2003	free-ranging
104.1	98.87	98.60	102.46	102.65	101.45	25.54	-1.53	3.95	5	female	caracal	2004	free-ranging
105.1	55.05	31.52	13.80	2.98	0.49	-2.31	-0.20	1.92	0	female	jackal	2004	free-ranging

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106.1	90.89	95.97	96.48	98.76	98.53	100.44	52.07	50.34	6.5	male	caracal	2004	free-ranging
107.1	97.55	99.96	99.56	102.97	101.49	103.28	4.81	5.02	6	male	caracal	2008	free-ranging
108.1	65.26	38.49	19.96	11.67	3.07	0.84	-0.84	1.94	0	male	jackal	2008	free-ranging
109.1	98.30	99.08	99.43	94.67	101.53	101.35	3.54	5.81	6	female	caracal	2008	free-ranging
110.1	101.70	101.47	101.30	101.39	100.37	102.76	-0.18	6.21	6	male	caracal	2008	free-ranging
110.2	101.13	98.41	98.27	101.17	100.48	100.02	0.36	4.45	6	male	caracal	2008	free-ranging
111.1	14.26	10.35	5.93	0.96	3.35	1.19	-0.29	12.10	0	male	jackal	2008	free-ranging
112.1	32.77	14.92	5.10	6.63	-2.04	1.30	3.06	-0.74	0	male	jackal	2009	free-ranging
113.1	9.15	7.61	4.44	2.62	0.06	0.52	1.92	-2.92	0	male	jackal	2009	free-ranging
114.1	97.77	102.54	100.95	99.10	101.26	65.61	-0.35	-2.76	5.5	female	caracal	2009	captive
115.1	29.98	20.19	-5.16	-0.22	0.01	-1.20	0.52	-2.55	0	male	jackal	2009	free-ranging
116.1	25.64	16.56	4.11	1.60	0.29	5.04	-1.95	2.16	0	NA	jackal	2011	free-ranging
117.1	99.87	99.77	99.00	99.36	101.77	-2.92	1.14	2.48	5	NA	caracal	2011	free-ranging
118.1	97.92	98.90	100.03	100.28	6.49	2.38	2.94	0.48	4	female	brown hyena	2012	free-ranging
119.1	95.35	97.30	100.39	101.41	6.02	-0.76	0.53	2.94	4	male	brown hyena	2012	free-ranging
120.1	97.41	98.59	97.97	100.08	100.54	101.31	-3.07	4.17	6	male	caracal	2012	free-ranging
121.1	94.41	98.63	99.05	100.76	4.50	0.65	-1.25	-2.41	4	female	brown hyena	2012	free-ranging
122.1	88.99	97.80	99.56	98.63	53.81	2.18	-4.91	-2.32	4.5	male	brown hyena	2012	free-ranging
123.1	94.51	99.15	52.09	0.07	6.68	5.40	6.36	9.02	2.5	female	brown hyena	2013	free-ranging
124.1	25.44	21.48	7.70	8.14	2.71	5.42	4.88	8.98	0	male	jackal	2013	free-ranging
125.1	92.59	96.80	98.17	99.19	6.13	-2.97	-2.53	-3.12	4	male	brown hyena	2013	free-ranging
126.1	99.02	100.45	51.80	1.87	4.22	5.54	11.27	2.33	2.5	female	brown hyena	2013	free-ranging
127.1	100.60	99.94	100.14	101.67	2.12	7.54	6.11	2.33	4	male	brown hyena	2013	free-ranging
128.1	97.44	102.47	102.39	106.37	109.26	6.01	1.50	0.89	5	male	cheetah	2002	free-ranging
129.1	90.74	92.89	93.66	96.13	97.06	98.32	4.19	2.85	6	male	cheetah	2002	free-ranging
130.1	93.37	97.70	98.17	99.35	102.18	14.95	-0.04	2.13	5	male	cheetah	2002	free-ranging

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animal ID	BKA (%) at dilution 1:2	BKA (%) at dilution 1:4	BKA (%) at dilution 1:8	BKA (%) at dilution 1:16	BKA (%) at dilution 1:32	BKA (%) at dilution 1:64	BKA (%) at dilution 1:128	BKA (%) at dilution 1:256	BKA rank	sex	species	year of sampling	roaming status
131.1	97.66	96.71	96.98	100.14	99.05	49.49	-3.26	-5.47	5.5	female	cheetah	2002	free-ranging
132.1	85.15	97.30	100.79	97.54	101.97	53.44	0.57	2.80	5.5	male	cheetah	2002	free-ranging
133.1	94.32	97.25	93.91	95.21	97.08	100.74	7.74	5.06	6	female	cheetah	2003	free-ranging
134.1	97.68	98.28	98.63	99.92	99.88	55.41	1.21	1.94	5.5	male	cheetah	2010	free-ranging
134.2	94.17	97.64	97.69	98.60	100.63	99.47	-1.59	5.73	6	male	cheetah	2013	free-ranging
135.1	93.48	95.75	97.53	97.88	96.79	63.38	8.24	6.51	5.5	male	cheetah	2013	free-ranging
136.1	97.71	97.45	99.21	86.57	99.60	-5.05	-3.69	-5.36	5	male	leopard	2003	free-ranging
137.1	96.83	97.84	98.81	99.38	58.04	-5.27	-7.17	-12.01	4.5	male	leopard	2003	free-ranging
138.1	100.14	101.79	100.57	102.96	51.56	-1.19	-3.45	-9.91	4.5	female	leopard	2004	free-ranging
139.1	99.54	102.39	104.62	106.59	107.82	59.47	1.59	3.86	5.5	male	leopard	2004	free-ranging
140.1	42.72	17.22	19.98	14.72	8.19	8.19	2.59	0.17	0	female	leopard	2010	free-ranging
141.1	96.18	93.61	97.69	93.12	95.94	2.62	4.79	-2.24	5	female	leopard	2011	free-ranging
142.1	96.63	96.75	95.08	96.67	95.36	95.69	4.42	-4.32	6	female	leopard	2011	free-ranging
143.1	102.49	102.23	100.46	100.03	101.50	49.54	-0.73	1.21	5.5	male	leopard	2011	free-ranging
144.1	99.73	98.11	97.12	99.14	99.32	3.62	0.61	-1.78	5	male	leopard	2011	free-ranging
145.1	NA	100.48	98.45	98.98	100.68	49.61	-0.35	-0.06	5.5	male	leopard	2011	free-ranging
146.1	94.42	97.25	98.62	96.55	98.29	1.93	-2.55	-0.52	5	male	leopard	2011	free-ranging
147.1	101.44	101.36	103.26	103.26	103.00	10.88	3.89	4.67	5	female	leopard	2011	free-ranging
148.1	101.61	104.02	103.05	102.75	103.22	5.24	3.80	1.30	5	female	leopard	2011	free-ranging
149.1	94.94	95.61	97.65	97.91	98.28	6.48	2.22	-5.14	5	female	leopard	2011	free-ranging
150.1	91.48	96.62	100.16	99.32	101.79	56.95	0.44	0.80	5.5	female	leopard	2012	free-ranging
151.1	103.44	104.87	99.75	101.59	99.19	4.48	-1.80	-2.86	5	male	leopard	2012	free-ranging
152.1	100.89	101.60	103.97	104.24	103.27	7.10	3.49	2.87	5	male	leopard	2012	free-ranging
153.1	98.19	98.40	97.36	96.78	98.07	8.03	5.95	1.13	5	female	leopard	2012	free-ranging
154.1	100.36	101.02	104.85	104.72	103.36	6.57	1.51	1.64	5	female	leopard	2012	free-ranging
155.1	97.65	96.65	97.82	97.36	98.98	4.62	1.26	-2.23	5	male	leopard	2012	free-ranging

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animal ID	BKA (%) at dilution 1:2	BKA (%) at dilution 1:4	BKA (%) at dilution 1:8	BKA (%) at dilution 1:16	BKA (%) at dilution 1:32	BKA (%) at dilution 1:64	BKA (%) at dilution 1:128	BKA (%) at dilution 1:256	BKA rank	sex	species	year of sampling	roaming status
156.1	97.14	102.81	101.83	102.25	106.57	109.08	3.88	6.20	6	male	leopard	2013	free-ranging
157.1	97.10	102.41	102.25	102.87	101.47	5.46	0.62	2.96	5	female	leopard	2013	free-ranging
158.1	109.92	98.65	97.35	99.42	98.51	97.06	-1.93	8.53	6	female	leopard	2013	free-ranging
159.1	96.75	102.72	103.51	104.63	14.03	1.96	-2.75	-3.59	4	male	leopard	2013	free-ranging
160.1	101.63	103.03	97.00	25.69	4.63	3.59	-1.35	-1.35	3	male	leopard	2013	free-ranging
161.1	95.59	106.41	105.58	104.54	103.24	3.22	7.70	-0.10	5	male	leopard	2013	captive
162.1	99.88	103.94	100.51	99.39	99.68	11.17	-3.61	-6.50	5	male	leopard	2013	free-ranging
163.1	97.97	100.05	101.87	103.04	104.21	53.47	7.05	1.51	5.5	male	leopard	2013	free-ranging
164.1	100.61	58.58	NA	4.91	-3.90	-6.74	-0.33	-2.63	1.5	female	leopard	2013	free-ranging
165.1	88.55	17.10	-1.82	-2.19	-6.11	-3.70	-9.65	-2.00	1	male	leopard	2013	free-ranging
166.1	92.18	95.48	96.66	100.48	14.03	0.68	-10.59	-5.87	4	male	leopard	2013	free-ranging
167.1	89.97	92.65	94.54	97.37	55.58	-2.10	-9.55	-5.26	4.5	female	leopard	2013	free-ranging
168.1	92.23	95.06	97.13	96.85	-0.59	-5.54	-10.31	-5.87	4	female	leopard	2013	free-ranging
169.1	98.17	98.61	99.24	101.00	99.98	4.81	-2.39	-4.59	5	female	leopard	2013	free-ranging
170.1	94.37	98.29	102.90	103.51	69.15	8.81	-0.23	3.08	4.5	female	leopard	2013	free-ranging
171.1	95.44	103.37	104.91	105.51	77.40	7.83	-1.31	-1.31	4.5	female	leopard	2013	free-ranging
172.1	99.22	108.54	101.36	103.74	110.27	15.43	3.82	1.12	5	female	leopard	2013	free-ranging
173.1	100.77	104.11	102.86	102.48	105.64	-0.58	2.39	2.76	5	male	leopard	2013	free-ranging
174.1	91.60	95.43	96.45	91.77	97.80	95.98	2.28	-1.90	6	male	cheetah	2002	free-ranging
175.1	NA	96.19	98.50	98.60	99.82	-4.28	-0.79	1.10	5	female	cheetah	2002	free-ranging
176.1	97.58	97.58	95.81	100.28	100.93	2.43	4.34	5.36	5	female	cheetah	2002	free-ranging
177.1	95.86	96.84	97.12	98.09	98.09	-0.78	-5.80	0.90	5	female	cheetah	2002	free-ranging
178.1	91.65	96.89	98.15	100.26	101.56	1.91	-0.41	0.93	5	male	cheetah	2002	free-ranging
179.1	100.94	102.82	104.36	107.51	109.22	112.55	0.62	3.08	6	male	cheetah	2002	free-ranging
180.1	96.68	97.82	98.05	99.09	99.19	62.37	-1.68	1.63	5.5	male	cheetah	2003	free-ranging
180.2	92.02	94.72	94.93	96.66	99.44	7.72	-4.26	-1.31	5	male	cheetah	2003	free-ranging

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animal ID	BKA (%) at dilution 1:2	BKA (%) at dilution 1:4	BKA (%) at dilution 1:8	BKA (%) at dilution 1:16	BKA (%) at dilution 1:32	BKA (%) at dilution 1:64	BKA (%) at dilution 1:128	BKA (%) at dilution 1:256	BKA rank	sex	species	year of sampling	roaming status
181.1	99.00	98.95	102.30	102.11	102.63	51.94	-0.31	-2.48	5.5	male	cheetah	2003	free-ranging
181.2	97.57	97.61	97.34	99.50	98.96	99.41	-0.47	-4.57	6	male	cheetah	2004	free-ranging
182.1	94.51	98.56	97.75	97.84	97.97	98.69	1.60	-0.61	6	male	cheetah	2003	free-ranging
182.2	91.71	89.75	93.93	95.02	96.85	99.61	-4.72	-5.02	6	male	cheetah	2004	free-ranging
183.1	94.43	94.68	93.47	98.48	98.07	3.51	-1.63	-1.88	5	female	cheetah	2003	free-ranging
184.1	88.42	89.81	91.04	90.96	88.58	89.93	1.11	-4.81	6	male	cheetah	2003	free-ranging
185.1	89.49	94.04	94.80	93.41	94.25	97.08	-1.31	-1.64	6	male	cheetah	2003	free-ranging
186.1	98.10	101.90	103.83	106.68	108.39	58.29	-1.78	0.27	5.5	male	cheetah	2003	free-ranging
187.1	77.32	91.86	92.35	93.67	95.80	6.29	3.13	-0.77	5	female	cheetah	2003	free-ranging
188.1	90.09	91.96	93.59	97.08	98.55	99.85	5.14	3.88	6	male	cheetah	2003	free-ranging
189.1	98.93	99.71	101.70	100.01	100.53	100.10	-4.27	-1.28	6	male	cheetah	2003	free-ranging
190.1	86.82	91.43	101.22	98.78	99.32	13.85	14.08	3.84	5	female	cheetah	2003	free-ranging
191.1	91.75	93.34	95.81	95.60	98.74	100.57	-0.04	-1.21	6	male	cheetah	2003	free-ranging
192.1	91.04	90.88	91.92	92.63	93.43	42.04	-3.98	-0.52	5.5	male	cheetah	2004	free-ranging
192.2	100.45	99.77	104.20	103.56	105.01	8.62	8.48	7.72	5	male	cheetah	2004	free-ranging
192.3	96.43	99.16	98.50	99.91	100.92	28.89	-4.39	-3.25	5	male	cheetah	2004	free-ranging
193.1	96.43	97.94	99.26	102.13	103.45	102.79	-3.05	4.77	6	male	cheetah	2004	free-ranging
194.1	91.68	93.34	94.16	95.78	97.65	100.90	4.25	-3.64	6	female	cheetah	2004	free-ranging
195.1	84.06	88.04	90.05	92.60	93.95	48.30	2.80	-3.40	5.5	male	cheetah	2004	free-ranging
195.2	96.22	97.66	95.63	96.06	97.95	5.62	2.58	1.28	5	male	cheetah	2005	free-ranging
195.3	100.49	102.54	101.41	104.59	103.46	104.74	-0.97	-0.56	6	male	cheetah	2006	free-ranging
195.4	97.51	100.61	96.61	100.74	100.94	13.25	5.49	4.47	5	male	cheetah	2007	free-ranging
196.1	104.01	105.63	109.04	110.66	104.71	5.57	2.11	-0.95	5	male	cheetah	2004	free-ranging
196.2	91.05	92.42	98.80	89.50	97.30	54.97	-0.46	-4.02	5.5	male	cheetah	2005	free-ranging
197.1	95.25	97.41	97.70	98.50	98.67	3.30	3.51	-2.98	5	male	cheetah	2005	free-ranging
197.2	98.65	97.95	99.95	98.73	98.82	98.52	0.57	-2.02	6	male	cheetah	2006	free-ranging

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197.3	97.16	97.91	100.62	103.03	101.12	102.86	5.94	3.98	6	male	cheetah	2007	free-ranging
198.1	97.07	96.50	97.63	98.64	97.28	4.28	1.61	-1.75	5	female	cheetah	2004	free-ranging
199.1	92.82	95.10	93.33	94.04	93.71	50.02	-1.35	-0.59	5.5	male	cheetah	2004	free-ranging
200.1	98.02	100.00	98.67	98.62	99.82	46.82	-2.42	1.08	5.5	male	cheetah	2005	free-ranging
201.1	95.97	97.29	94.98	101.96	98.42	98.51	4.78	0.47	6	male	cheetah	2006	free-ranging
202.1	101.71	104.55	99.68	101.32	99.08	101.42	100.02	3.26	7	female	cheetah	2011	free-ranging
203.1	98.61	86.90	102.75	99.40	102.59	56.99	-2.43	-1.83	5.5	female	cheetah	2006	free-ranging
204.1	97.62	98.33	99.63	101.06	99.84	49.60	2.47	2.26	5.5	male	cheetah	2006	free-ranging
205.1	98.89	99.09	99.98	100.17	101.21	101.50	46.90	1.76	6.5	male	cheetah	2006	free-ranging
205.2	94.38	99.46	97.41	96.88	99.11	51.41	0.54	2.23	5.5	male	cheetah	2008	free-ranging
206.1	94.47	98.08	91.74	98.75	99.51	51.54	-0.58	-3.17	5.5	female	cheetah	2007	free-ranging
207.1	100.75	102.12	101.54	100.96	104.02	102.60	3.58	6.43	6	male	cheetah	2007	free-ranging
208.1	96.60	99.50	97.79	98.35	52.38	53.49	-8.35	-6.28	5.5	male	cheetah	2007	free-ranging
209.1	95.45	95.98	97.43	97.39	98.55	98.89	1.83	1.73	6	male	cheetah	2007	free-ranging
210.1	97.39	98.34	97.39	96.74	97.22	47.86	-4.41	-3.80	5.5	male	cheetah	2008	free-ranging
211.1	96.87	98.74	98.79	100.16	100.91	100.33	1.82	7.27	6	male	cheetah	2008	free-ranging
212.1	95.86	97.82	97.53	97.65	98.48	50.47	1.59	1.38	5.5	male	cheetah	2008	free-ranging
213.1	93.11	95.07	93.07	97.92	96.98	98.38	1.22	-2.27	6	male	cheetah	2009	free-ranging
214.1	102.42	104.97	105.86	97.79	108.41	68.95	4.78	5.04	5.5	male	cheetah	2009	free-ranging
214.2	99.40	97.79	99.06	97.94	98.96	98.91	4.58	-0.60	6	male	cheetah	2011	free-ranging
214.3	97.01	97.70	97.57	98.30	98.42	98.81	2.13	-1.25	6	male	cheetah	2012	free-ranging
215.1	98.33	99.79	99.55	98.96	98.23	101.60	2.77	1.31	6	male	cheetah	2009	free-ranging
215.2	94.83	95.89	95.89	97.24	97.49	98.76	5.59	-0.38	6	male	cheetah	2010	free-ranging
216.1	92.64	96.51	95.19	97.92	97.02	99.32	0.03	-1.88	6	male	cheetah	2009	free-ranging
217.1	95.44	99.66	101.99	100.96	101.46	100.80	10.01	-0.83	6	male	cheetah	2009	free-ranging
218.1	93.37	95.36	93.79	96.81	97.58	99.11	1.65	0.16	6	male	cheetah	2009	free-ranging

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219.1	102.96	104.02	103.26	105.84	105.08	5.96	4.90	3.42	5	male	cheetah	2009	free-ranging
219.2	83.22	83.48	83.96	83.52	88.54	4.19	1.74	3.53	5	male	cheetah	2012	free-ranging
220.1	99.53	97.94	98.25	98.47	99.88	50.67	-3.47	-3.99	5.5	male	cheetah	2011	free-ranging
220.2	101.06	106.73	104.23	106.43	107.32	55.48	4.94	1.43	5.5	male	cheetah	2011	free-ranging
221.1	98.13	98.17	97.23	98.34	87.32	53.01	-1.82	-6.16	5.5	male	cheetah	2010	free-ranging
222.1	100.29	100.62	103.88	104.11	105.29	103.73	0.20	0.29	6	male	cheetah	2010	free-ranging
222.2	-4.19	5.00	10.28	4.24	1.96	5.71	3.78	3.63	0	male	cheetah	2012	free-ranging
223.1	101.91	99.43	101.17	102.26	97.14	101.57	22.04	9.97	6	female	cheetah	2010	free-ranging
224.1	99.98	96.77	97.06	97.80	98.89	57.21	-3.76	-3.22	5.5	female	cheetah	2010	free-ranging
224.2	NA	103.50	109.99	101.00	105.71	104.63	-2.07	10.20	6	female	cheetah	2011	free-ranging
225.1	102.13	101.20	103.87	103.20	100.95	102.23	52.32	0.20	6.5	male	cheetah	2010	free-ranging
225.2	93.59	100.00	96.71	98.79	98.46	98.71	5.26	1.64	6	male	cheetah	2011	free-ranging
226.1	95.49	96.47	97.56	97.51	100.02	26.97	-1.69	4.28	5	female	cheetah	2010	free-ranging
226.2	NA	103.20	109.04	101.26	100.35	98.75	2.42	-4.36	6	female	cheetah	2011	free-ranging
227.1	NA	106.70	108.43	107.31	101.26	106.44	8.04	-1.21	6	male	cheetah	2010	free-ranging
228.1	97.35	100.84	101.27	103.34	104.42	101.19	5.14	4.11	6	male	cheetah	2010	free-ranging
229.1	100.44	100.80	104.75	102.96	105.72	102.44	0.63	1.76	6	female	cheetah	2011	free-ranging
230.1	97.26	100.31	99.39	98.64	100.23	100.10	5.85	3.34	6	male	cheetah	2011	free-ranging
231.1	94.83	93.44	92.87	91.73	97.24	92.05	4.91	-4.61	6	female	cheetah	2011	free-ranging
232.1	96.99	97.91	99.51	100.05	99.93	52.12	6.33	-2.28	5.5	male	cheetah	2012	free-ranging
233.1	79.99	81.05	81.81	82.68	81.88	82.50	0.15	5.71	6	female	cheetah	2012	free-ranging
234.1	98.08	100.20	100.91	102.70	102.20	103.20	5.32	6.65	6	female	cheetah	2012	free-ranging
235.1	78.64	75.63	77.60	85.68	76.23	81.66	0.70	1.81	6	female	cheetah	2012	free-ranging
236.1	98.58	102.34	102.78	102.78	102.69	103.08	6.39	3.37	6	female	cheetah	2012	free-ranging
237.1	92.40	94.19	94.63	97.97	100.28	96.86	-2.79	1.12	6	male	cheetah	2012	free-ranging
238.1	98.52	98.16	98.62	99.39	100.90	-1.68	0.51	2.99	5	male	cheetah	2012	free-ranging

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239.1	95.75	96.36	98.27	96.92	98.44	4.52	-0.48	-0.22	5	male	cheetah	2012	free-ranging
239.2	95.62	99.33	99.71	99.66	102.07	98.80	-5.06	5.39	6	male	cheetah	2013	free-ranging
240.1	86.28	82.94	83.12	84.06	82.90	83.55	2.01	6.37	6	female	cheetah	2012	free-ranging
241.1	83.85	82.55	80.84	82.51	82.85	5.65	0.81	3.00	5	female	cheetah	2012	free-ranging
242.1	94.78	96.94	101.12	99.36	101.40	8.72	-2.71	-1.59	5	male	cheetah	2012	free-ranging
243.1	92.91	96.14	100.32	101.28	103.67	5.89	-1.43	-4.46	5	female	cheetah	2012	free-ranging
244.1	83.18	83.67	82.77	83.26	83.52	83.67	2.78	3.23	6	male	cheetah	2012	free-ranging
245.1	81.92	80.76	82.07	84.17	82.90	83.45	0.37	2.88	6	male	cheetah	2012	free-ranging
246.1	96.67	99.02	100.92	99.69	99.80	99.80	6.19	-4.17	6	male	cheetah	2012	free-ranging
247.1	90.82	94.66	96.67	97.56	98.63	1.94	-3.86	-5.96	5	male	cheetah	2012	free-ranging
248.1	92.92	102.62	102.39	107.01	107.10	6.53	3.31	3.68	5	male	cheetah	2013	free-ranging
249.1	93.96	97.42	98.35	99.52	99.61	3.48	-2.41	-5.07	5	male	cheetah	2013	free-ranging
250.1	90.67	94.14	94.72	99.41	100.59	100.85	-5.90	6.46	6	male	cheetah	2013	free-ranging
251.1	91.55	96.94	96.74	97.18	98.02	60.91	7.79	4.98	5.5	male	cheetah	2013	free-ranging
252.1	93.67	96.63	17.29	3.34	1.20	4.02	3.75	-11.38	2	female	cheetah	2013	free-ranging
253.1	96.14	103.98	105.51	104.18	105.45	106.78	4.36	-2.00	6	male	cheetah	2013	free-ranging
254.1	91.49	96.63	96.49	100.76	100.31	3.25	6.11	-5.70	5	male	cheetah	2013	free-ranging
255.1	93.87	97.37	99.40	99.40	100.32	1.04	2.67	1.55	5	male	cheetah	2013	free-ranging
256.1	90.76	100.72	98.53	99.72	99.72	55.55	-0.75	-4.43	5.5	male	cheetah	2013	free-ranging
257.1	92.81	92.76	70.29	NA	-10.27	-6.37	-10.47	-9.09	2.5	male	lion	2013	free-ranging
258.1	105.44	111.35	69.84	107.25	3.17	2.63	-0.27	2.57	4	male	lion	2013	free-ranging
259.1	91.18	90.69	91.83	63.52	44.50	-6.03	-10.22	-9.48	3.5	female	lion	2013	free-ranging
260.1	107.08	106.21	106.81	106.54	78.26	-3.77	-4.16	1.09	4.5	female	lion	2013	free-ranging
261.1	171.14	137.28	133.89	122.19	114.59	8.65	0.34	-6.22	5	male	lion	2013	free-ranging
262.1	100.78	94.61	99.91	50.68	6.80	0.08	6.09	5.68	3.5	female	lion	2013	free-ranging
263.1	89.17	88.73	91.04	89.97	88.61	90.05	-2.82	0.20	6	male	cheetah	2003	free-ranging

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264.1	97.06	98.34	98.86	103.06	102.06	101.50	4.13	1.30	6	male	cheetah	2003	free-ranging
265.1	85.45	89.68	91.33	93.75	96.50	49.45	-1.30	0.13	5.5	male	cheetah	2003	free-ranging
266.1	53.33	64.54	78.37	85.22	89.94	47.01	3.88	-0.47	5.5	female	cheetah	2003	free-ranging
267.1	92.65	76.14	83.62	90.62	95.82	99.60	3.09	-1.13	6	female	cheetah	2003	free-ranging
268.1	97.74	93.27	96.84	99.34	99.88	-0.27	-3.26	-9.91	5	female	cheetah	2004	free-ranging
269.1	86.70	92.34	94.51	96.35	98.74	0.96	-2.17	-1.96	5	female	cheetah	2004	free-ranging
270.1	93.63	88.89	91.80	91.20	88.97	43.95	-5.25	-1.15	5.5	male	cheetah	2004	free-ranging
271.1	96.68	97.06	95.65	97.63	99.47	4.65	2.15	3.37	5	female	cheetah	2004	free-ranging
272.1	98.19	99.23	102.27	104.13	102.53	101.53	-7.40	-6.22	6	male	cheetah	2004	free-ranging
272.2	88.59	91.67	94.63	96.45	99.37	99.21	-1.17	-7.00	6	male	cheetah	2004	free-ranging
273.1	102.66	101.88	102.70	101.92	100.75	100.57	0.11	-5.96	6	male	cheetah	2004	free-ranging
273.2	90.58	94.14	95.56	93.43	98.65	101.54	-2.51	-5.48	6	male	cheetah	2004	free-ranging
274.1	92.16	97.34	97.90	88.99	99.74	52.28	4.21	-8.90	5.5	male	cheetah	2005	free-ranging
275.1	98.04	99.02	98.64	100.01	99.97	49.59	1.19	-1.64	5.5	male	cheetah	2005	free-ranging
276.1	94.62	95.55	95.55	96.98	99.43	-1.13	3.68	-1.63	5	female	cheetah	2005	free-ranging
277.1	97.88	97.70	100.15	99.92	99.15	101.87	5.27	5.73	6	female	cheetah	2005	free-ranging
278.1	99.27	94.73	96.62	97.82	91.30	56.39	5.54	-4.83	5.5	male	cheetah	2005	free-ranging
279.1	99.94	97.61	98.52	99.56	99.36	3.96	3.54	0.92	5	male	cheetah	2005	free-ranging
280.1	98.35	99.41	101.52	100.97	101.89	100.92	1.77	-1.91	6	male	cheetah	2005	free-ranging
280.2	99.69	101.29	102.59	102.02	102.02	0.83	-0.38	-0.81	5	male	cheetah	2008	free-ranging
281.1	97.02	98.12	98.12	100.94	101.31	9.13	4.51	-3.90	5	male	cheetah	2005	free-ranging
282.1	99.03	97.87	97.63	98.16	99.52	99.85	-3.26	3.38	6	male	cheetah	2005	free-ranging
283.1	99.82	100.69	102.33	102.02	102.33	103.00	-4.15	-1.43	6	male	cheetah	2006	free-ranging
284.1	97.43	97.30	92.50	97.77	97.90	66.63	-0.20	-7.10	5.5	male	cheetah	2006	free-ranging
285.1	97.36	101.62	96.89	100.60	100.89	99.45	-3.91	2.56	6	male	cheetah	2009	free-ranging
286.1	101.76	101.97	101.81	100.86	101.02	102.18	8.01	-0.01	6	male	cheetah	2009	free-ranging

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286.2	99.41	103.41	103.56	103.36	101.87	104.84	-1.38	2.00	6	male	cheetah	2010	free-ranging
287.1	94.05	99.51	95.82	93.48	94.44	102.03	-3.66	-3.44	6	male	cheetah	2009	free-ranging
287.2	96.83	93.35	98.29	100.40	103.02	99.15	4.63	-5.43	6	male	cheetah	2010	free-ranging
287.3	98.33	97.11	99.51	96.32	97.68	52.83	4.01	-1.61	5.5	male	cheetah	2011	free-ranging
287.4	90.78	96.40	95.43	53.79	-3.95	0.80	-2.48	-5.91	3.5	male	cheetah	2013	free-ranging
288.1	93.47	93.19	100.70	101.26	99.08	100.48	6.69	1.65	6	male	cheetah	2009	free-ranging
289.1	101.95	98.51	101.09	104.83	103.71	103.49	4.50	0.51	6	male	cheetah	2009	free-ranging
289.2	93.29	94.80	97.39	96.88	95.37	97.06	-2.17	-0.19	6	male	cheetah	2012	free-ranging
289.3	94.73	100.11	100.33	100.37	101.07	100.90	2.54	6.43	6	male	cheetah	2012	free-ranging
289.4	94.64	96.94	98.16	98.53	98.74	97.91	3.34	-1.67	6	male	cheetah	2012	free-ranging
289.5	92.64	98.72	100.69	100.04	101.44	51.18	0.87	2.97	5.5	male	cheetah	2012	free-ranging
290.1	92.91	96.27	99.73	101.76	105.06	103.46	8.97	0.17	6	female	cheetah	2010	free-ranging
291.1	97.20	98.59	98.51	99.70	98.67	56.26	4.28	-2.63	5.5	male	cheetah	2010	free-ranging
292.1	96.87	97.18	101.48	97.54	99.69	0.53	-5.34	1.82	5	female	cheetah	2010	free-ranging
293.1	93.44	97.00	97.14	99.57	99.10	52.30	-2.13	-8.58	5.5	male	cheetah	2013	free-ranging
294.1	100.04	101.06	100.14	100.14	101.01	9.48	7.28	3.14	5	male	cheetah	2013	free-ranging
295.1	94.26	99.58	99.76	101.17	102.26	57.05	-1.02	-11.56	5.5	male	cheetah	2013	free-ranging
296.1	99.07	99.40	98.65	94.00	101.19	82.06	-3.31	1.95	5.5	male	cheetah	2002	captive
297.1	110.12	109.13	104.40	109.08	107.26	1.24	-3.08	-1.73	5	female	cheetah	2002	captive
298.1	109.60	101.52	102.69	100.67	105.74	9.80	NA	2.75	5	male	cheetah	2002	captive
299.1	34.06	32.15	26.30	15.24	8.21	0.53	-1.59	-0.16	0	male	cheetah	2003	free-ranging
300.1	96.09	96.94	99.49	99.41	100.14	3.42	0.66	-13.57	5	female	cheetah	2002	captive
301.1	105.53	108.15	103.56	99.88	99.45	6.67	4.02	0.85	5	male	cheetah	2002	captive
302.1	97.70	99.23	105.91	103.75	40.75	5.10	-0.90	2.08	4.5	male	cheetah	2002	captive
303.1	97.71	102.74	103.35	104.24	100.25	105.42	-3.45	-5.76	6	male	cheetah	2002	captive
304.1	102.21	97.28	97.15	96.94	98.68	102.94	-2.29	4.74	6	male	cheetah	2002	captive

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305.1	93.98	93.62	97.06	97.22	100.47	3.82	-1.41	-1.29	5	male	cheetah	2002	captive
306.1	94.30	94.99	99.14	101.72	101.27	3.96	1.54	-2.69	5	female	cheetah	2002	captive
307.1	102.49	101.70	102.23	102.44	103.55	103.97	1.73	5.90	6	female	cheetah	2004	captive
307.2	97.90	100.62	103.16	98.98	97.00	64.25	-1.48	-1.34	5.5	female	cheetah	2009	captive
308.1	NA	110.20	107.35	103.50	65.07	3.47	2.10	2.41	4.5	female	cheetah	2003	captive
308.2	98.03	100.18	100.09	98.77	97.98	98.47	2.92	1.94	6	female	cheetah	2006	captive
308.3	106.43	109.76	108.51	100.55	107.16	85.82	2.38	3.53	5.5	female	cheetah	2009	captive
309.1	97.62	101.89	102.92	102.97	100.67	56.31	-2.56	-1.43	5.5	male	cheetah	2004	captive
310.1	100.63	102.17	103.29	102.53	97.29	106.54	9.84	5.82	6	male	cheetah	2004	captive
311.1	95.25	96.37	97.39	99.02	99.35	100.47	-1.38	4.15	6	male	cheetah	2004	captive
312.1	96.04	103.96	106.30	100.89	101.11	101.02	2.56	6.74	6	male	cheetah	2004	captive
312.2	97.16	101.44	100.65	101.12	104.51	55.43	1.13	2.06	5.5	male	cheetah	2004	captive
313.1	94.73	99.43	99.87	99.77	104.18	99.38	-0.32	1.09	6	female	cheetah	2013	captive
314.1	90.84	96.29	98.61	99.09	103.10	105.42	-1.21	-3.09	6	male	cheetah	2013	captive
315.1	100.76	99.64	99.37	100.60	50.83	-1.98	5.63	2.54	4.5	female	cheetah	2013	captive
316.1	99.43	101.66	100.64	104.86	104.67	2.11	-9.43	1.33	5	female	cheetah	2013	captive
317.1	94.97	96.52	96.76	97.39	-0.51	-0.32	-1.04	5.07	4	male	cheetah	2013	captive
318.1	98.99	99.73	99.87	101.34	100.23	77.69	2.51	2.42	5.5	female	cheetah	2007	captive
318.2	98.01	97.39	95.88	97.11	99.33	2.88	1.41	-1.41	5	female	cheetah	2009	captive
318.3	97.88	98.39	99.76	101.04	99.03	104.25	2.12	3.17	6	female	cheetah	2012	captive
319.1	103.53	105.90	105.90	103.20	105.49	16.11	13.12	0.49	5	female	cheetah	2007	captive
320.1	98.13	100.18	101.01	98.57	101.55	98.86	0.53	-0.50	6	male	cheetah	2007	captive
320.2	93.79	100.51	100.08	99.47	97.52	2.55	-2.92	-3.70	5	male	cheetah	2009	captive
320.3	96.16	97.10	101.84	98.98	101.47	63.08	6.23	-5.33	5.5	male	cheetah	2011	captive
320.4	97.25	100.99	101.36	100.67	101.68	100.90	-3.00	-0.92	6	male	cheetah	2012	captive
321.1	100.82	102.00	104.61	101.60	99.86	5.50	2.49	0.04	5	male	cheetah	2007	captive

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321.2	90.70	100.42	100.03	98.99	99.25	2.55	3.85	0.25	5	male	cheetah	2009	captive
321.3	96.22	97.35	98.77	100.61	99.73	104.83	2.59	3.93	6	male	cheetah	2011	captive
321.4	97.45	98.33	101.20	102.45	103.24	103.28	1.86	1.90	6	male	cheetah	2011	captive
321.5	97.74	100.80	101.70	101.70	102.02	102.60	3.21	2.73	6	male	cheetah	2012	captive
322.1	97.15	97.36	96.85	97.23	101.32	106.68	-4.76	-0.50	6	male	cheetah	2009	free-ranging
323.1	32.31	97.65	104.60	55.34	102.86	104.41	-5.07	11.45	6	male	cheetah	2013	captive
324.1	98.68	99.21	99.59	100.60	100.55	0.52	-3.69	6.69	5	male	cheetah	2013	captive
325.1	90.06	98.03	99.67	102.96	107.88	106.68	3.24	2.46	6	male	cheetah	2013	captive
326.1	99.21	100.65	100.17	100.07	99.48	100.55	-3.63	2.17	6	male	cheetah	2013	captive
327.1	120.48	114.13	102.78	105.49	41.73	106.32	-1.05	-8.65	6	male	cheetah	2009	captive
328.1	96.55	99.70	100.58	103.63	104.15	-0.04	0.84	-5.41	5	female	cheetah	2013	captive
329.1	NA	102.77	105.62	103.16	102.38	61.76	9.25	6.91	5.5	malecastr	cheetah	2011	captive
330.1	95.45	96.57	101.95	101.00	101.77	101.04	2.40	0.98	6	male	cheetah	2011	captive
330.2	98.60	99.20	99.02	99.62	100.14	5.00	2.99	-4.68	5	male	cheetah	2012	captive
331.1	89.35	90.85	93.47	93.43	95.13	3.39	2.02	-0.94	5	male	cheetah	2011	captive
331.2	100.46	107.64	104.88	105.18	104.88	107.02	3.28	7.70	6	male	cheetah	2012	captive
331.3	99.21	100.71	102.20	102.09	52.85	5.31	-4.86	-7.31	4.5	male	cheetah	2013	captive
332.1	97.11	98.51	99.07	96.59	96.15	2.49	3.32	-1.40	5	male	cheetah	2011	captive
332.2	96.88	99.24	99.52	99.47	101.45	49.76	1.15	-0.37	5.5	male	cheetah	2012	captive
333.1	109.66	110.59	99.61	99.56	100.65	15.39	-0.79	2.80	5	female	cheetah	2011	captive
333.2	98.88	98.74	98.01	101.51	100.10	0.47	-0.85	-0.22	5	female	cheetah	2012	captive
334.1	97.24	98.39	97.15	100.26	98.71	51.92	5.05	4.91	5.5	male	cheetah	2012	captive
335.1	82.81	81.96	82.74	83.00	82.66	44.34	1.48	4.60	5.5	male	cheetah	2012	captive
336.1	95.14	95.49	96.10	97.97	99.10	51.55	-3.56	3.22	5.5	male	cheetah	2012	free-ranging
337.1	82.94	81.96	82.03	84.75	84.93	48.36	3.21	3.46	5.5	female	cheetah	2012	free-ranging
338.1	98.58	104.69	102.91	105.81	105.56	106.98	2.89	0.65	6	male	cheetah	2013	captive

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339.1	93.74	104.62	101.26	100.57	104.52	107.99	-2.29	-1.99	6	male	cheetah	2013	captive
340.1	95.60	100.84	101.71	104.38	105.06	0.80	2.64	5.70	5	female	cheetah	2013	captive
341.1	88.71	97.02	98.90	100.06	101.94	102.76	6.23	2.37	6	male	cheetah	2013	captive
342.1	95.36	101.13	93.85	101.66	105.44	3.32	1.96	-4.00	5	male	cheetah	2013	captive
343.1	13.62	98.71	99.58	94.99	48.87	50.85	-1.64	6.95	4.5	male	cheetah	2013	captive
344.1	98.46	97.22	100.94	101.36	54.61	4.09	-5.46	-10.21	4.5	female	cheetah	2013	captive
345.1	58.92	99.80	99.64	98.95	99.91	-10.71	-5.28	11.54	5	female	cheetah	2013	captive
346.1	99.34	100.37	101.51	104.25	62.36	-4.22	-0.35	-6.39	4.5	female	cheetah	2013	captive
347.1	97.64	100.06	104.51	102.80	104.35	0.12	0.12	-8.97	5	female	cheetah	2013	captive
348.1	99.09	101.16	103.44	104.48	101.56	-0.90	-1.15	-3.87	5	female	cheetah	2013	captive
349.1	100.74	97.98	99.90	100.30	100.84	5.32	2.36	-9.00	5	male	lion	2013	captive

CHAPTER 3 – A COMPARISON WITH THE LEOPARD

Second Manuscript

Cheetahs have a stronger constitutive innate immunity than leopards

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Cheetahs have a stronger constitutive innate immunity than leopards

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As a textbook case for the importance of genetics in conservation, absence of genetic variability at the major histocompatibility complex (MHC) is thought to endanger species viability, since it is considered crucial for pathogen resistance. An alternative view of the immune system inspired by life history theory posits that a strong response should evolve in other components of the immune system if there is little variation in the MHC. In contrast to the leopard (*Panthera pardus*), the cheetah (*Acinonyx jubatus*) has a relatively low genetic variability at the MHC, yet free-ranging cheetahs are healthy. By comparing the functional competence of the humoral immune system of both species in sympatric populations in Namibia, we demonstrate that cheetahs have a higher constitutive innate but lower induced innate and adaptive immunity than leopards. We conclude (1) immunocompetence of cheetahs is higher than previously thought; (2) studying both innate and adaptive components of immune systems will enrich conservation science.

Conservation science is a discipline that can help to slow down global biodiversity loss[1,2]. The integration of life history theory and other facets of evolutionary ecology into conservation science has the potential to provide new conservation management tools[3]. This is because an evolutionary approach uses a theoretical or empirical framework which provides testable predictions on the diversity of physiological responses to disturbances in individuals under natural selection[4]. With such knowledge, conservation management activities can be derived with more predictable outcomes than with the alternative trial and error approach[5].

One of the strongest natural selection pressures are pathogens that challenge the immune system of individuals and can lead to diseases and in many cases to death[6,7]. The usually highly polymorphic multigene family of the major histocompatibility complex (MHC) is part of the adaptive immunity and encodes key receptor molecules that recognise and bind foreign peptides for presentation to immune cells[8]. It is generally assumed that resistance to infection is more effective the more MHC loci and alleles exist in a host individual[9]. This is because a heterozygous individual with many loci is more likely to detect and respond adequately to a wider range of pathogens which will increase its fitness than an individual which is homozygous at one or more loci[10]. The more alleles are present in a population, the more it is likely that an individual will be heterozygous. Thus, balancing selection is likely to maintain high allelic diversity at the MHC because of selection pressures exerted by infections[11–13].

There is considerable evidence that high variability of MHC genes improves pathogen resistance. This comes mostly from humans or animals under laboratory conditions[14], although recent studies considered free-ranging populations (for example refs 15–20). The evidence is less clear on whether low MHC variability necessarily reduces population viability[21]. Examples of populations with low MHC variability and low susceptibility to diseases were described for, e.g., Chillingham cattle (*Bos taurus*)[22], North American and European moose (*Alces alces*)[23,24] and mountain goats (*Oreamnos americanus*)[25]. There are also examples of populations with high MHC variability and a high susceptibility to diseases, as in bighorn sheep (*Ovis canadensis*)[26]. These studies provide evidence in contrast to the expectation that low MHC variation results in an impaired immune response and vice versa[12,27,28].

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With an evolutionary ecology approach, one possible explanation of these constellations would be that there would have been selection pressure to strengthen other components of the immune system to provide an adequate compensatory immune response[29,30]. Thus, if a reduction in immunogenetic variability in a species impairs an immune component such as adaptive immunity, other components of the immune response might compensate any reduction in functionality[30]. This suggests that components of the immune system have the potential to interact, inhibit or compensate each other in species-specific ways, as has been theoretically discussed and empirically demonstrated[29–31], and that similar levels of protection against pathogens may be accomplished by different combinations of protective systems. Compensatory responses could, for instance, be channeled through a rise in the energetic investment in non-impaired immune components, or be the consequence of the evolution of increased genetic variation in non-impaired immune components.

To assess the potential diversity in investment and the overall immunocompetence of individuals, it is important to simultaneously measure several components of the immune response[32]. Here we combine functional assays and measurements of several effectors of the immune system in a species with relatively low genetic variability, the cheetah (*Acinonyx jubatus jubatus*), and compare them with measures from a species with relatively high genetic variability, the African leopard (*Panthera pardus pardus*) from sympatric free-ranging populations in Namibia.

Cheetahs exhibit a relatively low genetic variability[33,34], including the loci of the MHC[35,36], although this is actually higher than previously thought[37]. It was previously assumed that the consequence of this relatively low genetic variability was a high susceptibility to diseases and a poor reproductive performance[33–35,38]. However, such susceptibility and poor performance have only been reported from cheetahs in captivity[39,40]. Free-ranging cheetahs showed no clinical or pathological evidence for diseases, even when tested seropositive for several infectious diseases[41], and they successfully reproduced[42]. The findings in captivity were later shown to be a consequence of unfavorable husbandry conditions and breeding management rather than the relatively low genetic variability[43,44].

The African leopard is sympatric with the cheetah in the same habitat in Namibia. Individuals of both species live solitarily or in small groups consisting of mothers with their offspring. In cheetahs, independent litter mates or unrelated adult males may also form long-term coalitions of two to three animals[45,46]. Cheetahs and leopards generally hunt the same prey animals[46,47], although the leopard has a wider dietary breadth and also scavenges regularly. Leopards might therefore be exposed to the same pathogens but at higher contact rates than cheetahs. In contrast to cheetahs, leopards exhibit a relatively high genetic diversity; the leopard in Namibia is the subspecies which displays the highest diversity in mitochondrial DNA and microsatellite markers of all big cat species[48,49]. Consequently, the MHC diversity of Namibian leopards is higher than that of cheetahs[50]. Thus, the MHC dependent adaptive immune response of cheetahs might be weaker than that of leopards[51]. If immune components can compensate each other, e.g. through an increase in energetic investment[29,30], we expect Namibian cheetahs to invest more in the innate immune response than Namibian leopards.

Here we report the results of our measurements of humoral immunity generated by the three major components of the immune system, by assessing (1) adaptive immunity, (2) induced innate immunity and (3) constitutive innate immunity. Adaptive immunity is highly specific towards pathogen recognition and can only fight against a pathogen that was encountered previously and for which a specific antibody or T-cell was developed. The predominant antibody isotype in mammals is immunoglobulin G (IgG)[52], which was chosen as a representative for adaptive immunity in this study. Induced innate immunity, most importantly the acute phase response, is unspecific towards pathogen recognition and thus can react quickly in case of a challenge. It serves to restore homeostasis in the host and therefore increases in concentration shortly after tissue injuries or inflammations, or the experience of short-term stressors[53,54]. Serum amyloid A (SAA) is the most important acute phase protein in domestic cats[55–58] and in cheetahs[59]. Thus, SAA was chosen as a representative for induced innate immunity. Constitutive innate immune effectors provide a rapid first line of defense against intruders. The bacterial killing capacity of plasma and serum is primarily mediated by the complement and other antibacterial proteins and is a functional measurement of the constitutive innate immunity. Lysozyme is a major part of the constitutive innate antibacterial immunity which acts by digesting peptidoglycans of bacterial cell walls, especially of gram-positive bacteria[60]. The haemagglutination/haemolysis assay is a method that quantifies two interrelated parts of the constitutive innate immune system, natural antibodies (haemagglutination titer) and the complement system (haemolysis titer). Natural antibodies recognize epitopes of various antigens and initiate the complement system, a group of proteins that trigger a signaling cascade which finally leads to pathogen lysis[61,62]. We therefore chose the bacterial killing assay, the lysozyme assay and the haemagglutination/haemolysis assay to characterize constitutive innate immunity.

Because morphological, immunological and endocrinological parameters may be affected by differences in allostatic load ('stress')[63,64], we measured the impact of trapping cheetahs and leopards in box traps in terms of their glucocorticoid concentrations in a quasi-experimental setup. This permitted us to take into account possible differences in allostatic load between individuals or species[65,66].

Results

We first reduced the dimensionality of the data for an overall comparison of the immunity of cheetahs and leopards by summarizing the six immune measurements into the two first principal components (PC) of a principal component analysis (PCA, Fig. 1), as suggested by Buehler *et al.*[67]. To maximize the use of information contained within the data, we used an expectation-maximization algorithm to perform multiple imputation for incomplete data (see methods) before running the PCA. We ran PCAs on both the larger data set with imputed data (Model 1) and the original raw data set (Model 3) and found that the outcome was qualitatively very similar (see Supplementary Results). We therefore report here the results for the complete data set with imputed data.

PC1 captured 40.1% of the total variance in immune measurements and mainly reflected constitutive innate immunity, because three of the four variables for constitutive innate immunity aligned along the x-axis, which

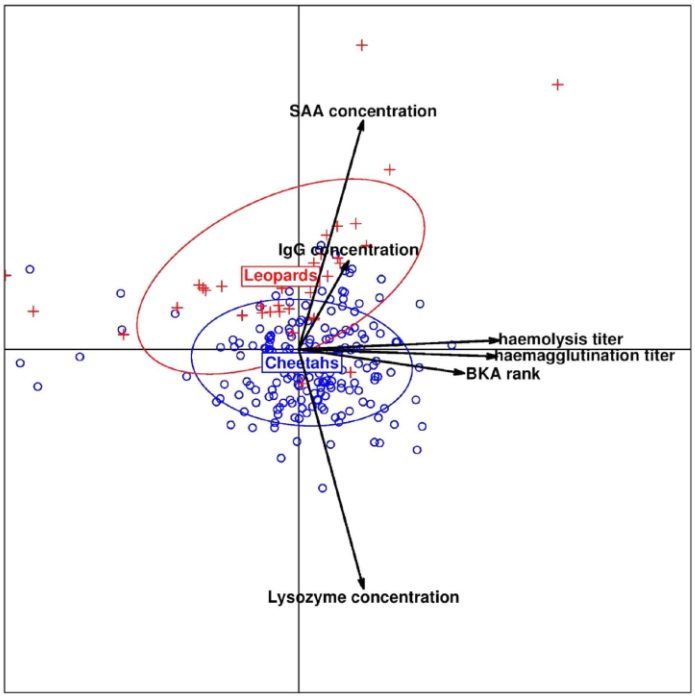

Figure 1. Immune differences between cheetahs and leopards. Position of all cheetahs (small circles) and leopards (plus signs) projected into the space defined by the first two principal components (PC1 in x-axis and PC2 in y-axis) of a principal component analysis performed on all six immune parameters. Together PC1 and PC2 capture nearly 64% of the total variance. Arrows represent the contribution of each immune parameter to PC1 and PC2. For each species, 1.5 inertia ellipses are depicted.

represents PC1, in Fig. 1 – these were bacterial killing assay (BKA) ranks, haemagglutination titer and haemolysis titer (see Table S3 for PCA loadings). Table S2 summarizes the logistic regression model that assesses the reliability of predicting species identity from the PCA. Cheetahs presented significantly higher scores (mean PC1 value: 0.042) than leopards (mean PC1 value: −0.230) on the first principal component (logistic regression, likelihood ratio test (LRT) = 7.45, $df = 1$, $P = 0.006$).

PC2 captured 23.8% of the total variance in immune measurements and mainly reflected the induced innate and adaptive immunity in terms of SAA and IgG concentrations, but also the constitutive innate immunity in terms of lysozyme concentrations (Fig. 1; Table S3). On PC2, cheetahs (mean PC2 value: −0.237) presented significantly lower scores than leopards (mean PC2 value: 1.299, logistic regression, LRT = 63.86, $df = 1$, $P < 0.001$).

There were no differences in immune values between the sexes in cheetahs (LRT = 0.19, $df = 2$, P = 0.91) or in leopards (LRT = 1.81, $df = 2$, P = 0.40), nor between adult and sub-adult cheetahs (LRT = 0.39, $df = 2$, P = 0.82) or leopards (LRT = 4.03, $df = 2$, P = 0.13).

We then characterized differences between cheetahs and leopards by performing pairwise comparisons for all immune variables (Fig. 2a–f) using only the actual measurements, i.e., without imputing missing values. In terms of adaptive immunity and induced innate immunity, cheetahs ($mean_{cheetahs} = 37.2 \pm 11.0$ mg/l) had lower IgG concentrations than leopards ($mean_{leopards} = 42.1 \pm 10.5$ mg/l, Mann-Whitney U-test, $W = 1998.5$, $N_{cheetahs} = 161$, $N_{leopards} = 35$, $P = 0.007$). Cheetahs ($mean_{cheetahs} = 213.9 \pm 303.1$ mg/l) also had lower SAA concentrations than leopards ($mean_{leopards} = 605.3 \pm 842.6$ mg/l, $W = 1462$, $N_{cheetahs} = 143$, $N_{leopards} = 34$, $P < 0.001$). Concerning constitutive innate immunity, cheetahs had higher BKA ranks than leopards ($W = 4427.5$, $P < 0.001$, $N_{cheetahs} = 180$, $N_{leopards} = 34$), with median ranks of 6.0 and 5.0, respectively, indicating a bacterial killing capacity of cheetahs which was twice as high as that of leopards. Serum lysozyme concentrations in cheetahs ($mean_{cheetahs} = 2.34 \pm 0.64$ mg/l) were higher than in leopards ($mean_{leopards} = 1.01 \pm 0.37$ mg/l, $W = 5658$, $N_{cheetahs} = 167$, $N_{leopards} = 35$, $P < 0.001$). There was no difference between species in haemagglutination titer ($W = 2850.5$, $P = 0.37$, $median_{cheetahs} = 5$, $median_{leopards} = 5$, $N_{cheetahs} = 158$, $N_{leopards} = 33$) or haemolysis titer ($W = 2927.5$, $P = 0.21$, $median_{cheetahs} = 4$, $median_{leopards} = 4$, $N_{cheetahs} = 157$, $N_{leopards} = 33$). Note that all of the aforementioned significant pairwise comparisons would remain significant if p-values were corrected for multiple testing using the Bonferroni procedure or more powerful alternatives to this method.

Serum cortisol concentrations in cheetahs ($mean_{cheetahs} = 19.8 \pm 18.3$ ng/ml) were significantly lower than in leopards ($mean_{leopards} = 57.0 \pm 18.1$ ng/ml, $W = 338$, $N_{cheetahs} = 166$, $N_{leopards} = 34$, $P < 0.001$), suggesting a higher allostatic load caused by the capture and handling procedure of the latter. There was no difference in the levels of

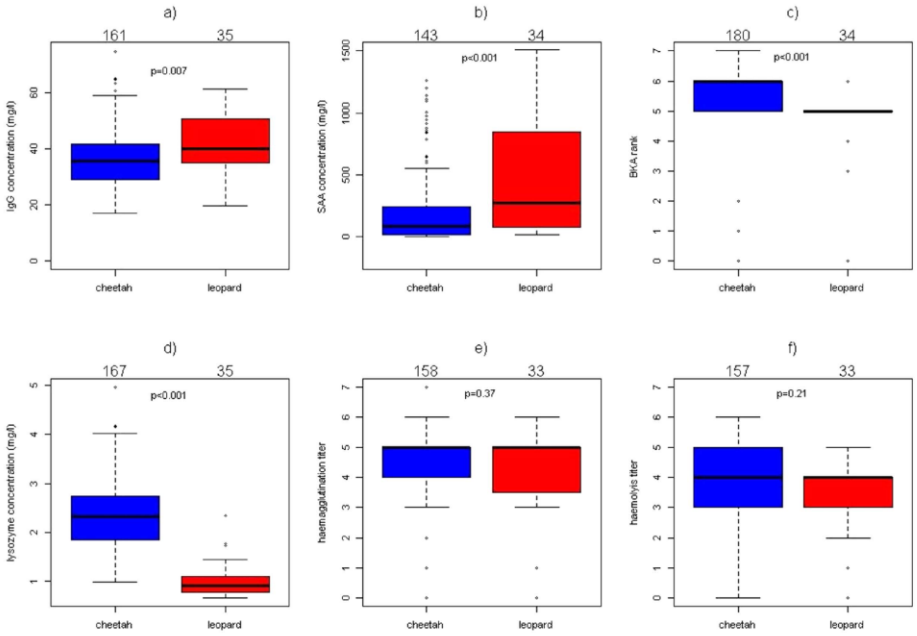

Figure 2. Pairwise comparison of immune parameters of cheetahs with leopards. (**a**) Immunoglobulin G concentration (**b**) Serum amyloid A (SAA) concentration (**c**) Bacterial killing assay (BKA) ranks (**d**) Lysozyme concentration, (**e**) Haemagglutination titer (**f**) Haemolysis titer. (**a**) is part of the adaptive immunity, (**b**) of the induced innate immunity and (**c**–**f**) are part of the constitutive innate immunity. Boxplots depict medians with 25% and 75% quartiles, *P*-values are indicated above the bars and samples sizes above the graphs.

injuries incurred by both species in the box traps (levels of injuries ranged from 1 to 4, Pearson's Chi-squared-test, $X^2 = 2.67$, $df = 3$, $P = 0.446$, $N_{cheetahs} = 182$, $N_{leopards} = 32$). Cortisol concentrations were influenced by age in cheetahs (Mann-Whitney-U-Test, $W = 1278.5$, $P = 0.038$, $\text{cortisol}_{adult} = 18.8 \pm 17.6$, $\text{cortisol}_{subadult} = 25.0 \pm 19.2$), but not in leopards (Mann-Whitney U-test, $W = 53.5$, $P = 0.18$, $\text{cortisol}_{adult} = 55.5 \pm 17.7$, $\text{cortisol}_{subadult} = 66.4 \pm 19.0$). Cortisol concentrations were not influenced by sex in leopards, (Mann-Whitney U-test, $W = 145.5$, $P = 0.97$, $\text{cortisol}_{males} = 56.7 \pm 20.3$, $\text{cortisol}_{females} = 57.3 \pm 15.9$) but were influenced by sex in cheetahs (Mann-Whitney-U-Test, $W = 3131$, $P = 0.009$, $\text{cortisol}_{males} = 17.9 \pm 16.2$, $\text{cortisol}_{females} = 26.1 \pm 21.9$).

When correcting for the potential influence of cortisol concentrations and then summarizing the modified immune parameters through a PCA (Model 2), we obtained qualitatively very similar results as those described above for the PCA for Model 1 (see Supplementary Results and Supplementary Table S3). We used a logistic regression with PC1 and PC2 as input to check whether both species could be reliably distinguished by immune parameters and showed that this was the case (Supplementary Results). When correcting for the possible effect of cortisol concentrations in the original, non-imputed dataset ($N_{cheetahs} = 80$, $N_{leopards} = 29$) before summarizing the modified immune parameters through a PCA (Model 4), we obtained qualitatively very similar results (see Supplementary Results and Table S3) as those from the PCA where no such correction took place (see Model 3 above).

Discussion

In this study we characterize the immunity of free-ranging Namibian cheetahs and demonstrate that despite presenting a relatively low MHC variability as previously described for this population[36] other parts of their immune response are not as impaired or reduced as previously thought. Our results show that cheetahs have a higher constitutive innate immune response than leopards, although their induced innate and adaptive immune response is lower. Thus, cheetahs might compensate the potential lack of immunocompetence in the adaptive immune system caused by their low MHC variability with a competent humoral constitutive immune system. If so, our findings could be an explanation as to why free-ranging cheetahs do not suffer from infectious diseases, particularly when tested seropositive for several virulent feline viruses, canine distemper virus and rabies virus[41,68–70]. The presence of antibodies previously measured in our cheetah study population[41,68–70] demonstrates exposure to pathogens, and subsequent monitoring of the life histories of our individually recognized study animals demonstrate their long-term survival after exposure.

The effect of allostatic load is known to differ between species and type of immune components measured. The full acute phase response is an example of an immune component that can be triggered by a short-term stressor[53]. Bacterial killing capacity was not influenced by stress in common noctules (*Nyctalus noctula*)[65], red knots (*Calidris canutus*)[71], bluecrowned motmots (*Momotus momota*) and claycolored robins (*Turdus grayi*)[66] but was

depressed by acute stress in three other bird species[66]. The level of natural antibodies (assessed by the hemagglutination assay) is not sensitive to stress[61] and accordingly, hemagglutination and hemolysis assays were not affected by handling stress in red knots (*Calidris canutus*)[71]. We used alternative models (see Supplementary Material) in which we corrected for the potential influence of allostatic load on immune measurements in both the full, imputed data set and the original raw data, and obtained results very similar to the original models. We therefore conclude that our results were not influenced by the differences in allostatic load observed between species.

Immunological measurements may also vary with the time of day[72] or month of year[72–74]. Our traps were open throughout the year and cheetahs and leopards entered the traps at any day of the year. Animals usually entered the traps between dusk and dawn and therefore were mostly sampled in the morning. Deviation from this sampling scheme was random for the species, thus no systematic bias in our data or results were expected in this respect.

MHC variability primarily affects the functionality of the adaptive immune response[51]. In our study, the adaptive part of the immune system was assessed by the predominant circulating antibody isotype in mammals, the concentration of IgG[52]. Lower levels of IgG have been associated with recurrent opportunistic bacterial infections in domestic animals[75] and lower overwinter survival in barn swallows[76]. Thus, higher levels of IgG can be regarded as a higher protective level of humoral adaptive immunity and a greater allocation of resources to this part of immunity[77]. Cheetahs had significantly lower concentrations of IgG than leopards. Compared to leopards, this might indicate a reduction of investment by cheetahs into adaptive immunity associated with their lower MHC diversity[36,50]. However, IgG is also produced in response to dietary and environmental antigens[77]. Higher concentrations of IgG of leopards might therefore also be a consequence of dietary differences between the two species, as leopards have a greater dietary breadth than cheetahs and are frequent scavengers. They might therefore encounter a larger abundance of pathogens and parasites, including repeated contact with pathogens colonizing carrion[78], both suggested to increase selection pressure on adaptive immunity. In contrast, cheetahs hardly ever scavenge and predominantly feed on freshly killed meat[46,78].

Regarding induced innate immunity, we detected lower concentrations of SAA in cheetahs than in leopards, even though it can increase 10 to 50 fold during illness in cheetahs[55,59]. SAA is the main acute phase protein in domestic cats. Concentrations of acute phase protein rapidly increase when inflammation occurs and rapidly decrease after elimination of the inflammation[59], or in response to a short-term stressor[58]. Thus, leopards either suffered from acute inflammation at the time of capture or, perhaps more likely, capture and immobilisation induced an increase in cortisol concentration followed by an increase in SAA concentration. Leopards also exhibited higher cortisol concentrations, although both cheetahs and leopards were exposed to the same capture and handling procedures. We interpret these findings to suggest that leopards might respond more strongly to such short-term challenges than cheetahs and as a consequence mount a stronger acute phase response when captured and handled than cheetahs.

The constitutive innate immunity is a first line defense against pathogens and can be activated quickly in case of infection. The bacterial killing capacity determines the ability to remove a bacterial pathogen that could be encountered in the wild and thus is (1) a functional test of the immune system, and (2) provides an environmentally relevant immune response[79]. In wild birds, mimicking a bacterial infection[80,81] or manipulating parasite loads[82] resulted in an increase in bacterial killing capacity, providing good evidence for the value of this assay. The higher bacterial killing capacity and lysozyme concentration of cheetahs indicates a higher investment into this immune branch by cheetahs than by leopards. Studies of natural antibody titers in mammals are scarce, but a study on free-ranging herbivores revealed large differences between species[83].

A focus on innate immunity was suggested for insular birds with lower genetic variability than continental birds[30]. As in island populations, at least two scenarios explain a shift of investment towards constitutive innate immunity in cheetahs. Either the relatively low genetic variability of cheetahs is a characteristic of the species and cheetahs invested throughout their evolutionary history more into their constitutive immunity than leopards, or cheetahs lost their previous adaptive immunity as a result of a demographic bottleneck(s) and in response changed their immune investment.

The first scenario is consistent with a recent genetic study on phylogeography and divergence time of extinct and extant African and Asian cheetah populations[37]. Previously, the relatively low MHC variability of cheetahs was thought to be the result of a first demographic bottleneck at the end of the late Pleistocene, approximately 10,000 years ago, and a second one as a result of direct and indirect anthropogenic actions in the past 200 years[33]. However, many mammal species went through a demographic bottleneck at the end of the late Pleistocene, including the leopard[84], which has a high genetic variability also at the MHC genes[48–50].

It was also suggested that a strong adaptive immunity may impede the evolution of genetic disease resistance in mammals by reducing selection pressure on the evolution of innate resistance traits[85]. Genetic disease resistance provides a structural basis to prevent particular pathogens to enter and harm a host. Therefore, a weaker[30] adaptive immunity may improve the chance that alleles for genetic disease resistance go to fixation in a population[85]. As adaptive immunity has substantial energetic costs[86], a strong innate immunity or genetic disease resistance should reduce selection pressure on strengthening adaptive immunity, which may have occurred in the cheetah[43].

Alternatively, if cheetahs had a higher genetic variability in adaptive immunity in the past, genetic drift may have been responsible for the possible loss of variability[87]. This is likely if balancing selection on MHC alleles is not particularly strong, e.g., if the benefits did not outweigh the high costs of MHC diversity and expression[88]. For instance, adaptive immunity has been suggested to be more important for social than solitary species because the probability of being repeatedly exposed to the same pathogens rises with higher contact rates with conspecifics, a core feature of group life[86,89]. As cheetahs are a solitary species[46], they would therefore be under less selection pressure to do so. If so, the most frequent MHC allele may get fixated and drift may prevent the fixation of subsequent mutations, keeping MHC diversity at a low level[90].

Overall, our results are compatible with a focus of immune investment by cheetahs on constitutive innate immunity rather than adaptive immunity when compared to leopards. However, this study compares the immune profiles of only two species. Different life histories in different species may drive immune profiles into different directions and thus the detected differences may have been caused by other factors we are unaware of or which are not linked to each other.

Although the maintenance of the immune system is an important aspect of disease resistance and thus contributes to the survival of the individual, each of its parts has its own inherent costs and protective values[91]. An evolutionary view of the immune system derived from life history theory would therefore argue that individuals have to trade-off these costs with other life-history traits such as growth and reproduction[88,92–94]. Cheetahs would therefore be expected to have their species-specific combination of immune defenses and other protective systems optimized for their ecological niche and life history. Our results suggest that such a protective immune phenotype is achieved in cheetahs by investing in constitute innate immunity, whereas leopards focus on the induced branches of the immune system.

The constitutive innate immunity is regarded as relatively cheap, whereas the induced innate immunity has high energetic and potentially pathological costs[88]. Therefore, cheetahs may invest more than leopards in the cheaper immune parts, perhaps because they have fewer energy reserves in form of fat depots[95]. Cheetahs have a slim body built for high speed chases to catch prey[96] where maximum maneuverability[97] is essential, but they lack the power and body mass to defend kills from other carnivore predators. The costs of induced adaptive immune responses which generate antigen-specific antibodies are assumed to be comparably low, although they involve high developmental costs generated by complicated and time-consuming lymphocyte diversification processes[88]. These processes are mostly restricted to the developmental period of the animal and require a substantial investment of energy and nutrients during ontogeny[98]. Cheetahs might not have the energy available to invest so heavily in this immune branch during development, since cheetah females usually raise litters of three to six cubs, whereas leopards usually only raise one or two[46].

Regardless of whether the low MHC variability of cheetahs is a consequence of the loss of high variability through a population bottleneck, genetic drift or selection against high variability, this study demonstrates high investment (lysozyme concentration) and functionality (bacterial killing capacity) of the cheetah's constitutive innate immune system. It has been suggested that half of the genetic variability for resistance to infections is attributable to non-MHC genes[99]. We suggest that the investment of free-ranging cheetahs and leopards in different immune branches might be equally successful in this habitat and that the immunocompetence of cheetahs might be higher than previously thought.

Our study highlights the importance of an evolutionary approach to the immune system derived from life history concepts, which argues that different parts of the immune system may evolve to cope with species-specific challenges within the trade-offs imposed by the resources available to an organism. This study might inspire new research that test predictions derived from our hypothesis for additional species. We used two large sympatric mammals that contrast in one important immune component, MHC variability. Other species also need to trade-off their resource allocations, thus a phylogenetic approach to compare immune investment across additional species would be useful. This requires that future studies simultaneously measure many or all parts of the immune system and do not limit themselves to the induced adaptive immune response (i.e., MHC variability). This would help to elucidate species-specific and/or habitat and environment-specific adaptations of the immune system.

Material and Methods

Study animals. Between 2002 and 2013 we captured 197 (49 female, 148 male) adult (>2 years of age) and sub-adult (>1–2 years of age) free-ranging cheetahs and 36 (19 female, 17 male) adult (>2 years of age) and sub-adult (>1–2 years of age) free-ranging leopards in box traps on farmland in east-central Namibia and immobilized them as previously described[68]. The animals were captured throughout the year. Once captured, they were kept in the box traps in the shade for several hours or overnight until the research team met at the box trap which was normally in the morning hours. The animals were immobilized with a dart gun and blood samples were taken between 20 min and 35 min after darting. After approximately 45 min to 60 min, the animals were given an antidote and observed until they had fully recovered from anaesthesia. From animals that were captured and sampled more than once ($N = 30$ cheetahs), one sample was randomly selected in order to avoid pseudo-replication in the analyses. The proportions of males (75.1% in cheetahs, 47.2% in leopards) differed between the species (Fisher's exact test, $P = 0.001$), whereas the proportion of sub-adults (15.2% in cheetahs, 16.6% in leopards) did not (Fisher's exact test, $P = 0.61$).

Animals in traps often acquired small injuries caused by their behavior in the trap. We recorded injuries and assessed the level of injuries as level 1 if they presented no injuries or only had old scars, as level 2 if they presented bloody claws, a bloody nose or one small abrasion, as level 3 if they presented several abrasions, small wounds or one abrasion and a bloody nose or bloody claws and as level 4 if they presented larger abrasions or larger wounds or injuries.

All experimental procedures described in the material and methods were approved by the Internal Ethics Committee of the Leibniz Institute for Zoo and Wildlife Research (IZW, Berlin, Germany) (permit number: 2002-04-01) and the Ministry of Environment and Tourism of Namibia (permit numbers: 525/2002, 700/2003, 764/2004, 939/2005, 1089/2006, 1194/2007, 1300/2008, 1392/2009, 1514/2010, 1514/2011, 1689/2012, 1813/2013), and all experiments were carried out in accordance with the approved guidelines of the IZW.

Blood sampling and storage. Blood was taken with serum and heparin Vacutainer tubes (Becton Dickinson, Franklin Lakes, USA), transported to the field laboratory in a cool box and centrifuged within 12 hours, very rarely within 24 hours after sampling. Results of immunological tests conducted with serum and

plasma from whole blood centrifuged after 2 hours, 4 hours, 6 hours and 12 hours from blood collection and after 2 hours and 4 hours from blood collection, respectively, do not differ[100]. There was no systematic bias for samples centrifuged later than 12 h after sampling, thus all samples were included for immunological tests. Serum and plasma were aliquoted and stored in liquid nitrogen. Samples were transported to Germany in full compliance with the Convention on International Trade in Endangered Species (CITES) and stored at −80 °C until laboratory analysis.

ELISA for Immunoglobulin G. Immunoglobulin G (IgG) concentration were measured in plasma samples with a protein A enzyme-linked immunosorbent assay (ELISA)[101]. Plasma samples were diluted 1:20.000 with 50 mM $NaHCO_3$. As a standard, we diluted purified cat IgG with a starting concentration of 1 mg/ml (Bethyl Laboratories, Montgomery, USA, Catalog N° P20-105) with 50 mM $NaHCO_3$ and created standard concentrations of 4.0 μg/ml, 2.0 μg/ml, 1.0 μg/ml, 0.5 μg/ml, 0.25 μg/ml, 0.125 μg/ml and 0.0625 μg/ml.

We pipetted 100 μl of diluted samples or standards in duplicates into each well of 96-well ELISA plates. Plates were incubated for 1 hour at 37 °C and washed twice with Tris-Buffered-Saline-Tween-20 (TSB-T20). Gelatine was added to TSB-T20 solution and 200 μl of 1% of this mixture was pipetted to each well to block non-specific reaction bindings. Plates were incubated for 30 min at 37 °C and washed twice with TSB-T20. Then, 100 μl of 1:12.000 solution of protein-A-horseradish-peroxidase (Protein A- HRP; Invitrogen; Catalog N° 10-1023) in TSB-T20 was added to each well and plates were incubated for 30 min at room temperature. Plates were washed with TSB-T20 and 100 μl of phosphate-citrate-buffer containing a 1% dilution of 3,3′,5,5′-Tetramethylbenzidin (TMB; TMB One Component Microwell Substrate; SouthernBiotech; Catalog N° 0411-01), dimethylsulfoxide (DMSO) and H_2O_2 was added to each well. The reaction was stopped after 5 min with 100 μl of 1% H_2SO_4. We measured the absorbance of the wells in the plates at 450 nm in a photometric microplate reader (Biotek; μQuant Microplate Spectrophotometer). A linear standard curve was calculated for each plate using the standard concentrations. IgG concentrations were then calculated based on the standard curve.

ELISA for serum amyloid A (SAA). We measured and calculated SAA concentrations with a commercial solid phase sandwich SAA Multispecies ELISA kit (Tridelta, Phase Range; Multispecies SAA ELISA kit; Catalog N° TP-802) following the instructions of the manufacturer. Cheetah and leopard plasma samples were diluted 1:1,500 and 1:2,000, respectively, with sample diluent (provided with the ELISA kit) prior to the assay. A linear standard curve was calculated using the calibrator standard for cats (100.0 ng/ml, 50.0 ng/ml, 25.0 ng/ml, 12.5 ng/ml, 6.25 ng/ml and 0.0 ng/ml). SAA concentrations were calculated according to the standard curve on each plate. Sixty-two samples had a higher absorbance than measurable with our spectrophotometer (Biotek; μQuant Microplate Spectrophotometer). These samples were either rerun ($N = 42$) with a higher dilution of 1:5,000 for cheetahs or 1:8,000 for leopards (samples were thawed again) or the entire plate was re-measured directly after discarding half of the volume in each well ($N = 20$).

Bacterial killing assay (BKA). We measured the *in vitro* bacterial killing ability of serum against *Escherichia coli*. The method was previously described in detail in ref. 102. Briefly, serum samples were serially diluted with phosphate-buffered-saline (PBS), resulting in eight dilutions from 1:2 to 1:265. Each well of a 96-well-plate was filled with 44 μl and mixed with 10 μl of a bacterial working solution of ~1.5×10^5 colony-forming units (CFU)/ml. After incubation for 30 min at 37 °C, tryptic soy broth was added to each well. Absorbance was measured with a spectrophotometer (Biotek; μQuant Microplate Spectrophotometer) to determine background absorbance and again after the plates had been incubated for 12 hours at 37 °C. Bacterial killing capacity was calculated for each dilution of serum against a positive control (wells that contain only bacteria without serum). Ranks were assigned to each dilution before killing capacity dropped from 100% to 0%, such that dilution 1:2 corresponded to rank 1, the usually lowest rank, dilution 1:4 to rank 2, etc. If bacterial killing did not reach 100% even at dilution 1:2, rank 0 was assigned.

Lysoplate assay. To measure the concentration of lysozyme we used the lysoplate assay method[103]. We prepared 1% noble agar (Sigma Aldrich; St. Louis, USA, Catalog N° A5431-250G) with PBS at pH = 6.3 and added the required amount of lysozyme-sensitive bacteria *Micrococcus lysodeikticus* (Sigma Aldrich; M3770) to reach a bacterial concentration of 25 mg/100 ml in the agar for cheetahs and 12.5 mg/100 ml in the agar for leopards. Plates had a diameter of 14.2 cm and were put on a pre-heated surface (50 °C), horizontally leveled with a water spirit to avoid quick and uneven cooling of the 30 ml noble agar on the plates. After cooling, 25 holes with diameters of 4.5 mm were punched into the agar and filled with 25 μl of serum samples (18 holes) or standards (7 holes). Standards with concentrations of 10.0 μg/ml, 7.5 μg/ml, 5.0 μg/ml, 2.5 μg/ml, 2.0 μg/ml, 1.25 μg/ml and 1.0 μg/ml were prepared using lysozyme form chicken egg white (Sigma Aldrich; St. Louis, USA, Catalog N° L6876). Plates were incubated at room temperature for 18 hours.

Micrococcus lysodeikticus is particularly sensitive to lysozyme, thus the bacterial lysis of the samples and standards creates a clear zone around the inoculated wells. The diameter of this clear zone is proportional to the logarithmic (basis of 10) lysozyme concentration in the samples and standards[103]. We photographed each plate in a photobox (Imaging system; peqlab) with a ruler next to it as a reference scale. The diameter of the lytic areas was measured digitally using the software ImageJ (version 1.48, http://imagej.nih.gov/ij/). Each lytic area was measured three times and the mean was used for calculations. The measurements of the lysis standards were plotted as a linear function of the log lysozyme concentration. This regression line was then used to infer the lysozyme concentrations of the cheetah and leopard samples.

Haemagglutination/haemolysis assay. The haemagglutination/haemolysis titers represent the levels of natural antibodies and complement[61]. Although the method was originally developed for avian species, it

has recently been modified for mammals by using chicken erythrocytes as target cells[62]. After pipetting 25 µl of plasma in the wells of the first two columns of a U-shaped 96-well microtitre plate, 25 µl sterile PBS was added to the 2nd–12th columns. Using a multi-channel pipette, the content of the second column wells was serially diluted until the 11th column, resulting in a dilution series for each sample from 1:2 to 1:1024. We used the last column of the plate as negative controls containing only PBS. We then added 25 µl of 1% chicken red blood cells suspension to all wells, covered them with Parafilm M (Pechiney Plastic Packaging, Chicago, USA), vortexed gently and incubated at 37 °C for 90 min. After incubation the plates were tilted at a 45° angle to increase the visualization of agglutination and kept at room temperature until analyses.

Agglutination and lysis, which reflect the activity of natural antibodies and the interaction between natural antibodies and complement[61,104], were recorded after 20 min (haemagglutination titre) and 90 min (haemolysis titre), respectively. Haemagglutination is characterized by the appearance of clumped red blood cells as a result of antibodies binding multiple antigens, whereas during haemolysis red blood cells are destroyed by complement. Haemagglutination/haemolysis titers were given as the $\log_2$ of the reciprocal of the highest dilution (i.e. lowest concentration) of plasma showing positive haemagglutination or haemolysis, respectively[62,104].

Measurement of cortisol concentration. Although cheetahs and leopards were captured in the same type of traps and therefore exposed to the same capture conditions, the two species might respond differently to these short-term challenges. Such challenges increase the allostastic load ('stress') and thereby may influence various immune parameters, as has been shown for SAA concentrations in rats[53]. To rule out the possibility that differences in immune parameters between the two species were caused by differences in allostatic load induced by different responses to the capture procedure, we measured the concentration of native cortisol, an indicator of allostatic load which rapidly increases after a stressful stimulus[105], in blood samples of cheetahs and leopards. Cortisol (hydrocortisone) was quantified as described earlier[106] by an enzyme immunoassay (EIA) using a polyclonal antibody (rabbit) against hydrocortisone-21-hemisuccinate-BSA and hydrocortisone-21-hemisuccinate-peroxidase as label. The inter-assay coefficient of variation of two biological samples was 7.3 and 8.1% (n = 14), respectively.

Statistical analyses. The dataset consisted of 251 captures and sampling events for 197 cheetahs and 36 captures and sampling events for 36 leopards. Sample sizes varied slightly for different immunological measurements because the bacterial killing assay and the hemagglutination/hemolysis assay should be limited to samples which are thawed for the first time only, otherwise they become unreliable. Some samples had been previously thawed for other studies and been frozen again and therefore could not be used. Other samples were too small to provide material for all analyses. We therefore expect that there was no systematic bias in the sets of available samples for the analysis of various immune parameters of cheetahs and leopards. The highest number of missing data was for SAA concentration with 68 missing measurements (26.8%). For all other immunological parameters the percentage of missing measurements was below 20%. We used principal component analysis (PCA), a statistical procedure that uses an orthogonal transformation to convert a set of possibly correlated variables into a set of linearly uncorrelated variables. PCA reduces the dimensionality of data and through the loadings of the original variables on the principal components helps to identify the true sources of variation in the data. Because PCA requires the removal of all samples with missing data, we used the R package Amelia II version 1.7.3, which allows for the imputation of missing values in the dataset[107]. Amelia II uses the expectation-maximization algorithm to perform multiple imputations for incomplete data. An examination of the quality of imputation is presented in the supplementary information (Figure S1, Table S1 and Supplementary Results).

To test whether both species differ in their immune characteristics, we used the dataset complemented by the imputed values and reduced the dimensionality of immune parameters by performing a mean-centered, scaled PCA with the R package ade4 version 1.7-2[108]. We then fitted a logistic regression model predicting species identity (a binary variable) as a function of the first two principal components. We tested the effect of each covariate by performing a likelihood ratio test between this model and a model that only contained the intercept and the other principal component. We ruled out the possibility that differences between species were a consequence of possible differences in the proportion of males and females sampled in the two species. To do so, we fitted one logistic regression per species predicting the sex (binary variable) as a function of the two principal components of the PCA, and compared the likelihood of the fit to a model with an intercept only. A non-significant result of this analysis would suggest that sex does not influence the outcome of the PCA analysis which uses the immune components to distinguish species. To control for the possible effects of allostatic load we fitted linear models predicting each immune variable by cortisol concentrations to extract the residuals of these models to be used in another PCA. We again predicted the species identity (binary variable) as a function of the two principal components from this new PCA with a logistic regression model and compared the results obtained with those from the first PCA. All statistical analyses were performed using R version 3.0.3[109].

Following the PCA analyses, we compared the median of each immune parameter separately with non-parametric Mann-Whitney-U-tests for not normally distributed data. This allowed us to use the original non-imputed dataset. Levels of injuries caused by the behavior of the animals in the box traps were analyzed with a chi-square-test for independence.

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Author Contributions

S.K.H. designed the study, collected many of the samples, carried out most of the laboratory work, analysed the data and drafted the manuscript. G.Á.C. designed the study, carried out laboratory work and contributed to writing the manuscript. BW designed the study, helped with data analysis and writing of the manuscript. A.C. helped with statistical analysis and improved the manuscript. M.D. carried out laboratory work. J.M. provided additional samples. H.H. helped design the study and contributed to writing the manuscript. All authors gave final approval for publication.

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Cheetahs compensate low major histocompatibility complex variability with a strong innate immune system

Electronic supplementary material

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1. Supplementary methods: Statistical analyses

Missing data in our dataset were imputed with the R package Amelia II version 1.7.3. Amelia II uses the expectation-maximization algorithm to perform imputation for incomplete data. Because our dataset had missing values amongst all immune variables, we used the method of multiple imputation to replace missing values and thereby increase the power of the statistical models.

Figure S1 presents a comparison of the observed, original dataset with the dataset which included imputed values. During imputation, BKA, haemagglutination and haemolysis were considered as continuous variables in order to improve imputation performance, which explains some differences in the distribution between original and imputed data (Fig. S1). Table S1 presents the results of the statistical comparison of imputed and original data, using Mann-Whitney U-tests for differences in the median of both data sets and Levene's test for differences in the variance.

To correct results for differences in allostatic load, we performed a linear model predicting each immune variable from glucocorticoid concentrations. We then extracted the residuals of these linear models to obtain the variation in allostatic load that is unrelated to variation in glucocorticoid concentrations. The residual variation associated with each immune variable constituted a set of new variables, which were used for another principal component analysis (PCA). The new principal component 1 (PC1) and principal component 2 (PC2) derived from this PCA were then used as input parameters for alternative models.

Figure S1 Comparison of distributions of observed and imputed values for all immune variables. The observed values are drawn in black, imputed* values in red.

*Imputation is the process of replacing missing data with substituted values. The R package "Amelia II"[1] implements a bootstrapping-based algorithm for multiple imputation of missing values.

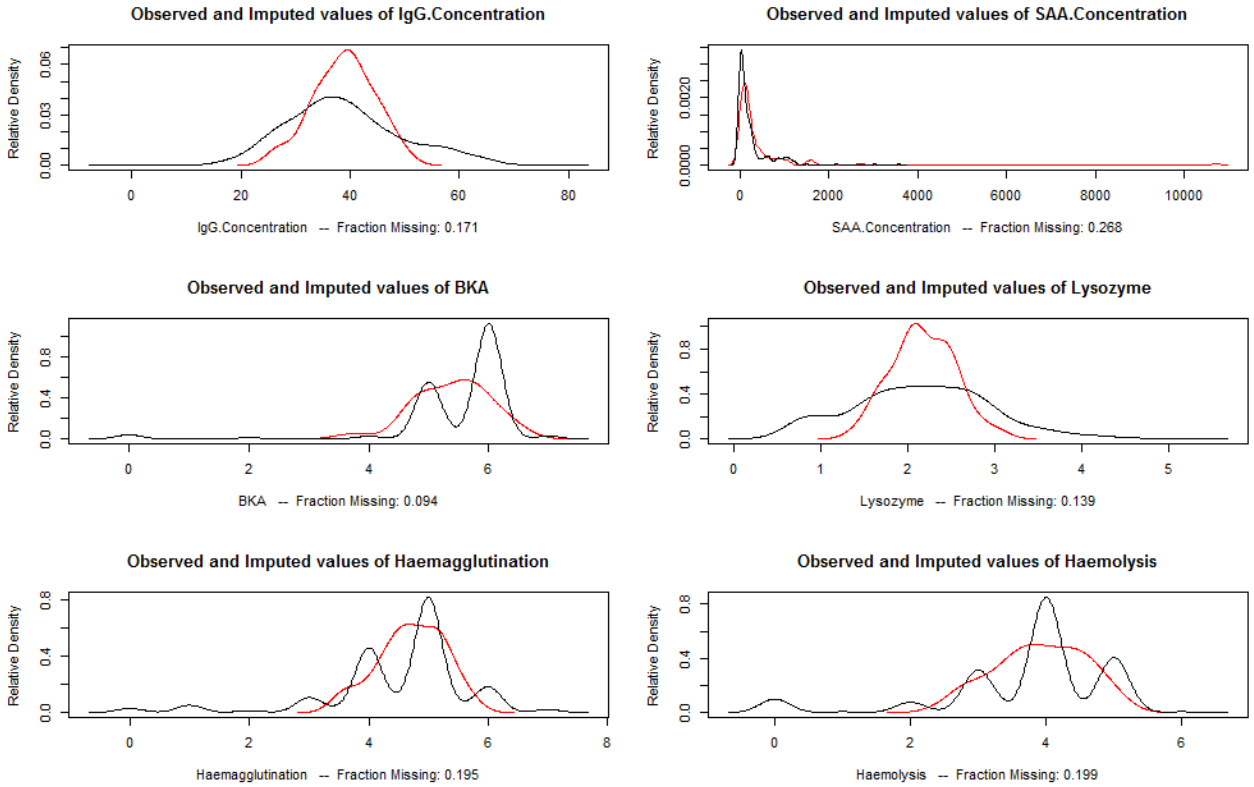


Table S1 Comparison of original data and imputed data.

Variable	Mann-Whitney U-test	Median original data	Median imputed data	Levene's test	variance original data	variance imputed data
IgG concentration[a]	W=5433; p=0.452	37.45	37.29	F=19.35; p<0.001	118.70	26.75
SAA concentration[b]	W=9779, p=0.007	86.77	130.49	F=1.53; p=0.21	2026631	210465.2
BKA rank[c]	W=3596; p=0.816	6.00	5.33	F=0.10; p=0.75	1.20	0.35
Lysozyme concentration	W=4698; p=0.620	2.16	2.16	F=16.09; p<0.001	0.63	0.15
Haemagglutination titer	W=6874; p=0.453	5.00	4.76	F=8.10; p=0.005	1.44	0.22
Haemolysis titer	W=7187; p=0.244	4.00	4.10	F=4.88; p=0.028	1.48	0.33

[a]IgG: Immunoglobulin G

[b]SAA: Serum Amyloid A

[c]BKA: Bacterial killing assay

The PCAs using the imputed dataset (1) without correction for allostatic load provided the basis for Model 1, described in the main text, with detailed factor loadings specified in Table S3, and (2) with correction for allostatic load provided the basis for Model 2, described below, with detailed factor loadings specified in Table S3. The PCAs using the smaller datset of observed, original data without imputation (1) without correction for allostatic load provided the basis for Model 3, described below, with detailed factor loadings specified in Table S4, and (2) with correction for allostatic load provided the basis for Model 4, described below, with detailed factor loadings specified in Table S4.

2. Supplementary results: distinguishing species

Table S2 presents the results of a logistic regression to check whether the two species can be reliably distinguished by the results of the PCA performed on imputed data (Model 1).

Table S2 | Summary table of the logistic regression model (Model 1) predicting whether the species is a cheetah (value = 1) or a leopard (value = 0). Estimates and associated standard error are expressed on the logit scale. P-values are here computed using a simple z-test on the estimates. See main text for likelihood-ratio tests.

	Estimate	Standard error	z-value	p-value
Intercept	2.811	0.364	7.715	<0.001
PC1[a]	0.389	0.194	2.007	0.045
PC2	-2.273	0.368	-6.170	<0.001

[a]PC: Principal component

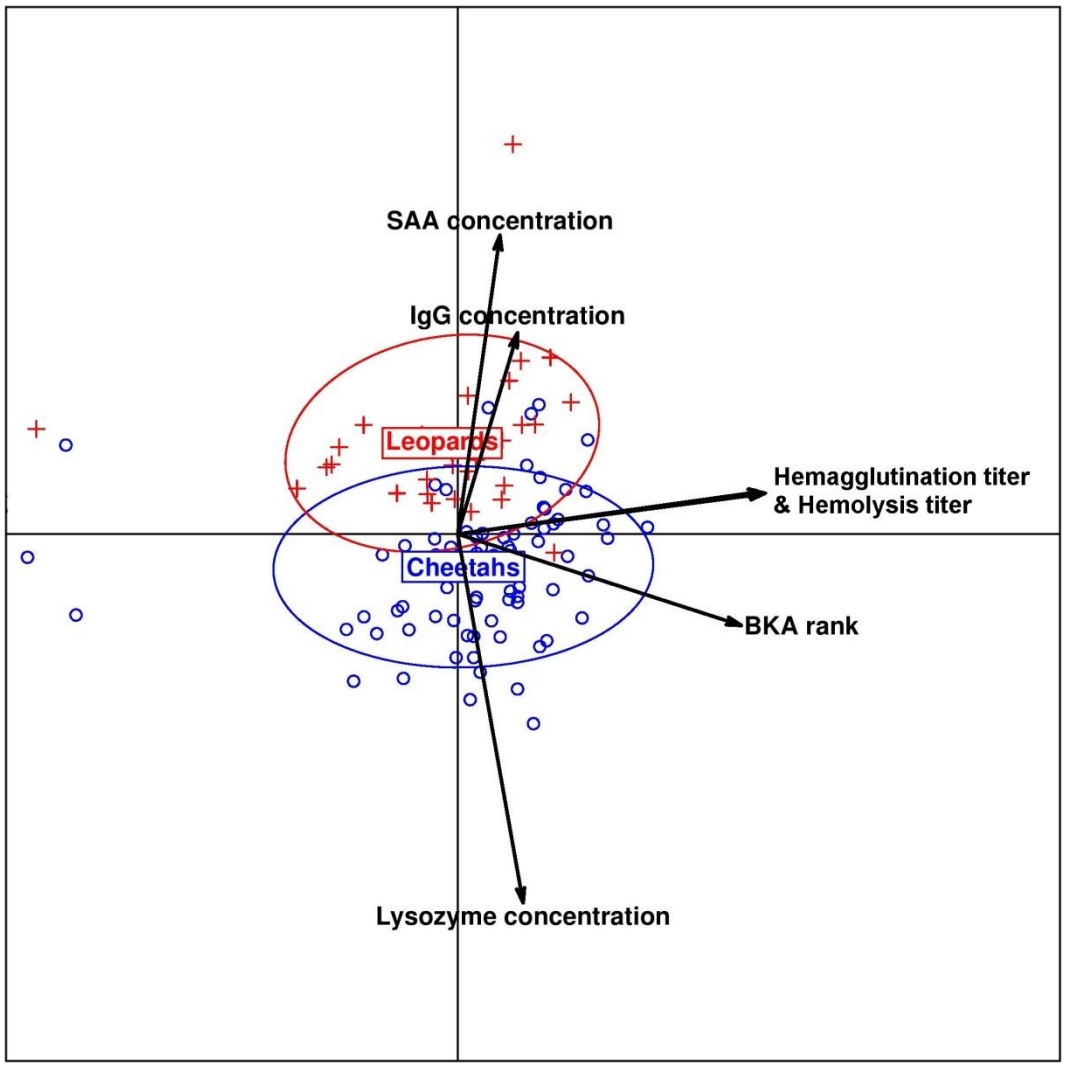
3. Supplementary results: the effect of imputation

Imputation significantly shifted (increased) the median only in the case of SAA concentrations but not for the other immunology variables (Table S1). Imputation also

reduced the variance from the original data for all immunology variables except SAA concentration, and reduced it significantly for IgG concentration, haemagglutination titer, haemolysis titer and lysozyme concentration (Table S1). A lower variance reduces the sensitivity of our analysis to imputed values, which makes the analysis therefore conservative.

Figure S2 presents the results of a PCA on the raw dataset, i.e. without imputing missing values ($N_{cheetahs}$ = 80, $N_{leopards}$ = 29). The results were qualitatively similar. Here PC1 captured 43.4% of the total variance in immune measurements (as compared to 40.1% with imputed data), PC2 captured 22.6% of the total variance (as compared to 23.8% with imputed data). The detailed factor loadings are listed in Table S4. In the logistic regression model for this non-imputed dataset (Model 3), cheetahs presented higher, but not significantly higher, scores on PC1 (mean PC1 value = 0.046) than leopards (mean PC1 value = -0.127, logistic regression, likelihood ratio test (LRT) = 0.42, df =1, P =0.51) and significantly lower scores on PC2 (mean PC2 value = -0.375) than leopards (mean PC2 value = 1.035, logistic regression, LRT = 55.65, df = 1, $P < 0.001$). In comparison, with imputed data, cheetahs presented significantly higher scores on PC1 and significantly lower scores on PC2 than leopards (see main text).

Figure S2 Immune differences between cheetahs and leopards based on original (non-imputed) data (Model 3). Scores of all cheetah (small circles) and leopard individuals (plus signs) on the first two principal components (PC1 on x-axis and PC2 on y-axis) of a principal component analysis performed on all six immune parameters. Arrows represent the contribution of each immune parameter to PC1 and PC2. For each species, 1.5 inertia ellipses are depicted.



4 Supplementary results: the importance of allostatic load

Table S3 specifies the detailed factor loadings for each original immune variable on the PCs for both the original model (Model 1) and the alternative model (Model 2) where we accounted for the variation in cortisol concentration between species. Note that both models used imputed data. The strongest shift in loadings affected the SAA concentration (halving the loading on PC1 and thereby reducing its impact on PC1), followed by an increase in the loading of IgG concentration on PC2 and a decrease of the loading of BKA rank on PC2, each by approximately a third of the original value.

Table S3 PCA loadings for Principal Component 1 and 2 using imputed data

Immune variable	Principal component 1		Principal component 2	
	Model 1	**Model 2**	**Model 1**	**Model 2**
Correcting for allostatic load?	No	Yes	No	Yes
IgG concentration[a]	0.2318	0.222	0.2935	0.3824
SAA concentration[b]	0.2991	0.1571	0.7578	0.7450
BKA rank[c]	0.7652	0.7693	-0.0799	0.0016
Lysozyme concentration	0.3005	0.3744	-0.7946	-0.7174
Haemagglutination titer	0.9170	0.9208	0.0243	0.0093
Haemolysis titer	0.9273	0.9227	0.0296	0.0615

[a]IgG: Immunoglobulin G

[b]SAA: Serum Amyloid A

[c]BKA: Bacterial killing assay

5 Supplementary results: the combined effect of imputation and of allostatic load

Table S4 specifies the results from two PCAs of the observed, original dataset without or with correction for allostatic load, by listing detailed factor loadings for each original immune variable. The main difference between the two PCAs is the increase in the loading of SAA concentrations on PC1, otherwise the loadings are very similar between both PCAs. The results from these PCAs are qualitatively similar to the respective PCAs run on the imputed data set (Table S3), in that the relative contribution of the original immune variables to PC1 and PC2 were preserved, even if in the imputed data set some of the loadings were stronger.

In a logistic regression model of the PCA using the non-imputed dataset ($N_{cheetahs}$ = 80, $N_{leopards}$ = 29) corrected for allostatic load (Model 4, depicted in Table S4), PC1 was not

significantly different between cheetahs (mean PC1 value = 0.039) and leopards (mean PC1 value = -0.108) (logistic regression predicting the species from PC values; LRT = 0.41, *df* = 1, *P* = 0.522). There was a significant difference between cheetahs (mean PC2 value = -0.168) and leopards (mean PC2 value = 0.463) for PC2 (logistic regression predicting the species from PC values; LRT = 9.38, *df* = 1, *P* = 0.002). For this PCA (Model 4), PC1 captured 44.1% of the total variance in immune measurements and PC2 captured 23.1%.

Table S4 PCA loadings for Principal Component 1 and 2 using non- imputed data

Immune variable	**Principal component 1**		**Principal component 2**	
	Model 3	**Model 4**	**Model 3**	**Model 4**
Correcting for allostatic load?	No	Yes	No	Yes
IgG concentration[a]	0.1836	0.1751	0.4446	0.4581
SAA concentration[b]	0.1294	0.1899	0.6602	0.6817
BKA rank[c]	0.8690	0.8719	-0.2021	-0.1605
Lysozyme concentration	0.2005	0.2359	-0.8151	-0.8235
Haemagglutination titer	0.9407	0.9395	0.0890	0.0618
Haemolysis titer	0.9354	0.9370	0.0944	0.0710

[a]IgG: Immunoglobulin G

[b]SAA: Serum Amyloid A

[c]BKA: Bacterial killing assay

CHAPTER 4 – TRADE-OFFS IN IMMUNE FUNCTION WITHIN THE CHEETAH

Third Manuscript

Trade-off in immunity of free-ranging and captive cheetahs – a quasi-experiment to determine priorities in immune investment

(in preparation)

Trade-offs in immunity of free-ranging and captive cheetahs: a quasi-experiment to determine priorities in immune investment

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Summary

1. The immune system is crucial for host defense against pathogens but requires energetic investment which has to be traded-off against other investments such as reproduction and growth. The immune system of cheetahs (*Acinonyx jubatus*) is particularly interesting because for decades it has been a textbook example of apparently low immunocompetence associated with low genetic variability at the major histocompatibility complex (MHC).
2. In this study we assess the immunocompetence of cheetahs by measuring six parameters of the humoral immune system of free-ranging and captive cheetahs: the concentration of immunoglobulin G (IgG) as a representative of the adaptive immune system, the concentration of serum amyloid A (SAA) to represent the induced innate immune system, and the bacterial killing capacity of the serum, the concentration of lysozyme and the capacity of natural antibodies and complement system to represent the constitutive innate immune system.
3. We measured immunocompetence in a large number of cheetahs either living in a free-ranging population on commercial farmland in Namibia, or as captive animals held by farmers or a private organization in large enclosures in their natural habitat, a quasi-experimental setup where animals had access to regular food resources but were not allowed to reproduce, and thus may have allocated investment into immune resources in a different manner.
4. We also measured allostatic load ('stress') associated with capture and handling using serum cortisol concentrations and neutrophil to lymphocyte (N/L) ratios in both free-ranging and captive cheetahs and investigated its influence on immune parameters.
5. Male cheetahs had a stronger bacterial killing capacity than females. Older animals had a higher IgG concentration and lower levels of complement than younger animals.
6. Free-ranging cheetahs had a lower IgG concentration, higher SAA concentration, a stronger bacterial killing capacity, higher cortisol concentrations and higher N/L ratios than captive individuals. Three out of six immune parameters were affected by allostatic load caused by handling, suggesting that 'stress' plays an important role when measuring immunity in free-ranging and captive cheetahs.
7. Our results suggest that contrary to previous hypotheses, cheetahs are not generally impaired in their immunocompetence, but adapt their investment according to their specific needs in function of life history stages.

Key words: age differences, captivity, cheetah, immunity, sex differences, stress

Introduction

The immune system is an important component of the body functions that contribute to the survival of an individual. It also requires energetic investment, which may play an important role in mediating life history trade-offs (Lochmiller & Deerenberg 2000). The energetic costs of mounting an immune response include the costs of development, maintenance and use (Klasing 2004). These costs vary substantially for different parts of the immune system and different stages in life. For example, recombination, gene conversion and mutation processes necessary for generating a diverse repertoire of lymphocytes for a potent adaptive immune response take several months to develop and require a major energetic investment during ontogeny. This will pay off later in life as this immune response can be activated quickly and effectively for familiar pathogens at a low effort (Lee 2006). Development and maintenance of protective cells and proteins for the innate immune response is regarded as energetically cheap, but the activation of the innate immune response also stimulates a systemic inflammatory response that can lead to high energetic and pathological costs (Lee 2006). It is likely that individuals invest in different parts of their immune system based on evolved reaction norms (Roff 1992) which take into account the availability and limits of their resources (Norris & Evans 2000) and the evolutionary history of their species in terms of pathogen encounters. If resource limitation becomes severe, individuals should alter their investment such that they maximize their fitness under these conditions (Norris & Evans 2000). (Schmid-Hempel 2003; Schmid-Hempel & Ebert 2003; Klasing 2004) and (Lee 2006) established an immune defense component model of the humoral immune system amongst which trade-offs in terms of investment are likely. To investigate immune investment we used a modified version of their model as shown in Table 1.

Cheetahs (*Acinonyx jubatus*) are an interesting species to study investment strategies into different parts of the immune system because (1) their immunocompetence may be impaired by relatively low genetic variability at the genes of the major histocompatibility complex (MHC) (O'Brien *et al.* 1985; Castro-Prieto, Wachter & Sommer 2011) and (2) trade-offs in investment may become clearer than in other species because the resources they can invest in the immune system may be limited, because their slim body is built for high speed chases and high maneuverability (Wilson *et al.* 2013) and lacks energy reserves in form of fat depots (Laurenson 1995). MHC genes are important for the induced adaptive immune system

and incite a cascade of responses to protect the body with pathogen recognizing antibodies and cytotoxic T-cells (Janeway *et al.* 2001). If relatively low MHC variability decreases cheetah immunocompetence, shifting investment towards other parts of the immune system may compensate for the loss of function by the impaired component (Matson 2006). We have recently provided the first evidence in this direction by demonstrating that the constitutive immune system of cheetahs is stronger than those of other carnivores and vertebrates and might thus compensate for the relatively low MHC diversity (Heinrich *et al.* 2016; Heinrich *et al.* 2017). This is in line with previous studies which demonstrate that free-ranging cheetahs showed no clinical or pathological evidence for diseases, even when they tested seropositive for several infectious diseases (Munson *et al.* 2004; Thalwitzer *et al.* 2010). Here, we ask to which extent cheetahs may vary their investment into different parts of the immune system between the sexes, across different life history stages (age) and between a free-ranging and captive environment, i.e. a lower and higher likelihood of exposure to pathogens, respectively (see below). We also check whether the 'stress' and increase in allostatic load, i.e. the cumulative energetic cost of maintaining homeostasis through behavioral or physiological responses to challenges (Romero, Dickens & Cyr 2009; McEwen & Wingfield 2010), associated with capture and handling (Hofer & East 1998) may affect measures of the immune response.

In mammals, females invest more energy in producing and raising offspring than males (Trivers 1972; Clutton-Brock 1988). Females might also maximize fitness through increased longevity (Bateman 1948; Rolff 2002), requiring a good immune response. Cheetah females substantially invest in their offspring through lactation and food provisioning, because the offspring stay with their mother for approximately 18 months but do not contribute to the acquisition of food and might even lower the hunting success of the mother by disturbing the hunt (Caro 1994). When food intake is insufficient to sustain both lactation and self-maintenance, resource allocation trade-offs are expected in terms of decreased investment in immune processes (Viney, Riley & Buchanan 2005; Houston *et al.* 2007). Consistent with this idea, studies of wild and domestic ungulates (e.g. (Festa-Bianchet 1989; Coop & Kyriazakis 1999; Houdijk, Jessop & Kyriazakis 2001; Turner *et al.* 2012)), bats (e.g.(Christe, Arlettaz & Vogel 2000; Plowright *et al.* 2008)), rabbits (Cattadori *et al.* 2005) and carnivores (East *et al.* 2015) demonstrated an increase in pathogen infection during lactation. Experimental studies also showed that improved nutrition during lactation (e.g. (Houdijk *et al.* 2003; Jones *et al.* 2011) and reduced lactation effort following the experimental reduction of litter size in rats (*Rattus norvegicus)* boosts specific immune

processes and reduces parasite load (Jones *et al.* 2012). Cheetah females may therefore have relatively few resources to invest into their immune system and focus on its cheaper components. The costs of humoral effectors of adaptive immunity such as immunoglobulins are considered to be relatively low once the developmental period is completed whereas the maintenance costs of the constitutive part of the innate immune system is relatively high (see Table 1, (Lee 2006)).

Mammalian males might maximize fitness through a high mating rate rather than longevity (Bateman 1948; Rolff 2002) and therefore might be able to afford to have a weaker immune response. Several studies report mammalian males to have higher parasite burdens (Poulin 1996; Zuk & McKean 1996; Moore & Wilson 2002), weaker antibody responses (Daniels & Belosevic 1994) or lower antibody titres (Kacprzak-Bergman 1994; Olsen & Kovacs 1996) than females. Previous studies demonstrated that testosterone had a suppressive effect on immunoglobulin concentration (Hirota *et al.* 1976; Grossman 1985; Saino, Møller & Bolzerna 1995; Duffy *et al.* 2000; Casto, Nolan Jr & Ketterson 2001) which forms part of adaptive immunity. Thus, we expect cheetah males to exhibit lower immunoglobulin concentration than cheetah females. At the same time males might need to rely on a strong innate immune system which is important for wound healing, because they have a higher risk of injury than females as they regularly fight over territories (Caro, Fitzgibbon & Holt 1989). We therefore expect adult females to have a higher concentration of immunoglobulins and adult males to invest more into the innate immune system than females.

As juveniles develop, the immune system is likely to change because young animals need time to develop their adaptive immunity. Thus, we predict younger animals to have a weaker adaptive immune response than older (adult) animals. As the innate immune system should protect its host from early life stages onwards, we expect the innate immune part to be similar in younger and older animals.

Cheetahs kept in Namibia in large enclosures in the same habitat as free-ranging individuals are fed regularly with meat and thus their energetic resources should be less limited than for free-ranging cheetahs. In Namibia, captive cheetahs are not allowed to reproduce, thus female cheetahs may invest more energetic resources in their immunity. Thus, we expect a stronger overall immunity in captivity, especially for females. Additionally, captive cheetahs are likely to have higher concentration of antibodies such as immunoglobulin G (IgG) than free-ranging ones because the former are vaccinated on a yearly basis against the most common infectious feline diseases such as rabies, feline panleukemiavirus, feline calicivirus and feline rhinotracheitis virus. Thus, young captive cheetahs should already have

high IgG concentration whereas in free-ranging cheetahs young animals should have low antibody concentration which increase towards adulthood. Moreover, Namibian captive cheetahs in their enclosures often live in close proximity to other captive cheetahs or carnivore species as well as to domestic dogs and domestic cats of the associated farms. Thus, it is likely that captive cheetahs experience a higher pathogen pressure than free-ranging ones and therefore have higher concentration of antibodies such as IgG.

Free-ranging and captive cheetahs in commercial farmland in Namibia do not differ in their levels of chronic allostatic load ('stress') in terms of the size of their adrenal glands as measured by ultrasonography, a proxy for chronic stress (Wachter *et al.* 2011), but the procedures of capture and handling of free-ranging cheetahs differed from the handling of captive cheetahs in their large enclosures. Free-ranging cheetahs were captured in box traps and often spent several hours up to one night in the trap before immobilization. This differed from captive cheetahs which were immobilized in their enclosures without previous confinement. Captive cheetahs were also accustomed to the presence of people whereas free-ranging cheetahs were not. Capture and handling therefore provided an excellent opportunity to assess how an acute stressor might influence the immune response (Padgett & Glaser 2003). We therefore expected free-ranging cheetahs to exhibit higher serum cortisol concentrations and higher neutrophil to lymphocyte (N/L) ratios than captive ones, and a correspondingly stronger change in our measures of the immune response.

Material and Methods

Study area and study animals

The study area was located on commercial farmland in east-central Namibia, ranging from 16°30'E to 19°00'E and from 21°30'S to 23°00'S. Captive animals were kept in the same area or further north at the Africat Foundation, a non-profit conservation facility for carnivores.

Between 2002 and 2013 we captured and handled 231 free-ranging cheetahs (63 females, 168 males) in box traps and immobilized them as previously described (Thalwitzer *et al.* 2010). Of the 63 free-ranging female cheetahs, five were in oestrus, four were pregnant, two were lactating, 15 were caught with weaned cubs, 30 were sub-adults or cubs, and of seven the reproductive status at capture was not known. Additionally, we sampled 96 cheetahs (38 females, 58 males) kept in large enclosures on private farms or at Africat Foundation which were immobilized in their enclosures. Of the 38 captive female cheetahs, 37 were adult but not reproductively active and one was a cub. Because in Namibia captive cheetahs are not

allowed to reproduce, we used this as a quasi-experimental setup to investigate the priorities of free-ranging and captive cheetahs in their immune investment. The age of captive cheetahs, in years, was provided by the managers of the captive facilities. The age of free-ranging cheetahs, in years, was estimated according to (Caro 1994). Age was entered as a continuous variable in statistical analyses and included cubs (2 - 12 months, n = 37), sub-adults (13 – 23 months, n = 29), adults (≥ 2 – 7 years, n = 210) and old individuals (>7 years, n = 35).

Blood sampling and storage

Blood was taken with Vacutainer® tubes (Becton Dickinson), transported to the field laboratory in a cooling box equipped with ice packs and centrifuged within 12 hours, very rarely within 24 hours after sampling. Before centrifugation, an aliquot of the EDTA full blood was used to prepare blood smears on glass slides. Serum and plasma samples were collected after centrifugation, stored in liquid nitrogen, transported to Germany and then stored at -80°C until analysis.

Measurements of the allostatic load ('stress')

We assessed the level of acute allostatic load ('stress') by serum cortisol concentrations (Sapolsky 2002) and the neutrophil to lymphocyte (N/L) ratio in blood smears (Davis, Maney & Maerz 2008). Serum cortisol (hydrocortisone) concentration was quantified as described earlier (Voigt *et al.* 2004) by an enzyme immunoassay (EIA) using a polyclonal antibody (rabbit) against hydrocortisone-21-hemisuccinate-BSA and hydrocortisone-21-hemisuccinate-peroxidase as label. The inter-assay coefficient of variation of two biological samples was 7.3 % and 8.1 % (n = 14), respectively.

The N/L ratio is a second indicator for allostatic load because glucocorticoids lead to an increase in the number of neutrophils and a decrease in the number of lymphocytes in the blood (Davis, Maney & Maerz 2008). We expect these two indices to be highly correlated and associated with measures of the immune response in a similar way.

Blood smears were stained with May-Gruenwald's solution (#T863.2, Carl Roth GmbH) and Giemsa solution (#T862.1, Carl Roth GmbH). Differential white blood cell counts were performed by counting 100 leucocytes under oil-immersed 1000x magnification and N/L ratios were subsequently calculated. All differential cell counts were performed by the same person (SKH) and were conducted in a blind manner with respect to individual identity.

Measurements of immunocompetence

To assess trade-offs and compensatory investment in the immune system of cheetahs, we measured (1) the concentration of IgG to estimate the strength of the adaptive immune system, (2) the concentration of acute phase protein serum amyloid A (SAA) to determine the strength of the induced innate immune system, and (3) the bacterial killing capacity of the serum, the concentration of lysozyme, the capacity of natural antibodies and complement as proxies for the constitutive innate immune system (Table 1).

ELISA for Immunoglobulin G (IgG)

IgG concentration (in mg/ml) was measured in plasma samples using a protein A enzyme-linked immunosorbent assay (ELISA) (Ross *et al.* 1993). The method is described in detail in (Heinrich *et al.* 2017). In short, plasma samples were diluted 1:20.000 and a standard curve was created using seven different concentrations of purified cat IgG (Bethyl Laboratories, Montgomery, USA, Catalog N° P20-105). After pipetting 100 µl of diluted samples or standards in duplicates in 96-well ELISA plates, plates were incubated for 1 hour at 37°C and washed twice with Tris-Buffered-Saline-Tween-20 (TSB-T20). After blocking the unspecific binding with 1% gelatine-TSB-T20, we added a 1:12,000 solution of protein-A-horseradish-peroxidase in TSB-T20 and incubated the plates at room temperature. After washing with TSB-T20, phosphate-citrate-buffer containing Tetramethylbenzidine was added and the reaction stopped after 5 min with 100 µl H_2SO_4. We measured the absorbance of the wells in the plates at 450 nm in a photometric microplate reader (Biotek; µQuant Microplate Spectrophotometer). A linear standard curve was calculated for each plate using the standard concentrations. IgG concentrations were then calculated based on the standard curve.

ELISA for serum amyloid A (SAA)

SAA concentrations (in ng/ml) were measured with a commercial solid phase sandwich SAA Multispecies ELISA kit (Tridelta, "PHASE"^{TM} RANGE; Multispecies SAA ELISA kit; Catalog N° TP-802) following the manufacturer instructions using 1:1,500 diluted plasma samples. SAA concentrations were calculated according to the standard curve on each plate. Thirty-four samples had a higher absorbance than measurable with the photometer (Biotek; µQuant Microplate Spectrophotometer). These samples were rerun with a higher dilution of 1:5,000. Eighteen samples still had a higher absorbance, therefore the entire plate was re-measured directly after discarding half of the volume in each well, after which absorbance could be measured for those 18 samples. In this case, all concentrations of the plate were

recalculated and compared to the previously calculated concentrations. The results were not different from the previously calculated concentrations (Wilcoxon signed-ranks test, $V = 660$, $p = 0.48$).

Bacterial killing assay (BKA)

We measured the *in vitro* bacterial killing capacity (BKA) of serum against *Escherichia coli* (ATCC #8739). The method is described in detail in (Heinrich *et al.* 2016). In short, serum samples were serially diluted in 96-well plates with PBS, resulting in 8 dilutions from 1:2 to 1:256. Each diluted sample was mixed with a bacterial working solution of approximately 1.5×10^5 colony-forming units/ml. After incubation for 30 min at 37°C, tryptic soy broth (Sigma Aldrich, Germany, Catalog N° 22092) was added, the background absorbance was measured and the plates were incubated again for 12 hours at 37°C. After the incubation, we measured the absorbance, and the bacterial killing capacity was calculated for each dilution of serum against a positive control. Ranks were assigned to each dilution before killing capacity dropped from 100% to 0%, such that dilution 1:2 corresponded to rank 1, dilution 1:4 to rank 2, all the way to the highest dilution of 1:256 corresponding to rank 8 (Heinrich *et al.* 2016). The higher the rank (and the higher the dilution of the serum), the higher the bacterial killing capacity.

Lysoplate assay

We measured the concentration of lysozyme (in µg/ml) using the lysoplate assay method (Osserman & Lawlor 1966). The method is fully described in (Heinrich *et al.* 2017). In short, we prepared 1% noble agar (Sigma Aldrich, Germany, Catalog N° A5431) plates that contained the lysozyme-sensitive bacteria *Micrococcus lysodeikticus* (Sigma Aldrich, Germany, Catalog N° M3770) at a pH of 6.3. Twenty-five holes were punched into the agar and filled with serum samples (18 holes) or standards (7 holes) with concentrations of 10.0 µg/ml, 7.5 µg/ml, 5.0 µg/ml, 2.5 µg/ml, 2.0 µg/ml, 1.25 µg/ml and 1.0 µg/ml, using lysozyme from chicken egg white (Sigma Aldrich, Germany, Catalog N° L6876). The diameter of the clear zone created by bacterial lysis after incubation is proportional to the logarithmic lysozyme concentration in the samples and standards (Osserman & Lawlor 1966). The measurements of the lysis standards were plotted as a linear function of the $\log_{10}$ lysozyme concentration. This regression line was used to infer the lysozyme concentrations of the samples. The range of lyzozyme concentration was between 0.35 µg/ml and 4.97 µg/ml.

Haemagglutination-haemolysis assay

We assessed the capacity of natural antibodies and complement by using the haemagglutination-haemolysis assay (Matson, Ricklefs & Klasing 2005). The method is described in detail in (Heinrich *et al.* 2017). In short, after plasma was serially diluted from 1:2 to 1:1024 with sterile PBS in a U-shaped 96-well plate, the same amount of 1% chicken red blood cell suspension was added to all wells and the plate was incubated at 37°C for 90 min. Haemagglutination and haemolysis, which reflect the activity of the natural antibodies and the interaction between natural antibodies and complement (Matson, Ricklefs & Klasing 2005; Pap *et al.* 2010), was recorded after 20 min and 90 min, respectively. The capacities of natural antibodies and complement were given as rank with the highest dilution of plasma showing full haemagglutination or haemolysis, respectively, such that dilution 1:2 corresponded to rank 1, dilution 1:4 rank 2, all the way to the highest dilution of 1:1024 corresponding rank 10 (Pap *et al.* 2010; Gilot-Fromont *et al.* 2012). .

Statistical analysis

Analysis of allostatic load ('stress')

We used Spearman's rank correlation coefficient to assess the correlation between cortisol concentration and N/L ratio. We fitted linear models for both allostatic load measures as a function of sex, age and captivity status (free-ranging or captive) and the interactions thereof. We used the transformation with the natural logarithm if the assumptions of linear models were not met by the original data set. For cortisol concentration, we excluded one data point from the data set which lay outside the calibration curve. The removal of this data point improved the model, but did not significantly alter the results.

Analysis of immunity

For each of the continuous immune responses – IgG concentration, SAA concentration and lysozyme concentration – we fitted a linear model as a function of cortisol concentration, N/L ratio, sex, age and captivity status and the interactions between the last three predictors. We used the transformation with the natural logarithm if the assumptions of linear models were not met by the original data set. To test the overall effect of both measures of allostatic load on each measure of immune response, we fitted a reduced linear model, predicting immune responses only as a function of sex, age and captivity status and their interactions. We then compared the two models using function anova in the R package car version 3.2.1 (Fox *et al.*

2009) to test whether the fit by the reduced model was significantly worse than the fit by the full model.

For the ordinal immune response of the bacterial killing capacity, we fitted a logistic regression model predicting the probability of the variable to be in the range of values including the median or higher as a function of cortisol concentration, the natural logarithm of the N/L ratio, sex, age and captivity status. For bacterial killing capacity the median rank was 5.5, thus the lower value range encompassed the dilutions 1:2 to 1:16, equivalent to ranks 1 to 4 (if there was no bacterial killing at dilution 1:2 the corresponding rank was 0), and the higher value range encompassed the dilutions 1:32 and 1:128, equivalent to ranks 5 to 7. For haemagglutination and haemolysis capacities, sample sizes of captive animals were too small to consider the effect of captivity in the models. We therefore fitted these models only for free-ranging animals and predicted haemagglutination and haemolysis capacities by cortisol concentration, the natural logarithm of the N/L ratio, sex and age. For the haemagglutination capacity, the median rank was 5, thus the lower value range encompassed the dilutions 1:2 to 1:16, equivalent to ranks 1 to 4 and the higher value range covered the dilutions 1:32 to 1: 128, equivalent to ranks 5 to 7. For the haemolysis capacity, the median rank was 4, thus the lower range value encompassed the dilutions 1:2 to 1:8, equivalent to ranks 1 to 3 and the higher value range covered the dilutions 1:16 to 1:64, equivalent to ranks 4 to 6.

To test the overall effect of both measures of allostatic load, we fitted a reduced logistic regression model, predicting immune responses as a function of only sex and age. We then compared the two models with a likelihood ratio test to check whether the fit by the reduced model was significantly worse than the fit by the full model that included the two measures of allostatic load. Sample sizes varied slightly for different immunological measurements because the bacterial killing assay and the hemagglutination / hemolysis assay should be limited to samples which are thawed for the first time only, otherwise they become unreliable. Some samples had been previously thawed for other studies and been frozen again and therefore could not be used. Sample volumes of other samples were too small to provide material for all analyses. Therefore, we included information on sample size for each reported analysis.

All statistical analyses were performed using the open software R version 3.1.3 (R-Core-Team 2015). Package car version 2.0-25 (Fox *et al.* 2009) was used to test the significance of models, nortest version 3.2.2 (Gross & Ligges 2015) and lmtest version 3.2.2 (Zeileis & Hothorn 2002) were used to test the assumptions of linear models, and effects

version 3.2.2 (Fox 2003) was used to plot the figures. The significance threshold for all tests was fixed at 5 % and all tests were two-tailed. Data are presented as means ± S.D.

Ethics statement

Animal immobilizations and sample collections were authorized by the Ministry of Environment and Tourism (MET) of Namibia and complied with the laws of the country. Samples were transported in full compliance with the Convention on International Trade in Endangered Species (CITES). The methods applied and the study design were approved by the Internal Ethics Committee of the Leibniz Institute for Zoo and Wildlife Research (IZW, Berlin, Germany; permit #2002-04-01).

Results

Five of six immune parameters were significantly affected by life history parameters and stress. This applied to IgG (general linear model, $F_{8,145} = 8.896$, $p < 0.0001$), SAA (general linear model, $F_{8,119} = 2.447$, $p=0.017$), bacterial killing capacity (logistic regression, log-likelihood ratio test [LRT] = 29.80, df = 8, n = 173, p = 0.00023), lysozyme concentrations (general linear model, $F_{8,156} = 4.566$, $p < 0.0001$), and haemolysis capacity (logistic regression, LRT = 10.20, df = 4, n = 145, p = 0.037). Haemagglutination capacity was not significantly influenced by life history parameters and stress (logistic regression, LRT = 7.33, df = 4, n = 145, p = 0.12). An overview of the dataset used for the linear models and of the entire raw dataset is summarized in tables 2 and 3, respectively. The dataset of the former is smaller than the one of the latter because for some cheetahs not all life history parameter and stress measurements were available, a requirement that had to be fulfilled for the linear models. The dataset for cortisol concentration and N/L ratio is the same for table 3.

Effect of sex on immunity

Cheetah males had a significantly higher bacterial killing capacity (logistic regression, likelihood ratio test statistic [LR] = 6.42, df = 1, p = 0.011, Fig. 1) and a significantly higher lysozyme concentration ($F_{1, 158} = 4.49$, $p = 0.036$) than females. There were no differences between males and females in IgG concentration ($F_{1, 152} = 0.82$, $p = 0.37$), SAA concentration ($F_{1, 119} = 2.40$, $p = 0.12$) and haemolysis capacity (LR = 1.37, df = 1, p = 0.24). Additionally, there was no interaction between sex and captivity status for any of the immune measures.

Effect of age on immunity

IgG concentration significantly increased with age ($F_{1,152}$ = 15.24, p < 0.001, Fig. 2). The effect was significantly stronger for free-ranging cheetahs than captive ones (interaction between age and captivity status, $F_{1,152}$ = 7.19, p = 0.008): in captive cheetahs, younger animals already had a high IgG concentration which increased with age at only a shallow slope whereas in free-ranging cheetahs the increase in IgG concentration from younger to older animals increased substantially (Fig. 2). Older animals were also significantly less likely to have a high haemolysis capacity (LR = 4.75, df = 1, p = 0.029). Age did not affect SAA concentration ($F_{1, 119}$ = 1.38, p = 0.24), bacterial killing capacity (LR = 0.91, df = 1, p = 0.34) or lysozyme concentration ($F_{1, 158}$ = 1.43, p = 0.23).

Effect of captivity status on immunity

Cheetahs in captivity had significantly higher IgG concentration than free-ranging individuals ($F_{1,152}$ = 4.65, p = 0.033). Free-ranging cheetahs had significantly higher SAA concentrations ($F_{1,119}$ = 8.23, p = 0.005) and a significantly higher bacterial killing capacity than captive animals (LR = 5.71, df = 1, p = 0.017, Fig. 1). For lysozyme concentration there was a non-significant trend with free-ranging cheetahs showing higher lysozyme concentrations than captive ones ($F_{1, 158}$ = 3.43, p = 0.066).

Effect of allostatic load on immunity

The six measures of the immune response were modeled with (full model) and without (reduced model) the two measures of allostatic load, serum cortisol concentration and N/L ratio, in order to gauge the impact of allostatic load on measures of the immune response. The comparison of the full and the reduced models revealed that allostatic load in terms of the combined measure of cortisol concentration and N/L ratio significantly decreased IgG concentration ($F_{2,152}$ = 3.21, p = 0.043), significantly increased lysozyme concentration ($F_{2,158}$ = 9.38, p < 0.001) and significantly affected haemagglutination capacity ($LR_{2, 145}$ = 6.49, p = 0.039). An increase in serum cortisol concentration was associated with a significant increase in haemagglutination capacity, whereas an increase in the N/L ratio was associated with a non-significant trend of decrease in haemagglutination capacity. There was a non-significant trend for allostatic load to affect haemolysis capacity ($LR_{2, 145}$ = 5.61, p = 0.06). Here, an increase in serum cortisol concentration was also associated with an increase in haemolysis capacity, whereas an increase in the N/L ratio was associated with a significant decrease in

haemolysis capacity. The natural logarithm of SAA concentration ($F_{2,119}$ = 2.12, p = 0.124) and bacterial killing capacity ($LR_{2, 173}$ = 3.07, p = 0.215) were not affected by allostatic load.

Effect of sex, age and captivity status on allostatic load

Cortisol concentrations and N/L ratios were significantly positively correlated (Spearman's rank correlation ρ = 0.35, *n* = 195, p < 0.001). Cortisol concentrations ($F_{6,232}$ = 33.04, p < 0.0001) and N/L ratios ($F_{6,230}$ = 10.13, p < 0.0001) were significantly affected by sex, age and captivity status and their interactions.

Free-ranging cheetahs had significantly higher ($F_{1,232}$ = 167.73, p < 0.00001) cortisol concentrations than captive ones. Also, females had significantly higher ($F_{1,232}$ = 4.50, p = 0.035) cortisol concentrations than males, although the difference between the sexes was small. Age ($F_{1,232}$ = 0.79, p = 0.37), the interaction of age with sex ($F_{1,232}$ = 0.16, p = 0.69), the interaction of age with captivity status ($F_{1,232}$ = 0.05, p = 0.82) and the interaction of sex with captivity status ($F_{1,232}$ = 0.13, p = 0.72) were not significant.

Free-ranging cheetahs also had significantly higher N/L ratios ($F_{1,230}$ = 28.19, p < 0.00001) than captive ones. N/L ratio significantly increased with age ($F_{1,216}$ = 28.61, p < 0.00001), and significantly more strongly in free-ranging animals (interaction between age and captivity status, $F_{1,230}$ = 9.10, p = 0.0029). Sex ($F_{1,230}$ = 2.64, p = 0.11) and the interactions of sex and age ($F_{1,230}$ = 0.39, p = 0.53) and of sex and captivity ($F_{1,230}$ = 1.35, p = 0.25) had no effect. An effect plot of the models for cortisol concentration and N/L ratio by captivity status and age is presented in Fig 3.

Discussion

The immune system is a complex defense system against pathogens which may have a strong effect on the survival of an individual. The investment of an individual into the different immune branches changes during life and may depend on age, sex (Lee 2006) and reproductive condition (e.g. East *et al.* 2015). Here we examined the humoral part of the adaptive and innate immune system of the cheetah, a species suggested to be impaired in its immune response because of its relatively low genetic variability at the MHC. This study demonstrates that cheetahs vary their investment in different immune branches with sex, life history stage as indicated by age, access to resources, pathogen exposure and/or vaccinations. We also demonstrate that the allostatic load caused by capture and handling affected some measures of the immune response, which should be taken into account when interpreting

measurements of immune responses in free-ranging and captive cheetahs, and possibly other species (Hofer & East 1998).

Effect of sex on immunity

As predicted, males were significantly more likely to have a higher bacterial killing capacity and had a significantly higher lysozyme concentration than females. Thus, they invested more in the overall constitutive innate immune system than females. This difference is probably mediated by sex steroid hormones (Olsen & Kovacs 1996), especially by testosterone (Muehlenbein & Bribiescas 2005). According to the immunocompetence-handicap-hypothesis, testosterone is acting in opposing ways by promoting reproduction-relevant traits, which enhance reproductive success, at the cost of immunocompetence, which may reduce their health status as a consequence (Folstad & Karter 1992). Thus, several studies suggested that males have a general lower immunity than females (Klein 2000). However, studies that tested predictions of this hypothesis produced contradictory results, particularly when considering other factors such as season, energy budget or the specific measure of the immune response (Deerenberg & Arpanius 1997; Pap *et al.* 2010; Ruiz *et al.* 2010). A meta-analysis found little support for a general suppression of immunocompetence in males (Roberts, Buchanan & Evans 2004). It seems more likely that testosterone suppresses some parts of the immune system while it enhances others (Hasselquist *et al.* 1999; Ezenwa, Stefan Ekernas & Creel 2012). A stronger innate immunity, also important for local inflammation and for wound healing, is expected in males because they regularly fight over territories (Caro, Fitzgibbon & Holt 1989).

We expected cheetah females to show higher concentration of IgG than males because the maintenance costs of this adaptive immune component are relatively low once it has been established during ontogeny, but this was not the case. Females show lower constitutive innate immunity in terms of bacterial killing capacity and lysozyme concentration than males. Female cheetahs roam alone or with their offspring, avoid encounters with other carnivores and do not scavenge food, thereby minimizing their chances of encountering pathogens (Caro 1994; Altizer *et al.* 2003; Vitone, Altizer & Nunn 2004). In comparison to males, they do not engage in fights over territories, minimizing their chances of injury. Moreover, pregnant and lactating females and females with dependent offspring invest a substantial amount of energy into their offspring and might have few reserves to invest in their innate immunity during these periods (Festa-Bianchet 1989; Coop & Kyriazakis 1999; Beechler *et al.* 2012). However, there was no interaction between sex and captivity status in any of the immune

measures. Thus, regular access to food resources in captivity did not change the immune investment allocation of cheetah females. However, our sample size for free-ranging females, which were reproductively active at the time of capture, was small; only four females were pregnant, two females were lactating and 15 females were accompanied by their offspring.

Effect of age on immunity

As predicted, IgG concentrations of younger cheetahs were lower than those of older individuals. This is an indication for a functioning adaptive immune response despite the low MHC diversity of cheetahs (Castro-Prieto, Wachter & Sommer 2011). Whether the amount and diversity of antibodies accumulated through life is lower than for species with higher MHC diversity has yet to be tested, but a recent comparison with leopards suggests that this might be the case (Heinrich *et al.* 2017). We suspect that the increase in IgG concentration with age goes hand in hand with the development of a repertoire of antibodies against encountered antigens. This is consistent with previous studies which demonstrated that free-ranging cheetahs do have antibodies against the most common feline diseases (Munson *et al.* 2004; Thalwitzer *et al.* 2010).

In parallel with the age-related increase in IgG concentration there was a decrease in haemolysis capacity. This suggests that as the adaptive immune system becomes stronger with increasing age (here higher IgG concentrations), some parts of the innate immune system (here the complement) are reduced. This is most probably a consequence of differential resource allocation because the costs and benefits of different immune branches may change with age (Nebel *et al.* 2013). Similar results were obtained in other vertebrates such as barn swallows (*Hirundo rustica*) (Møller & Haussy 2007), tree swallows (*Tachicineta bicolor*) (Palacios *et al.* 2007) and snakes (Ujvari & Madsen 2011), indicating that immunosenescence does not affect the different immune branches in the same way.

As it is the function of the innate immune system to protect the body in a generic and immediate way, the functioning of the complete innate immune system may be a priority in younger cheetahs. If so, they might prioritise investment into this immune part despite the demands of other energetically costly processes such as growth. In adult cheetahs these resources may be allocated to reproduction. This immune investment strategy might provide higher fitness when adaptive immunity is reduced by relatively low MHC diversity.

Effect of captive status on immunity

Captive cheetahs in large enclosures in Namibian farmland with regular access to food resources and no reproductive burden did not have an overall stronger immunity than free-ranging cheetahs, but a stronger adaptive immunity. As predicted, captive cheetahs exhibited higher IgG concentrations than free-ranging ones, which is likely a consequence of high energetic resources, vaccinations and/or higher pathogen encounter rates in the captive environment. This differs from spotted hyenas where captive individuals had lower IgG concentrations than free-ranging ones (Flies *et al.* 2015). However, the captive spotted hyenas were born and housed in a facility with an artificial environment in California, USA, which was regularly cleaned and disinfected to minimize the exposure of the animals to pathogens. This was very different for the captive cheetahs in our study, which lived in large enclosures in unchanged, natural habitats in a range country. Furthermore, free-ranging cheetahs had a higher bacterial killing capacity than captive cheetahs, a difference not observed in spotted hyenas (Flies *et al.* 2015). This implies again that trade-offs in immune responses exist, i.e. when one part of the immunity is emphasized, another part may be reduced (Adamo 2004; Matson *et al.* 2006). Because of the vaccinations and/or higher pathogen exposure, captive animals might have to invest more energetic resources in adaptive immunity and thus invest less in constitutive innate immunity.

Moreover, young captive cheetahs had higher IgG concentrations than free-ranging ones. Also here, this result is likely a consequence of high energetic resources in captivity and vaccinations. Thus, young captive cheetahs might not have to trade-off their energy budget between growth and immunity, which provides them with the resources to develop a strong adaptive immune response earlier than free-ranging individuals. The high IgG concentration of captive animals is also an indication of a functioning adaptive immune system in this species with relatively low genetic variability.

We also measured higher serum amyloid A (SAA) concentrations in free-ranging cheetahs than in captive ones. This is unexpected because the high incidence of amyloidosis in captive cheetahs, at least in zoos, suggests the contrary (Papendick *et al.* 1997; Zhang *et al.* 2008; Serizawa, Chambers & Une 2011; Depauw *et al.* 2014). There are at least two, non-exclusive explanations for this result, both related to ‘stress’-induced effects (see below for further discussion). Firstly, the difference could be a consequence of the different timing (time difference between entering the trap and darting in free-ranging animals vs. direct darting in captive animals) of immobilization and thus uneven ‘stress’-related secretion of SAA between free-ranging and captive cheetahs. Alternatively, it is possible that constant secretion of SAA

and the increase in the baseline SAA levels in cheetahs in captivity has reduced the ability of captive animals to mount a strong reaction to a stressor through overstimulation of the system, as has been previously suggested (Romero, Dickens & Cyr 2009; Romero 2012).

Effect of allostatic load on immunity

We assessed acute allostatic load by serum cortisol concentrations (Sapolsky 2002) and N/L ratio in blood smears (Davis, Maney & Maerz 2008). This ratio is a second indicator for acute increases in allostatic load because glucocorticoids increase the number of neutrophils and decrease the number of lymphocytes in the blood (Davis, Maney & Maerz 2008). We expected these two indices of allostatic load to be highly correlated and associated with the measures of immune response in a similar way.

An increase in acute allostatic load ('stress') has a broad effect on the immune system which is mostly inhibiting, but sometimes also enhancing immune responses (Romero & Butler 2007). Increases of glucocorticoid concentrations generally start after 3 to 5 min of the acute stressor and negative feedback will start reducing glucocorticoid concentrations after 30 to 60 min (Romero & Butler 2007). Thus, baseline levels of glucocorticoids can only be measured directly during the first 3 min after capture (Romero & Reed 2005). In our study, the blood samples were collected between 20 and 35 min after the cheetahs were darted for immobilization, thus at approximately the peak serum glucocorticoid levels.

Glucocorticoids interact with intracellular receptors and initiate gene transcription, which has a delayed but sustained effect on the body (Romero & Reed 2005; Romero & Butler 2007). Glucocorticoids bind to glucocorticoid receptors and either initiate the production of new proteins or inhibit protein production (Buttgereit & Scheffold 2002; Romero & Butler 2007). Hours or days may pass before an effect on the cellular, tissue or organismic level can be measured (Buttgereit & Scheffold 2002). Thus, because free-ranging individuals, in contrast to captive ones, spent several hours in the trap before they were darted, this confinement might have had consequences on their immune response. It is unlikely that free-ranging cheetahs had elevated glucocorticoid levels when being captured and triggered transcription processes that affected our measures of the immune response. This is because free-ranging and captive cheetahs in Namibia do not differ in their chronic allostatic load measured in terms of the size of adrenal glands (Wachter *et al.* 2011) nor in their acute allostatic load measured in terms of faecal glucocorticoid metabolites (Terio *et al.* 2003, 2004).Thus, the link between glucocorticoid concentrations measured after darting and

the observed immunosuppressive effects are likely to originate from the different handling procedures.

Of all six measures of the immune response, lysozyme concentration was the measure most substantially affected by acute allostatic load. Some studies consider lysozyme as an antibacterial enzyme and part of the constitutive innate immune response (Lee 2006), whereas others view it as an acute phase protein (Owen-Ashley & Wingfield 2007). Lysozyme is a plasma protein with the ability to digest the surface of gram-positive bacteria (Demers & Bayne 1997). In a previous study, lysozyme concentration increased after an acute stressor was applied, i.e. a handling stressor which lasted for 10 min (Demers & Bayne 1997). Our results are consistent with this finding; lysozyme can be considered as an acute phase protein in the cheetah, which may explain the strong association with increases in allostatic load and the tendency of free-ranging cheetahs to have higher lysozyme concentrations.

Interestingly, haemagglutination capacity increased but IgG concentrations declined as serum cortisol concentrations increased. Haemagglutination capacity measures the levels of natural antibodies which serve as recognition molecules capable of opsonizing, i.e. marking, invading microbes for elimination by phagocytes (Matson, Ricklefs & Klasing 2005). Most natural antibodies are pentameric immunoglobulin M (IgM) molecules, although some IgG also have opsonizing characteristics (Janeway *et al.* 2001). While there is an extensive literature on the effect of chronic levels of increased allostatic load on different antibody concentrations, previous studies in pigs and in birds did not find any effect of acute increases in allostatic load on antibody concentrations (Hicks *et al.* 1998; Matson, Ricklefs & Klasing 2005). Both immunoglobulin isotypes require days to weeks to be produced even during secondary infections (Tizard 2013) and the average half-life of IgG antibodies is 18 days in humans (Levy *et al.* 1970). Thus, our results on the effect of an acute increase in allostatic load on the antibody concentration are unexpected.

SAA concentration, bacterial killing capacity and haemolysis capacity were independent of our two measures of the allostatic load. Acute phase proteins, including SAA, are mainly produced by hepatocytes and triggered by different stimuli, including increases in allostatic load (Cray, Zaias & Altman 2009). The mechanisms of the stressor-induced increase of acute phase proteins are unknown. Catecholamines and glucocorticoids have been suggested as mediators, because an increase in adrenocorticotropic hormone (ACTH) does not influence the release of SAA (Van Der Kolk *et al.* 1992). Moreover, different stressors induce different signaling cascades (Alsemgeest *et al.* 1995; Saco *et al.* 2008), which is consistent with our finding on the lack of association between SAA concentration and our two measures

of allostatic load. Although in cheetahs both acute phase (SAA) and neuroendocrine stress effectors (cortisol) were elevated in free-ranging animals, different stressors could have contributed to their respective increases. In line with this argument, the differences in signaling cascades triggered by the various stressors could also explain the strong association between another acute phase protein, the lysozyme, and increases in allostatic load (see above).

Bacterial killing capacity was not influenced by the acute increase in allostatic load, similar to a recent study in bats, in which bacterial killing capacity was also not affected by such an acute increase (Strobel, Becker & Encarnação 2015) and a study in birds, in which bacterial killing capacity was also not affected when sampling took place up to 30 min after darting (Buehler *et al.* 2008). The bacterial killing capacity is an integrative measurement of constitutive innate immunity, and killing of *E. coli* is mainly complement dependent (Moore *et al.* 2011). In line with this, the haemolysis assay, representing the strength of the complement system (Matson, Ricklefs & Klasing 2005), was also not affected by the acute increase in allostatic load.

Effect of sex, age and captivity status on allostatic load

The correlation between both measures of allostatic load was highly significant but only moderate. A stronger correlation might have been expected because glucocorticoids increase the number of neutrophils and decrease the number of lymphocytes and thus should reliably indicate glucocorticoid levels (Davis, Maney & Maerz 2008). However, in several studies in birds heterophil (the equivalent to neutrophils in mammals) to lymphocyte ratio (H/L ratio) was not significantly correlated with corticosterone concentrations (Vleck *et al.* 2000; Ilmonen *et al.* 2003; Clinchy *et al.* 2004) and it has been proposed that these two measurements do not indicate the same type of stress (Müller, Jenni-Eiermann & Jenni 2011). In the mentioned study H/L ratio was a better indicator for environmental stress factors whereas corticosterone was more sensitive to internal factors such as fat reserves (Müller, Jenni-Eiermann & Jenni 2011). In any case, the N/L ratio is considered to be a reliable method to assess acute increases in allostatic load (Dhabhar 2002; Davis, Maney & Maerz 2008). It is inexpensive, easily done with minimal equipment and tiny samples (Davis, Maney & Maerz 2008) and can be integrated into most eco-immunological studies.

Female cheetahs had slightly higher cortisol concentration than male cheetahs. The former were often captured together with cubs. Additionally, female cheetahs rarely visit the marking tress at which they were captured and may feel threatened not only by humans but

also by the male cheetah(s) which hold the territory. Together these factors may have resulted in a higher allostatic load in female cheetahs than in males.

As predicted, free-ranging cheetahs exhibited higher levels of acute increases in allostatic load in cortisol concentrations and N/L ratios than captive ones, probably a consequence of the former being captured in traps for several hours and not being accustomed to people and handling. Older cheetahs had a stronger stress response in terms of N/L ratio but not cortisol concentrations than younger ones. It is difficult to distinguish between the perception of a stressor by an individual and the capacity of an individual to respond appropriately at the physiological level to this stressor (Dickens & Romero 2013). We therefore cannot conclude whether older cheetahs were more stressed or mounted a stronger stress response in terms of N/L ratio.

Conclusion

Cheetahs invest their energy resources differently into the branches of their immune system, depending on their sex, age and captivity status. Male cheetahs invest more in their innate immune system than females, whereas older and captive cheetahs invest more in their adaptive immune system than younger and free-ranging cheetahs, respectively. Handling influences some measures of the immune response, underlining the importance of assessing allostatic load when the immune system of free-ranging animals is evaluated. Contrary to previous ideas, our results demonstrate that cheetahs are not generally impaired in their immunocompetence because of their relatively low variability at the MHC and are likely to mount adequate immune responses. This is encouraging for planning cheetah conservation management or re-introduction programs.

Author Contributions

Conceived and designed the experiments: SKH, BW, GÁC. Collected the samples and data: SKH, BW. Performed the experiments: SKH, GÁC, MD. Analysed the data: SKH. Wrote the manuscript: SKH, BW, GÁC. Commented on the manuscript: HH, MD.

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Table 1: Immune parts with their suggested costs and functions, and the tests used for investigating them (modified from (Schmid-Hempel & Ebert 2003), (Klasing 2004) and (Lee 2006)).

Immune part	**Costs**			**Functions**		**Measures used in this study**
	Development	**Maintenance**	**Use**			
Adaptive immunity	high	low	low	Specific response to known pathogens		IgG concentration
Innate immunity Constitutive part	low	medium	low	First line of defense Opsonize, i.e. mark, neutralize and destroy pathogens Destroy infected host cells	}	Bacterial killing capacity Lysozyme concentration Haemagglutination titre Haemolysis titre
Innate immunity Induced part	low	low	high	Increased strength of many immunological processes Sequester nutrients from pathogens		acute phase protein serum amyloid A (SAA) concentration

Table 2 Results of measurements of six immune parameters and stress measurements of free-ranging and captive cheetahs in Namibia used for the linear models.

Groups	Statistic	IgG concentration [mg/ml]	SAA concentration [ng/ml]	Bacterial killing capacity [rank]	Lysozyme concentration [µg/ml]	Haemolysis capacity [rank]	Haem-agglutination capacity [rank]	Cortisol concentration [ng/ml]	Neutrophil / lymphocyte ratio [–]
all	median	36.8	66.5	5.5	2.2	4	5	12.0	5.1
	mean ± SD	38.7 ±11.6	181.7 ± 269.9	5.2 ± 1.3	2.3 ± 0.7	3.7 ± 1.3	4.0 ± 1.8	16.4 ± 17.5	8.3 ±9.9
	N	161	128	182	167	139	139	240	237
male	median	37.9	58.5	5.5	2.4	4	5	11.6	5.6
	mean ± SD	39.3 ± 12.2	164.8 ±248.3	5.3 ±1.3	2.4 ± 0.7	3.8 ±1.2	4.5 ±1.2	15.8 ± 16.9	9.0 ±10.5
	N	118	98	133	121	114	114	173	167
female	median	35.0	99.6	5	2.1	4	5	13.5	4.3
	mean ± SD	37.1 ±9.5	236.9 ±329.7	5.1 ±1.4	2.1 ±0.6	3.5 ±1.5	4.2 ±1.6	17.9 ± 19.0	6.7 ±8.2
	N	43	30	49	46	36	36	66	70
free-ranging	median	35.5	77.9	5.5	2.32	4	5	14.4	6.1
	mean ± SD	36.4 ± 10.9	191.6 ± 274.4	5.2 ±1.4	2.4 ±0.7	3.7 ±1.3	4.46 ±1.3	19.4 ± 18.2	9.2 ± 10.7
	N	133	121	157	144	150	150	192	194
captive	median	48.7	2.7	5	1.86	-	-	2.5	3.9
	mean ± SD	49.6 ± 8.2	10.8 ± 20.7	5.3 ±0.5	1.87 ±0.52	-	-	4.3 ± 5.3	4.5 ± 3.4
	n	28	7	25	23	-	-	47	43

Table 3 Results of measurements of six immune parameters of all free-ranging and captive cheetahs in Namibia.

Groups	Statistic	IgG concentration [mg/ml]	SAA concentration [ng/ml]	Bacterial killing capacity [rank]	Lysozyme concentration [µg/ml]	Haemolysis capacity [rank]	Haem-agglutination capacity [rank]
all	median	37.4	45.6	5.5	2.2	4	4
	mean ± SD	38.6 ± 11.4	170.9 ± 280.0	5.3 ± 1.2	2.3 ± 0.7	3.2 ± 1.6	4.0 ± 1.7
	N	274	208	257	248	273	273
male	median	39.1	49.4	6	2.3	4	5
	mean ± SD	39.7 ± 12.1	162.1 ± 260.0	5.4 ± 1.1	2.3 ± 0.7	3.4 ± 1.6	4.1 ± 1.6
	N	182	144	181	175	195	195
female	median	34.6	33.4	5.5	2.1	4	4
	mean ± SD	36.2 ± 9.2	190.8 ± 321.8	5.1 ± 1.2	2.1 ± 0.6	3.1 ± 1.8	3.75 ± 1.9
	N	82	64	76	73	78	78
free-ranging	median	35.1	74.4	5.5	2.2	4	5
	mean ± SD	35.9 ± 10.8	197.3 ± 288.3	5.3 ± 1.2	2.3 ± 0.7	3.8 ± 1.3	4.5 ± 1.3
	N	192	170	213	197	191	191
captive	median	46.0	4.3	5	1.9	-	-
	mean ± SD	45.9 ± 9.5	53.0 ± 203.1	5.4 ± 0.5	2.0 ± 0.5	-	-
	N	72	38	44	51	-	-

Figure legends

Figure 1: Effect plot of a logistic regression model predicting BKA rank to be higher than the rank of 5 by sex and captivity status.

Figure 2: Effect plot of a linear model predicting IgG concentration by captivity status and the natural logarithm of age.

Figure 3: Effect plot of the linear model predicting (a) the natural logarithm of cortisol concentration, (b) the natural logarithm of neutrophil to lymphocyte (N/L) ratio by age and captivity status.

Figure 1

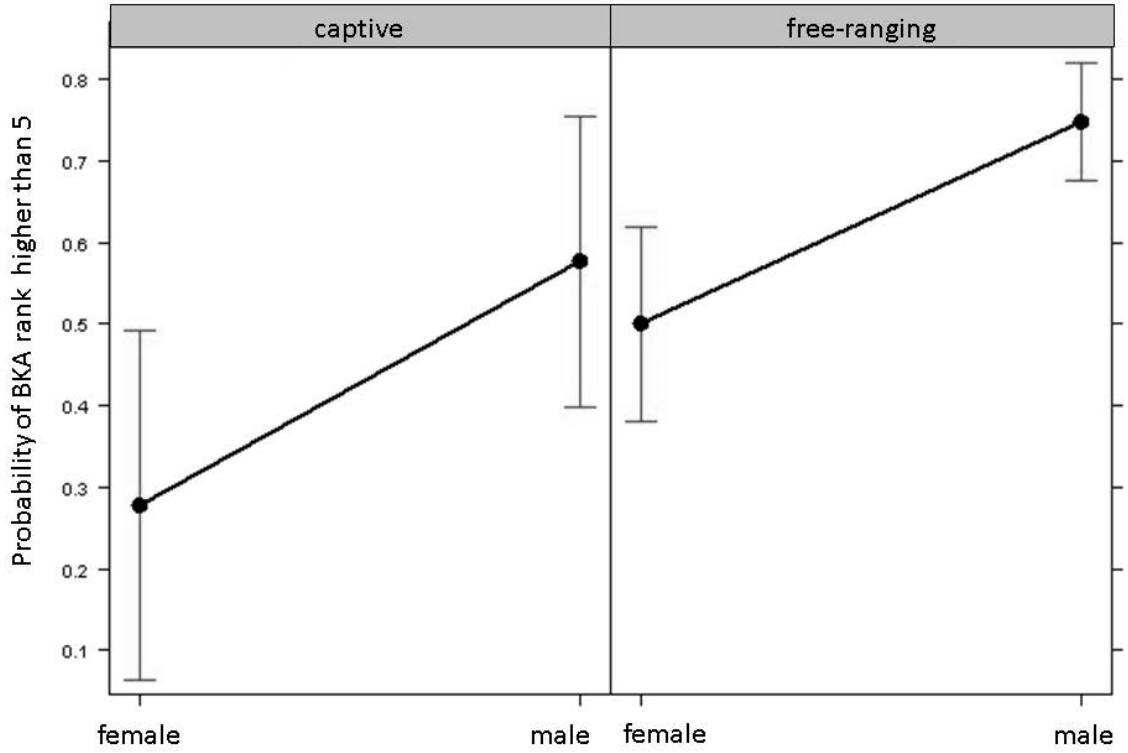

captive
free-ranging
Probability of BKA rank higher than 5
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
female
male
female
male

Figure 2

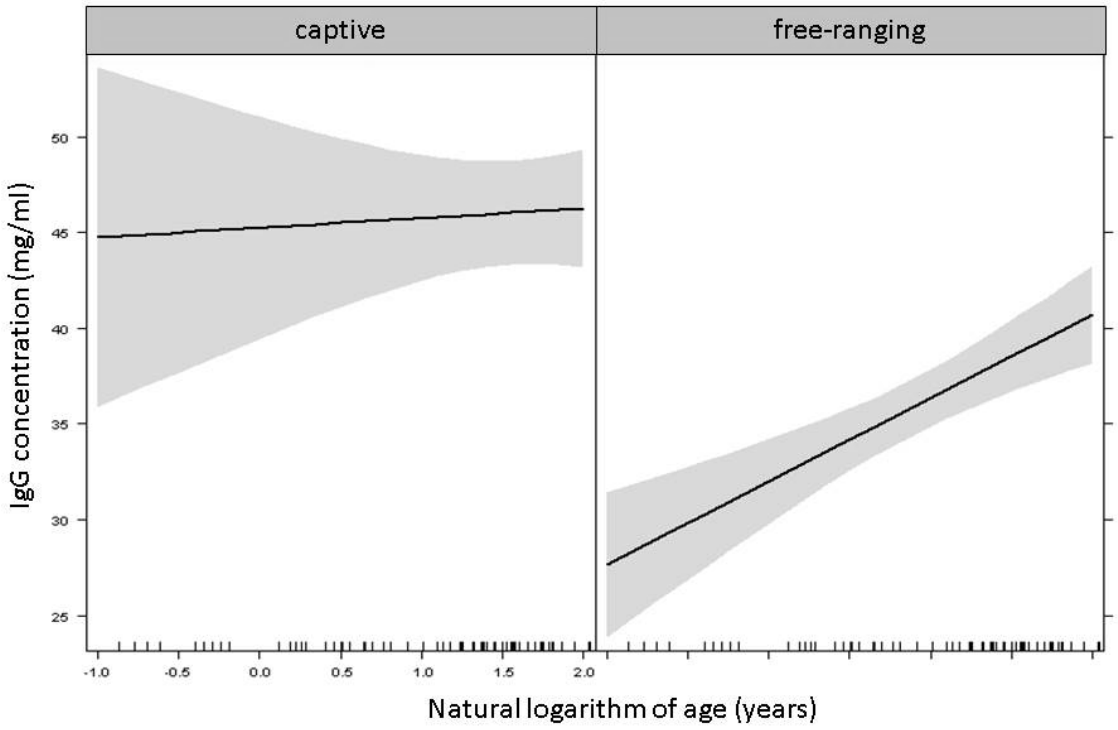

captive
free-ranging
IgG concentration (mg/ml)
50
45
40
35
30
25
-1.0
-0.5
0.0
0.5
1.0
1.5
2.0
Natural logarithm of age (years)

Figure 3a

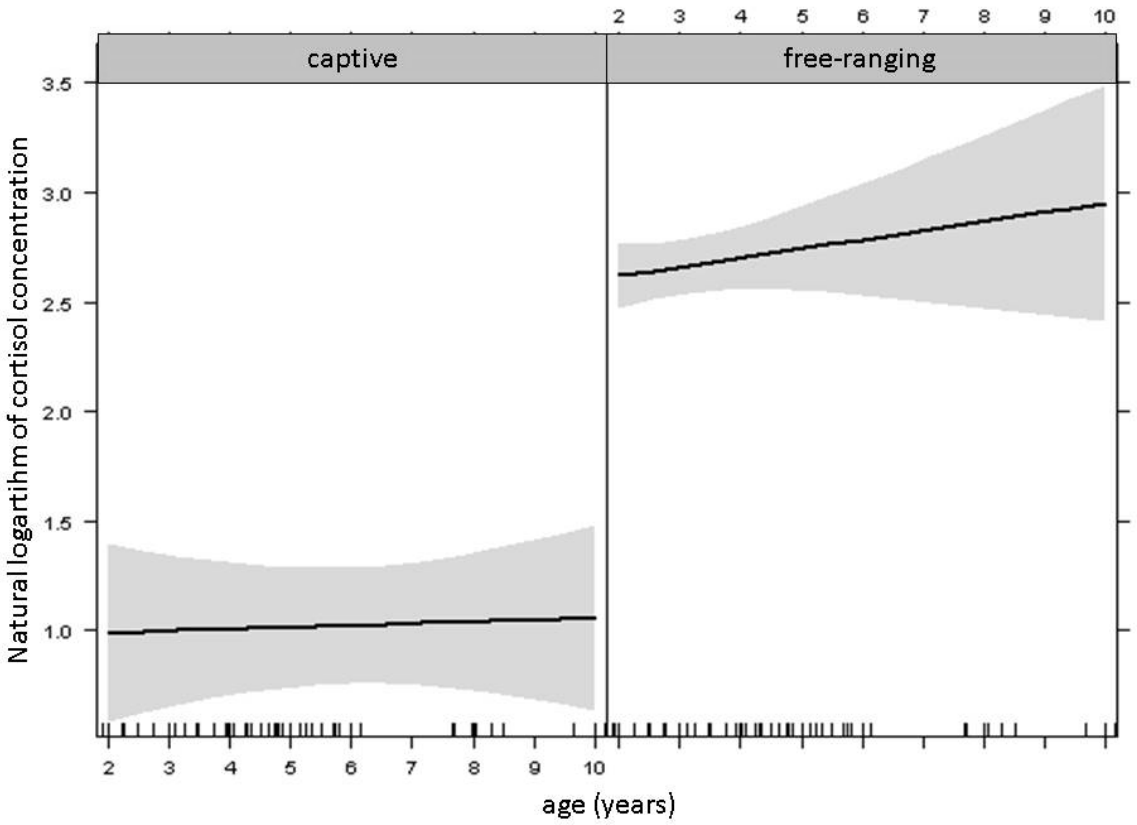
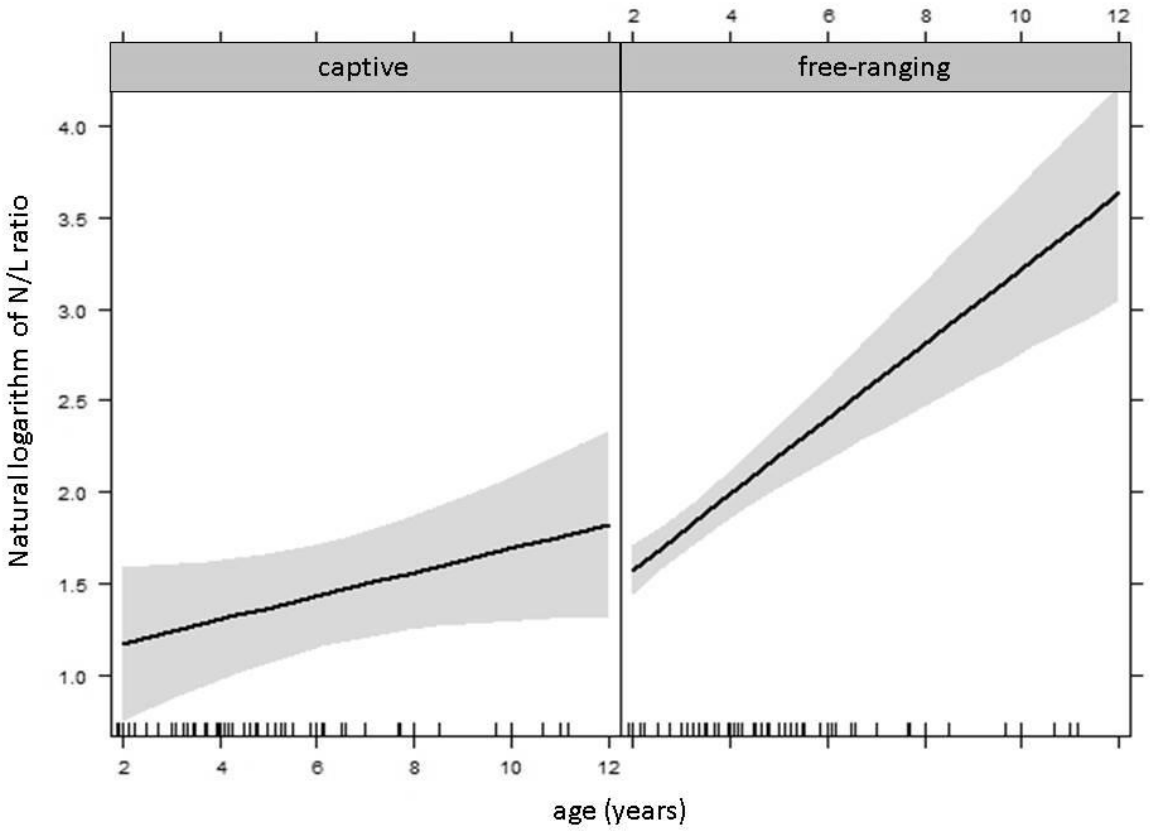

captive
free-ranging
Natural logartihm of cortisol concentration
3.5
3.0
2.5
2.0
1.5
1.0
2
3
4
5
6
7
8
9
10
age (years)

Figure 3b


captive
free-ranging
Natural logarithm of N/L ratio
4.0
3.5
3.0
2.5
2.0
1.5
1.0
2
4
6
8
10
12
age (years)

CHAPTER 5 – GENERAL DISCUSSION

The general purpose of this thesis was to improve our understanding of the immune responses of the cheetah, a species whose immune system might be highly compromised by low genetic diversity. More specifically, I investigated whether the apparent low MHC diversity necessarily leads to impaired immune responses and whether different parts of immunity may compensate each other. If functional compensation is possible, cheetahs might invest in those parts of the immune system which provide the highest fitness returns for a given life history state. For this purpose I measured several aspects of the innate immune response of cheetahs and compared their immunocompetence in detail with that of another sympatric felid, the leopard, which has a higher genetic diversity than cheetahs, and for one measure, the bacterial killing assay, with a range of carnivore species from different carnivore families. I also compared the immune responses of male and female, juvenile and adult, as well as free-ranging and captive Namibian cheetahs, which are exposed to different pathogen pressures.

5.1 Cheetahs in comparison with other carnivore species

The first aim of my thesis was to investigate whether immune measurements of cheetahs differed significantly from those of other sympatric carnivore species suspected to have a higher genetic diversity.

In a comparison with five other carnivore species the constitutive innate immunity, as assessed by the bacterial killing assay (chapter 2), the cheetah, together with the caracal, exhibited the highest bacterial killing capacity. Their capacities and the ones of the other feliform species were an order of magnitude higher than the one of the investigated canid, the black backed jackal, and other vertebrates which had been previously examined (French and Neuman-Lee 2012).

Contrary to my expectation, the strength of constitutive innate immunity did not reflect pathogen transmission risk, assessed by social organisation, foraging behaviour or body mass. This is surprising because pathogens impose a strong selection pressure on their hosts by reducing host fitness (Lehmann 1993), which in turn should influence the strength of their immune responses. Epidemiological theory predicts that disease risk increases with group size as a result of more opportunities for transmission (Anderson and May 1979). This is confirmed by a meta-analysis that revealed that both the intensity and the prevalence of

parasitism increases with the degree of sociality (Côté and Poulin 1995). This was reflected in the immune system in birds, where highly colonial species of swallows and martins (family Hirundinidae) had higher levels of T-cell and B-cell responses than solitary species, suggesting a higher investment in cell-mediated immunity in social species (Møller et al. 2001). Foraging behaviour should also impose selection pressures on immune functions, particularly in scavengers. Not only do scavengers take up microbes and toxins that colonize carrion (Janzen 1977), but carrion is also expected to host more virulent pathogens (Janzen 1977, Bull 1994, Blount et al. 2003). Body mass is expected to correlate with encounter rates of pathogens because larger bodies need more food and thus are likely to ingest more pathogens (Vitone et al. 2004). Additionally, larger bodies present more niches for colonization (Kuris et al. 1980), roam larger areas (Lindstedt et al. 1986) and have longer lifespans, which increases the diversity of encountered pathogens (Pacala and Dobson 1988). In a comparative study of primates, larger bodied species harboured a greater diversity of helminths and nematodes (Vitone et al. 2004). This was confirmed in comparative studies of the immune system of rodents (Tian et al. 2015) and bats (Schneeberger et al. 2013). In both studies, a higher body mass was positively correlated with higher white blood cell counts. However, there was no significant effect of body mass on bacterial killing capacity in the bat study (Schneeberger et al. 2013). Such differences in association with two immune measurements probably reflect the variability of the immune system, in this case cellular versus humoral immunity.

Because the immune system consists of different parts, which are connected and work together in a complex arrangement, it has been suggested that the different parts might compensate each other (Norris and Evans 2000). The bacterial killing capacity represents the strength of constitutive innate immunity. In comparison with adaptive immunity, innate immunity is the evolutionarily “older” part of the immune system (Janeway et al. 2001), and might thus be more related to the phylogeny of the species. Thus, I assessed whether the strength of constitutive innate immunity was related to the phylogenetic relatedness of the species. I found that phylogeny was a good predictor of the strength of constitutive innate immunity. I suggest that innate immunity might be more influenced by phylogeny, whereas adaptive immunity might be more influenced by ecological factors such as social organization, foraging behaviour or body mass.

This study was a first indicator that cheetahs may focus more of their immune investment on the constitutive innate immunity than other species and that their immunity was not generally impaired.

In chapter 3 I demonstrated that cheetahs had a stronger constitutive innate immunity but a weaker adaptive immunity and induced innate immunity than leopards. I suggested a shift to constitutive innate immunity in cheetahs in comparison to leopards. This could be explained by at least two scenarios.

On the one hand the relatively low genetic diversity of cheetahs could be a species characteristic and throughout their evolutionary history cheetahs invested more in constitutive innate immunity. Thus it may be a genetic adaptation. Similarly, a study on immune systems of continental and insular birds revealed an increase in innate induced immunity in insular compared to continental birds, implying a reorganization of immune function in insular birds (Matson 2006). Insular populations are expected to exhibit limited immune system function because of the suspected attenuation in pathogen pressure on islands (Frankham 1997). Thus, reduced benefits of immunologically relevant genetic diversity might lead to the loss of such genetic diversity through drift (Matson 2006), an effect generally known amongst island populations and not limited to the genetic diversity of the immune system (Frankham 1997, Frankham 1998). This first scenario is supported by a recent study on the phylogeography and divergence time of cheetah populations (Charruau et al. 2011), which did not support the widely circulated idea of a bottleneck (O'Brien et al. 1987) in the southern, northern and East African cheetah populations.

On the other hand such a shift to innate immunity may have happened by means of an up-regulation of innate immunity to compensate for a lack of adaptive defence. An up-regulation may be necessary if cheetahs lost a previously higher genetic diversity, including such diversity at the MHC, through demographic bottlenecks as has been previously suggested (O'Brien et al. 1987). Variation at the MHC is thought to reflect the ability to recognize a wide variety of intracellular (MHC class I) and extracellular (MHC class II) pathogens (Bernatchez and Landry 2003). Low variation at the MHC is therefore associated with an impaired ability of the adaptive immune system to recognize and thus defend against an invasion by pathogens (Sommer 2005). In genetically engineered mice lacking this form of adaptive immunity, an up-regulation of innate immunity was documented (Råberg et al. 2002).

It is widely acknowledged that the innate and adaptive immune system are intimately entwined, interacting with and complementing each other (Janeway et al. 2001). The innate immune system is the evolutionarily older part of immunity and forms a first line of defence, triggering and guiding the slower adaptive immune response (Fearon and Locksley 1996). An optimality approach to the evolution of the adaptive immune system must take into consideration that it needed to take over some of the roles of innate defence to be energetically affordable (Råberg et al. 2002). This is supported by the finding of negative genetic correlations between the two parts (Cheng et al. 1991). Thus, it has been suggested that a strong adaptive immunity may impede the evolution of innate resistance traits (Harding et al. 2005). Conversely, a very strong innate immunity may reduce selection pressure to improve adaptive immunity. A reliance on high constitutive levels of immunity might be beneficial for the cheetah because it provides the most rapid and effective defence any immune branch can muster (Klasing 2004). Adaptive immune defences are particularly useful in case of repeated infections with the same pathogen (Lee 2006). An investment in this part of immunity should therefore be more advantageous for social species than for mainly solitary species such as the cheetah.

Vertebrates may have evolved the adaptive immune system because the combination of innate and adaptive defenses confers better protection against infectious organisms than innate defense alone. However, both parts have their own inherent costs which have to be traded off against each other (Cheng et al. 1991, Råberg et al. 2002), as well as against other life history traits (Nordling et al. 1998, Lee 2006, Stahlschmidt et al. 2013). The costs of adaptive immune responses involve high developmental costs generated by complex and time consuming lymphocyte diversification processes (Butler et al. 2006, Lee 2006). These processes are mostly restricted to the ontogeny of the animal and require a substantial investment of energy and nutrients (Klasing and Leshchinsky 1999).

It is thus possible that for cheetahs the benefits of an immunologically relevant high genetic diversity do not outweigh the costs. Reasons could be that they reduce contact with pathogens by avoiding to feed on carcasses, that cheetahs live at low densities or that cheetahs avoid contact with other carnivore species (Caro 1994). A previous study on seroprevalence and sources of transmission for feline viruses found very low prevalence for all viruses and suggested that intraspecific contacts are not frequent enough to facilitate viral transmission (Thalwitzer et al. 2010). Environments with low levels of pathogen abundance likely produce

relatively low selection pressures for maintaining polymorphisms at MHC compared with environments with high rates of infectious disease (Miller et al. 2001).

The comparison with the leopard confirmed previous findings in that the immune system of the cheetah demonstrates a higher investment in constitutive innate immunity, and a lower investment in other parts. This does not confirm the previously held believe that the immune system of cheetahs is generally impaired.

Further support that cheetahs have a well functioning immune system comes from two personal observations in the field in Namibia (unpublished data). In both cases the cheetahs exhibited extreme wound healing. One case was a thin adult female cheetah of 46 kg that was killed by a farmer. She had fleas on her ears, but apart from that she was in a good condition. Necropsy revealed that she was heavily infested with tape worms (*Cestoda*). As a consequence, her jejunum had a hole where the infestation with worms was strongest. The hole was surrounded by a lot of fibrine. This implies that the body had already started repairing the jejunum and may have even been successful if the farmer had not killed the cheetah. The other case was a young captive male cheetah of approximately 2 years of age that had been attacked by baboons (*Papio ursinus*) which had entered his enclosure. The baboons heavily bit him on the body and inflicted deep wounds on his head. They bit off his scalp and removed part of the scull. This cheetah was treated by a local veterinarian and survived without persistent damages. Both cases demonstrate a good capacity for wound healing in cheetahs, which is consistent with the suggested competence immunity.

5.2 Trade-offs in the immune system of cheetahs

Protection against pathogenic challenges requires an investment in an immune system that responds to all potential pathogens as specifically and as fast as possible (Klasing and Leshchinsky 1999). However, the maintenance, activation and operation of the immune system entails costs which prevents any organism to maximise immune responses at all times. Thus, these costs have to be traded off against other life history traits such as reproduction and growth (Lochmiller and Deerenberg 2000, Lee 2006).

In chapter 4 I compared the immunity of male and female, juvenile and adult, as well as free-ranging and captive cheetahs. Innate immunity is the predominant effector mechanism of neonates and permits a fast response to novel pathogens (Klasing and Leshchinsky 1999). As

predicted, innate immune responses were similar for juvenile and adult cheetahs, and adaptive immunity increased with age. However, the latter effect was significantly stronger for free-ranging cheetahs than captive ones. In captive cheetahs, younger animals already had a high adaptive immunity, which only increased at a shallow slope. One reason for this might be the higher pathogen pressure all captive cheetahs are exposed to because of the close proximity of domestic cats and dogs on the associated farms, as well as the higher proximity of other cheetahs and/or their regular vaccinations. All these sources induce antibody production, which is part of the adaptive immunity, and which are likely to accumulate with age. However, the result could also originate from differences in energy budgets between free-ranging and captive cheetahs. Captive cheetahs have regular, predictable access to food and may thus have more resources available to invest into adaptive immunity already at an early age, whereas free-ranging cheetahs have to divide their limited energetic resources between developing adaptive immunity and other processes such as growth. The results indicate that the immune system of cheetahs was effective in its response to challenges and are consistent with previous studies which showed that cheetahs develop antibodies against antigens they encounter (Munson et al. 2004, Thalwitzer et al. 2010).

Male and female cheetahs have different life histories, which were partly reflected in differences in their immune responses. As predicted, males had a stronger constitutive innate immunity than females as expressed by higher bacterial killing capacity and a higher lysozyme concentration than females. A stronger innate immunity, which is important for local inflammation and wound healing, may benefit males more than females because males may suffer more injuries from regular fights over territorial ownership (Caro and Collins 1987). This result is also consistent with my prediction from the point of view of females who should invest less in innate immunity because it is energetically the more expensive part.

However, contrary to the expectation that females invest more in the energetically cheaper adaptive immunity, there was no difference in adaptive immunity between males and females as measured by immunoglobulin concentrations. There are several possible explanations for this result. Females, but also males, may not invest a significant amount of energy into this part of the immune system because its scope may be weakened by low MHC diversity. The energy budget of females may also be limited in that they save some energy by investing less in innate immunity, but do not have the resources to invest more in adaptive immunity than males. Sample size for adult female cheetahs was relatively small, reducing the power of finding a difference if there really was one between the sexes. Differences between the sexes

may also have been obscured if females change investment between different levels of reproductive status. The sample size for this analysis consisted of 33 adult females, of which five were in oestrus, four were pregnant, two were lactating, 15 were accompanied by their weaned cubs and for seven reproductive status was unknown. The statistical power to detect differences between females at different reproductive stages was therefore low.

5.3 Immunity and allostatic load (stress)

Cheetahs often do not cope well with captivity in the western world. This is explained by their special dietary requirements (Setchell et al. 1987, Munson 1993), restricted movements (Merola 1994), behavioural changes (Caro 1993) and immunosuppression caused by chronic stress (Terio et al. 2004). However, in a range state like Namibia, captive cheetahs live in large enclosures in their natural habitat. Previous examinations of adrenal gland sizes with ultrasonography as a proxy for chronic stress revealed no differences between free-ranging and captive Namibian cheetahs in adrenal gland sizes and thus in levels of chronic stress (Wachter et al. 2011). Furthermore, captive Namibian cheetahs live in close proximity to people and their domestic animals and therefore are more accustomed to people than free-ranging ones. Thus, acute stress caused by capture and handling is likely to influence immune parameters in free-ranging and captive cheetahs to different degrees. Therefore, I assessed levels of allostatic load as a possible confounding factor.

Glucocorticoid concentrations and the ratio of neutrophils to lymphocytes were lower in captive than free-ranging cheetahs, indicating that captive cheetahs were less stressed by capture and handling. Of the six immune parameters measured in this study three were associated with variations in allostatic load. As expected, the bacterial killing assay was not influenced by allostatic load. In the bacterial killing assay we used *E.coli* as bacterium, which is efficiently killed by the complement system. The strength of the complement system, as assessed by the haemolysis assay, was also not affected by allostatic load. Contrary to expectation, concentrations of the acute phase protein SAA were also not influenced by allostatic load. This is surprising because the production of acute phase proteins in the liver is triggered by different stimuli including increases in allostatic load (Cray et al. 2009). However, the exact mechanism and timing of the stressor induced increase in acute phase proteins are unknown and may differ between species.

Overall, my study demonstrates the importance of including a measurement for allostatic load in immunological studies, to be able to statistically control for this confounding factor.

5.4 Conclusion and perspectives

In this thesis I assessed the immunological phenotype of the cheetah with several immunological tests. I demonstrated that, contrary to widespread belief, the immune system of the cheetah is competent, not generally impaired, and strongest in its constitutive innate immunity. My results provide significant insights in the complexity of immune responses in non-domestic carnivore species. They underline that genetic studies of the immune system can provide valuable information but do not replace functional assessments of immune responses. Measurements of allostatic load are useful in combination with immunological studies, because stress is a possible confounding factor for some of the immune parameters. My study demonstrates that a comprehensive understanding of the immunity can be gained by combining the assessment of the immune system with its confounding factors.

My findings of the immune status of cheetahs could be extended by further assessing their immune responses with additional immunological tests, particularly functional tests. This study primarily measured humoral immunity. Cellular immunity is another important aspect of pathogen defence that needs to be assessed. Such a functional test is the lymphocyte proliferation assay (LPA) which measures the ability of lymphocytes to proliferate in response to a challenge by mitogens or anti-receptor antibodies (Ahmed et al. 1994). For LPAs, mitogens such as lipopolysaccharids can be used to artificially stimulate B-cells, concanavalin A or phytohaemagglutinin can be used to stimulate T-cells or pokeweed mitogen applied to stimulate B- and T-cells (Cunnick et al. 1994, Demas et al. 2011).

Additionally, the BKA can also be performed with fresh whole blood and different type of bacteria (e.g. *Staphylococcus aureus*) to induce and test also the cellular innate immune response (Tieleman et al. 2005, French and Neuman-Lee 2012). Under field conditions, both the LPA and BKA are challenging to perform because fresh blood is the best source of material to conduct the tests.

Investigations on trade-offs concerning energetic allocations into different immune branches would profit from a larger sample size of females than were available in this study. The sample sizes between non-breeding, pregnant and lactating females were too small to provide

any valuable results. Comparing reproducing and non-reproducing females, would be an important first step. Additionally, the data could be analysed from the perspectives of capital and income breeders. The concepts of these contrasting reproductive strategies have served as explanations for behavioural and physiological diversity (Jönsson 1997, Houston et al. 2007, Stephens et al. 2009), and might also explain immunological diversity in females. The two concepts are defined from the temporal distribution of resource acquisition relative to resource use. Capital breeders provide their offspring, from the foetal state to weaning and sometimes to independence, with energy accumulated and stored already before conception in fat depots or additional muscle mass, whereas income breeders provide offspring with energy resources gained concurrently and continuously (Houston et al. 2007). Cheetahs are most likely income breeders, because their slim body lacks large fat depots. Thus, they are likely to adjust their energy intake while they are pregnant or lactating. Their immunological set-up and investment in their immunity could be compared to a mixed breeder such as the spotted hyena (*Crocuta crocuta*) (Hofer et al. 2016) or a distinct capital breeder, such as the grey seal (Beck et al. 2003, Houston et al. 2007).

Overall, my study highlights the importance of an evolutionary approach to the immune system and I hope that it will encourage other studies to test analogous hypotheses in additional species.

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Die Immunantwort von Geparden (*Acinonyx jubatus)* aus evolutionärer, vergleichender und lebensgeschichtlicher Perspektive

ZUSAMMENFASSUNG

Geparde *(Acinonyx jubatus,* Familie: Felidae, Ordnung: Carnivora*)* waren einst weit verbreitet in Afrika und Asien, wurden aber in weiten Teilen ausgerottet. Als Hauptgefährdungsfaktoren gelten Verlust und Fragmentierung des Lebensraumes, Konkurrenz durch andere Karnivorenarten, Tötung aufgrund der Bedrohung von Nutztierbeständen und eine hohe Anfälligkeit für Krankheiten, die in Zusammenhang mit geringer genetischer Vielfalt steht. Geringe genetische Vielfalt kann Populationen angreifbarer für neue Pathogene machen, vor allem wenn die geringe genetische Vielfalt auch im Bereich der Immungene des Haupthistokompatibilitätskomplexes (MHC) auftritt. Geparde gelten als klassisches Beispiel für die negativen Auswirkungen genetischer Uniformität, welches in vielen Lehrbüchern über den genetischen Artenschutz sowie in zahlreichen wissenschaftlichen Veröffentlichungen genannt wird. Eine kürzlich veröffentlichte Studie bestätigte mit der *single-strand conformation polymorphismus analysis* frühere Studien und wies eine geringe genetische Vielfalt im Bereich von MHC Klasse I und Klasse II nach. Eine hohe Empfänglichkeit für Krankheiten wurde jedoch bisher nur für in menschlicher Obhut gehaltene Geparde nachgewiesen, wohingegen freilebende Geparde einen guten Gesundheitszustand aufweisen.

Es ist stark umstritten, ob die geringe Vielfalt im MHC ein Problem für die Arterhaltung des Gepards ist. Diese Dissertation wurde durchgeführt, um einen Einblick in das Immunsystem der Geparde zu gewinnen und betrachtet dessen Immunparameter sowohl im Vergleich zu denen anderer, im gleichen Gebiet lebender (sympatrischer) Karnivorenarten (**Kapitel 2 und 3**), als auch als Funktion verschiedener lebensgeschichtlicher Attribute und Lebensumstände (**Kapitel 4**). Die Arbeit ist eingebettet in ein Langzeit-Forschungsprojekt von Geparden in Zentralnamibia, in dessen Rahmen seit 2002 Blutproben von etwa 400 Geparden und anderen Karnivorenarten gesammelt wurden, die für diese Arbeit zur Verfügung standen.

In **Kapitel 2** vergleiche ich den konstitutiven angeborenen Teil des Immunsystems zwischen sechs sympatrischen afrikanischen Karnivorenarten mit einem funktionalen Test, dem

bacterial killing assay (BKA). Um verschiedene Arten vergleichen zu können, adaptierte ich zunächst das gängige Protokoll des BKAs so, dass die gleiche Bakterienkonzentration und gleichen Plasmaverdünnungen für alle Arten verwendet werden konnten. Es zeigte sich, dass die konstitutive angeborene Immunantwort von Katzenartigen (Felidae) mindestens eine Größenordnung stärker war als die von Hundeartigen (Canidae). Geparde und Karakale (*Caracal caracal*) hatten die stärkste konstitutive angeborene Immunantwort im Vergleich zu den anderen vier untersuchten Karnivorenarten und Arten anderer Gattungen, die in früheren Studien untersucht wurden.

In **Kapitel 3** vergleiche ich sechs Parameter des humoralen Immunsystems von Geparden mit denen sympatrisch lebender Leoparden (*Panthera pardus*), die unter Katzen die größte genetische Vielfalt, auch im MHC Bereich, aufweisen. Ich konnte zeigen, dass die konstitutive angeborene Immunantwort bei Geparden stärker ist als bei Leoparden. Andererseits verfügten Leoparden über eine stärkere induzierte angeborene, sowie eine stärkere adaptive Immunantwort als Geparde. Diese Ergebnisse deuten auf unterschiedliche Strategien der beiden Arten in Bezug auf die Investition in verschiedene Bereiche des Immunsystems hin, die möglicherweise mit der unterschiedlichen Vielfalt im MHC der beiden Arten zusammenhängen. Zudem zeigt diese Studie, dass das Immunsystem der Geparde von der geringen genetischen Vielfalt nicht so stark beeinträchtigt zu sein scheint, wie bisher vermutet wurde.

In **Kapitel 4** untersuche ich den Einfluss verschiedener Faktoren, wie Alter, Geschlecht und Pathogendruck auf sechs Immunparameter von Geparden. Zusätzlich untersuche ich den Einfluss der allostatischen Belastung („Stress“) bei der Untersuchung der unter Narkose gesetzten Tiere auf diese Immunparameter. Die gefundenen Unterschiede ordne ich den energetischen Kosten der einzelnen Immunparameter zu, die sich in unterschiedlichen Lebensphasen unterscheiden können. Männliche Tiere zeigten eine stärkere angeborene konstitutive Immunantwort als weibliche Tiere. Ältere Tiere wiesen höhere Konzentrationen von Immunoglobulin G (IgG) Antikörpern auf aber niedrigere Konzentrationen von Komplementproteinen als jüngere Tiere. In menschlicher Obhut lebende Tiere sind durch ihre räumliche Nähe zu Menschen, deren Haustieren und zu anderen Artgenossen einem höheren Pathogendruck ausgesetzt als freilebende Tiere. Erstere wiesen höhere Konzentrationen von IgG Antikörpern als freilebende Geparde auf. Freilebende Tiere zeigten höhere Messwerte der Stressparameter (als Maß für die allostatische Belastung) bei der Untersuchung unter Narkose als in menschlicher Obhut lebende Tiere in Namibia. Drei der sechs Immunparameter wurden

von der allostatischen Belastung beeinflusst. Dies bedeutet, dass Messungen der allostatischen Belastung wichtig sind, wenn das Immunsystem von Geparden evaluiert wird.

Zusammenfassend vergleicht diese Arbeit 1) einen Teil des Immunsystems der Geparde mit dem mehrerer sympatrischer Karnivorenarten, 2) sechs Immunparameter bei Geparden und sympatrisch vorkommenden Leoparden und 3) sechs Immunparameter zwischen unterschiedlichen Altersgruppen, Geschlechtern und Pathogendrucke bei Geparden, und misst allostatische Belastung als mögliche Einflußgröße für Immunparameter. Damit ist diese Studie eine der umfassendsten Studien des Immunsystems bei einem freilebenden Wildtier.

Immunity of cheetahs (*Acinonyx jubatus*):

an evolutionary, comparative and life-history perspective

SUMMARY

The cheetah (*Acinonyx jubatus,* family: Felidae order: Carnivora) was once widely distributed across Africa and Asia but has been extirpated from a large portion of this area. Major threats are considered to be habitat loss and fragmentation, competition with other carnivore species, their killing as livestock predators and high disease susceptibility associated with low genetic diversity. Low genetic diversity may result in populations being more vulnerable to new pathogens, especially if the immune genes of the major histocompatibility complex (MHC) are affected. Cheetahs have been described as a classic example for the negative consequences of genetic uniformity in the MHC in numerous textbooks on conservation genetics and in scientific publications. A recent study using single-strand conformation polymorphism analysis has confirmed earlier studies and reported low genetic diversity at MHC class I and MHC class II in cheetahs. However, high disease susceptibility has only been reported for captive cheetahs, whereas free-ranging cheetahs exhibit a good overall health status.

Whether low genetic diversity at the MHC is of relevance for the conservation of the cheetah has been controversially discussed. This thesis has been designed to examine the immunological architecture of the cheetah and assess its immune parameters both in comparison with other sympatric carnivore species (**chapter 2 and 3**) as well as a function of life history attributes and its living environment (**chapter 4**). This thesis is embedded in a long-term research project on cheetahs in central Namibia, through which blood samples from approximately 400 cheetahs and other carnivore species were collected since 2002 and made available for this study.

In **chapter 2** I compare the constitutive innate immune part of six sympatric African carnivore species using a functional test, the bacterial killing assay (BKA). To compare different species, I first adapted the classical BKA protocol in such a way that the same dilutions and bacterial concentrations could be applied to all species. I demonstrated that feline species had a constitutive innate immune response of at least one magnitude higher than

those of canine species. Cheetahs and caracals (*Caracal caracal*) had a higher bacterial killing capacity than the other four carnivore species studied and also than species of other taxa which had been investigated in previous studies.

In **chapter 3** I compare six humoral immune parameters of the cheetah to those of the sympatric leopard *(Panthera pardus)*, which exhibits the highest genetic diversity amongst felines, including the MHC. The strength of the constitutive innate immune system was higher in cheetahs than in leopards. In contrast, leopards exhibited a stronger induced innate and adaptive immune system than cheetahs. These results suggest different immune investment strategies employed by the two species, possibly as a consequence of the differences in MHC diversity in the two species. My analyses indicate that the immune system of the cheetah might not be as impaired by the low genetic diversity as has been previously suggested.

In **chapter 4** I study how different factors such as age, sex and pathogen pressure influence six immune parameters in cheetahs. Additionally, I evaluate the influence of handling-associated allostatic load ("stress") on these immune parameters. I link the measured differences to the underlying energetic costs of the immune system, which may change during different life history stages. Male cheetahs showed a stronger constitutive innate immune response than female cheetahs. Older animals had higher concentrations of immunoglobulin G (IgG) antibodies but lower concentrations of complement than younger animals. Cheetahs in captivity experienced higher pathogen pressure than free-ranging cheetahs because of the proximity to people, their companion animals and other cheetahs. Cheetahs in captivity had higher concentrations of IgG antibodies than free-ranging individuals in the same habitat. Free-ranging cheetahs had higher values of handling-associated allostatic load, as measured by glucocorticoid concentrations, than cheetahs kept in captivity and in the proximity of people in the same habitat. I demonstrated that three of six immune parameters were influenced by stress parameters, suggesting that allostatic load needs to be taken into account when evaluating immunity in cheetahs.

In summary, this study compares (1) one part of the immune system of the cheetah with several sympatric species, (2) six immune parameter in cheetahs and sympatric leopards and (3) six immune parameters between different age classes, sexes and pathogen pressures in cheetahs, and estimates allostatic load as a possible factor influencing immunocompetence. Altogether, this makes this study one of the most comprehensive studies on the immune system of a free-ranging wild mammal.

This thesis is based on the following manuscripts:

1. Heinrich, S.K., Wachter, B., Aschenborn O.H.K., Thalwitzer, S., Melzheimer, J., Hofer, H., Czirják, G.À. (2016) **Feliform carnivores have a distinguished constitutive innate immune response.** Biology Open 5: 550-555.

   Own contribution to this publication: fieldwork for sample collection, including trapping and immobilising of animals, laboratory work, data analysis, drafting and writing of manuscript.

2. Heinrich, S.K., Hofer, H., Courtiol, A., Melzheimer, J., Dehnhard, M., Czirják, G.À., Wachter, B. (2017) **Cheetahs have a stronger constitutive innate immunity than leopards.** Scientific Reports 7: 44837

   Own contribution to this publication: co-design of study, fieldwork for sample collection, including trapping and immobilizing of animals, laboratory work, data analysis, drafting and writing of manuscript.

3. Heinrich, S.K., Czirják, G.À., Hofer, H., Dehnhard, M., Wachter, B. (2017) **Trade-offs in immunity of free-ranging and captive cheetahs – a quasi-experimental set-up to determine priorities in immune investment.** In preparation.

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DECLARATION/SELBSTSTÄNDIGKEITSERKLÄRUNG

I hereby declare, that this thesis was written by me independently and I used no other than the mentioned sources.

Hiermit versichere ich, dass ich die vorliegende Dissertation eigenständig verfasst habe und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe.

Berlin, 20.10.2017 Sonja Heinrich

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