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Serological and microbiological evaluation of the health status of free-ranging and captive cheetahs (*Acinonyx jubatus*) on Namibian farmland

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In den Wissenschaften ist viel Gewisses, sobald man sich von den Ausnahmen nicht irre machen lässt und die Probleme zu ehren weiß. (Johann Wolfgang von Goethe)

Meiner Familie…

The research described in this thesis was conducted at the Leibniz Institute of Zoo and Wildlife Research (IZW), Berlin, Germany, in the Clinical Laboratory, Vetsuisse Faculty, University of Zurich, Zurich, Switzerland and in the Central Veterinary Laboratory, Windhoek, Namibia. Financial support was provided by the Messerli Foundation, the Vetsuisse Faculty, the German Academic Exchange Service (DAAD) and the IZW.

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Abbreviations

ABCD ..... European Advisory Board on Cat Diseases

AKR-MuLV ..... AKR murine leukemia virus

BeRV ..... baboon endogenous retrovirus

bp ..... base pair

CDV ..... canine distemper virus

CITES ..... Convention on International Trade in Endangered Species

CMhm ..... *Candidatus Mycoplasma haemominutum*

CMt ..... *Candidatus Mycoplasma turicensis*

CRP ..... Cheetah Research Project (IZW)

DAAD ..... German Academic Exchange Service

DNA ..... deoxyribonucleic acid

EDTA ..... ethylenediaminetetraacetic acid

EIA ..... enzyme immunoassay

ELISA ..... enzyme-linked immunosorbent assay

FCV ..... feline calicivirus

FCoV ..... feline coronavirus

FeLV ..... feline leukemia virus

FeRD114eRV ..... feline RD114 endogenous retrovirus

fGAPDH ..... feline glyceraldehyde-3-phosphate dehydrogenase

fGCM ..... faecal glucocorticoid metabolite

FHV ..... feline herpesvirus 1

Fig. ..... Figure

FIV ..... feline immunodeficiency virus

FPV ..... feline parvovirus

fTM ..... faecal testosterone metabolite

HBSS ..... Hanks' balanced salt solution

HPLC ..... high performance liquid chromatography

IUCN ..... International Union for the Conservation of Nature

IZW ..... Leibniz Institute for Zoo and Wildlife Research, Berlin, Germany

MHC ..... major histocompatibility complex

Mhf ..... *Mycoplasma haemofelis*

Mhc ..... *Mycoplasma haemocanis*

PBMC ..... peripheral blood mononuclear cells

PBS ..... phosphate-buffered saline

PCR ..... polymerase chain reaction

PLV ..... puma lentivirus

PERT ..... product-enhanced reverse transcriptase (assay)

RIA ..... radioimmunoassay

RMuLV ..... Rauscher murine leukemia virus

RNA ..... ribonucleic acid

RT ..... reverse transcriptase

SPF ..... specific-pathogen-free

ssRNA ..... single-stranded RNA

TNA ..... total nucleic acid

WB ..... western blot

XMRV ..... xenotropic murine leukemia virus

This thesis is based on two publications in the main body of the thesis and one publication in the appendix.

In the main body of the thesis:

1. Krengel, A., Cattori, V., Meli, M.L., Wachter, B., Böni, J., Bisset, L., Thalwitzer, S., Melzheimer, J., Jago, M., Hofmann-Lehmann, R., Hofer, H., Lutz, H. (2015) Gammaretrovirus-specific antibodies in free-ranging and captive Namibian cheetahs. Clinical and Vaccine Immunology 22: 611-617. Doi: 10.1128/CVI.00705-14.

Own contributions to this publication: field work including capture, anaesthesia and sampling of most study animals, all laboratory work including preparation of the shipments and handling of samples, writing main parts of the manuscript, coordination of the co-authors and obtaining funding for part of the study (DAAD).

2. Krengel, A., Meli, M.L., Cattori, V., Wachter, B., Willi, B., Thalwitzer, S., Melzheimer, J., Hofer, H., Lutz, H., Hofmann-Lehmann, R. (2012) First evidence of hemoplasma infection in free-ranging Namibian cheetahs (*Acinonyx jubatus*). Veterinary Microbiology, 162: 972-976. Doi: 10.1016/j.vetmic.2012.10.009.

Own contributions to this publication: field work including capture, anaesthesia and sampling of most study animals; all laboratory work, including preparation of the shipments and handling of samples; evolving the study design and building the cooperation with RHL; writing of the manuscript, coordination of the co-authors and obtaining funding for part of the study (DAAD).

In the appendix:

3. Pribbenow S, Wachter B, Ludwig C, Weigold A, Dehnhard M 2016: Validation of an enzyme-immunoassay for the non-invasive monitoring of faecal testosterone metabolites in male cheetahs (*Acinonyx jubatus*). General and Comparative Endocrinology 228: 40-47. Doi: http://dx.doi.org/10.1016/j.ygcen.2016.01.015.

Own contribution to this publication: field work including capture, anaesthesia and sampling of most study animals; preparatory laboratory work, including preparation of the shipments and handling of samples; compiling a preparatory study; assisting in the writing of the manuscript and obtaining funding for part of the study (DAAD).

1. General Introduction

1.1. The Cheetah Research Project (CRP)

The cheetah research project of the Leibniz Institute for Zoo and Wildlife Research was founded in 2002 to address a series of research questions regarding health, reproduction, genetics, nutrition and space use of free-ranging and captive cheetahs (*Acinonyx jubatus*) on Namibian farmland. By collecting scientific data on these disciplines of this largest cheetah population in the world, the project aims to develop sustainable solutions to mitigate the existing conflict between farmers and free-ranging cheetahs and to support a healthy captive population.

In the past, large numbers of cheetahs have been hunted by the farmers, because they were perceived as a threat to their livestock and co-existing wildlife. During the last 16 years the collaboration with the famer communities has grown substantially, and as a consequence of continuous exchange of knowledge, the researchers of the CRP and the farmers have developed and tested changes in their management to coexist with cheetahs.

1.2. The structure of the thesis

This doctoral thesis is embedded in the discipline of veterinary medicine with a focus on the health status of free-ranging and captive Namibian cheetahs. The thesis consists of four chapters and one appendix. Chapter 1 introduces the project and gives a general introduction to the study animals, my study aims and the topics I investigated. Chapter 2 presents the serological and immunogenetic examinations of several gammaretroviruses in free-ranging and captive cheetahs, as well as a study on vaccinations against the oncogenic gammaretrovirus feline leukemia virus (FeLV) in captive cheetahs, published in Clinical and Vaccine Immunology (Krengel et al. 2015). Chapter 3 describes the first detection of hemoplasma in free-ranging cheetahs, a blood parasite which can lead to anemia and even death, particularly if the host is co-infected with an immunosuppressive gammaretrovirus such as FeLV or feline immunodeficiency virus (FIV), published in Veterinary Microbiology (Krengel et al. 2013). The thesis concludes with chapter 4, in which the findings, other important diseases in exotic carnivore species and limitations of my and other studies in free-ranging feline populations are discussed.

The appendix includes an introduction on non-invasive enzyme immunoassays (EIA) and the publication on the development and validation of such an EIA for faecal testosterone metabolites (fTM) in cheetah males, published in General and Comparative Endocrinology (Pribbenow et al. 2016). The hormone challenges for this study were conducted in two German zoological gardens and the validation of the EIA for cheetah faeces collected under

field conditions was conducted with faecal samples from free-ranging and captive cheetahs in Namibia. I am a co-author of this publication and have contributed to the study by (1) providing faecal samples from Namibia and (2) collecting sub-samples of cheetah faeces in captivity in pre-set time intervals up to 72 hours after faeces deposition to determine the stability of fTM concentrations in faeces being exposed to the natural environment.

1.3. The cheetah as a study species

The cheetah is a large feline species that belongs to the family Felidae and subfamily Felinae within the mammalian order of Carnivora. It is the only member of the genus *Acinonyx*. There are five subspecies recognized which inhabit different parts of Africa and Iran in Asia (Marker 1998; Krausman and Morales 2005; Durant et al. 2008). The study area of the CRP is located in central and eastern Namibia and is inhabited by the subspecies *Acinonyx jubatus jubatus*. Within the last decades, cheetahs have disappeared from vast areas of their former range (Figure 1; Durant et al. 2008). As a consequence, they were listed in Appendix I of the Convention on International Trade in Endangered Species of Fauna and Flora in 1975 (CITES 1984) and classified as vulnerable in the red list by the International Union for the Conservation of Nature (IUCN) in 1986. The cheetah has since remained on this red list and was last re-evaluated in 2008 (Durant et al. 2008). It is estimated that cheetahs have gone extinct in 76% of their historic habitat in Africa (Ray et al. 2005). Nonetheless, they persist on commercial farmland in Namibia, i.e. outside protected areas (Nowell 1996; Hanssen and Stander 2003). This stronghold is mainly a consequence of the eradication of the main carnivore competitors and predators of the cheetah, the African lion (*Panthera leo*) and spotted hyena (*Crocuta crocuta*), several decades ago (Marker-Kraus et al. 1996; Kelly et al. 1998; Purchase et al. 2007).

Because cheetahs are very elusive and difficult to detect, a census of the worldwide population and an estimate of the density of this species is challenging. Nevertheless, several surveys have been conducted in the past. Approximately 100,000 animals were estimated to have lived in 1900 (Myers 1975), and the population was estimated to have declined to approximately 15,000 individuals living in 29 African countries in the 1970s (Marker 1998). Subsequent surveys of selected countries were conducted in the 1980s (Gros 1996; Gros 1998; Gros and Rejmanek 1999; Gros 2002). In Namibia, the free-ranging cheetah population was estimated to range between 3,100 and 5,800 individuals (Hanssen and Stander 2004) and was judged to be the country with the highest number of cheetahs (Purchase et al. 2007).

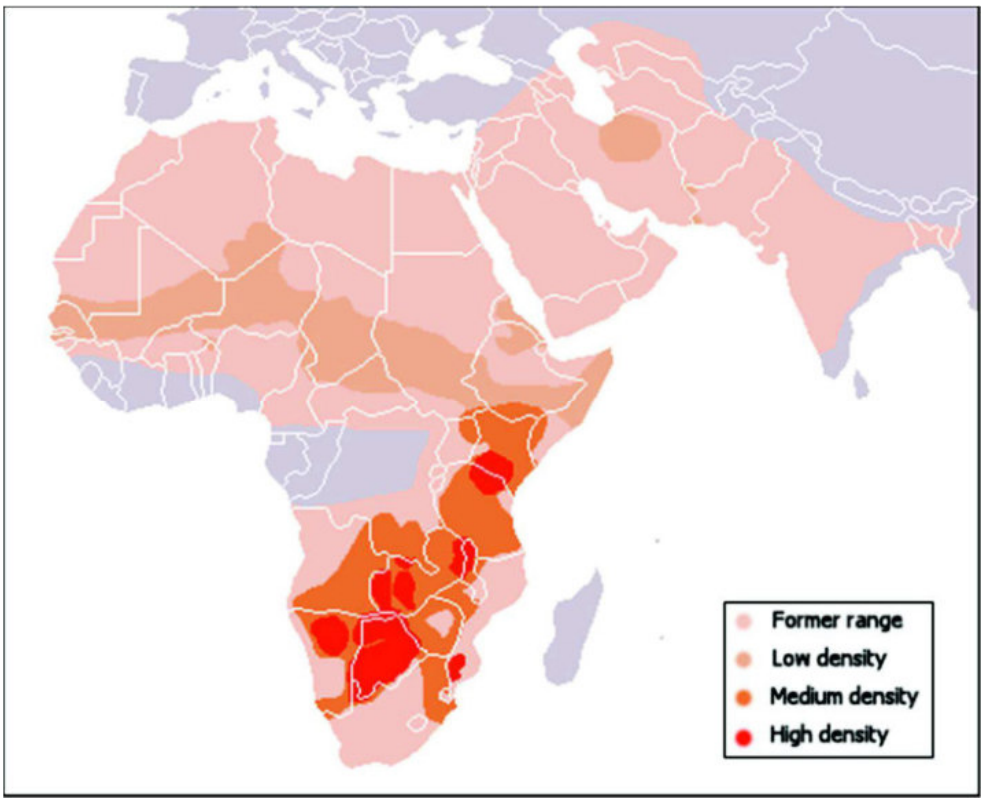

Figure 1: Distribution map of cheetahs in Africa by IUCN downloaded 2015 (http://maps.iucnredlist.org/). Used with permission by the IUCN red list unit.

Living on commercial farmland has some consequences for the probability to become infected by diseases from or to transmit diseases to herbivores or other carnivores. Cheetahs on commercial farmland in Namibia might come into contact with unvaccinated feral domestic cats and domestic dogs or with other free-ranging carnivore species such as the leopard (*Panthera pardus*), caracal (*Felis caracal*), serval (*Leptailurus serval*), brown hyena (*Hyaena brunnea)*, black-backed jackal (*Canis mesomelas*) and other, smaller carnivore species (Schneider 1994; Marker-Kraus et al. 1996; Lindsey et al. 2013). The CRP therefore collects data and samples from all free-ranging carnivore species captured, found or reported dead in the study area to compare serological titres and to test samples for infectious agents.

Cheetah males exhibit two spatial tactics. They either defend small territories or roam in large home ranges. They are solitary or form coalitions of two or three males, both when defending a territory or when roaming in large areas (Caro and Durant 1991; Caro 1994) Females have large and overlapping home ranges that encompass several male territories (Caro 1994). During oestrous, females might mate with several males and mixed paternities were described (Gottelli et al. 2007). This social system indicates a higher contact rate between individual cheetahs than might be expected from a purely solitary species and could thus assist in the possible spread of infectious diseases.

The cheetah has been one of the most frequently cited examples for a high level of inbreeding depression, as evidenced by a lack of genetic variability, after a proposed genetic bottleneck (O'Brien et al. 1983; 1985; 1986; O'Brien and Evermann 1988). The proposed and widely discussed consequences of this idea were: high susceptibility to pathogens, low reproductive performance, high infant mortality, morphological abnormalities and a feeble immune system. However, most impairments of these kinds were described in captive cheetahs but not free-ranging ones (O'Brien et al. 1985; Evermann 1986; Evermann et al. 1988; Marker and O'Brien 1989; Heeney et al. 1990; Laurenson et al. 1992; Caro and Laurenson 1994; Laurenson 1994; Marker-Kraus 1997; Marker and Dickman 2004; Thalwitzer et al. 2010; Castro-Prieto et al. 2011; Wachter et al. 2011; Castro-Prieto et al. 2012), and have, more recently, been attributed to unfavourable captive husbandry conditions or breeding management plans (Munson 1993; Wildt et al. 1993; Laurenson et al. 1995; Wielebnowski 1996; 2002; Terio et al. 2004; Wachter et al. 2011; Terio et al. 2012).

Studies by the CRP of the reproductive performance of cheetah females revealed that neither the lack of genetic variability nor the allostatic load ("stress") of being held in captivity impairs reproduction in captivity, but rather the age at first reproduction and pathologies on the reproductive tract that develop after a few years in nulliparous females (Wachter et al. 2011). This phenomenon is known as asymmetric reproductive aging and can be prevented by early breeding of captive females (Hermes et al. 2004). It does not occur in free-ranging females because they breed early in life (Laurenson et al. 1992; Wachter et al. 2011). Asymmetric reproductive aging is also known in other mammals such as captive African and Asian elephants (*Loxodonta africana, Elephas maximus*) and southern and northern white rhinoceroses (*Ceratotherium simum simum*, *Ceratotherium simum cottoni*) (Hildebrandt et al. 2000; Hermes et al. 2004; 2006). Further studies of the CRP have investigated the number of the immune gene alleles and detected ten MHC I and four MHC II alleles, which is a relatively small number of alleles compared to other carnivore species but more than previously identified (Castro-Prieto et al. 2011; 2012). Although such low variability of the MHC suggests that the potential of the adaptive immune system to respond appropriately to infectious disease might be compromised, the cheetah may compensate this with a high capacity of the constitutive innate immune as measured with a bacterial killing assay (Heinrich et al. 2016).

1.4. The oncogenic gammaretrovirus feline leukemia virus (FeLV) in the Namibian cheetah

In this thesis, I analysed blood samples from cheetahs for evidence of contact and infection with FeLV. FeLV infection is well described in the domestic cat and is of world-wide importance in this species (e.g. Hartmann 2006; Bolin and Levy 2011; Hartmann 2012; Willett and Hosie 2013). FeLV is a type C retrovirus, also known as gammaretrovirus, that causes suppressive effects on the bone marrow and the immune system, leading to neoplasia, facilitating secondary infections and hence infected animals can be presenting diverse clinical signs (Hartmann 2012). The transmission of FeLV is usually direct, although faecal transmission has been proven as well (Gomes-Keller et al. 2009; O'Brien et al. 2012). Despite the genome of FeLV being rather simple as it includes only information for its structure and replication, after replication *in vivo* or by recombination with endogenous FeLV sequences new and closely related viruses are formed (Bolin and Levy 2011). These different variants form a heterogeneous family and are divided into four subgroups based on the surface glycoprotein sequence. The progression and severity of the infection is strongly dependent on the virus subtype and the general condition of the infected animal (Bolin and Levy 2011). FeLV leads to a decreased life expectancy, but with medical care a certain quality of life for the infected animal can be preserved for a few years (Levy et al. 2006). FeLV has different stages of disease, which can strongly alter the outcome and the duration of the disease. All infections are considered to be chronic and usually have an asymptomatic phase, which can vary in length (Hartmann 2012). Currently four stages of FeLV infection are proposed: abortive, regressive, progressive and focal or atypical infection (Torres et al. 2005; Hofmann-Lehmann et al. 2007; 2008; Levy et al. 2008). Because FeLV provirus is integrated into the host genome, it is highly unlikely to be fully cleared in the course of time irrespective of the infection stage of the animal (Hartmann 2012). Within the last twenty years, the prevalence of FeLV infections in domestic cats is proposed to have decreased due to a combination of testing and identifying infected animals and vaccinating uninfected animals as preventative measures (Little et al. 2011). FeLV can also affect non-domestic feline species, including critically endangered ones (Brown et al. 2008; Meli et al. 2009). In 1995, a captive cheetah in Namibia died from a FeLV infection after being in contact with another captive cheetah which tested positive for FeLV antigens (Marker et al. 2003). There was circumstantial evidence that the source of this infection was a feral domestic cat. In free-ranging Florida panthers (*Puma concolor coryi*) and Iberian lynxes (*Lynx pardinus*), a similar route with lethal course of infection was described in all FeLV antigen positive panthers and in nearly 50% of the provirus positive lynxes (Brown et al. 2008; Meli et al. 2009).

Chapter 2 presents the first evidence of FeLV seropositivity in free-ranging cheetahs. Depending on the antibody test, up to 19% of the 88 tested cheetahs had to be considered positive for FeLV. A subsample of six FeLV seropositive cheetahs was also tested against antibodies of Rauscher murine leukemia virus and revealed seropositive results for all samples. Despite various antigen tests we did not identify proviral DNA, similar to previous FeLV antigen studies (Munson et al. 2004; Thalwitzer et al. 2010). We also demonstrated that a vaccination against FeLV with a commercial cat vaccine induced measurable antibody values in captive cheetahs. For this study several assays, cell cultures and tests on serum, plasma, peripheral blood mononuclear cells (PBMCs) and total nucleic acid (TNA) extracted from whole blood and from plasma were used (see Material and Methods in Chapter 2).

Cheetahs in Namibia come into contact with numerous other viral infectious agents. Antibody prevalence of feline herpesvirus 1 (FHV), feline calicivirus (FCV), feline parvovirus (FPV), feline coronavirus (FCoV), canine distemper virus (CDV), FIV, puma lentivirus (PLV) and rabies virus in free-ranging cheetahs in Namibia revealed up to 65% seroprevalence for FCV (Munson et al. 2004; Thalwitzer et al. 2010). All seropositive cheetahs were in good health status when immobilised and sampled (Munson et al. 2004; Thalwitzer et al. 2010). Thus, there is currently not too much concern that cheetah might seriously suffer from infectious diseases, although increasing pressure by people and their companion animals and livestock as well as potential newly emerging diseases might increase their exposure to pathogens and co-infection with unknown outcomes.

1.5. A novel pathogen in the cheetah: Hemoplasma

In this study I investigated the prevalence of hemotropic mycoplasmas (hemoplasmas) in free-ranging Namibian cheetahs using quantitative real-time PCR on blood samples. Hemoplasmas are cell wall-free bacteria that parasitise red blood cells and circulate between domestic cats or free-ranging feline species and their invertebrate hosts. The exact mode of transmission is still not fully understood and blood-sucking vectors or direct transmission are proposed (Willi et al. 2007). In felids, three *Hemoplasma* species are known: *Mycoplasma haemofelis* (Mhf), '*Candidatus Mycoplasma turicensis*' (CMt) and '*Candidatus Mycoplasma haemominutum*' (CMhm) (Foley and Pedersen 2001; Neimark et al. 2001; Willi et al. 2005; 2006). Mhf is the causative agent of feline infectious anemia, which leads to severe macrocytic normochromic anemia and induces acute hemolysis associated with anorexia, lethargy and death (Foley et al. 1998; Westfall et al. 2001). Similar to infections with FeLV, the disease progression and outcome strongly depends on the *Hemoplasma* species involved, the type of infection (acute or chronic) and the health status of the host (Willi et al. 2007). Some aspects of the agent remain difficult to assess because it is not possible to culture hemoplasmas outside the host (Neimark and Kocan 1997; Tasker et al. 2003). For

the detection of *Hemoplasma* infections, PCR is the method of choice and for further isolate identification the 16S rRNA gene and the RNAse P gene need to be sequenced (Birkenheuer et al. 2002; Willi et al. 2007).

Infections with *Hemoplasma* have been described in numerous captive feline species and in free-ranging Iberian lynxes, Eurasian lynxes (*Lynx lynx*), European wildcats (*Felis silvestris silvestris*) and African lions from Tanzania (Willi et al. 2007; Munson et al. 2008; Meli et al. 2009). In one study, free-ranging animals had higher prevalence than captive animals (Willi et al. 2007), stressing the importance of studying *Hemoplasma* infections in free-ranging felid species, particularly in vulnerable and endangered ones. Previously, this agent had not yet been documented in free-ranging felids from southern Africa. Hemoplasmas usually pose a small threat for a healthy animal, but if the animal is co-infected with an agent that induces immunosuppressive effects such as FeLV or FIV, the progression of infectious anemia and the deterioration of the animal is likely (Tasker 2010). However, serious effects of anemia can also be observed in seemingly healthy animals (de Bortoli et al. 2012).

1.6. Aims of thesis

Due to the importance and possible devastating effects FeLV infections can have in exotic felids, each hint for its presence should be pursued. A previous study conducted in the same study area as the work reported in this thesis revealed inconsistent and inconclusive results for FeLV, i.e. some animals tested positive for antibodies and/or antigens in some but not other tests (Thalwitzer 2007). Two possible explanations were discussed: (1) a low dose infection of FeLV, leading to negative antigen but positive antibody tests, or (2) a possible infection with another gammaretrovirus, for example the exogenous murine leukemia virus (MuLV), which may have induced positive FeLV antibody results through cross-reactivity. These conflicting findings triggered a series of new research questions which are the basis of chapter 2 of this thesis (Krengel et al. 2015). I analysed blood samples collected between 2002 and 2009 of 88 free-ranging and 56 captive cheetahs held in large enclosures in their natural habitat in Namibia. As vaccination against FeLV is one key element in the prevention of the spread of infection, the efficacy of the vaccinations performed in the past and during the course of this study were also evaluated. The samples of the animals held in captivity included samples of first time and repeatedly vaccinated as well as non-vaccinated animals. I used these samples to (1) (re-)test for antibodies against FeLV using ELISAs and Western blots that included the vaccine induced antibodies, (2) extract total nucleic acids (TNA) from whole blood for real-time PCR evaluation, and (3) perform product-enhanced reverse transcriptase assays (PERTs) after growing cell cultures of the isolated cheetah PBMCs to screen for reverse transcriptase (RT) activity.

If animals are infected with a blood parasite and co-infected with an immunosuppressive agent such as FeLV or FIV, chronic disease manifestation can be the consequence (Breitschwerdt and Kordick 1995). I thus screened the free-ranging Namibian cheetah population for hemoplasma to investigate whether this blood parasite poses a possible threat to the health of cheetahs.

The aim of this doctoral thesis was to (1) follow up on the previous findings of inconclusive FeLV antibody tests, (2) evaluate the immune response to FeLV vaccinations and (3) screen the samples for hemoplasmas.

1.7. References

Birkenheuer, A. J., E. B. Breitschwerdt, A. R. Alleman and C. Pitulle (2002). "Differentiation of *Haemobartonella canis* and *Mycoplasma haemofelis* on the basis of comparative analysis of gene sequences." Am J Vet Res 63: 1385-1388.

Bolin, L. L. and L. S. Levy (2011). "Viral determinants of FeLV infection and pathogenesis: Lessons learned from analysis of a natural cohort." Viruses 3: 1681-1698.

Breitschwerdt, E. B. and D. L. Kordick (1995). "Bartonellosis." J Am Vet Med Assoc 206: 1928-1931.

Brown, M. A., M. W. Cunningham, A. L. Roca, J. L. Troyer, W. E. Johnson and S. J. O'Brien (2008). "Genetic characterization of feline leukemia virus from Florida panthers." Emerg Infect Dis 14: 252-259.

Caro, T. M. (1994). Cheetahs of the Serengeti Plains: Group living in an asocial species, University of Chicago Press.

Caro, T. M. and S. M. Durant (1991). "Use of quantitative analyses of pelage characteristics to reveal family resemblances in genetically monomorphic cheetahs." J Hered 82: 8-14.

Caro, T. M. and M. K. Laurenson (1994). "Ecological and genetic factors in conservation: a cautionary tale." Science 263: 485-486.

Castro-Prieto, A., B. Wachter, J. Melzheimer, S. Thalwitzer, H. Hofer and S. Sommer (2012). "Immunogenetic variation and differential pathogen exposure in free-ranging cheetahs across Namibian farmlands." PLoS ONE 7(11): e49129.

Castro-Prieto, A., B. Wachter and S. Sommer (2011). "Cheetah Paradigm Revisited: MHC Diversity in the World's Largest Free-Ranging Population." Mol Biol Evol 28: 1455-1468.

CITES (1984). Convention on International Trade in Endangered Species of Wild Fauna and Flora, Part of the Endangered Species Act. CODE FEDERAL REGISTER, Part 23.

de Bortoli, C. P., M. R. Andre, M. C. Seki, A. A. Pinto, T. Machado Sde and R. Z. Machado (2012). "Detection of hemoplasma and *Bartonella* species and co-infection with retroviruses in cats subjected to a spaying/neutering program in Jaboticabal, SP, Brazil." Rev Bras Parasitol Vet 21: 219-223.

Durant, S., L. Marker, N. Purchase, F. Belbachir, L. Hunter, C. Packer, C. Breitenmoser-Wursten, E. Sogbohossou and H. Bauer (2008). *Acinonyx jubatus*, The IUCN Red List of Threatened Species. Version 2014.3.

Evermann, J. F. (1986). "Feline coronavirus infection of cheetahs." Feline Practice 16: 21-30.

Evermann, J. F., J. L. Heeney, M. E. Roelke, A. J. McKeirnan and S. J. O'Brien (1988). "Biological and pathological consequences of feline infectious peritonitis virus infection in the cheetah." Arch Virol 102: 155-171.

Foley, J. E., S. Harrus, A. Poland, B. Chomel and N. C. Pedersen (1998). "Molecular, clinical, and pathologic comparison of two distinct strains of *Haemobartonella felis* in domestic cats." Am J Vet Res 59: 1581-1588.

Foley, J. E. and N. C. Pedersen (2001). ",*Candidatus* Mycoplasma haemominutum', a low-virulence epierythrocytic parasite of cats." Int J Syst Evol Microbiol 51: 815-817.

Gomes-Keller, M. A., E. Gönczi, B. Grenacher, R. Tandon, R. Hofman-Lehmann and H. Lutz (2009). "Fecal shedding of infectious feline leukemia virus and its nucleic acids: A transmission potential." Vet Microbiol 134: 208-217.

Gottelli, D., J. Wang, S. Bashir and S. M. Durant (2007). "Genetic analysis reveals promiscuity among female cheetahs." Proc R Soc B 274: 1993-2001.

Gros, P. M. (1996). "Status of the cheetah in Malawi." Nyala 19: 33-36.

Gros, P. M. (1998). "Status of the cheetah *Acinonyx jubatus* in Kenya: a field-interview assessment." Biol Cons 85: 137-149.

Gros, P. M. (2002). "The status and conservation of the cheetah *Acinonyx jubatus* in Tanzania." Biol Cons 106: 177-185.

Gros, P. M. and M. Rejmanek (1999). "Status and habitat preferences of Uganda cheetahs: an attempt to predict carnivore occurrence based on vegetation structure." Biodiv Cons 8: 1561-1583.

Hanssen, L. and P. Stander (2003). Namibia large carnivore atlas. Report 2, Predator Conservation Trust: 1-12.

Hanssen, L. and P. Stander (2004). Namibia large carnivore atlas. P. C. Trust. Windhoek, Namibia, http://www.desertlion.info//reports/atlas_july2004.pdf.

Hartmann, K. (2006). Feline leukeima virus infection. In: Infectious diseases of the dog and cat. E. C. Greiner (ed), 3: 105-131. St. Louis, MO, USA, Saunders Elsevier.

Hartmann, K. (2012). "Clinical aspects of feline retroviruses: A review." Viruses 4: 2684-2710.

Heeney, J. L., J. F. Evermann, A. J. McKeirnan, L. Marker-Kraus, M. E. Roelke, M. Bush, D. E. Wildt, D. G. Meltzer, L. Colly, J. Lukas and et al. (1990). "Prevalence and implications of feline coronavirus infections of captive and free-ranging cheetahs (*Acinonyx jubatus*)." J Virol 64: 1964-1972.

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

Hermes, R., T. B. Hildebrandt and F. Göritz (2004). "Reproductive problems directly attributable to long-term captivity -- asymmetric reproductive aging." Anim Reprod Sci 82: 49-60.

Hermes, R., T. B. Hildebrandt, C. Walzer, F. Göritz, M. L. Patton, S. Silinski, M. J. Anderson, C. E. Reid, G. Wibbelt, K. Tomasova and F. Schwarzenberger (2006). "The effect of long non-reproductive periods on the genital health in captive female white rhinoceroses (*Ceratotherium simum simum*, *C. s. cottoni*)." Theriogenology 65: 1492-1515.

Hildebrandt, T. B., F. Göritz, N. C. Pratt, J. L. Brown, R. J. Montali, D. L. Schmitt, G. Fritsch and R. Hermes (2000). "Ultrasonography of the urogenital tract in elephants

(*Loxodonta africana* and *Elephas maximus*): An important tool for assessing female reproductive function." Zoo Biol 19: 321-332.

Hofmann-Lehmann, R., V. Cattori, R. Tandon, F. S. Boretti, M. L. Meli, B. Riond and H. Lutz (2008). "How molecular methods change our views of FeLV infection and vaccination." Vet Immunol Immunopathol 123: 119-123.

Hofmann-Lehmann, R., V. Cattori, R. Tandon, F. S. Boretti, M. L. Meli, B. Riond, A. C. Pepin, B. Willi, P. Ossent and H. Lutz (2007). "Vaccination against the feline leukaemia virus: Outcome and response categories and long-term follow-up." Vaccine 25: 5531-5539.

Kelly, M. J., M. K. Laurenson, C. D. FitzGibbon, D. A. Collins, S. M. Durant, G. W. Frame, B. C. R. Bertram and T. M. Caro (1998). "Demography of the Serengeti cheetah (*Acinonyx jubatus*) population: the first 25 years." J Zool Lond 244: 473-488.

Krausman, P. R. and S. M. Morales (2005). "*Acinonyx jubatus*." Mammalian Species 771: 1-6.

Krengel, A., V. Cattori, M. L. Meli, B. Wachter, J. Boni, L. R. Bisset, S. Thalwitzer, J. Melzheimer, M. Jago, R. Hofmann-Lehmann, H. Hofer and H. Lutz (2015). "Gammaretrovirus-specific antibodies in free-ranging and captive namibian cheetahs." Clin Vaccine Immunol 22: 611-617.

Krengel, A., M. L. Meli, V. Cattori, B. Wachter, B. Willi, S. Thalwitzer, J. Melzheimer, H. Hofer, H. Lutz and R. Hofmann-Lehmann (2013). "First evidence of hemoplasma infection in free-ranging Namibian cheetahs (*Acinonyx jubatus*)." Vet Microbiol 162: 972-976.

Laurenson, M. K. (1994). "High juvenile mortality in cheetahs (*Acinonyx jubatus*) and its consequences for maternal care." J Zool Lond 234: 387-408.

Laurenson, M. K., T. M. Caro and M. Borner (1992). "Female cheetah reproduction." Natl Geogr Res Explor 8: 64-75.

Laurenson, M. K., N. Wielebnowski and T. M. Caro (1995). "Extrinsic factors and juvenile mortality in cheetahs." Cons Biol 9: 1329-1331.

Levy, J., C. Crawford, K. Hartmann, R. Hofmann-Lehmann, S. Little, E. Sundahl and V. Thayer (2008). "2008 American Association of Feline Practitioners' feline retrovirus management guidelines." J Fel Med Sur 10: 300-316.

Levy, J. K., H. M. Scott, J. L. Lachtara and P. C. Crawford (2006). "Seroprevalence of feline leukemia virus and feline immunodeficiency virus infection among cats in North America and risk factors for seropositivity." J Am Vet Med Assoc 228: 371-376.

Lindsey, P. A., C. P. Havemann, R. Lines, L. Palazy, A. E. Price, T. A. Retief, T. Rhebergen and C. Van der Waal (2013). "Determinants of persistence and tolerance of carnivores on Namibian ranches: implications for conservation on Southern African private lands." PLoS ONE 8(1): e52458.

Little, S., D. Bienzle, L. Carioto, H. Chisholm, E. O'Brien and M. Scherk (2011). "Feline leukemia virus and feline immunodeficiency virus in Canada: Recommendations for testing and management." Can Vet J 52: 849-855.

Marker-Kraus, L. (1997). Morphological abnormalities reported in Namibian cheetahs (*Acinonyx jubatus*). 50th Anniversary Congress of VAN and 2nd African Congress of the WVA. Windhoek, Namibia, Veterinary Association of Namibia: 9-18.

Marker-Kraus, L., D. Kraus, D. Barnett and S. Hurlbut (1996). "Cheetah survival on Namibian farmlands". Windhoek, Cheetah Conservation Fund.

Marker, L. (1998). "Current status of the cheetah (*Acinonyx jubatus*)". Proceedings of a Symposium on cheetahs as Game Ranch Animals, Onderstepoort, South Africa.

Marker, L., L. Munson, P. A. Basson and S. Quackenbush (2003). "Multicentric T-cell lymphoma associated with feline leukemia virus infection in a captive namibian cheetah (*Acinonyx jubatus*)." J Wildl Dis 39: 690-695.

Marker, L. and S. J. O'Brien (1989). "Captive breeding of the cheetahs (*Acinonyx jubatus*) in North American Zoos (1871-1986)." Zoo Biol 8: 3-16.

Marker, L. L. and A. J. Dickman (2004). "Dental anomalies and incidence of palatal erosion in Namibian cheetahs (*Acinonyx jubatus jubatus*)." J Mammal 85: 13-18.

Meli, M. L., V. Cattori, F. Martinez, G. Lopez, A. Vargas, M. A. Simon, I. Zorrilla, A. Munoz, F. Palomares, J. V. Lopez-Bao, J. Pastor, R. Tandon, B. Willi, R. Hofmann-Lehmann and H. Lutz (2009). "Feline leukemia virus and other pathogens as important threats to the survival of the critically endangered Iberian lynx (*Lynx pardinus*)." PLoS ONE 4: e4744.

Munson, L. (1993). "Diseases of captive cheetahs (*Acinonyx jubatus*): Results of the cheetah research council pathology survey, 1989–1992." Zoo Biology 12: 105-124.

Munson, L., L. Marker, E. Dubovi, J. A. Spencer, J. F. Evermann and S. J. O'Brien (2004). "Serosurvey of viral infections in free-ranging Namibian cheetahs (*Acinonyx jubatus*)." J Wildl Dis 40: 23-31.

Munson, L., K. A. Terio, R. Kock, T. Mlengeya, M. E. Roelke, E. Dubovi, B. Summers, A. R. Sinclair and C. Packer (2008). "Climate extremes promote fatal co-infections during canine distemper epidemics in African lions." PLoS ONE 3(6): e2545.

Myers, N. (1975). The cheetah *Acinonyx jubatus* in Africa: Report of a survey in Africa from the Sahara southwards, IUCN/WWF Joint Project. W. W. F. & International Union for Conservation of Nature and Natural Resources. Morges, Switzerland.

Neimark, H., K. E. Johansson, Y. Rikihisa and J. G. Tully (2001). "Proposal to transfer some members of the genera *Haemobartonella* and *Eperythrozoon* to the genus *Mycoplasma* with descriptions of ‚*Candidatus Mycoplasma haemofelis*', ‚*Candidatus Mycoplasma haemomuris*', ‚*Candidatus Mycoplasma haemosuis*' and ‚*Candidatus Mycoplasma wenyonii*'." Int J Syst Evol Microbiol 51: 891-899.

Neimark, H. and K. M. Kocan (1997). "The cell wall-less rickettsia *Eperythrozoon wenyonii* is a *Mycoplasma*." FEMS Microbiol Let 156: 287-291.

Nowell, K. (1996). Namibian cheetah conservation strategy, Ministry of Environment and Tourism, Windhoek

O'Brien, S. J. and J. F. Evermann (1988). "Interactive influence of infectious disease and genetic diversity in natural populations." Trends Ecol Evol 3: 254-259.

O'Brien, S. J., M. E. Roelke, L. Marker, A. Newman, C. A. Winkler, D. Meltzer, L. Colly, J. F. Evermann, M. Bush and D. E. Wildt (1985). "Genetic basis for species vulnerability in the cheetah." Science 227: 1428-1434.

O'Brien, S. J., D. E. Wildt, D. Gildman, C. R. Merril and M. Bush (1983). "The cheetah is depauperate in genetic variation." Science 221: 459-462.

O'Brien, S. J., J. L. Troyer, M. A. Brown, W. E. Johnson, A. Antunes, M. E. Roelke and J. Pecon-Slattery (2012). "Emerging Viruses in the *Felidae*: Shifting Paradigms." Viruses 4: 236-257.

O'Brien, S. J., D. E. Wildt and M. Bush (1986). "The cheetah in genetic peril." Scient Am 254: 84-92.

Pribbenow, S., B. Wachter, C. Ludwig, A. Weigold and M. Dehnhard (2016). "Validation of an enzyme-immunoassay for the non-invasive monitoring of faecal testosterone metabolites in male cheetahs (*Acinonyx jubatus*)." Gen Comp Endocrinol 228: 40-47.

Purchase, G., L. Marker, K. Marnewick, R. Klein and S. Williams (2007). "Regional assessment of the status, distribution and conservation needs of the cheetah in southern Africa." CAT News 3 (Special Issue): 44-46.

Ray, J. C., L. Hunter and J. Zigouris (2005). Setting conservation and research priorities for larger African carnivores. Report No.24, New York: Wildlife Conservation Society.

Schneider, H. P. (1994). Animal health and veterinary medicine in Namibia. Agrivet. Windhoek, Namibia.

Tasker, S. (2010). "Haemotropic mycoplasmas: What's their real significance in cats?" Journal of Feline Medicine & Surgery 12: 369-381.

Tasker, S., S. H. Binns, M. J. Day, T. J. Gruffydd-Jones, D. A. Harbour, C. R. Helps, W. A. Jensen, C. S. Olver and M. R. Lappin (2003). "Use of a PCR assay to assess the prevalence and risk factors for *Mycoplasma haemofelis* and *'Candidatus Mycoplasma haemominutum'* in cats in the United Kingdom." Vet Rec 152: 193-198.

Terio, K. A., L. Marker and L. Munson (2004). "Evidence for chronic stress in captive but not free-ranging cheetahs (*Acinonyx jubatus*) based on adrenal morphology and function." J Wildl Dis 40: 259-266.

Terio, K. A., L. Munson and P. F. Moore (2012). "Characterization of the gastric immune response in cheetahs (*Acinonyx jubatus*) with *Helicobacter*-associated gastritis." Vet Pathol 49: 824-833.

Thalwitzer, S. (2007). Reproductive activity in cheetah females, cub survival and health of male and female cheetahs on Namibian farmland. Berlin, Freie Universität, Berlin, Germany; Mensch&Buch Verlag. ISBN-10: 3-86664-320-9; ISBN-13: 978-3-86664-320-8.

Thalwitzer, S., B. Wachter, N. Robert, G. Wibbelt, T. Muller, J. Lonzer, M. L. Meli, G. Bay, H. Hofer and H. Lutz (2010). "Seroprevalences to viral pathogens in free-ranging and captive cheetahs (*Acinonyx jubatus*) on Namibian farmland." Clin Vaccine Immunol 17: 232-238.

Torres, A. N., C. K. Mathiason and E. A. Hoover (2005). "Re-examination of feline leukemia virus: host relationships using real-time PCR." Virology 332: 272-283.

Wachter, B., S. Thalwitzer, H. Hofer, J. Lonzer, T. B. Hildebrandt and R. Hermes (2011). "Reproductive history and absence of predators are important determinants of reproductive fitness: the cheetah controversy revisited." Cons Lett 4: 47-54.

Westfall, D. S., W. A. Jensen, W. J. Reagan, S. V. Radecki and M. R. Lappin (2001). "Inoculation of two genotypes of *Hemobartonella felis* (California and Ohio variants) to induce infection in cats and the response to treatment with azithromycin." Am J Vet Res 62: 687-691.

Wielebnowski, N. (1996). "Reassessing the relationship between juvenile mortality and genetic monomorphism in captive cheetahs." Zoo Biology 15: 353-369.

Wielebnowski, N. C., K. Ziegler, D. E. Wildt, J. Lukas and J. L. Brown (2002). "Impact of social management on reproductive, adrenal and behavioural activity in the cheetah (*Acinonyx jubatus*)." Anim Cons 5: 291-301.

Wildt, D. E., J. L. Brown, M. Bush, M. A. Barone, K. A. Cooper, J. Grisham and J. G. Howard (1993). "Reproductive status of cheetahs (*Acinonyx jubatus*) in North American Zoos: The benefits of physiological surveys for strategic planning." Zoo Biol 12: 45-80.

Willett, B. J. and M. J. Hosie (2013). "Feline leukaemia virus: Half a century since its discovery." The Vet J 195: 16-23.

Willi, B., F. S. Boretti, C. Baumgartner, S. Tasker, B. Wenger, V. Cattori, M. L. Meli, C. E. Reusch, H. Lutz and R. Hofmann-Lehmann (2006). "Prevalence, risk factor analysis,

and follow-up of infections caused by three feline *Hemoplasma* species in cats in Switzerland." J Clin Microbiol 44: 961-969.

Willi, B., F. S. Boretti, V. Cattori, S. Tasker, M. L. Meli, C. Reusch, H. Lutz and R. Hofmann-Lehmann (2005). "Identification, molecular characterization, and experimental transmission of a new *Hemoplasma* isolate from a cat with hemolytic anemia in Switzerland." J Clin Microbiol 43: 2581-2585.

Willi, B., F. S. Boretti, S. Tasker, M. L. Meli, N. Wengi, C. E. Reusch, H. Lutz and R. Hofmann-Lehmann (2007). "From *Haemobartonella* to *Hemoplasma*: molecular methods provide new insights." Vet Microbiol 125: 197-209.

Willi, B., C. Filoni, J. L. Catao-Dias, V. Cattori, M. L. Meli, A. Vargas, F. Martinez, M. E. Roelke, M. P. Ryser-Degiorgis, C. M. Leutenegger, H. Lutz and R. Hofmann-Lehmann (2007). "Worldwide occurrence of feline *Hemoplasma* infections in wild felid species." J Clin Microbiol 45: 1159-1166.

2. Gammaretrovirus-specific antibodies in free-ranging and captive Namibian cheetahs

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Gammaretrovirus-Specific Antibodies in Free-Ranging and Captive Namibian Cheetahs

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The cheetah population in Namibia is the largest free-ranging population in the world and a key population for research regarding the health status of this species. We used serological methods and quantitative real-time PCR to test free-ranging and captive Namibian cheetahs for the presence of feline leukemia virus (FeLV), a gammaretrovirus that can be highly aggressive in populations with low genetic diversity, such as cheetahs. We also assessed the presence of antibodies to other gammaretroviruses and the responses to a FeLV vaccine developed for domestic cats. Up to 19% of the free-ranging cheetahs, 27% of the captive nonvaccinated cheetahs, and 86% of the captive vaccinated cheetahs tested positive for FeLV antibodies. FeLV-antibody-positive free-ranging cheetahs also tested positive for Rauscher murine leukemia virus antibodies. Nevertheless, FeLV was not detectable by quantitative real-time PCR and no reverse transcriptase activity was detectable by product-enhanced reverse transcriptase assay in the plasma of cheetahs or the supernatants from cultures of peripheral blood mononuclear cells. The presence of antibodies to gammaretroviruses in clinically healthy specimens may be caused either by infection with a low-pathogenic retrovirus or by the expression of endogenous retroviral sequences. The strong humoral immune responses to FeLV vaccination demonstrate that cheetahs can respond to the vaccine and that vaccination against FeLV infection may be beneficial should FeLV infection ever become a threat, as was seen in Iberian lynx and Florida panthers.

The cheetah population in Namibia is the largest free-ranging population of this vulnerable species (1). For more than 2 decades, cheetahs have been considered highly susceptible to infectious diseases because of low genetic variability, which is assumed to impair immune responses to viral challenges (2–5). Evidence for fatal viral infections in cheetahs comes from an outbreak of feline infectious peritonitis (FIP), a consequence of feline coronavirus (FCoV) infections, in a captive population in the United States that was kept at nonethologically high density (6–8) and from a single case of very rapid feline leukemia virus (FeLV) disease progression in a captive Namibian cheetah in 1995 (9). No disease outbreaks have been reported in any free-ranging cheetah population, but several studies have identified antibodies against viruses such as feline herpesvirus (FHV), feline calicivirus (FCV), feline parvovirus (FPV), FCoV, canine distemper virus (CDV), feline immunodeficiency virus (FIV), and rabies virus (10–13). In Namibia, free-ranging cheetahs are generally in good health; no clinical signs of viral infections were detected during sampling, and none of the histopathological examinations conducted after necropsies showed lesions related to viral infections (12–15). A recent study on major histocompatibility complex (MHC) class I and class II confirmed the relatively low genetic variability in cheetahs (2). Despite the small number of MHC class I alleles (10 alleles), Namibian cheetahs can still mount effective immune responses against some viral challenges, although their immunocompetence might be limited when they are confronted with new pathogens (2, 16). Thus, it is important to continuously monitor the free-ranging cheetah population in Namibia, particularly for viruses for which no antibodies have been reported so far, such as FeLV, an oncogenic gammaretrovirus (10–13).

FeLV is of particular interest because, in the 1995 case, a cheetah experienced rapid deterioration and died from an infection transmitted from a captive cheetah that tested positive for FeLV antigens. Circumstantial evidence indicated that the origins of the infection were nonvaccinated feral and domestic cats viremic with FeLV (9). Such a method of transmission and a course of disease were also observed in Florida panthers (*Puma concolor coryi*) (17) and Iberian lynx (*Lynx pardinus*), the most endangered free-ranging felid species (18). In Namibia, cheetahs roam mainly on commercially used farmland, where they might come into contact with nonvaccinated feral and domestic cats and dogs, as well as free-ranging leopards (*Panthera pardus*) and smaller carnivores (19, 20). Previous studies documented that cheetahs in north-central Namibia have higher seroprevalences of FHV, FCV, FCoV, and CDV than do cheetahs in east-central Namibia (12, 13). This is probably a consequence of greater contact frequency, due to a likely higher density of mostly nonvaccinated feral and domestic cats and dogs in regions with greater human populations (13, 21, 22).

The goals of the present report were to study and to discuss the nature of antibodies to FeLV or a closely related retrovirus that were present in a portion of serum samples from free-ranging and

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captive Namibian cheetahs. We also examined the efficacy of vaccination against FeLV to induce antibodies to FeLV in captive cheetahs.

MATERIALS AND METHODS

Study animals and samples. In this retrospective study, blood samples that had been collected between June 2002 and July 2009 from 88 free-ranging cheetahs (63 males and 25 females) on commercial farmland in east-central Namibia were analyzed. To study samples from animals living close to human settlements, with potential contact with domestic cats, we also sampled 56 cheetahs (37 males and 19 females) held in enclosures within their natural habitat. Thirty of the captive cheetahs were housed at the AfriCat Foundation, a nonprofit conservation facility for carnivores in central Namibia, and 26 on privately owned farms. We collected heparinized plasma samples from the free-ranging cheetahs, and we collected heparinized plasma and/or serum samples and EDTA-treated whole-blood samples from the cheetahs housed at AfriCat and the 26 captive cheetahs housed on private farms. Because samples collected over time may provide evidence of seroconversion, some animals were repeatedly used for sample collection. Of the free-ranging cheetahs, five were sampled twice, one was sampled three times, and one was sampled four times. Among the captive cheetahs, seven were sampled twice, two were sampled three times, and one was sampled four times. One animal was sampled as a free-ranging cheetah and later as a captive cheetah and was counted in both categories. The intervals between repeated sampling times ranged between 1 month and 28 months. In this study, we used 72 serum and 26 heparinized plasma samples from free-ranging cheetahs and 29 serum and 45 heparinized plasma samples from captive cheetahs. In addition, we used 26 EDTA-treated whole-blood samples from free-ranging cheetahs and 15 from captive cheetahs. Depending on the question examined, different sample sets were used (see "Statistical analysis," below).

Free-ranging and captive animals were captured, anesthetized, sampled, clinically examined (body size determination, weight and temperature measurements, body palpation, and visual check for signs of infection), and released again, as described previously (13, 23). Captive animals were examined as part of the annual health check-up, which included vaccinations developed for domestic cats against FHV, FCV, FPV, and FeLV (Tricat [Nobivac], containing attenuated FHV, FCV, and FPV and the nonglycosylated envelope gene product expressed in *Escherichia coli*, or Fel-o-Vax Lv-K, containing killed FeLV) and rabies (Rabisin [Merial, Midrand, South Africa], containing inactivated rabies virus). Thirty of the plasma samples originated from cheetahs that had been vaccinated for several years. Five plasma samples originated from nonvaccinated cheetahs that were vaccinated for the first time on the day of examination and were tested again between 23 months and 28 months later.

Samples were collected from the vena saphena or vena cephalica into heparin, serum, and EDTA blood collection tubes (BD Vacutainer Systems, Plymouth, United Kingdom). The samples were kept at 4°C during transport to the field station or the Central Veterinary Laboratory in Windhoek, Namibia. All plasma, serum, and whole-blood samples were stored in liquid nitrogen. Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood samples from 35 captive and 22 free-ranging cheetahs through a Ficoll gradient using Histopaque (Sigma-Aldrich, Buchs, Switzerland) and Hanks' balanced salt solution (HBSS) (Invitrogen, Basel, Switzerland), as described previously (24), and were stored in liquid nitrogen. Plasma, serum, and whole-blood samples and PBMCs were transported to Switzerland at −80°C on dry ice or at −196°C in a dry shipper (Voyageur 12; Air Liquide, Paris, France), in full compliance with the Convention on International Trade in Endangered Species (CITES).

Enzyme-linked immunoabsorbent assays. Serum and plasma samples were tested for FeLV p27 antigen using a sandwich enzyme-linked immunoabsorbent assay (ELISA), as described previously (25, 26). Samples that reached an optical density (OD) of >20% of the positive-control value were considered true-positive samples, samples that reached ≥4% to 20% of the positive-control value were considered questionable, and samples with values below 4% were considered true-negative samples (25, 27). We also determined the presence of antibodies against FeLV p45 (the nonglycosylated form of the gp70 surface unit of the envelope glycoprotein) and whole FeLV, purified by ultracentrifugation (FL-74), by ELISA as described (28, 29), at a dilution of 1:200. Antibody levels were assessed by comparison with predefined positive-control sera, which consisted of pooled sera collected from FeLV regressor cats (29). To determine FL-74 ELISA cutoff values, we measured the mean plasma OD values for specific-pathogen-free (SPF) cats ($n = 15$, assayed in duplicate), as a percentage of the positive-control value (assigned to be 100%). The cutoff value was set at the mean value plus 2.58 times the standard deviation (99% confidence interval for all negative results). Determination of the cutoff value for the p45 ELISA was performed in the same way, with 5 SPF cats. A compilation of the numbers of animals and samples from free-ranging, captive nonvaccinated, and captive vaccinated cheetahs used for each test is presented in Table 1.

Western blot analysis. Western blotting (WB) was used to determine the presence of antibodies against FeLV gp70, p58, p27, and the two fragments of p15(E), using 1:100 dilutions of the samples (30). We used 24 serum and 26 plasma samples from 45 free-ranging cheetahs and 11 serum and 45 plasma samples from 45 captive cheetahs; depending on the question examined, different sample sets were used (see "Statistical analysis," below). Samples that showed antibodies to FeLV p27 plus one or two p15(E) fragments or to both p15(E) fragments only were considered positive (29, 31).

Antibody reactivity against other retroviruses was tested with serum samples from six randomly chosen FeLV-WB-positive and six FeLV-WB-negative free-ranging cheetahs. Samples were tested for reactivity against the following retroviruses (a generous gift to H.L. from the U.S. National Cancer Institute): baboon endogenous retrovirus, feline RD114 endogenous retrovirus, Rauscher murine leukemia virus (RMuLV), and AKR murine leukemia virus (AKR-MuLV). Antibodies were visually assessed by WB using 0.5 μg of antigen per strip for each virus preparation, with 1:50 dilution of the samples.

Total nucleic acid extraction. Total nucleic acids (TNA) were extracted from 100 μl EDTA-treated blood ($n = 41$) and 100 μl heparinized plasma ($n = 71$) after the addition of 100 μl of $MgCl_2$- and $CaCl_2$-free phosphate-buffered saline (PBS) (Invitrogen) to each volume. For this, the MagNA Pure LC TNA isolation kit (Roche Diagnostics, Rotkreuz, Switzerland) was used, following the manufacturer's instructions. TNA were eluted with 100 μl buffer (Roche) and stored at −20°C until further analysis.

Real-time PCR. The presence of amplifiable DNA in each of the 41 whole-blood TNA samples and the extraction controls was tested by a quantitative real-time PCR assay for feline glyceraldehyde-3-phosphate dehydrogenase (fGAPDH), as described previously (32). The presence of amplifiable exogenous FeLV viral RNA or DNA was determined by real-time reverse transcriptase (RT)-PCR or real-time PCR, respectively, in whole-blood and plasma TNA. Whole-blood TNA were tested for amplifiable endogenous FeLV sequences as described previously (33–35).

Product-enhanced reverse transcriptase assay. PBMCs were cultured in 5 ml medium consisting of RPMI 1640 medium (Sigma), 10% inactivated fetal calf serum (Invitrogen), 1% L-glutamine (Invitrogen), and 1% antibiotic/antimycotic (Invitrogen), in a 25-cm^2 cell culture flask (TPP, Trasadingen, Switzerland), and 10 μl of concanavalin A (5 mg/ml; Sigma) was added. After 1 day, 40 U/ml of interleukin 2 (IL-2) (Sandoz Pharmaceuticals AG, Cham, Switzerland) was added to the culture. Five milliliters of medium and IL-2 at 8 U/ml were added to the culture at day 3, and then 5 ml of medium was replaced and IL-2 at 8 U/ml was added to the culture every 3 to 4 days. Once a week, 5 ml of supernatant was collected, centrifuged for 10 min at 1,500 × *g*, and stored in 1-ml aliquots at −80°C. After 4 weeks, cells were frozen again and stored in liquid nitrogen. RT activity was assessed in supernatants collected in the fourth week of the PBMC cultures, at the Swiss National Center for Retroviruses, Uni-

TABLE 1 Serological results of ELISAs for the presence of FeLV p27 antigens and antibodies against FeLV p45 and FeLV whole virus (FL-74) in free-ranging, captive nonvaccinated, and captive vaccinated cheetahs

ELISA[a]	Free-ranging		Captive nonvaccinated		Captive vaccinated	
	No. (%) of animals	No. (%) of samples	No. (%) of animals	No. (%) of samples	No. (%) of animals	No. (%) of samples
P27						
>20% (positive)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
≥4% to ≤20%	6 (7)	6 (6)	3 (11)	3 (8)	2 (6)	2 (6)
<4% (negative)	82 (93)	92 (94)	25 (89)	36 (92)	33 (94)	33 (94)
Total tested	88	98	26	39	35	35
Sum	88 (100)	98 (100)	28 (100)[b,c]	39 (100)	35 (100)	35 (100)
P45						
>20% (positive)	1 (1)	1 (1)	3 (11)	6 (20)	25 (71)	25 (71)
≤20% (negative)	69 (99)	74 (99)	24 (89)	24 (80)	10 (29)	10 (29)
Total tested	70	75	26	30	35	35
Sum	70 (100)	75 (100)	27 (100)[b,c]	30 (100)	35 (100)	35 (100)
FL-74						
>22% (positive)	17 (19)	21 (21)	7 (25)	9 (23)	30 (86)	30 (86)
≤22% (negative)	72 (81)	77 (79)	21 (75)	30 (77)	5 (14)	5 (14)
Total tested	88	98	26	39	35	35
Sum	89 (100)[b]	98 (100)	28 (100)[b]	39 (100)	35 (100)	35 (100)

[a] Samples with OD values of ≥20% (P27 and P45) or ≥22% (FL-74) were considered positive; samples with OD values of ≥4% and ≤20% were considered questionable and samples with OD values of <4% were considered negative for p27 antigens.
[b] The sum of the animals is greater than the total number of animals because animals captured and tested repeatedly were classified in different OD levels (see the text).
[c] The sum of the animals is greater than the total number of animals because animals tested once with plasma and once with serum were classified in different OD levels (see the text).

versity of Zurich (Zurich, Switzerland), using the product-enhanced reverse transcriptase (PERT) assay (36). RT activity was also assessed in 12 heparinized plasma samples from WB- and ELISA-negative animals. Potential inhibition by heparin was excluded by testing a positive RT sample together with aliquots with and without heparin. As no inhibition was observed, no correction of the measured RT values was necessary.

Statistical analysis. All tests were performed using Systat 13.0 (Systat Software Inc., Richmond, VA). To assess whether vaccinated animals differed from nonvaccinated animals in the serological assays, we used the three categories of free-ranging, captive nonvaccinated, and captive vaccinated cheetahs. For nonvaccinated animals sampled repeatedly, we randomly selected one test result for the analyses, so that each animal was represented only once in the analyses. For the five captive animals tested both as nonvaccinated and as vaccinated, we used only the nonvaccinated results, because all other vaccinated animals had been vaccinated for several years (see Results). As serological data were not normally distributed (Lilliefors test, $P < 0.05$), comparisons were performed using nonparametric Kruskal-Wallis tests. WB results were assessed using a chi-square test. Results for male and female animals of each group and for each test were compared with Fisher's exact test.

RESULTS

Study animals. None of the study animals showed any clinical signs of an infectious disease, such as fever, enlarged lymph nodes, pale mucus membranes, anemia, or prolonged capillary refill time, or was lethargic or dehydrated.

Serological assays. (i) P27 ELISA. None of the free-ranging, captive nonvaccinated, or captive vaccinated cheetahs tested positive for p27 (Table 1). All seven repeatedly tested free-ranging cheetahs and five of the seven repeatedly tested captive nonvaccinated cheetahs (71%) were negative in all tests. None of the five captive cheetahs that were sampled as nonvaccinated animals and 2 years later as vaccinated against FeLV turned positive. Three of the 30 AfriCat cheetahs (10%) kept at the facility of the AfriCat Foundation, all of which had been vaccinated for several years, tested questionable; all others tested negative.

(ii) P45 ELISA. One free-ranging (1%), three captive nonvaccinated (11%), and 25 captive vaccinated (71%) cheetahs tested positive (Table 1). All three repeatedly tested free-ranging cheetahs and two of the three repeatedly tested captive nonvaccinated cheetahs revealed the same results for the repeated tests. Two of the five captive cheetahs that were sampled as nonvaccinated animals and 2 years later as vaccinated animals tested positive for both samples, and two tested negative for both samples; only one of the animals turned from negative to positive. Twenty-two of the AfriCat cheetahs (73%) tested positive. The OD values differed for the three categories (Kruskal-Wallis test statistic, 58.46; $P < 0.0001$; free-ranging, $n = 70$; nonvaccinated, $n = 25$; vaccinated, $n = 30$), with captive vaccinated cheetahs having higher values than free-ranging cheetahs (Conover-Inman test for pairwise comparisons, $P < 0.0001$) or captive nonvaccinated cheetahs ($P < 0.0001$). Free-ranging cheetahs had OD values similar to those for captive nonvaccinated cheetahs ($P = 0.11$) (Fig. 1).

(iii) FL-74 whole-virus ELISA. Seventeen free-ranging (19%), seven captive nonvaccinated (27%), and 30 captive vaccinated cheetahs (86%) tested positive (Table 1). Six of the seven repeat-

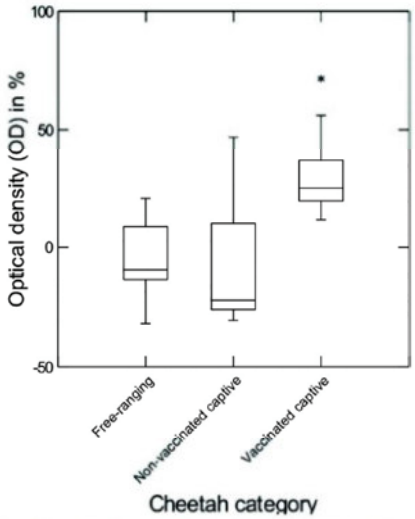

FIG 1 Optical densities obtained for samples from free-ranging, captive nonvaccinated, and captive vaccinated cheetahs in antibody tests for FeLV p45. Captive vaccinated cheetahs had higher OD levels than free-ranging ($P < 0.0001$) and captive nonvaccinated ($P < 0.0001$) cheetahs, whereas the latter two had similar OD levels ($P = 0.11$). Box and whisker plot: lower line, 25th percentile; middle line, 50th percentile; upper line, 75th percentile; whiskers, 5th and 95th percentiles, respectively. *, outlier.

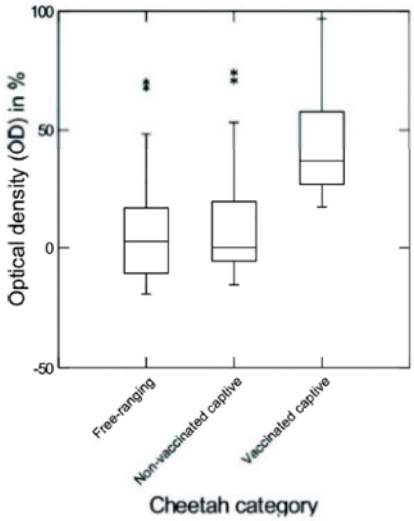

FIG 2 Optical densities obtained for samples from free-ranging, captive nonvaccinated, and captive vaccinated cheetahs in antibody tests for FeLV FL-74. Captive vaccinated cheetahs had higher OD levels than free-ranging ($P < 0.0001$) and captive nonvaccinated ($P < 0.0001$) cheetahs, whereas the latter two had similar OD levels ($P = 0.20$). Box and whisker plot: lower line, 25th percentile; middle line, 50th percentile; upper line, 75th percentile; whiskers, 5th and 95th percentiles, respectively. *, outliers.

edly tested free-ranging cheetahs (86%) and five of the seven repeatedly tested captive nonvaccinated cheetahs (29%) revealed the same results for the repeated tests. Two of the five captive cheetahs that were sampled as nonvaccinated animals and 2 years later as vaccinated animals tested positive for both samples, and two tested negative for both samples; only one of the animals turned from negative to positive. Twenty-seven of the AfriCat cheetahs (90%) tested positive. The OD values differed for the three categories (Kruskal-Wallis test statistic, 46.38; $P < 0.0001$; free-ranging, $n = 88$; nonvaccinated, $n = 25$; vaccinated, $n = 30$), with captive vaccinated cheetahs having higher values than free-ranging cheetahs (Conover-Inman test for pairwise comparisons, $P < 0.0001$) or captive nonvaccinated cheetahs ($P < 0.0001$). Antibodies to FL-74 virus increased with the time of presence in the AfriCat facility ($R = 0.37$, $P = 0.044$). Free-ranging cheetahs had OD values similar to those for captive nonvaccinated cheetahs ($P = 0.20$) (Fig. 2).

Western blotting. Twenty-seven free-ranging (60%), 10 captive nonvaccinated (60%), and 24 captive vaccinated cheetahs (69%) were positive (Table 2). All three repeatedly tested free-ranging cheetahs revealed the same results for the repeated tests. Two of five captive cheetahs that were sampled as nonvaccinated animals and 2 years later as vaccinated animals tested positive for both samples, and two tested negative for both samples; one animal turned, contrary to expectation, from positive to negative. The vaccinated AfriCat cheetahs did not differ from captive nonvaccinated and free-ranging cheetahs in their proportions of positive and negative samples ($\chi^2 = 1.43$; free-ranging positive, $n = 27$; free-ranging negative, $n = 18$; nonvaccinated positive, $n = 10$; nonvaccinated negative, $n = 5$; vaccinated positive, $n = 22$; vaccinated negative, $n = 8$; $P = 0.49$).

None of the cheetahs tested positive for all four tests performed. Of the 34 free-ranging, 13 captive nonvaccinated, and 34 captive vaccinated cheetahs that tested positive for at least one of the tests, nine (27%), five (39%), and nine (27%), respectively, were positive for a second test and four (12%), one (8%), and 18 (53%), respectively, were positive for three tests. There were no significant differences in the proportions of seropositive or seronegative males and females for p45 ELISA, FL-74 ELISA, and WB.

Of the six FeLV-WB-positive and six FeLV-WB-negative free-ranging animals that were selected for further WB evaluation for four other retroviruses, the six WB-positive animals also demonstrated antibodies against the two p15(E) fragments of RMuLV. The six WB-negative animals tested negative for all four retroviruses.

Real-time PCR. Forty of 41 whole-blood TNA samples showed acceptable amounts of genomic DNA (>10,000 copies/reaction [33]) and were selected for further analysis. PCR assays for endogenous and exogenous FeLV were negative for all animals.

PERT assay. We tested PBMC cultures from seven WB-positive samples and one WB-negative sample from free-ranging animals. The PERT assays were negative. The RT activity of cell culture supernatants was 0 to 16 nU/ml, and that of plasma from 12 WB- and ELISA-negative animals was 0 to 1 nU/ml. Positive-control samples had an activity range of 10,000 to 20,000 nU/ml.

DISCUSSION

FeLV infecting free-ranging felids can threaten entire populations (18, 37). To date, the free-ranging Namibian felid population has been spared retroviral infections (12, 13, 38, 39); the Kalahari Desert may act as a natural barrier, keeping the Namibian populations naive to pathogens present in adjacent countries (10, 11,

TABLE 2 Western blot results for the presence of antibodies against FeLV p27 and FeLV p15(E) in free-ranging, captive nonvaccinated, and captive vaccinated cheetahs

Western blot	Free-ranging		Captive nonvaccinated		Captive vaccinated	
	No. (%) of animals	No. (%) of samples	No. (%) of animals	No. (%) of samples	No. (%) of animals	No. (%) of samples
FeLV[a]						
Positive for both p15(E) fragments	13 (29)	16 (32)	6 (37)	10 (47)	23 (66)	23 (66)
Positive for p27 + p15(E)	14 (31)	15 (30)	4 (26)	5 (24)	1 (3)	1 (3)
Negative	18 (40)	19 (38)	6 (37)	6 (29)	11 (31)	11 (31)
Total tested	45	50	15	21	35	35
Sum	45 (100)	50 (100)	16 (100)[b]	21 (100)	35 (100)	35 (100)
Positive for p15(E) of[c]						
BeRV	0 (0)	0 (0)	ND	ND	ND	ND
FeRD114eRV	0 (0)	0 (0)	ND	ND	ND	ND
RMuLV	6 (50)[d]	6 (50)[d]	ND	ND	ND	ND
AKR-MLV	0 (0)	0 (0)	ND	ND	ND	ND
Total tested	12	12				

[a] Samples were considered positive when antibodies were detected against FeLV p27 plus one or two p15(E) fragments or against both p15(E) fragments only.
[b] The sum of the animals is greater than the total number of animals because animals captured and tested repeatedly were positive for different fragments (see the text).
[c] Six FeLV-p15(E)-positive and six FeLV-p15(E)-negative samples from 12 free-ranging cheetahs were used to test antibody reactivity against four additional retroviruses. BeRV, baboon endogenous retrovirus; FeRD114eRV, feline RD114 endogenous retrovirus; RMuLV, Rauscher murine leukemia virus; AKR-MLV, AKR murine leukemia virus; ND, not done.
[d] All six RMuLV-p15(E)-positive samples were also FeLV-p15(E)-positive samples.

40, 41). In our study, we detected antibodies against FeLV and RMuLV but no FeLV viral DNA or reverse transcriptase activity in PBMCs or plasma samples. If the Kalahari Desert is a natural barrier to pathogens, then the undetected retrovirus triggering measurable antibodies against FeLV and RMuLV in Namibian cheetahs might be an endemic one. Previous studies testing carnivores for FeLV used only antigen tests (12, 13), whereas we also used antibody tests in this study. Using antibody tests also in countries across the Kalahari Desert might provide new insights into the distribution of retroviruses in southern Africa.

Because antibodies that are able to recognize FeLV can cross-react with RMuLV (29, 42) but not with the more distantly related baboon or feline RD114 endogenous retrovirus, we speculate that Namibian cheetahs are either infected with a retrovirus or harbor a differentially expressed endogenous retrovirus that belongs to the gammaretrovirus mammalian group subfamily. No antibody reactivity to p15(E) of AKR-MuLV was detected, although AKR-MuLV p15(E) differs from FeLV and RMuLV p15(E) at only one amino acid position each. Thus, the observation that cheetah antibodies do not recognize AKR-MuLV p15(E) could be explained by the fact that AKR-MuLV p15(E) is different in a conformational epitope not present in FeLV and RMuLV p15(E).

Alternatively, the presence of reactive antibodies against RMuLV in all six FeLV-WB-positive samples might be due to the activation of endogenous gammaretroviruses not directly related to an infectious disease. A similar observation has been reported recently for humans diagnosed with recent-onset psychosis (43). Those subjects developed antibodies cross-reactive to MuLV and the Mason-Pfizer monkey virus (a simian D-type betaretrovirus), whereas patients with long-term schizophrenia or control subjects did not exhibit such cross-reactive antibodies (43). Although this underlying cause is unlikely to be important for cheetahs, aberrant endogenous retrovirus expression might also be triggered by other factors and was reported to be involved in defective mice spermatogenesis (44). Defective spermatogenesis can result in teratospermia, i.e., the production of >60% morphologically abnormal sperm per ejaculate, which is a well-known phenomenon in felids, particularly cheetahs (45–47). However, the capacities of ejaculates from proven and unproven cheetah male breeders to inseminate oozytes *in vitro* do not differ (46), suggesting that teratospermia in cheetahs is not of major concern for captive or free-ranging populations, particularly not for the world's largest population in Namibia. Still, aberrant endogenous retrovirus expression might be involved in the production of teratospermic ejaculates in cheetahs, eventually becoming problematic for particular individuals if teratospermia reaches levels that lead to infertility.

In domestic cats viremic with FeLV, antibody-positive samples may show low levels of replicating virus, whereas viremic cats with samples with low or undetectable antibody levels may have productive infections with high levels of replicating virus (48). Therefore, samples from FeLV-WB-positive and WB-negative animals were used in the PERT assay. The negative PERT results may be due to complete repression of the virus in the antibody-positive animals or the absence of infection in the antibody-negative animals. Animals with productive infections might exist in the wild only rarely or infections might have only a very short phase of viremia, with such animals having a rather low probability of being captured and sampled. Additional analyses using, for example, targeted high-throughput sequencing of genomic DNA would be required to identify the unknown retroviral strain.

The fact that all of the animals were healthy also suggests that this virus may not be very pathogenic and/or has a short course of infection. This hypothesis is supported by the recent demonstration of nearly asymptomatic experimental infection of *Mus pahari* (a mice strain lacking endogenous murine leukemia viruses and gammaretrovirus restriction factor fv1 and seen as a transspecies

transmission model for gammaretroviruses) with xenotropic murine leukemia virus-related gammaretrovirus (XMRV) (49, 50); while viral RNA was detected in the plasma of only 1/10 infected mice, 10/12 infected mice developed persistent neutralizing antibodies directed against Gag and Env proteins.

Therefore, in analogy to the situation in *Mus pahari*, we speculate that cheetahs, in the context of hunting (22), become exposed to a gammaretrovirus present in wild mice. This virus might be able to transiently infect cheetahs, which develop an effective immune response, seroconvert, and are able to overcome this retroviral infection. Alternatively, cheetahs may react to low-level exposure to FeLV like domestic cats. In domestic cats exposed experimentally to low levels of FeLV, seroconversion to FeLV was observed but FeLV proviral DNA was found to be present only in very small amounts in some internal organs (31, 51).

The good health status of the seropositive cheetahs further suggests that, despite the relatively low MHC class I variability, the cheetah population can mount effective immune responses against the gammaretrovirus investigated in this study. Low MHC variability, however, might limit the immunocompetence of individuals when confronted with new pathogens (2, 16).

Because Namibian cheetahs are potentially susceptible to FeLV (9), captive animals were preventively vaccinated with a vaccine widely used for domestic cats. We attempted to assess the magnitude of the immune responses after cheetahs have been vaccinated according to a protocol used for domestic cats (28). The cheetahs housed at AfriCat that had been vaccinated for several years had higher OD values for p45 and FL-74 than did free-ranging and captive nonvaccinated cheetahs but similar OD values for p27 and similar patterns of antigen recognition in WB. The increased levels of antibodies to FL-74 virus in cheetahs with long residence at AfriCat can be explained by repeated FeLV vaccinations. Thus, tests for antibodies to p45 and FL-74 virus seem to be useful to assess the effects of FeLV vaccination on antibody induction also in cheetahs. From the 71% and 86% positive responses to p45 and FL-74 antigens, respectively, in the vaccinated cheetahs at AfriCat, it can be concluded that cheetahs readily respond to FeLV vaccination. However, of the five captive animals that were vaccinated once and retested 2 years later, only one animal (20%) demonstrated higher OD values for p45 and FL-74 in the second test. This supports the concept that boosting may induce increased levels of antibodies. However, increased levels of antibodies are not necessarily identical to protection. In view of the presence of domestic cats infected with FeLV, it might be useful to continue with the vaccination program in captive animals.

In conclusion, we detected the presence of antibodies reacting with a FeLV-related gammaretrovirus in Namibian cheetahs. As the animals were in good health, we conclude that this infection of unknown origin may be endemic in Namibian cheetahs and that the affected animals can mount efficient immune responses and cope with the virus despite low MHC variability. Because no replicating virus was detected, further studies are required to identify its origin. As most of the FeLV-vaccinated cheetahs could mount immune responses to the vaccine, it appears to be meaningful to continue with the vaccination program against FeLV as a preventive measure in captive animals.

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REFERENCES

1. **Durant S, Marker L, Purchase N, Belbachir F, Hunter L, Packer C, Breitenmoser-Wursten C, Sogbohossou E, Bauer H.** 2008. *Acinonyx jubatus*, *on* The IUCN Red List of Threatened Species. http://www.iucnredlist.org/details/219/0. Accessed 24 July 2011.
2. **Castro-Prieto A, Wachter B, Sommer S.** 2011. Cheetah paradigm revisited: MHC diversity in the world's largest free-ranging population. Mol Biol Evol 28:1455–1468. http://dx.doi.org/10.1093/molbev/msq330.
3. **O'Brien SJ, Roelke ME, Marker L, Newman A, Winkler CA, Meltzer D, Colly L, Evermann JF, Bush M, Wildt DE.** 1985. Genetic basis for species vulnerability in the cheetah. Science 227:1428–1434. http://dx.doi.org/10.1126/science.2983425.
4. **O'Brien SJ, Wildt DE, Bush M.** 1986. The cheetah in genetic peril. Sci Am 254:84–92. http://dx.doi.org/10.1038/scientificamerican0586-84.
5. **O'Brien SJ, Evermann JF.** 1988. Interactive influence of infectious disease and genetic diversity in natural populations. Trends Ecol Evol 3:254–259. http://dx.doi.org/10.1016/0169-5347(88)90058-4.
6. **Evermann JF, Roelke ME, Briggs MB.** 1986. Feline coronavirus infection of cheetahs. Feline Pract 16:21–30.
7. **Evermann JF, Heeney JL, Roelke ME, McKeirnan AJ, O'Brien SJ.** 1988. Biological and pathological consequences of feline infectious peritonitis virus infection in the cheetah. Arch Virol 102:155–171. http://dx.doi.org/10.1007/BF01310822.
8. **Marker L, O'Brien SJ.** 1989. Captive breeding of the cheetahs (*Acinonyx jubatus*) in North American zoos (1871–1986). Zoo Biol 8:3–16. http://dx.doi.org/10.1002/zoo.1430080103.
9. **Marker L, Munson L, Basson PA, Quackenbush S.** 2003. Multicentric T-cell lymphoma associated with feline leukemia virus infection in a captive Namibian cheetah (*Acinonyx jubatus*). J Wildl Dis 39:690–695. http://dx.doi.org/10.7589/0090-3558-39.3.690.
10. **Olmsted RA, Langley R, Roelke ME, Goeken RM, Adger-Johnson D, Goff JP, Albert JP, Packer C, Laurenson MK, Caro TM, Scheepers L, Wildt DE, Bush M, Martenson JS, O'Brien SJ.** 1992. Worldwide prevalence of lentivirus infection in wild feline species: epidemiologic and phylogenetic aspects. J Virol 66:6008–6018.
11. **Brown EW, Miththapala S, O'Brien SJ.** 1993. Prevalence of exposure to feline immunodeficiency virus in exotic felids. J Zoo Wildl Med 24:357–364.
12. **Munson L, Marker L, Dubovi E, Spencer JA, Evermann JF, O'Brien SJ.** 2004. Serosurvey of viral infections in free-ranging Namibian cheetahs (*Acinonyx jubatus*). J Wildl Dis 40:23–31. http://dx.doi.org/10.7589/0090-3558-40.1.23.
13. **Thalwitzer S, Wachter B, Robert N, Wibbelt G, Muller T, Lonzer J, Meli ML, Bay G, Hofer H, Lutz H.** 2010. Seroprevalences to viral pathogens in free-ranging and captive cheetahs (*Acinonyx jubatus*) on Namibian farmland. Clin Vaccine Immunol 17:232–238. http://dx.doi.org/10.1128/CVI.00345-09.
14. **Thalwitzer S.** 2007. Reproductive activity in cheetah females, cub survival and health of male and female cheetahs on Namibian farmland. Ph.D. dissertation. Freie Universität, Berlin, Germany.
15. **Munson L, Terio KA, Worley M, Jago M, Bagot-Smith A, Marker L.** 2005. Extrinsic factors significantly affect patterns of disease in free-ranging and captive cheetah (*Acinonyx jubatus*) populations. J Wildl Dis 41:542–548. http://dx.doi.org/10.7589/0090-3558-41.3.542.
16. **Radwan J, Biedrzycka A, Babik W.** 2010. Does reduced MHC diversity

decrease viability of vertebrate populations? Biol Conserv 143:537–544. http://dx.doi.org/10.1016/j.biocon.2009.07.026.
17. Brown MA, Cunningham MW, Roca AL, Troyer JL, Johnson WE, O'Brien SJ. 2008. Genetic characterization of feline leukemia virus from Florida panthers. Emerg Infect Dis 14:252–259. http://dx.doi.org/10.3201/eid1402.070981.
18. Meli ML, Cattori V, Martinez F, Lopez G, Vargas A, Simon MA, Zorrilla I, Munoz A, Palomares F, Lopez-Bao JV, Pastor J, Tandon R, Willi B, Hofmann-Lehmann R, Lutz H. 2009. Feline leukemia virus and other pathogens as important threats to the survival of the critically endangered Iberian lynx (*Lynx pardinus*). PLoS One 4:e4744. http://dx.doi.org/10.1371/journal.pone.0004744.
19. Schneider HP. 1991. Animal health and veterinary medicine in Namibia. Agrivet, Windhoek, Namibia.
20. Marker-Kraus L, Kraus D, Barnett D, Hurlbut S. 1996. Cheetah survival on Namibian farmlands. Cheetah Conservation Fund, Otjiwarongo, Namibia.
21. Mendelsohn J, Jarvis A, Roberts C, Robertson T. 2002. Atlas of Namibia: a portrait of the land and its people. David Philip Publisher, Cape Town, South Africa.
22. Malan JS. 1995. Peoples of Namibia. Rhino Publisher, Pretoria, South Africa.
23. Wachter B, Thalwitzer S, Hofer H, Lonzer J, Hildebrandt TB, Hermes R. 2011. Reproductive history and absence of predators are important determinants of reproductive fitness: the cheetah controversy revisited. Conserv Lett 4:47–54. http://dx.doi.org/10.1111/j.1755-263X.2010.00142.x.
24. Pepin AC, Tandon R, Cattori V, Niederer E, Riond B, Willi B, Lutz H, Hofmann-Lehmann R. 2007. Cellular segregation of feline leukemia provirus and viral RNA in leukocyte subsets of long-term experimentally infected cats. Virus Res 127:9–16. http://dx.doi.org/10.1016/j.virusres.2007.03.008.
25. Lutz H, Pedersen NC, Theilen GH. 1983. Course of feline leukemia virus infection and its detection by enzyme-linked immunosorbent assay and monoclonal antibodies. Am J Vet Res 44:2054–2059.
26. Hofmann-Lehmann R, Huder JB, Gruber S, Boretti F, Sigrist B, Lutz H. 2001. Feline leukaemia provirus load during the course of experimental infection and in naturally infected cats. J Gen Virol 82:1589–1596.
27. Lutz H, Pedersen NC, Durbin R, Theilen GH. 1983. Monoclonal antibodies to three epitopic regions of feline leukemia virus p27 and their use in enzyme-linked immunosorbent assay of p27. J Immunol Methods 56:209–220. http://dx.doi.org/10.1016/0022-1759(83)90413-1.
28. Lehmann R, Franchini M, Aubert A, Wolfensberger C, Cronier J, Lutz H. 1991. Vaccination of cats experimentally infected with feline immunodeficiency virus, using a recombinant feline leukemia virus vaccine. J Am Vet Med Assoc 199:1446–1452.
29. Lutz H, Pedersen N, Higgins J, Hubscher U, Troy FA, Theilen GH. 1980. Humoral immune reactivity to feline leukemia virus and associated antigens in cats naturally infected with feline leukemia virus. Cancer Res 40:3642–3651.
30. Lutz H, Arnold P, Hubscher U, Egberink H, Pedersen N, Horzinek MC. 1988. Specificity assessment of feline T-lymphotropic lentivirus serology. Zentralbl Veterinarmed B 35:773–778.
31. Gomes-Keller MA, Gönczi E, Grenacher B, Tandon R, Hofman-Lehmann R, Lutz H. 2009. Fecal shedding of infectious feline leukemia virus and its nucleic acids: a transmission potential. Vet Microbiol 134:208–217. http://dx.doi.org/10.1016/j.vetmic.2008.08.011.
32. Leutenegger CM, Mislin CN, Sigrist B, Ehrengruber MU, Hofmann-Lehmann R, Lutz H. 1999. Quantitative real-time PCR for the measurement of feline cytokine mRNA. Vet Immunol Immunopathol 71:291–305. http://dx.doi.org/10.1016/S0165-2427(99)00100-2.
33. Tandon R, Cattori V, Willi B, Meli ML, Gomes-Keller MA, Lutz H, Hofmann-Lehmann R. 2007. Copy number polymorphism of endogenous feline leukemia virus-like sequences. Mol Cell Probes 21:257–266. http://dx.doi.org/10.1016/j.mcp.2007.01.003.
34. Tandon R, Cattori V, Willi B, Lutz H, Hofmann-Lehmann R. 2008. Quantification of endogenous and exogenous feline leukemia virus sequences by real-time PCR assays. Vet Immunol Immunopathol 123:129–133. http://dx.doi.org/10.1016/j.vetimm.2008.01.027.
35. Tandon R, Cattori V, Gomes-Keller MA, Meli ML, Golder MC, Lutz H, Hofmann-Lehmann R. 2005. Quantitation of feline leukaemia virus viral and proviral loads by TaqMan® real-time polymerase chain reaction. J Virol Methods 130:124–132. http://dx.doi.org/10.1016/j.jviromet.2005.06.017.
36. Pyra H, Böni J, Schüpbach J. 1994. Ultrasensitive retrovirus detection by a reverse transcriptase assay based on product enhancement. Proc Natl Acad Sci U S A 91:1544–1548. http://dx.doi.org/10.1073/pnas.91.4.1544.
37. Cunningham MW, Brown MA, Shindle DB, Terrell SP, Hayes KA, Ferree BC, McBride RT, Blankenship EL, Jansen D, Citino SB, Roelke ME, Kiltie RA, Troyer JL, O'Brien SJ. 2008. Epizootiology and management of feline leukemia virus in the Florida puma. J Wildl Dis 44:537–552. http://dx.doi.org/10.7589/0090-3558-44.3.537.
38. Spencer JA, Morkel P. 1993. Serological survey of sera from lions in Etosha National Park. S Afr J Wildl Res 23:60–61.
39. Letcher JD, O'Conner TP. 1991. Incidence of antibodies reacting to feline immunodeficiency virus in a population of Asian lions. J Zoo Wildl Med 22:324–329.
40. Roelke ME, Brown MA, Troyer JL, Winterbach H, Winterbach C, Hemson G, Smith D, Johnson RC, Pecon-Slattery J, Roca AL, Alexander KA, Klein L, Martelli P, Krishnasamy K, O'Brien SJ. 2009. Pathological manifestations of feline immunodeficiency virus (FIV) infection in wild African lions. Virology 390:1–12. http://dx.doi.org/10.1016/j.virol.2009.04.011.
41. Osofsky SA, Hirsch KJ, Zuckermann EE, Hardy WD. 1996. Feline lentivirus and feline oncovirus status of free-ranging lions (*Panthera leo*), leopards (*Panthera pardus*), and cheetahs (*Acinonyx jubatus*) in Botswana: a regional perspective. J Zoo Wildl Med 27:453–467.
42. Parks WP, Scolnick EM. 1972. Radioimmunoassay of mammalian type-C viral proteins: interspecies antigenic reactivities of the major internal polypeptide. Proc Natl Acad Sci U S A 69:1766–1770. http://dx.doi.org/10.1073/pnas.69.7.1766.
43. Dickerson F, Lillehoj E, Stallings C, Wiley M, Origoni A, Vaughan C, Khushalani S, Sabunciyan S, Yolken R. 2012. Antibodies to retroviruses in recent onset psychosis and multi-episode schizophrenia. Schizophr Res 138:198–205. http://dx.doi.org/10.1016/j.schres.2012.03.037.
44. Ollinger R, Childs AJ, Burgess HM, Speed RM, Lundegaard PR, Reynolds N, Gray NK, Cooke HJ, Adams IR. 2008. Deletion of the pluripotency-associated Tex19.1 gene causes activation of endogenous retroviruses and defective spermatogenesis in mice. PLoS Genet 4:e1000199. http://dx.doi.org/10.1371/journal.pgen.1000199.
45. Pukazhenthi BS, Wildt DE, Howard JG. 2001. The phenomenon and significance of teratospermia in felids. J Reprod Fertil Suppl 57:423–433.
46. Pukazhenthi BS, Neubauer K, Jewgenow K, Howard J, Wildt DE. 2006. The impact and potential etiology of teratospermia in the domestic cat and its wild relatives. Theriogenology 66:112–121. http://dx.doi.org/10.1016/j.theriogenology.2006.03.020.
47. Wildt DE, Bush M, Howard JG, O'Brien SJ, Meltzer D, Van Dyk A, Ebedes H, Brand DJ. 1983. Unique seminal quality in the South African cheetah and a comparative evaluation in the domestic cat. Biol Reprod 29:1019–1025. http://dx.doi.org/10.1095/biolreprod29.4.1019.
48. Hofmann-Lehmann R, Cattori V, Tandon R, Boretti FS, Meli ML, Riond B, Lutz H. 2008. How molecular methods change our views of FeLV infection and vaccination. Vet Immunol Immunopathol 123:119–123. http://dx.doi.org/10.1016/j.vetimm.2008.01.017.
49. Sakuma T, Tonne JM, Squillace KA, Ohmine S, Thatava T, Peng KW, Barry MA, Ikeda Y. 2011. Early events in retrovirus XMRV infection of the wild-derived mouse *Mus pahari*. J Virol 85:1205–1213. http://dx.doi.org/10.1128/JVI.00886-10.
50. Sakuma T, Tonne JM, Malcolm JA, Thatava T, Ohmine S, Peng KW, Ikeda Y. 2012. Long-term infection and vertical transmission of a gammaretrovirus in a foreign host species. PLoS One 7:e29682. http://dx.doi.org/10.1371/journal.pone.0029682.
51. Major A, Cattori V, Boenzli E, Riond B, Ossent P, Meli ML, Hofmann-Lehmann R, Lutz H. 2010. Exposure of cats to low doses of FeLV: seroconversion as the sole parameter of infection. Vet Res 41:17. http://dx.doi.org/10.1051/vetres/2009065.

3. First evidence of hemoplasma infection in free-ranging Namibian cheetahs (*Acinonyx jubatus*)

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Short communication

First evidence of hemoplasma infection in free-ranging Namibian cheetahs (*Acinonyx jubatus*)

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ABSTRACT

Infections with feline hemotropic mycoplasmas (hemoplasmas) have been documented in domestic cats and free-ranging feline species with high prevalences in Iberian lynxes (*Lynx pardinus*), Eurasian lynxes (*Lynx lynx*), European wildcats (*Felis silvestris silvestris*), African lions (*Panthera leo*) in Tanzania and domestic cats in South Africa. The prevalence of hemoplasmas has not yet been investigated in free-ranging felids in southern Africa. In this study we screened 73 blood samples from 61 cheetahs in central Namibia for the presence of hemoplasmas using quantitative real-time PCR. One of the cheetahs tested PCR-positive. Phylogenetic analysis based on partial sequencing of the 16S rRNA and RNAse P genes revealed that the isolate belongs to the *Mycoplasma haemofelis/haemocanis* group. This is the first molecular evidence of a hemoplasma infection in a free-ranging cheetah.

1. Introduction

Hemotropic mycoplasmas (hemoplasmas) are cell wall free bacteria that parasitize red blood cells. In felids three hemoplasma species were described: *Mycoplasma haemofelis* (Mhf), '*Candidatus* Mycoplasma turicensis' (CMt) and '*Candidatus* Mycoplasma haemominutum' (CMhm) (Foley and Pedersen, 2001; Neimark et al., 2001; Willi et al., 2005, 2006a). Mhf is the causative agent of feline infectious anemia, leading to severe macrocytic normochromic anemia and inducing acute hemolysis associated with anorexia, lethargy and death (Foley et al., 1998; Westfall et al., 2001).

Hemoplasma infections were previously detected in various captive feline species and in free-ranging Iberian and Eurasian lynxes (*Lynx pardinus* and *Lynx lynx*), European wildcats (*Felis silvestris silvestris*) and African lions (*Panthera leo*) from Tanzania (Willi et al., 2007; Munson et al., 2008; Meli et al., 2009). In one study free-ranging animals had higher prevalence than captive animals (Willi et al., 2007), high-lightening the importance of studying hemoplasma infections in free-ranging felids.

The present study examined the prevalence of hemoplasma infection in Southern Africa in the worldwide largest free-ranging cheetah (*Acinonyx jubatus*) population. This population inhabits the farmland in Namibia and it is important to identify the pathogens that might threaten this valuable cheetah population. Throughout Namibia, free-ranging cheetahs are in a good health status and no clinical signs of viral infections have been detected (Munson et al., 2005; Thalwitzer et al., 2010).

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2. Materials and methods

2.1. Study animals and sample collection

Seventy-three blood samples collected between June 2002 and July 2009 from 61 cheetahs in central Namibia were tested for hemoplasma infection. The ratio of adult males ($n = 24$) to adult females ($n = 20$) and juvenile males ($n = 5$) to juvenile females ($n = 12$) was similar ($\chi^2 = 3.106$; $P = 0.078$; $n = 61$).

Cheetahs were captured, anesthetized and radio-collared as previously described in Wachter et al. (2011). The animals were clinically examined and blood was collected. Samples were shipped to Switzerland on dry ice in full compliance with the Convention on International Trade in Endangered Species (CITES).

2.2. Nucleic acid isolation and hemoplasma PCR assays

Total nucleic acids (TNA) were extracted from 100 μl of EDTA-anticoagulated blood using the MagNA Pure LC® TNA isolation kit (Roche Diagnostics, Rotkreuz, Switzerland). The elution volume was 100 μl. Presence of amplifiable TNA was confirmed using a quantitative real-time PCR assay for feline glyceraldehydes-3-phosphate dehydrogenase (GAPDH) (Leutenegger et al., 1999). Real-time PCR assays for the detection of Mhf, CMt and CMhm were performed as previously described (Willi et al., 2005, 2006a). Owing to high sequence similarity, *Mycoplasma haemocanis* (Mhc) is also amplified in the Mhf assay (Willi et al., 2006a). To detect possible new strains a general SYBR Green PCR that detects a broad range of hemoplasmas was additionally conducted as previously described (Willi et al., 2009).

2.3. Sequencing of partial 16S rRNA and RNase P genes

We used the conserved 16S rRNA gene for a general classification of the isolate. A 1'313 bp-long stretch was derived by amplifying four overlapping segments of the 16S rRNA gene (Fig. 1), representing almost the entire 1'396 bp 16S rRNA gene. For amplification, primers previously described were used (Jensen et al., 2001; Willi et al., 2006a; Meli et al., 2010) and summarized in Table 1. In addition, we used the highly variable RNase P gene to differentiate between Mhf and Mhc (Birkenheuer et al., 2002). The RNA subunit (126 bp) of the RNase P gene was amplified as previously described (Tasker et al., 2003). PCR products were purified and submitted to Sanger fluorescent dideoxynucleotide sequencing (Microsynth; Balgach, Switzerland). Sequences were assembled using the Staden package 1.5 (http://staden.sourceforge.net/) and compared to those available in GenBank using ClustalW (Thompson et al., 1994). A bootstrap phylogenetic tree for the 16S rRNA gene and the RNase P gene, respectively, was created by the neighbor-joining method (Saitou and Nei, 1987) using a distance matrix that was corrected for nucleotide substitutions based on the Kimura 2 – parameter model (Kimura, 1980). The dataset was re-sampled 1000 times to generate bootstrap values. *Mycoplasma hyopneumoniae* was used as the root for both phylogenetic trees. Analyses were conducted using MEGA 4.0 (Tamura et al., 2007). The assembled 16S rRNA and the RNase P gene nucleotide sequences have been submitted to GenBank under accession numbers GU734681 and GU734682, respectively.

2.4. Statistical analysis

Fisher's exact and Chi-square tests were conducted with SYSTAT 12.0 (Systat Software GmBH, Erkrath, Germany) and the 95% Clopper-Pearson confidence interval of the observed seroprevalence with StatXact 8.0 (Cytel Inc., Cambridge, USA).

3. Results

3.1. Prevalence of hemoplasma infection

PCR for detection of Mhf/Mhc was positive in one adult female cheetah of approximately 6–8 years of age. The prevalence of Mhf/Mhc infection in the study population was 1.6% (95% confidence interval: 0.04–8.8%). PCRs for detection of CMt and CMhm and the SYBR Green PCR were negative in all 73 samples of the 61 cheetahs. No study animal showed signs of anemia, such as pale mucus membranes or a prolonged capillary refill time, had enlarged lymph nodes or was lethargic or dehydrated.

The Mhf/Mhc positive female was continuously radio-tracked over a period of 46 months until her carcass was found in the field. Due to the decomposed state of her body, the cause of death could not be determined.

3.2. Phylogenetic analyses

The assembled almost complete 16S rRNA gene (Fig. 1) showed 99% identity with a Mhf isolate from a domestic cat in Australia (AY150977), but also 99% identity with Mhc isolates from domestic dogs in Japan and Germany (AY529641, AY150973). Phylogenetic analyses based on the 16S rRNA gene of the hemoplasma isolate revealed no major subclustering within the *Haemofelis* group (which

Fig. 1. Diagram of length and position of the four amplified segments in relation to the assembled sequence of the 16S rRNA gene.

Table 1
Primers used to amplify four segments of the 16S rRNA and the RNA subunit of the RNase P genes of the isolate.

Target gene	Gene segment	Primer name	Sequence	Reference	Amplicon length (bp)
16S rRNA gene	Segment 1	(F) MychaeF	5′-ACG AAA GTC TGA TGG AGC AAT A-3′	Jensen et al. (2001)	132
		(R) MychaeRW	5′-ACG CCC AAT AAA TCC G(A/G)A TAA T-3′	Jensen et al. (2001)	132
	Segment 2	(F) MycCatNewF	5′-GAA AGT CTG ATG GAG CAA TAC CAT-3′	Willi et al. (2005)	917
		(R) Mhf_rev	5′-CAA ATG AAT GTA TTT TTA AATGCC CAC-3′	Willi et al. (2005)	917
	Segment 3	(F) MHBforw	5′-GAA TTA ATG CTG ATG GTATGC CTA A-3′	Meli et al. (2010)	413
		(R) MycCatNewRW	5′-CTG GCA CAT AGT T(A/T)G CTG TCA CTT A-3′	Willi et al. (2005)	413
	Segment 4	(F) MHBforw	5′-GAA TTA ATG CTG ATG GTATGC CTA A-3′	Willi et al. (2005)	469
		(R) MychaeRW	5′-ACG CCC AAT AAA TCC G(A/G)A TAA T-3′	Jensen et al. (2001)	469
RNase P gene	Subunit	(F) RNasePFor1	5′-CTG CGA TGG TCG TAA TGT TG-3′	Tasker et al. (2003)	126
		(R) RNasePRev1	5′-GAG GAG TTT ACC GCG TTT CA-3′	Tasker et al. (2003)	126

F = forward primer; R = reverse primer.

includes both Mhf and Mhc) in the phylogenetic tree (Fig. 2).

The 126 bp sequence of the RNA subunit of the RNase P gene showed 94% identity with a Mhf isolate from a wildcat in France (DQ859006) and 92% identity with a Mhf isolate from a lion in Tanzania (DQ859007), but also 95% identity with a Mhc isolate from a domestic dog in Germany (AY150989). Phylogenetic analyses based on the RNA subunit of the RNase P gene of the hemoplasma isolate positioned the isolate closer to the Mhc than the Mhf cluster(s) without clear assignment to one of the subclusters (Fig. 3). The isolate from the cheetah did not cluster with one of the two lion Mhf subclusters.

4. Discussion

This is the first evidence of a hemotropic mycoplasma infection in a free-ranging cheetah and the first report on hemoplasma in Namibia. The prevalence was low with only one adult female being positive, not showing any signs of impairment. Thus, our results suggest that hemoplasma infections might not pose a serious threat to the Namibian cheetah population.

The hemoplasma isolate of this cheetah could not be assigned to either Mhf or Mhc subcluster. This finding is surprising because most isolates from felids from all over the world could be clearly assigned to the Mhf subcluster

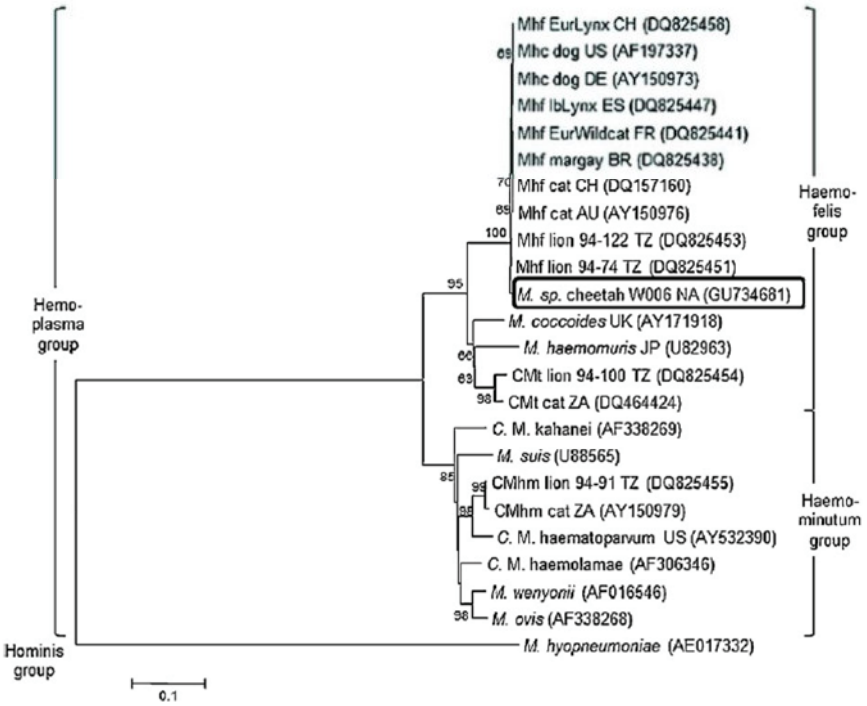

Fig. 2. Bootstrap phylogenetic analysis of the nearly complete 16S rRNA gene sequences of our cheetah isolate *Mycoplasma* species cheetah W006 NA (GU734681) and related organisms. The tree was constructed using the neighbor-joining method, and evolutionary distances are to the scale shown. Bootstrap percentage values are given at the nodes of the tree with only values ≥60 shown. The name of the bacteria, the animal species of the isolate origin, the ISO 3166 codes of the country of origin and the GenBank accession numbers are shown, unless the information was inaccessible. Note that the *Haemofelis* group comprises Mhf and Mhc.

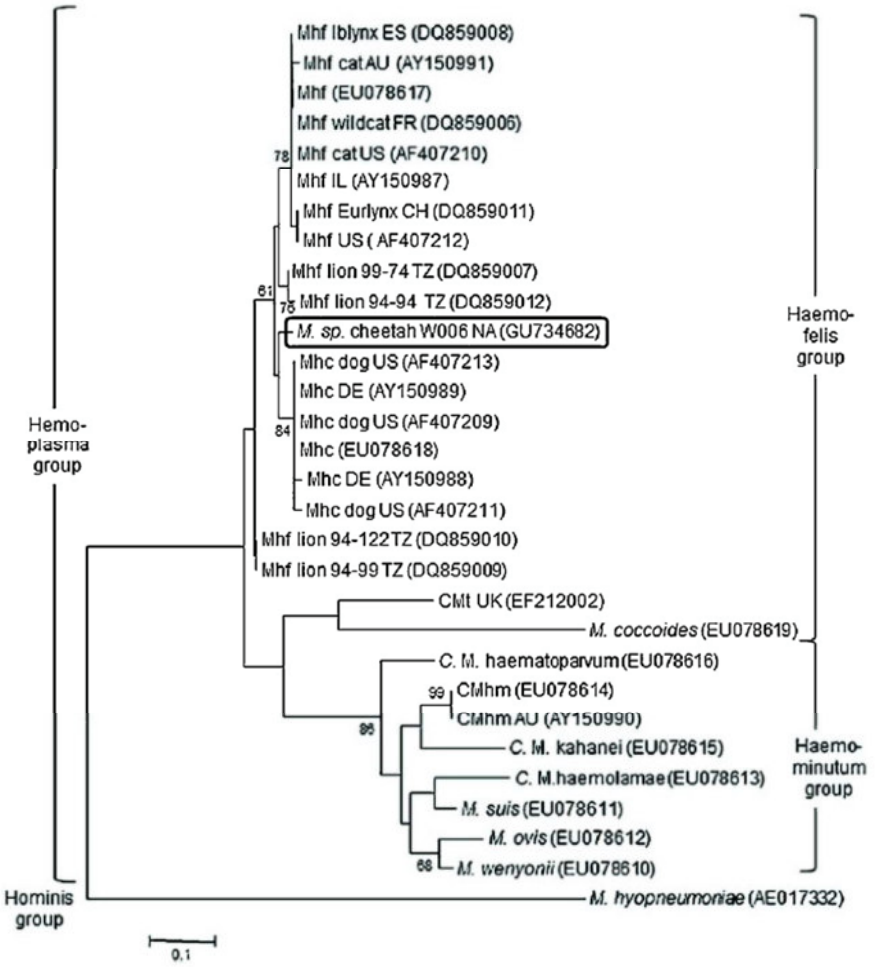

Fig. 3. Bootstrap phylogenetic analysis of the subunit of the RNase P gene of our cheetah isolate *Mycoplasma* species. cheetah W006 NA (GU734682) and related organisms. For details see Fig. 2.

using the RNase P gene (Tasker et al., 2003). It may be due to the fact that this is the first isolate characterized west of the Kalahari Desert. The Kalahari Desert has been suggested to be a barrier to infectious agents (Brown et al., 1993) and thus might have enabled Mhf in Namibia to evolve independently from the *Mycoplasma* previously identified in South Africa or Tanzania (Willi et al., 2006b, 2007) into a currently unknown strain.

Immunosuppressive viruses, such as feline immunodeficiency virus (FIV) and feline leukemia virus (FeLV), can increase the likelihood of co-infections with hemoplasma and severity of anemia in such cases can be aggravated (Foley and Pedersen, 2001; George et al., 2002). FIV and FeLV were found to be absent in Namibian cheetahs (Munson et al., 2004; Thalwitzer et al., 2010), thus co-infection with these pathogens was not expected. The hemoplasma PCR-positive cheetah tested negative for FIV and FeLV. However, it tested positive for antibodies to canine distemper virus (CDV) in a previous study (Thalwitzer et al., 2010). CDV also has an immunosuppressive effect and secondary infections are common (Sykes, 2010). This cheetah was the oldest free-ranging female sampled in the present study and lived for almost four years after the initial sampling, indicating that she was not impaired by the co-infection. Nonetheless, it seems important to verify the good health status of this worldwide largest free-ranging cheetah population and to continue the examination of previously not investigated pathogens in this and in sympatric Namibian carnivore populations.

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References

Birkenheuer, A.J., Breitschwerdt, E.B., Alleman, A.R., Pitulle, C., 2002. Differentiation of *Haemobartonella canis* and *Mycoplasma haemofelis* on the basis of comparative analysis of gene sequences. Am. J. Vet. Res. 63, 1385–1388.

Brown, E.W., Miththapala, S., O'Brien, S.J., 1993. Prevalence of exposure to feline immunodeficiency virus in exotic felids. J. Zoo Wildl. Med. 24, 357–364.

Foley, J.E., Pedersen, N.C., 2001. '*Candidatus* Mycoplasma haemominutum', a low-virulence epierythrocytic parasite of cats. Int. J. Syst. Evol. Microbiol. 51, 815–817.

Foley, J.E., Harrus, S., Poland, A., Chomel, B., Pedersen, N.C., 1998. Molecular, clinical, and pathologic comparison of two distinct strains of *Haemobartonella felis* in domestic cats. Am. J. Vet. Res. 59, 1581–1588.

George, J.W., Rideout, B.A., Griffey, S.M., Pedersen, N.C., 2002. Effect of preexisting FeLV infection or FeLV and feline immunodeficiency virus coinfection on pathogenicity of the small variant of *Haemobartonella felis* in cats. Am. J. Vet. Res. 63, 1172–1178.

Jensen, W.A., Lappin, M.R., Kamkar, S., Reagan, W.J., 2001. Use of a polymerase chain reaction assay to detect and differentiate two strains of *Haemobartonella felis* in naturally infected cats. Am. J. Vet. Res. 62, 604–608.

Kimura, M., 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J. Mol. Evol. 16, 111–120.

Leutenegger, C.M., Mislin, C.N., Sigrist, B., Ehrengruber, M.U., Hofmann-Lehmann, R., Lutz, H., 1999. Quantitative real-time PCR for the measurement of feline cytokine mRNA. Vet. Immunol. Immunopathol. 71, 291–305.

Meli, M.L., Cattori, V., Martínez, F., López, G., Vargas, A., Simón, M.A., Zorrilla, I., Muñoz, A., Palomares, F., López-Bao, J.V., Pastor, J., Tandon, R., Willi, B., Hofmann-Lehmann, R., Lutz, H., 2009. Feline leukemia virus and other pathogens as important threats to the survival of the critically endangered Iberian lynx (*Lynx pardinus*). PLoS ONE 4, e4744.

Meli, M.L., Willi, B., Dreher, U.M., Cattori, V., Knubben-Schweizer, G., Nuss, K., Braun, U., Lutz, H., Hofmann-Lehmann, R., 2010. Identification, molecular characterization, and occurrence of two bovine hemoplasma species in Swiss cattle and development of real-time TaqMan quantitative PCR assays for diagnosis of bovine hemoplasma infections. J. Clin. Microbiol. 48, 3563–3568.

Munson, L., Marker, L., Dubovi, E., Spencer, J.A., Evermann, J.F., O'Brien, S.J., 2004. Serosurvey of viral infections in free-ranging Namibian cheetahs (*Acinonyx jubatus*). J. Wildl. Dis. 40, 23–31.

Munson, L., Terio, K.A., Worley, M.B., Jago, M., Bagot-Smith, A., Marker, L., 2005. Extrinsic factors significantly affect patterns of disease in free-ranging and captive cheetah (*Acinonyx jubatus*) populations. J. Wildl. Dis. 41, 542–548.

Munson, L., Terio, K.A., Kock, R., Mlengeya, T., Roelke, M.E., Dubovi, E., Summers, B., Sinclair, A.R.E., Packer, C., 2008. Climate extremes promote fatal co-infections during canine distemper epidemics in African lions. PLoS ONE 3, e2545.

Neimark, H., Johansson, K.E., Rikihisa, Y., Tully, J.G., 2001. Proposal to transfer some members of the genera *Haemobartonella* and *Eperythrozoon* to the genus *Mycoplasma* with descriptions of '*Candidatus* Mycoplasma haemofelis', '*Candidatus* Mycoplasma haemomuris', '*Candidatus* Mycoplasma haemosuis' and '*Candidatus* Mycoplasma wenyonii'. Int. J. Syst. Evol. Microbiol. 51, 891–899.

Saitou, N., Nei, M., 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4, 406–425.

Sykes, J.E., 2010. Immunodeficiencies caused by infectious diseases. Vet. Clin. N. Am. Small Anim. Pract. 40, 409–423.

Tamura, K., Dudley, J., Nei, M., Kumar, S., 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol. Biol. Evol. 24, 1596–1599.

Tasker, S., Helps, C.R., Day, M.J., Harbour, D.A., Shaw, S.E., Harrus, S., Baneth, G., Lobetti, R.G., Malik, R., Beaufils, J.P., Belford, C.R., Gruffydd-Jones, T.J., 2003. Phylogenetic analysis of hemoplasma species: an international study. J. Clin. Microbiol. 41, 3877–3880.

Thalwitzer, S., Wachter, B., Robert, N., Wibbelt, G., Muller, T., Lonzer, J., Meli, M.L., Bay, G., Hofer, H., Lutz, H., 2010. Seroprevalences to viral pathogens in free-ranging and captive cheetahs (*Acinonyx jubatus*) on Namibian farmland. Clin. Diagn. Lab. Immunol. 17, 232–238.

Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22, 4673–4680.

Wachter, B., Thalwitzer, S., Hofer, H., Lonzer, J., Hildebrandt, T.B., Hermes, R., 2011. Reproductive history and absence of predators are important determinants of reproductive fitness: the cheetah controversy revisited. Conserv. Lett. 4, 47–54.

Westfall, D.S., Jensen, W.A., Reagan, W.J., Radecki, S.V., Lappin, M.R., 2001. Inoculation of two genotypes of *Hemobartonella felis* (California and Ohio variants) to induce infection in cats and the response to treatment with azithromycin. Am. J. Vet. Res. 62, 687–691.

Willi, B., Boretti, F.S., Cattori, V., Tasker, S., Meli, M.L., Reusch, C., Lutz, H., Hofmann-Lehmann, R., 2005. Identification, molecular characterization, and experimental transmission of a new hemoplasma isolate from a cat with hemolytic anemia in Switzerland. J. Clin. Microbiol. 43, 2581–2585.

Willi, B., Boretti, F.S., Baumgartner, C., Tasker, S., Wenger, B., Cattori, V., Meli, M.L., Reusch, C.E., Lutz, H., Hofmann-Lehmann, R., 2006a. Prevalence, risk factor analysis, and follow-up of infections caused by three feline hemoplasma species in cats in Switzerland. J. Clin. Microbiol. 44, 961–969.

Willi, B., Tasker, S., Boretti, F.S., Doherrm, M.G., Cattori, V., Meli, M.L., Lobetti, R.G., Malik, R., Reusch, C.E., Lutz, H., Hofmann-Lehmann, R., 2006b. Phylogenetic analysis of "*Candidatus* Mycoplasma turicensis" isolates from pet cats in the United Kingdom, Australia, and South Africa, with analysis of risk factors for infection. J. Clin. Microbiol. 44, 4430–4435.

Willi, B., Filoni, C., Catao-Dias, J.L., Cattori, V., Meli, M.L., Vargas, A., Martinez, F., Roelke, M.E., Ryser-Degiorgis, M.P., Leutenegger, C.M., Lutz, H., Hofmann-Lehmann, R., 2007. Worldwide occurrence of feline hemoplasma infections in wild felid species. J. Clin. Microbiol. 45, 1159–1166.

Willi, B., Meli, M.L., Luthy, R., Honegger, H., Wengi, N., Hoelzle, L.E., Reusch, C.E., Lutz, H., Hofmann-Lehmann, R., 2009. Development and application of a universal hemoplasma screening assay based on the SYBR green PCR principle. J. Clin. Microbiol. 47, 4049–4054.

4. General discussion of the findings and conclusions

Wildlife diseases are worldwide of great concern for species living in protected as well as non-protected areas (Ramsauer et al. 2007; Filoni et al. 2012; Goodrich et al. 2012; Foley et al. 2013). Particularly carnivore species are threatened by diseases, and within feline species the cheetah has been reported to be infected with a variety of viral and non-viral pathogens (Kennedy et al. 2003; Molia et al. 2004; Munson et al. 2004a; Millward and Williams 2005; Troyer et al. 2005; Thalwitzer et al. 2010).

Thus, it is important to monitor free-ranging cheetah populations for possible health threats and also captive populations, because disease outbreaks in captivity have been reported repeatedly (Evermann 1986; Junge et al. 1991; Munson et al. 2004b). Preventive measures, such as vaccinations, are therefore also an important research area for the animals held in captivity, including the effectiveness of vaccinations in captivity and the assessment of its application as a potential conservation management tool for free-ranging populations at risk.

Free-ranging and captive cheetahs in Namibia have been tested for antibodies and for antigens of various viral agents (Kennedy et al. 2003; Munson et al. 2004a; Thalwitzer 2007; Thalwitzer et al. 2010; Flacke et al. 2015). In chapter 2 of this thesis, I focused on the previously demonstrated hints for a possible FeLV presence in the Namibian cheetah population (Thalwitzer 2007), as well as on the effectiveness of FeLV vaccination in captive held cheetahs. To my knowledge such a comprehensive approach has not been conducted before, although in other vulnerable or endangered species such as Iberian lynx, FeLV infections can have disastrous effects. In the free-ranging Iberian lynx population, nearly half of the 14 provirus-positive animals died within a period of six months (Meli et al. 2009).

Co-infections of viruses, particularly of those that induce immunodeficiency, and blood parasites can result in more severe clinical effects than a single infection with only one pathogen in free-ranging carnivore populations (Willi et al. 2007). Previously, hemoplasma infections were described in free-ranging Iberian lynxes, European wildcats and African lions, without evidence of clinical signs or high mortality, (Willi et al. 2007; Munson et al. 2008; Meli et al. 2009). In chapter 3, I present the *Hemoplasma* results based on quantitative real-time-PCR of TNA extracted from blood samples from 61 free-ranging cheetahs (Krengel et al. 2013).

4.1 Importance of FeLV in free-ranging felids

Until recently, FeLV infections and disease progression in free-ranging and feline species were rarely reported, but two recent FeLV outbreaks in free-ranging Florida panthers and the critically endangered Iberian lynxes demonstrate that this virus can also be dangerous for non-domestic feline species (Brown et al. 2008; Cunningham et al. 2008; Meli et al. 2009; 2010; O'Brien et al. 2012). There are four FeLV strains which differ in their virulence, the A, B, C and T strains (Overbaugh and Bangham 2001). More virulent strains are formed when strain A, the only strain primarily transmitted from domestic cats to other felids, is recombined with endogenous FeLV, which is only present in the domestic cat lineage (Neil et al. 1991). Because exotic felid species lack endogenous FeLV, it was first thought that only infections with already recombined strains pose a threat (O'Brien et al. 2012). However, the FeLV outbreak in the Florida panther revealed new insights in the complex epidemiology of FeLV. This population was serosurveyed since 1978 for many viral and other infectious agents (Roelke et al. 1993). The outbreak was first discovered with an increase in the number of FeLV antibody positive animals, followed by five viremic animals, all of which died (Cunningham et al. 2008). The high virulence of this FeLV strain was unusual and it was identified as a FeLV-A strain with no evidence for recombination with endogenous FeLV, thus being a new pathogenic variant of the FeLV-A strain (Chandhasin et al. 2005; Brown et al. 2008). Noteworthy, some of the diseased animals were also FIV positive (Brown et al. 2008). Similarly, the FeLV outbreak in the Iberian lynx was also a variant of the FeLV-A strain and was fatal in half of the positive cases (Meli et al. 2009). In this case, the high virulence was attributed to host rather than viral factors, because the animals were all FIV negative (Meli et al. 2010).

These outbreaks indicate that the crossing of species barriers is possible for domestic cat virus strains and thus poses a threat to other feline species, especially endangered ones. Co-infection of FeLV and FIV was also detected in free-ranging guignas (*Leopardus guigna*) on Chiloé Island in Chile, where the anthropogenic perturbation of a natural landscape led to an increase in the contact between domestic cats and free-ranging felids (Mora et al. 2015). Recently, a jaguar (*Panthera onca*) in the Pantanal wetland of Brazil was tested FeLV-B positive with PCR from whole blood (Silva et al. 2016), the first FeLV-B strain in a free-ranging felid.

4.2 Importance of FIV and CDV in free-ranging felids

Apart from FeLV, also FIV and CDV are of potential concern for free-ranging felids (Kennedy-Stoskopf 1999). For both viruses, antibodies in several free-ranging feline species were detected, including the cheetah (Troyer et al. 2005; Thalwitzer et al. 2010).

FIV is a lentivirus that causes immunosuppression in most infected cat species, particularly in domestic cats, and is thus used as a model for HIV/AIDS in humans (Willett et al. 1997; Pecon-Slattery et al. 2008). The clinical consequences for the infected animal and the different FIV lineages seem to be species-specific, thus it is likely that the virus co-evolved with its particular host (Pecon-Slattery et al. 2008). This might explain why some felids exhibit few clinical symptoms, predominantly in Africa from where FIV is proposed to originate (Pecon-Slattery et al. 2008). Nevertheless, lions infected with the lion specific FIV strain, the FIVple strain, can have clinical signs coherent with AIDS defining conditions such as lymphadenopathy and lymphoid depletion (Roelke et al. 2009). Certain FIVple strains in lions can also cause elevated mortality in animals co-infected with CDV and *Babesia* (Troyer et al. 2011), but not in animals co-infected with bovine tuberculosis (Maas et al. 2012).

CDV, a morbillivirus, is a threat to a broad range of carnivore species including aquatic ones such as Caspian seals (*Phoca caspica*), feline ones such as lynxes (*Lynx canadiensis* and *Lynx rufus*), Amur tigers (*Panthera tigris altaica*) and African lions and canine ones such as the world's rarest canid, the Ethiopian wolf (*Canis simensis*) (Kuiken et al. 2006; Daoust et al. 2009; Seimon et al. 2013; Gordon et al. 2015). Detailed molecular analysis and experiments with different CDV strains demonstrated that different viral strains circulate within wildlife and within domestic animals, the latter often cited as sources of infection in wildlife animals (Munson et al. 2008; Nikolin et al. 2012b). It has also been shown that different CDV strains have different traits and thus seem to have coevolved within at least two different host systems, a group of specialist-type strains in the large and rather homogenous population of domestic dogs as opposed to several generalist-type strains in the smaller and heterogeneous host species-rich guild of smaller populations of different wild carnivore species (Nikolin et al. 2012a). These results suggest (1) infected domestic dogs are most likely to transmit their specialist strains to other canine species, (2) infected free-ranging non-canid carnivore species transmit their generalist strains to other non-canine carnivores, (3) it is possible that generalist-type strains are transmitted to canids, (4) it is unlikely that canids spread generalist-type strains to non-canid carnivore species (Nikolin et al. 2012a). Previously, CDV infections in lions and other carnivore species in the Serengeti National Park (NP) in Tanzania were attributed to a high density of domestic dogs close to the park (Roelke-Parker et al. 1996; Cleaveland et al. 2000), although this view is not universally accepted and alternative explanations are available (Haas et al. 1996; East et al. 2012). After a vaccination program of the domestic dog population in the vicinity of the Serengeti NP, infections in the lion populations still occurred and did not coincide with infection peaks in domestic dogs, thus further demonstrating that other factors or animal species contributed to the maintenance of the virus in this ecosystem (Viana et al. 2015). Recently two distinct viral strains were described to have circulated in (a) the lion and spotted hyena (*Crocuta crocuta*)

populations and (b) the canid, including the domestic dog, populations during the 1993/1994 CDV outbreak in the Serengeti NP, with the strain circulating in the non-canid, particularly lion and hyena, carnivore populations being of particular virulence (Nikolin et al. 2016). Thus, the fatalities in the lion population were not caused by a spill-over from CDV infected domestic dogs. Further infections in other regions of the world have been noted, for example a CDV outbreak in domestic dogs and black-backed jackals (*Canis mesomelas*) in the coastal region of Namibia (Gowtage-Sequeira et al. 2009) and one in at least six feline species in Costa Rica (Avendaño et al. 2016).

4.3 Importance of blood parasites in free-ranging felids

Numerous parasites are known to occur in feline species and the disease progression varies from unapparent to lethal. In the free-ranging Namibian cheetah, no fatal infections with parasites are known, yet several agents have been described, e.g. *Babesia*, nematodes and coccidias (Bosman et al. 2007; Meny et al. 2012). The importance of surveys of tick borne pathogens (TBP) such as *Babesia*, *Hepatozoon*, *Theileria* and *Ehrlichia* and the use of molecular methods has been recently stressed, because habitat fragmentation and thus population fragmentation are likely to render small host populations more susceptible to TBP (Kelly et al. 2014; Williams et al. 2014). Recently, a new method has been tested on blood slides from human blood to extract DNA and to screen for hemoplasma species (Tasker et al. 2010). This could help in detecting previous outbreaks and in screening old blood smears for pathogen history and past epidemics. Although fatalities of animals have rarely occurred by sole infection of a parasite, co-infections with viral diseases or stressful conditions can lead to the death of an animal (Penzhorn 2006; East et al. 2008). TBP occur in most climatic conditions, including harsh environments such as the Russian Far East, indicating a potential risk for carnivore species worldwide (Thomas et al. 2016). It is therefore important to screen many free-ranging species and gain knowledge on the occurrence and modes of transmission as part of a comprehensive approach to conservation.

4.4 Serosurveys in cheetahs

4.4.1. Serosurveys in the Namibian cheetah population

In a previous study in north-central Namibia, samples of 81 free-ranging cheetahs collected between 1992 and 1998 were screened for antibodies against FHV, FCV, FPV, FCoV, CDV and FIV as well as for antigens of FeLV (Munson et al. 2004). Antibody prevalence were 12% for FHV, 65% for FCV, 48% for FPV, 29% for FCoV, 24% for CDV and 0% for FIV and antigen prevalence was 0% for FeLV. During this time period, several incidences of local CDV outbreaks occurred in sub-Saharan Africa (Alexander et al. 1996; Roelke-Parker et al. 1996). Whether there was also an outbreak of CDV in Namibian cheetahs is presently unclear but unlikely, since there were no reports from other wildlife species or domestic animals in Namibia. At the time of sampling, no clinical signs of CDV or other infections were noted in the cheetahs (Munson et al. 2004a), consistent with the results of a subsequent study in which samples of necropsies and gastric biopsies of 78 free-ranging Namibian cheetahs, 80 cheetahs kept in South African enclosures and 147 cheetahs kept in North American zoos were investigated (Munson et al. 2005). Free-ranging cheetahs had no severe lesions and were in good health status, whereas approximately two-thirds of captive cheetahs in South African and North America had moderate to severe gastritis.

A subsequent study in east-central Namibia on free-ranging cheetahs also included non-vaccinated captive cheetahs and other free-ranging carnivores (Thalwitzer et al. 2010). The study used serum samples collected between 2002 and 2004 of 62 free-ranging cheetahs, 24 non-vaccinated captive cheetahs, four leopards, three caracals and one black-backed jackal. Samples were also analysed from necropsies of 15 free-ranging cheetahs, eight leopards, two black-backed jackals, one African wildcat (*Felis libyca*), one bat-eared fox (*Otocyon megalotis*), one honey badger (*Mellivora capensis*), one aardwolf (*Proteles cristatus*) and one captive cheetah. Antibody prevalence were measured for FHV, FCV, FPV, FCoV, CDV, FIV, PLV and rabies, and antigen prevalence for FeLV, while tissue samples were screened for rabies antigen. In contrast to the study performed in north-central Namibia, the prevalence for antibodies against all viruses in east-central Namibia was below 5%. Consistent with previous findings, no signs of infection or contact with FeLV or FIV were detected. A possible explanation for this difference might be the higher human population density and thus higher numbers of feral domestic animals, particularly domestic dogs and domestic cats in north-central than east-central Namibia. If in both areas the same proportion of dogs and cats are not vaccinated against common viruses, they might transmit more viruses to cheetahs in the former than latter area (Thalwitzer et al. 2010). Consistent with these results, and possibly as a consequence of differential selection pressure by these pathogens, genetic differentiation at class I loci of the MHC (relevant for combatting viruses)

also significantly differed between north-central and east-central Namibia in the expected direction (Castro-Prieto et al. 2012).

The geographic difference in the seroprevalence of cheetahs has a practical consequence when it comes to translocating cheetahs. Translocations of animals are sometimes used to mitigate conflicts between farmers and cheetahs (Weise et al. 2015). Often, the potential risk of pathogen transmission is not known in the original and the new area. The serosurvey in Namibia demonstrates that translocations from east-central to north-central Namibia might create the risk that naïve, seronegative cheetahs are exposed to viral contact, whereas potentially infected cheetahs from north-central Namibia might increase the risk of viral exposure to cheetahs in east-central Namibia (Thalwitzer et al. 2010). A good example of careful planning was the serosurvey of wild cats and feral cats in Portugal in preparation for the reintroduction of the Iberian lynx (Duarte et al. 2012).

In addition to serosurveys, the area into which a cheetah is planned to be translocated should be a suitable cheetah habitat without its main predators and without the threat from which it is removed (Weise et al. 2014; 2015). If it is private land, the land owners should agree to the translocation and the animals should be monitored for several months after release (Rust and Marker 2013; Weise et al. 2014) if only to check that the stress of translocation in itself did not have any fatal consequences (Hofer and East 1998). If this is not the case, the likelihood of success is low (Weise et al. 2014; 2015).

Case studies of cheetahs are also important to investigate because each case of sickness in this vulnerable species might (1) be an indicator for a ‘new’ disease, (2) mark the beginning of an outbreak, (3) show potential effects of agents known to occur in free-ranging animals, (4) be an indicator of unfavourable housing conditions in captive animals or (5) be the result of unknown co-infections. Three case studies are: A FHV-positive wild born captive cheetah in Namibia with a secondary bacterial skin infection had to be euthanised despite intensive treatment (Flacke et al. 2015). Another wild born captive cheetah in Namibia was FeLV positive, developed a FeLV induced multicentric T-cell lymphoma with all neoplastic tissues containing viral sequences and died within two months (Marker et al. 2003). A more recent report of a multicentric T-cell lymphoma and cutaneous hemangiosarcoma in a cheetah born and kept in a North American zoo was FeLV negative (Lindemann et al. 2015). The clinical progression of the disease was milder than in the Namibian case, but after developing clinical signs over four months, the animal was euthanised and the necropsy revealed profound lesions on the organs. These examples demonstrate that although free-ranging cheetah populations generally are healthy, cheetahs can deteriorate rapidly when infected in captivity.

4.4.2. Serosurveys in my studies

In the study on gammaretroviruses, no clinical signs of FeLV infection were noted and no positive results for the FeLV p27 antigen ELISA were measured in any free-ranging or captive Namibian cheetah. However, 19% of free-ranging cheetahs, 25% of captive non-vaccinated cheetahs and 86% of captive, vaccinated cheetahs tested positive for FeLV FL-74 antibodies. The FeLV p45 antibody ELISA revealed that 1% of free-ranging cheetahs, 11% of captive non-vaccinated animals and 71% of captive vaccinated animals were seropositive. Most free-ranging and captive non-vaccinated cheetahs were seropositive for only one of the antibody tests, but most of the captive vaccinated animals were seropositive for all three tests (Table 1).

Table 1: Number of free-ranging, captive non-vaccinated and captive vaccinated cheetahs with seropositive results for one, two or three antibody tests. Percentages in brackets refer to the number of animals seropositive for one antibody test.

Number of tests	Free-ranging	Captive, non-vaccinated	Captive, vaccinated
1	34	13	34
2	9 (27%)	5 (39%)	9 (27%)
3	4 (12%)	1(8%)	18 (53%)

Six randomly chosen WB positive and six randomly chosen WB negative samples were also used to test for reactivity against baboon endogenous retrovirus, feline RD114 retrovirus, Rauscher murine leukemia retrovirus (RMuLV) and AKR MuLV. The six WB positive samples were also seropositive against RMuLV. Perhaps cheetahs harbour a differentially expressed endogenous gammaretrovirus that belongs to a mammalian group subfamily or the results are due to the activation of endogenous gammaretrovirus not directly related to an infectious disease. Such mechanisms have been suggested for Gairdner's shrew-mice (*Mus pahari*) (Sakuma et al. 2011; 2012).

Forty of the 41 whole blood samples revealed sufficient amounts of total nucleic acid (TNA) and were screened for endogenous and exogenous FeLV using real-time PCRs. All results were negative. Similarly, RT activity measured with the PERT assay in PBMC cell culture supernatants from seven WB positive and one WB negative cheetahs and in plasma samples from 12 WB negative and ELISA negative cheetahs were all negative.

Thus, the Namibian cheetah population is exposed to a FeLV strain or a related gammaretrovirus strain that induces antibodies which bind to some but not to all FeLV antigens in the ELISA tests and also bind to RMuLV antigens. However, the gammaretrovirus strain is of yet unknown origin. The vaccination study in captive-held cheetahs demonstrates that the vaccine induces a strong humoral immune response and thus is likely to be useful for cheetahs.

In the study on hemoplasma, TNA from 73 EDTA whole blood samples obtained from 61 free-ranging cheetahs was extracted and screened for the presence of hemoplasmas using four different PCRs. The PCR for CMt, CMhm and the SYBR green PCR, which allows testing for a broader range of possibly new hemoplasmas, were all negative. One sample was positive in the PCR screening for Mhf/Mhc. To further characterise the isolate, the conserved 16S rRNA gene and the highly variable RNase P gene were sequenced. Bootstrap phylogenetic trees for both genes were created and the new isolate assigned to the *Haemofelis* group with no further attribution to the Mhf or the Mhc subcluster. The positive animal was a female cheetah aged approximately 6 to 8 years at the time of sampling. The female was monitored with a radio collar for another 48 months until her carcass was found decomposed in the field. Thus, the clinical consequences for infected cheetahs do not seem to be of concern for the survival of the animals. Because *Hemoplasma* infection occurred at low prevalence in the Namibian cheetah population, this blood parasite is unlikely to be a threat to the population, except if high proportions of co-infections with other pathogens occur in the future.

4.5 Serosurveys in other carnivore species

4.5.1. Serosurveys of viral diseases

Studies on seroprevalence are a useful tool to monitor pathogens with which animals came into contact, and may assist either in identifying environmental conditions or possible carriers within the same ecological guild of host species which may transmit the relevant pathogen. For example, the prevalence of FPV and FCV differed substantially between the lion population in the Serengeti NP and the one in the adjacent, but ecologically separated Ngorongoro Crater, with lions from the former area exhibiting higher seroprevalence than those from the latter area (Hofmann-Lehmann et al. 1996). Such differences are important to identify possible ‘vector species’ susceptible to these agents. Differences in seroprevalence were also described for FCoV in lions from Tanzania, Namibia and South Africa, with lions in Tanzania having substantially higher seroprevalence than lions in southern Africa (Hofmann-Lehmann et al. 1996). This might be a consequence of the drier climate in southern Africa than in Tanzania. The Kalahari in Botswana might also effectively act as a barrier for pathogens because lions from Botswana are seronegative for FCoV, FPV, FeLV, *Ehrlichia*

and *Anaplasma*, and have very low prevalence of CDV and FCV (Brown et al. 1993; Spencer and Morkel 1993; Ramsauer et al. 2007). However, FHV and FIV are present in Botswana (Ramsauer et al. 2007), indicating that some viruses might possibly be transmitted via feral or domestic animals. The first case of lethal CDV infection reported from Botswana was a case in 1994 in African wild dogs (*Lycaon pictus*) in the Chobe National Park (NP); the source of infection was suspected to be feral domestic dogs close to the Chobe NP (Alexander et al. 1996).

In Zambia, there is no evidence of CDV in lions, but there is in African wild dogs, spotted hyenas and domestic dogs close to the South Luangwa and Liuwa Plain NPs (Berentsen et al. 2013). However, 40 % of the lions in Zambia showed neutralising antibodies to rabies without any clinical signs of disease, implying the lions were exposed to rabies by consuming infected food (Berentsen et al. 2013), through exchange of saliva via friendly social (affiliative) behaviour, or a strain of low virulence (East et al. 2001). A similar 'non-bite' mode of rabies transmission was proposed for kudus in Namibia, as these animals are highly susceptible to the disease, and infected kudus exhibit very high virus titres in their saliva (Barnard et al. 1982; Mansfield et al. 2006). Antibodies against rabies with no clinical signs of infection were described in spotted hyenas in the Serengeti (East et al. 2001) and in black-backed jackals in the Etosha NP in Namibia (Bellan et al. 2012). Black-backed jackals also showed high levels of antibodies against *Bacillus anthracis* (BA), an agent endemic to this region, and against CDV, both without any clinical signs (Bellan et al. 2012). Hence, the modes of transmission, in particular the potential 'carrier' host species involved, and the consequences of exposure in terms of developing clinical symptoms are not yet fully understood, limiting the predictability of further disease progression or the stipulation of preventive management measures. A limiting factor may be that serosurveys usually do not provide insights to the identity of the current strain in circulation, do not demonstrate whether one or several strains are simultaneously in circulation, do not clarify how virulent a given strains for a specific host might be (e.g. East et al. 2001; Nikolin et al. 2012a; 2012b), nor do they suggest how species richness of the relevant host species guild will affect transmission (e.g. Keesing et al. 2006).

Antibodies of feline hosts against several viruses were also measured outside the African continent. Antibodies against CDV and rabies were measured in jaguars in Brazil, in mountain lions (*Puma concolor*) in California, USA, against FPV, FCV, FCoV, FHV and FeLV, in Geoffroy's cats (*Leopardus geoffroyi*) in Argentina against CDV, FCV, FCoV and FPV, and in Amur tigers in Russia against FCoV, CDV and FPV (Goodrich et al. 2012; Uhart et al. 2012; Foley et al. 2013; Furtado et al. 2013).

The risk for wildlife species to be affected by the respective diseases is likely to increase because of increasing anthropogenic disturbances such as habitat loss and fragmentation, encroachment of people into protected areas and climate change (Semenza and Menne 2009; Watson et al. 2015). Because CDV can be a major co-factor in disease progression in some carnivore species, caution is required when translocations or re-introductions are planned, particularly if the species involved is susceptible to CDV and rabies such as African wild dogs (Scheepers and Venzke 1995; Goller et al. 2010).

4.5.2. Serosurveys of non-viral diseases

There are fewer studies on the prevalence of non-viral than viral diseases in free-ranging or captive wildlife or domestic animals, but their importance as co-pathogens in the course of disease progression, as risk factor for wildlife and domestic animals or as zoonotic threat to humans is high (Noden and van der Colf 2013; Kelly et al. 2014; Noden and Soni 2015; Thomas et al. 2016).

In Zambia, *Babesia* was detected in African lions and spotted hyenas, but not in African wild dogs and domestic dogs, whereas *Hepatozoon* was detected in all four carnivore species, and *Ehrlichia* and *Bartonella* in none (Williams et al. 2014). Detailed analyses of the isolates identified three species of *Babesia* in two carnivore species, emphasising the importance of detailed pathogen identification to understand species susceptibility and co-evolution processes. In South Africa, up to 50% of free-ranging lions and domestic cats were infected with *Babesia*, whereas in Namibia only 7.5% of free-ranging cheetahs were infected with *Babesia* (Bosman et al. 2007).

It is important that state-of-the-art molecular methods are implemented and possibly adapted to wildlife species, because new isolates can hardly be detected with morphological methods, previously considered to be the standard for such investigations, and cannot be classified by relying on morphology alone (Bosman et al. 2007). In addition, sentinel individuals or species might provide an early-warning system for the arrival of new isolates, particularly if they are of zoonotic relevance. *Ehrlichia canis*, previously not described from Namibia, was detected in more than 50% of domestic dogs, including stray dogs, in the area of the capital Windhoek (Manyarara et al. 2015). Domestic dogs with clinical signs had higher numbers of tick and rickettsial infestation. These dogs predominantly roam the streets and outskirts of the city and may thus come into contact with wild animals. It was thus suggested to focus on the zoonotic potential of vector borne diseases in Namibia and to use sentinel animals in rural areas of the entire country to screen for zoonotic agents such as *Leishmania* (Noden and Soni 2015). A study from Brazil focussed on screening neotropic and exotic wild-born felids in zoological gardens as well as in conservation and breeding centres in Brazil for FeLV, FIV, FCV, FPV, FCoV, *Bartonella*, feline hemoplasmas and piroplasms (Filoni et al.

2012). This study emphasised the importance of screening animals in breeding centres, especially prior to movements or reintroduction into the wild, and also stresses the importance of human safety when working with potentially zoonotic threats such as *Bartonella*. Thus, non-viral agents are widespread and it is advisable to screen wildlife and domestic animals on a regular basis.

4.6 Vaccinations in free-ranging carnivore species

Viral diseases can have devastating effects on populations of wild carnivore species. Thus, it is not surprising that vaccinations have been applied many times as a “disease management” tool in an attempt to reduce potential outbreaks or prevent infections of wildlife. However, vaccinations are usually developed for a particular target host species, mainly livestock and domestic companion animals, and cannot easily be transferred to wildlife. Vaccinations of free-ranging and captive wild carnivore species have therefore met with varying success, depending on the infectious agent and the target species involved. To be able to perform a feasible and, of course, also sensible plan of action, it is necessary to establish relevant facts for each case as a first step of planning an intervention. The following examples of vaccinations used in different free-ranging and captive held carnivores show that rigorous work before and after interventions can profoundly aid in future procedures.

Although most cases of vaccinations in wild carnivores deal with viral agents, a study in captive Namibian cheetahs vaccinated against anthrax in 2000 because mortalities from anthrax were observed in the Etosha NP provides valuable knowledge for the planning and testing of a vaccination trial in a wild carnivore species (Turnbull et al. 2004). Following vaccinations, a successful trial with sera from cheetahs in live mice with a passive protection test was performed. Thus, this example showed that the cheetah mounts an effective immune response to this vaccine and no ill effects were noted. Sera from black rhinoceros (*Diceros bicornis*) vaccinated against anthrax and from African lions with naturally acquired antibodies also proved to be protective (Turnbull et al. 2004). This study thus combined test results from self-acquired antibodies and vaccine-induced ones, a rare and in my opinion valuable approach.

A more complex issue are viral agents and the vaccination regimes performed to prevent acquisition or spread of viral diseases in free-ranging as well as captive held animals. As previously mentioned, the infection of immunosuppressive viruses such as CDV can have profound effects on individual animals or whole populations and thus research on preventive measures are highly indicated for this agent. Within canids, marked differences in the effectiveness of CDV vaccines were detected. For example, a modified live vaccine (MLV) CDV vaccination in captive red wolves (*Canis rufus*) did not result in fatalities and revealed measurable antibody titres that were maintained at high levels three years post vaccination in

all 32 animals tested (Harrenstien et al. 1997; Anderson et al. 2014). Similar results were measured for canine parvo virus (CPV) vaccination, suggesting that a triennial vaccination scheme might be suitable for this species. Such a vaccination scheme might be feasible for free-ranging red wolves (Anderson et al. 2014). However, vaccination trials in captive African wild dogs in a North American and a German zoological garden with a MLV against CDV lead to the death of the animals through CDV (McCormick 1983; Durchfeld et al. 1990). Since then, new trials with two further developed vaccines have been conducted in captive African wild dogs. One vaccine was an experimentally developed immunostimulating complex (ISCOM) CDV vaccine and the other a commercially available canarypox-vectored recombinant CDV vaccine (Philippa et al. 2006). In these tests, both vaccines proved to be safe but were only efficacious for up to one year post vaccination. Practical use in the field remains thus limited because it would be challenging to conduct yearly booster vaccinations in free-ranging African wild dogs and also the assessment of antibody formation is hardly possible and requires further immobilisations and thus more stress for the animal and its pack. Several vaccines commercially available in Europe and developed for domestic dogs, including inactivated, ISCOM and MLV CDV vaccines, were also tested on Eurasian otters (*Lutra lutra*), Asian small-clawed otters (*Aonyx cinereus*) and North American river otters (*Lontra canadensis*). Only the MLV induced a satisfactory antibody response in any of the three species (Günther-Weigl 2009). These examples highlight the importance of experiments on the efficacy and safety of vaccines used in wild animal species and show that the application of vaccinations in free-ranging populations is difficult to predict.

African wild dogs are also susceptible to rabies and thus were subjected to measures in the past to proposedly prevent rabies infection (e.g. Gascoyne et al. 1993b). The outcome of these measures have been analysed very critically and can give us important information on the complexity of consequences when interventions are not carefully planned in advance. In 1989, a rabies outbreak almost wiped out an entire big pack in Kenya (Kat et al. 1996). The following activities of darting and vaccination, in an effort to save the remainder of the pack, resulted in further mortalities. Shortly after this incident, at least 4 of the 34 vaccinated African wild dogs in the adjacent Serengeti NP died, supposedly of rabies after a rabies vaccination programme, and the remaining animals all vanished within a year after the vaccination (Burrows 1992; Burrows et al. 1994; Gascoyne et al. 1993b). It was argued that the handling stress during this programme reduced the natural potential of the immune system of the rabies infected animals, which resulted in the outbreak of rabies of infected individuals a few months later, without a protective effect of the vaccination (Burrows et al. 1994). An alternative explanation was that the injection of just a single dose rabies vaccine was insufficient and that the timing of the vaccination was too late (Woodroffe 2001). Furthermore, the immobilisation of single animals from wild dog packs for radio collaring

only, and not for vaccination purposes, did not lead to a severe disturbance or elevated mortality rates of the rest of the pack, as did the vaccination and thus darting of each pack member of a pack within the Serengeti study area (Burrows et al. 1994). Interestingly, significant antibody titres against rabies were detected in samples taken from wild dogs in the Serengeti NP between 1988 and 1990, before the vaccination campaign was performed in the African wild dogs in the Serengeti NP in September 1990 (Gascoyne et al. 1993a; 1993b). Thus, the wild dog population must have been exposed to rabies, without apparently causing mortality, before the beginning of the vaccination campaign in Serengeti NP in September 1990 after which the vaccinated African wild dogs vanished within 9 months (Burrows et al. 1994; Gascoyne et al. 1993a; 1993b). This shows that the measures taken were not well planned and did not aid the African wild dogs, but most probably further contributed to their fatal fate.

The topic of rabies vaccination in African wild dogs continues to be a topical issue, and several studies still try to find a feasible protocol for free-ranging as well as captive animals. A recent study tested an inactivated rabies vaccination in African wild dogs in zoological gardens and concluded that oral vaccination is ineffective, whereas intramuscular vaccination induced antibody production (Connolly et al. 2013). A previous study used live oral vaccine, for which the titres of antibodies against rabies dropped already 100 days after being vaccinated at least three times and thus a fourth parenteral inactivated booster vaccine was administered (van Heerden et al. 2002). One intramuscular injection of a single or double dose of a newly developed recombinant vaccine yielded protective titres for at least 15 months and thus was suggested to be sufficient to provide protection (Connolly et al. 2015). The results of this new study differ from the results of previous attempts, according to a review of the performance of rabies vaccinations in free-ranging African wild dogs, because single dose intramuscular injections were not effective in the past (East and Burrows 2001). For the most common form of vaccination in the early 1990s, dart-vaccination, it has been difficult to determine whether the dart vaccination is really effective, due to incomplete injection of the vaccine for example, or vaccination is efficacious, and most importantly, the extensively dart-vaccinated study packs have all died (Burrows 1994; Burrows et al. 1994; East and Burrows 2001).

Thus, for free-ranging African wild dogs a single vaccination does not seem to induce an appropriate immune response, for most vaccines in most cases, and the mode of delivery of vaccine is very important, with dart-vaccination and oral delivery being substantially less efficacious than intramuscular injection. Therefore there remain significant doubts as to whether vaccination of African wild dogs against rabies is suitable as long as handling stress is a possible explanation of the disastrous vaccination programme in the Serengeti NP, the

efficacy of vaccination is not ameliorated and the natural infection of rabies can be averted by the animals themselves, at least in the cases that did produce antibodies and thus survived.

To counteract a viral outbreak, different strategies can be implemented and should be considered, especially if the zoonotic potential and threat of disease spread to humans is eminent. Here I discuss three prominent examples. First, in the case of rabies in European red fox (*Vulpes vulpes*), more than ten different attenuated live vaccines were administered via millions of oral baits over a period of more than 30 years (Freuling et al. 2013; Müller et al. 2015). What alternative options are there if such a long-term approach is not possible, as in the case of many endangered and rare species, particularly if they live in remote and hardly inhabited areas? In the case of the Ethiopian wolf, the metapopulation was proposed to be protected from future outbreaks by limiting vaccinations to subpopulations in corridors (Haydon et al. 2006). During the last rabies outbreak in this species in 2003 to 2004, over 75% of a subpopulation died or disappeared, with rabies confirmed as the causative agent (Randall et al. 2004). Subsequent rabies vaccination was conducted in almost 70 animals caught and all of the later recaptured animals (n=19) had seroconverted (Randall et al. 2004). This vaccination programme involved high financial costs and considerable stress for the targeted animals. Yet as the Ethiopian wolves were not subjected to extensive darting on a limited number of occasions, such as the African wild dog packs, and were immobilised for the handling procedure none of the study animals was noted to have shown negative signs after the handling, or has even died thereafter. Another approach exists when all animals in a population are individually known or distinguishable, because this makes it possible to individually test and vaccinate them. This was implemented with a subpopulation of the Iberian lynx. Here, almost an entire subpopulation was caught and evaluated for FeLV infection status (López et al. 2009). Seronegative animals were vaccinated against FeLV, whereas viremic animals were moved to captivity. With this regime, the spread of FeLV infection to other subpopulations was successfully averted, proving that testing and only vaccinating animals that do not provide a risk for other lynxes after being released are the right mode of action for this virus infection in this target species. The examples of rabies in red foxes and Ethiopian wolves as well as FeLV in the Iberian lynxes further show that each disease outbreak in each species needs a specific approach. A general conclusion from these examples is therefore that future vaccination campaigns will only be successful if the specific conditions are fully appreciated and the planning and the preparations are comprehensive and accurate.

In chapter 2 of this thesis, I demonstrated that vaccination of captive cheetahs against FeLV with a vaccine containing killed FeLV induced antibodies (Krengel et al. 2015). This was

similar to a vaccination trial using a recombinant subunit FeLV vaccine in captive cheetahs held in the Wildlife Safari Park in Winston (USA), servals and Bengal tigers (*Panthera tigirs tigirs*) in the Miami Metrozoo (USA), but in contrast to captive tigers and lions in the zoological garden Planète Sauvage (France), where no antibody response against FeLV was induced (Briggs and Ott 1986; Citino 1988; Risi et al. 2012). It is thus important to follow up on the efficacy of vaccinations, especially in captive animals, for which access to samples is usually easily possible. Such an approach has proven to be effective in a captive cheetah group, in which a supposed FeLV outbreak was mitigated through vaccination of the remaining animals (Briggs and Ott 1986). However, the detailed efficacy of vaccines can only be tested in challenge trials with laboratory animals, as a viral challenge of endangered species *in vivo* is not advisable due to obvious reasons. However, the passive protection tests performed with the cheetah serum showed that this test complements the measurement of antibody titres well and can provide a good idea of vaccination efficacy (Turnbull et al. 2004). A viral challenge study was recently conducted to evaluate a recombinant and an inactivated live FeLV vaccine in domestic cats (Stuke et al. 2014). The latter vaccine was significantly more effective than the former in preventing persistent p27 antigenemia and FeLV proviral DNA integration into the bone marrow cells after a challenge with a highly virulent FeLV strain.

The examples I discussed here demonstrate the importance of testing vaccines in terms of efficiency, efficacy and unwanted side-effects for the control of viral diseases in large carnivore species prior to any vaccination campaign. A further step can be to identify and maybe even vaccinate possible reservoir hosts for the different viral agents affecting the wild carnivore populations or posing a risk for human health. This is in line with the conclusion of a review on infectious diseases and conservation of large free-ranging carnivore species (Murray et al. 1999).

4.7 Extrinsic factors affecting the immune system

Some of the agents investigated in the Namibian cheetah such as FCoV and FHV have demonstrably caused disease or were fatal in captive animals but not in free-ranging ones (Evermann 1986; Munson et al. 2004a; Flacke et al. 2015). A study comparing disease prevalence of captive and free-ranging cheetahs confirmed that captivity had a substantial influence on the course of diseases in this species (Munson et al. 2005). Reasons for an increase in disease susceptibility might be allostatic load (“stress”) that suppresses an effective immune response when infected with a pathogen (Ramírez 1998; Munson et al. 2004b). A method to detect allostatic load is the measurement of ‘stress’ hormone metabolites, such as glucocorticoid metabolites, in faeces (Terio et al. 2004; Ludwig et al. 2013). This non-invasive method has the advantage that the animals are not disturbed when

sampled, thus their base stress level can be evaluated. Indeed, cheetahs kept in zoological gardens have higher faecal glucocorticoid metabolite concentrations than free-ranging ones (Terio et al. 2004), supporting the possibility that stress might reduce the immunocompetence of captive cheetahs.

Other extrinsic factors for increased disease susceptibility might be weather and climate conditions. On a larger scale, global climate change can lead to the emergence of diseases in new countries, if the vectors can prevail outside their endemic areas. This was observed for the sandfly (*Phlebotomus papatasi*); the vector needed for the transmission of the protozoa *Leishmania* causing leishmaniasis in dogs and humans. The disease was previously limited to the Mediterranean area, but recently has spread to European countries more distant at temperate latitudes (Semenza and Menne 2009). Such changes pose the potential risk of diseases emerging in hitherto naïve populations. Simulation models on the effects of climate change on vector borne diseases revealed varying, opposite effects for different pathogens and the diseases they are responsible for, thus predictions of the spread of diseases are tinged with a high level of uncertainty (Rogers and Randolph 2006).

4.8 Limitations of serosurveys and vaccinations in carnivores

Serosurveys usually cover one population across a part of the distributional range of a species and thus can only represent a sample of the total gene pool of the species (Walston et al. 2010; Goodrich et al. 2012). Nonetheless, such studies can serve as a good indicator of the physiological capacities of individuals of a species to mount an effective immune response against a pathogen (e.g. Heinrich et al. 2016). However, the transmission probability of pathogens might strongly differ between study areas and populations, thus the same species should be investigated in different ecosystems. For example, Namibian cheetahs mainly occur on farmland and might come into contact with people, livestock, domestic cats and domestic dogs either in a feral form or kept as companion animals, as well as carnivore species such as black-backed jackals, caracals and leopards, whereas in the Serengeti NP in Tanzania, cheetahs mainly come into contact with large carnivore species such as lions, spotted hyenas and leopards (Caro 1994; Schneider 1994; Marker-Kraus et al. 1996; Durant et al. 2007; Lindsey et al. 2013).

Viruses have many options to avoid the immune system of the host, thus it is likely that the impact of the virus on the host may differ in subsequent infections with the same virus (Altizer et al. 2003; Pedersen et al. 2007). The short generation times of viruses, their large population size and the strong selection pressure from the immune system of the host are suspected to accelerate adaptive changes of the virus (Altizer et al. 2003). For example, in the FeLV selection acts on the nucleotide sequence which determines the virulence of the

virus and thus the disease outcome (Overbaugh and Bangham 2001). New strains might therefore impose an increased risk to non-domestic feline species (O'Brien et al. 2012).

In the last decades, molecular methods to measure seroprevalence and detect agents have developed substantially (e.g. O'Brien et al. 2012). However, often the only references at hand are studies conducted many years ago with the methods available at that time (Murray et al. 1999). Sometimes it is possible to use established as well as novel methods in a study and thus compare results obtained with different tests. Such test series are limited by the amount of sample available, the feasibility of former methods and the available funding. However, for some agents, detection methods were only developed recently, such as for the 'new' genus *Kobuvirus* in felids, canids or hyaenids (Chung et al. 2013; Olarte-Castillo et al. 2015). This virus is rather unknown in animals, thus not much research in detection methods has been conducted. Since the new method was described, the presence of canine kobuviruses in African carnivores was detected in the golden jackal (*Canis aureus*), the side-striped jackal (*Canis adustus*), the spotted hyena and domestic dogs, and thus proved not to be as host specific as previously thought (Olarte-Castillo et al. 2015).

Although the impact on a species by a viral epidemic can be substantial, there are many constraints on vaccinations and unforeseen consequences can arise. When vaccinating an animal, it is often unknown whether the animal has an acute or chronic infection (Briggs and Ott 1986). Furthermore, the handling of the animal, including the capture, sampling and vaccination of free-ranging animals can induce stress and thus reduce the vaccination success, as was proposed for the rabies outbreaks in African wild dogs in East Africa (Burrows et al. 1994; Hofer and East 1998). It is thus not advisable to vaccinate species prone to severe reactions towards the stress of handling, which will not only imperil the life of the animal but also the efficacy of the vaccination.

A further limitation to the use of vaccinations in wildlife species is the unknown reaction and thus risk for them if the vaccine was not developed for this species (Cleaveland 2009). Even if a vaccine can be applied to another species, this might have some consequences for the population dynamics of species operating within the same ecological guild as competitors and/or as predators of each other. For example, a mathematical model was developed to forecast the consequences of a successful CDV vaccination campaign of lions with a domestic dog vaccine for the cheetah population in the Serengeti NP (Chauvenet et al. 2011). The improved survival of lions from such a hypothetical campaign was predicted to increase the risk of extinction of the cheetah population by a factor of two because in this model scenario CDV was assessed to be a 'natural' regulator of the lions which both kill and compete with cheetahs.

Individual differences in the efficacy, including lethal outcomes, of vaccinations can partially be explained by variable responses to the vaccine within a host population. For example, vaccines designed for domestic cats can have very diverse individual effects in terms of the induction of antibody formation in domestic cats (Stuke et al. 2014). Also, if an individual mounts an immune response against one component of a multi-agent vaccine, this does not imply a production of antibodies against all agents included in the vaccine (Risi et al. 2012). Furthermore, the detection of antibodies after a vaccination is usually performed with ELISAs developed for domestic cat serum and thus antibodies of other feline species might not always be detected. In young animals, neutralisation or cross-reaction of a vaccine with maternal antibodies is possible, thus the first vaccination should be conducted early in the life of kittens, e.g. when they are eight or nine weeks old, followed by booster vaccinations at the age of twelve weeks and again one year later (Radford et al. 2009). For cheetah cubs, vaccination against FHV, FCV and FPV was even recommended in two week intervals at age eight, ten and twelve weeks of age (Wack et al. 1993), a suggestion that is impractical outside a captive setting.

Thus, vaccinating free-ranging species bears many risks and should be planned and conducted with the utmost caution. Vaccines should be tested first in captive held animals, and the use of an attenuated live vaccine should always be seen as an option of last resort since it carries higher risks for the host than inactivated vaccines (Kennedy-Stoskopf 1999).

4.9 Conclusions and recommendations

My studies confirm that the general health status of the free-ranging and captive Namibia cheetahs is good and that they do not show any clinical signs of infectious diseases. Although up to 19% of the free-ranging cheetah population was FeLV seropositive, depending on the antibody test (Krengel et al. 2015), no infectious antigen or proviral DNA of FeLV was detected in any of the samples with the numerous tests performed. This is promising, because the effects of a FeLV epidemic can be disastrous (e.g. López et al. 2009). It would be valuable to characterise the origin of the antibodies. This could be done, for instance, by analysing tissue samples of antibody positive cheetahs for endogenous gammaretroviruses of feline and murine origin. It would also be useful to test for antibodies against FeLV in Botswana and South Africa from the other (eastern) side of the Kalahari Desert, to shed further light on the distribution of FeLV. The hemoplasma study demonstrated that it is beneficial to screen cheetah samples for new or potentially underestimated agents. With the current state-of-the-art-technique of analysing TNA by PCRs, several tests can be conducted in a cost and time effective manner.

Vaccinations against FeLV and other viruses adduce positive antibody titres in captive cheetahs. These antibodies are not a guarantee for the complete protection against a viral infection, but show that the immune system of the cheetah can mount measurable immune responses. For free-ranging cheetahs and other carnivore species in the vicinity of people and their livestock and companion animals, given that the same viral strains affect wildlife and domestic animals, it might be more feasible to vaccinate the possible domestic reservoir animals, such as cats and dogs, than the cheetahs and to inform local people on the possible risk of zoonotic diseases caused by agents such as rabies or *Bartonella*.

From a health point of view, the prospect for the free-ranging cheetah population in Namibia is promising. The main challenges lie in reducing human wildlife conflicts, habitat fragmentation, habitat destruction, removal of natural prey species, poaching and illegal trading. Translocations of cheetahs as a conflict mitigating action are questionable and thus might only be conducted if other mitigation efforts failed and if the seroprevalence of pathogens in the original and new area are known and are similar.

For future studies it is advisable to collect as many samples as possible to provide the opportunity for retrospective studies on pathogens, the validation of not yet established methods or methods not yet validated for this species. In the light of the potential drastic consequences of infections in vulnerable carnivore species, the need to screen for emerging pathogens is steadily growing. The collaboration of scientists working with free-ranging carnivores and those working with carnivores in a captive setting should also be rewarding, as demonstrated by vaccination trials (Risi et al. 2012; Anderson et al. 2014). Laboratory animals might be included to gain knowledge on the modes of transmission. This expertise will be advantageous for the development of effective action plans to manage possible outbreaks of various agents.

4.10 References

Alexander, K. A., P. W. Kat, L. A. Munson, A. Kalake and M. J. G. Appel (1996). "Canine distemper-related mortality among wild dogs (*Lycaon pictus*) in Chobe National Park, Botswana." J Zoo Wildl Med 27: 426-427.

Altizer, S., D. Harvell and E. Friedle (2003). "Rapid evolutionary dynamics and disease threats to biodiversity." Trends Ecol Evol 18: 589-596.

Anderson, K., A. Case, K. Woodie, W. Waddell and H. H. Reed (2014). "Duration of immunity in red wolves (*Canis rufus*) following vaccination with a modified live parvovirus and canine distemper vaccine." J Zoo Wildl Med 45: 550-554.

Avendaño, R., F. Barrueta, S. Soto-Fournier, M. Chavarría, O. Monge, G. A. Gutiérrez-Espeleta and A. Chaves (2016). "Canine distemper virus in wild felids of Costa Rica." J Wildl Dis 52: 373-377.

Barnard, B., R. Hassel, H. Geyer and W. De Koker (1982). "Non-bite transmission of rabies in kudu (*Tragelaphus strepsiceros*)." Onderstepoort J Vet Res 49: 191-192.

Bellan, S. E., C. A. Cizauskas, J. Miyen, K. Ebersohn, M. Küsters, K. C. Prager, M. Van Vuuren, C. Sabeta and W. M. Getz (2012). "Black-backed jackal exposure to rabies virus, canine distemper virus, and *Bacillus anthracis* in Etosha National Park, Namibia." J Wildl Dis 48: 371-381.

Berentsen, A. R., M. R. Dunbar, M. S. Becker, J. M'Soka, E. Droge, N. M. Sakuya, W. Matandiko, R. McRobb and C. A. Hanlon (2013). "Rabies, canine distemper, and canine parvovirus exposure in large carnivore communities from two Zambian ecosystems." Vector Borne Zoonotic Dis 13: 643-649.

Bosman, A. M., E. H. Venter and B. L. Penzhorn (2007). "Occurrence of *Babesia felis* and *Babesia leo* in various wild felid species and domestic cats in Southern Africa, based on reverse line blot analysis." Vet Parasitol 144: 33-38.

Briggs, M. B. and R. L. Ott (1986). "Feline leukemia virus infection in a captive cheetah and the clinical and antibody response of six captive cheetahs to vaccination with a subunit feline leukemia virus vaccine." J Am Vet Med Assoc 189: 1197-1199.

Brown, E. W., S. Miththapala and S. J. O'Brien (1993). "Prevalence of exposure to feline immunodeficiency virus in exotic felids." J Zoo Wildl Med 24: 357-364.

Brown, M. A., M. W. Cunningham, A. L. Roca, J. L. Troyer, W. E. Johnson and S. J. O'Brien (2008). "Genetic characterization of feline leukemia virus from Florida panthers." Emerg Infect Dis 14: 252-259.

Burrows, R. (1992). "Rabies in wild dogs." Nature 359: 277.

Burrows, R. (1994). "Rabies in African wild dogs of Tanzania." J Wildl Dis 30: 297-299.

Burrows, R., H. Hofer and M. L. East (1994). "Demography, extinction and intervention in a small population: the case of the Serengeti wild dogs." Proc R Soc Lond *B* Biol Sci 256: 281-292.

Caro, T. M. (1994). Cheetahs of the Serengeti Plains: Group living in an asocial species. Chicago, University of Chicago Press.

Castro-Prieto, A., B. Wachter, J. Melzheimer, S. Thalwitzer, H. Hofer and S. Sommer (2012). "Immunogenetic variation and differential pathogen exposure in free-ranging cheetahs across Namibian farmlands." PLoS ONE 7: e49129.

Chandhasin, C., P. N. Coan and L. S. Levy (2005). "Subtle mutational changes in the SU protein of a natural feline leukemia virus subgroup A isolate alter disease spectrum." J Virol 79: 1351-1360.

Chauvenet, A. L. M., S. M. Durant, R. Hilborn and N. Pettorelli (2011). "Unintended consequences of conservation actions: managing disease in complex ecosystems." PLoS ONE 6: e28671.

Chung, J.-Y., S.-H. Kim, Y.-H. Kim, M.-H. Lee, K.-K. Lee and J.-K. Oem (2013). "Detection and genetic characterization of feline kobuviruses." Virus Genes 47: 559-562.

Citino, S. B. (1988). "Use of a subunit feline leukemia virus vaccine in exotic cats." J Am Vet Med Assoc 192: 957-959.

Cleaveland, S. (2009). "Viral threats and vaccination: disease management of endangered species." Anim Conserv 12: 187-189.

Cleaveland, S., M. G. Appel, W. S. Chalmers, C. Chillingworth, M. Kaare and C. Dye (2000). "Serological and demographic evidence for domestic dogs as a source of canine distemper virus infection for Serengeti wildlife." Vet Microbiol 72: 217-227.

Connolly, M., P. Thomas, R. Woodroffe and B. L. Raphael (2013). "Comparison of oral and intramuscular recombinant canine distemper vaccination in African wild dogs (*Lycaon pictus*)." J Zoo Wildl Med 44: 882-888.

Connolly, M., P. Thomas, R. Woodroffe and B. L. Raphael (2015). "Single- versus double-dose rabies vaccination in captive African wild dogs (*Lycaon pictus*)." J Zoo Wildl Med 46: 691-698.

Cunningham, M. W., M. A. Brown, D. B. Shindle, S. P. Terrell, K. A. Hayes, B. C. Ferree, R. T. McBride, E. L. Blankenship, D. Jansen, S. B. Citino, M. E. Roelke, R. A. Kiltie, J. L. Troyer and S. J. O'Brien (2008). "Epizootiology and management of feline leukemia virus in the Florida puma." J Wildl Dis 44: 537-552.

Daoust, P.-Y., S. R. McBurney, D. L. Godson, M. W. G. v. d. Bildt and A. D. M. E. Osterhaus (2009). "Canine distemper virus-associated encephalitis in free-living lynx (*Lynx canadensis*) and bobcats (*Lynx rufus*) of eastern Canada." J Wildl Dis 45: 611-624.

Duarte, A., M. Fernandes, N. Santos and L. Tavares (2012). "Virological Survey in free-ranging wildcats (*Felis silvestris*) and feral domestic cats in Portugal." Vet Microbiol 158: 400-404.

Durant, S. M., S. Bashir, T. Maddox and M. K. Laurenson (2007). "Relating long-term studies to conservation practice: the case of the Serengeti Cheetah Project." Conserv Biol 21: 602-611.

Durchfeld, B., W. Baumgärtner, W. Herbst and R. Brahm (1990). "Vaccine-associated canine distemper infection in a litter of African hunting dogs (*Lycaon pictus*)." J Vet Med, Series B 37: 203-212.

East, M. L. and R. Burrows (2001). "The efficacy of inactivated rabies vaccine for the protection of African wild dogs (*Lycaon pictus*) against viral challenge - a review." Verh ber Erkrg Zootiere 40: 233-242.

East, M. L., H. Hofer, J. H. Cox, U. Wulle, H. Wiik and C. Pitra (2001). "Regular exposure to rabies virus and lack of symptomatic disease in Serengeti spotted hyenas." Proc Natl Acad Sci USA 98: 15026-15031.

East, M. L., J. W. Nyahongo, K. Goller and H. Hofer (2012). Does the vastness of the Serengeti limit human-wildlife conflicts? In: Fencing for Conservation: restriction of evolutionary potential or a riposte to threatening processes? M. J. Somers and M. W. Hayward (eds.): 125-151. Heidelberg, New York, Springer.

East, M. L., G. Wibbelt, D. Lieckfeldt, A. Ludwig, K. Goller, K. Wilhelm, G. Schares, D. Thierer and H. Hofer (2008). "A *hepatozoon* species genetically distinct from *H. canis* infecting spotted hyenas in the Serengeti ecosystem." J Wildl Dis 44: 45-52.

Evermann, J. F. (1986). "Feline coronavirus infection of cheetahs." Fel Pract 16: 21-30.

Filoni, C., J. L. Catão-Dias, V. Cattori, B. Willi, M. L. Meli, S. H. R. Corrêa, M. C. Marques, C. H. Adania, J. C. R. Silva, M. F. V. Marvulo, J. S. F. Neto, E. L. Durigon, V. M. de Carvalho, S. D. A. Coutinho, H. Lutz and R. Hofmann-Lehmann (2012). "Surveillance using serological and molecular methods for the detection of infectious agents in captive Brazilian neotropic and exotic felids." J Vet Diagn Invest 24: 166-173.

Flacke, G. L., A. Schmidt-Küntzel and L. Marker (2015). "Treatment of chronic herpesviral dermatitis in a captive cheetah (*Acinonyx jubatus*) in Namibia." J Zoo Wildl Med 46: 641-646.

Foley, J. E., P. Swift, K. A. Fleer, S. Torres, Y. A. Girard and C. K. Johnson (2013). "Risk factors for exposure to feline pathogens in California mountain lions (*Puma concolor*)." J Wildl Dis 49: 279-293.

Freuling, C. M., K. Hampson, T. Selhorst, R. Schröder, F. X. Meslin, T. C. Mettenleiter and T. Müller (2013). "The elimination of fox rabies from Europe: determinants of success and lessons for the future." Philos Trans R Soc B: Biol Sci 368: 20120142.

Furtado, M. M., J. D. de Ramos Filho, K. C. Scheffer, C. J. Coelho, P. S. Cruz, C. Y. Ikuta, A. T. de Almeida Jácomo, G. E. de Oliveira Porfírio, L. Silveira, R. Sollmann, N. M. Tôrres and J. S. Ferreira Neto (2013). "Serosurvey for selected viral infections in free-ranging jaguars (*Panthera onca*) and domestic carnivores in Brazilian Cerrado, Pantanal and Amazon." J Wildl Dis 49: 510-521.

Gascoyne, S., M. K. Laurenson, S. Lelo and M. Borner (1993a). "Rabies in African wild dogs (*Lycaon pictus*) in the Serengeti region, Tanzania." J Wildl Dis 29: 396-402.

Gascoyne, S. C., A. A. King, M. K. Laurenson, M. Borner, B. Schildger and J. Barrat (1993b). "Aspects of rabies infection and control in the conservation of the African wild dog (*Lycaon pictus*) in the Serengeti region, Tanzania." Onderstepoort J Vet Res 60: 415-420.

Goller, K. V., R. D. Fyumagwa, V. Nikolin, M. L. East, M. Kilewo, S. Speck, T. Müller, M. Matzke and G. Wibbelt (2010). "Fatal canine distemper infection in a pack of African wild dogs in the Serengeti ecosystem, Tanzania." Vet Microbiol 146: 245-252.

Goodrich, J. M., K. S. Quigley, J. C. M. Lewis, A. A. Astafiev, E. V. Slabi, D. G. Miquelle, E. N. Smirnov, L. L. Kerley, D. L. Armstrong, H. B. Quigley and M. G. Hornocker (2012). "Serosurvey of free-ranging Amur tigers in the Russian Far East." J Wildl Dis 48: 186-189.

Gordon, C. H., A. C. Banyard, A. Hussein, M. K. Laurenson, J. R. Malcolm, J. Marino, F. Regassa, A.-M. E. Stewart, A. R. Fooks and C. Sillero-Zubiri (2015). "Canine distemper in endangered Ethiopian wolves." Emerg Infect Dis 21: 824-832.

Gowtage-Sequeira, S., A. C. Banyard, T. Barrett, H. Buczkowski, S. M. Funk and S. Cleaveland (2009). "Epidemiology, pathology, and genetic analysis of a canine distemper epidemic in Namibia." J Wildl Dis 45: 1008-1020.

Günther-Weigl, A. (2009). “Humoral immune response in Eurasian otters (*Lutra lutra*), Asian small-clawed otters (*Aonyx cinereus*) and North American river otters (*Lontra canadensis*) after vaccination with different canine distemper virus vaccines.” Disseration, Freie Universität Berlin.

Haas, L., H. Hofer, M. East, P. Wohlsein, B. Liess and T. Barrett (1996). "Canine distemper virus infection in Serengeti spotted hyenas." Vet Microbiol 49: 147-152.

Harrenstien, L. A., L. Munson, E. C. Ramsay, C. F. Lucash, S. A. Kania and L. N. D. Potgieter (1997). "Antibody responses of red wolves to canine distemper virus and canine parvovirus vaccination." J Wildl Dis 33: 600-605.

Haydon, D. T., D. A. Randall, L. Matthews, D. L. Knobel, L. A. Tallents, M. B. Gravenor, S. D. Williams, J. P. Pollinger, S. Cleaveland, M. E. J. Woolhouse, C. Sillero-Zubiri, J. Marino, D. W. Macdonald and M. K. Laurenson (2006). "Low-coverage vaccination strategies for the conservation of endangered species." Nature 443: 692-695.

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

Hofer, H. and M. L. East (1998). "Biological conservation and stress." Adv Study Behav 27: 405-525.

Hofmann-Lehmann, R., D. Fehr, M. Grob, M. Elgizoli, C. Packer, J. S. Martenson, S. J. O'Brien and H. Lutz (1996). "Prevalence of antibodies to feline parvovirus, calicivirus, herpesvirus, coronavirus, and immunodeficiency virus and of feline leukemia virus antigen and the interrelationship of these viral infections in free-ranging lions in East Africa." Clin Diagn Lab Immunol 3: 554-562.

Junge, R. E., R. E. Miller, W. J. Boever, G. Scherba and J. Sundberg (1991). "Persistent cutaneous ulcers associated with feline herpesvirus type 1 infection in a cheetah." J Am Vet Med Assoc 198: 1057-1058.

Kat, P. W., K. A. Alexander, J. S. Smith, J. D. Richardson and L. Munson (1996). "Rabies among African wild dogs (*Lycaon pictus*) in the Masai Mara, Kenya." J Vet Diagn Invest 8: 420-426.

Keesing, F., R. D. Holt and R. S. Ostfeld (2006). "Effects of species diversity on disease risk." Ecol Lett 9: 485-498.

Kelly, P., L. Marabini, K. Dutlow, J. Zhang, A. Loftis and C. Wang (2014). "Molecular detection of tick-borne pathogens in captive wild felids, Zimbabwe." Parasit Vectors 7: 514.

Kennedy-Stoskopf, S. (1999). Emerging viral infections in large cats. In: Zoo and Wild Animal Medicine. M. E. Fowler and R. E. Miller (eds.); 4: 401-410. Philadelphia, PA, USA, W.B. Saunders.

Kennedy, M., S. Kania, E. Stylianides, H. Bertschinger, D. Keet and M. van Vuuren (2003). "Detection of feline coronavirus infection in Southern African nondomestic felids." J Wildl Dis 39: 529-535.

Krengel, A., V. Cattori, M. L. Meli, B. Wachter, J. Boni, L. R. Bisset, S. Thalwitzer, J. Melzheimer, M. Jago, R. Hofmann-Lehmann, H. Hofer and H. Lutz (2015). "Gammaretrovirus-specific antibodies in free-ranging and captive namibian cheetahs." Clin Vaccine Immunol 22: 611-617.

Krengel, A., M. L. Meli, V. Cattori, B. Wachter, B. Willi, S. Thalwitzer, J. Melzheimer, H. Hofer, H. Lutz and R. Hofmann-Lehmann (2013). "First evidence of hemoplasma infection in free-ranging Namibian cheetahs (*Acinonyx jubatus*)." Vet Microbiol 162: 972-976.

Kuiken, T., S. Kennedy, T. Barrett, M. W. G. Van de Bildt, F. H. Borgsteede, S. D. Brew, G. A. Codd, C. Duck, R. Deaville, T. Eybatov, M. A. Forsyth, G. Foster, P. D. Jepson, A. Kydyrmanov, I. Mitrofanov, C. J. Ward, S. Wilson and A. D. M. E. Osterhaus (2006). "The 2000 canine distemper epidemic in Caspian seals (*Phoca caspica*): pathology and analysis of contributory factors." Vet Pathol 43: 321-338.

Lindemann, D. M., J. W. Carpenter, J. C. Nietfeld, E. Gonzalez, M. Hallman and B. M. Hause (2015). "Multicentric T-cell lymphoma and cutaneous hemangiosarcoma in a captive cheetah (*Acinonyx jubatus*)." J Zoo Wildl Med 46: 961-966.

Lindsey, P. A., C. P. Havemann, R. Lines, L. Palazy, A. E. Price, T. A. Retief, T. Rhebergen and C. Van der Waal (2013). "Determinants of persistence and tolerance of carnivores on Namibian ranches: implications for conservation on Southern African private lands." PLoS ONE 8: e52458.

López, G., M. López-Parra, L. Fernández, C. Martínez-Granados, F. Martínez, M. L. Meli, J. M. Gil-Sánchez, N. Viqueira, M. A. Díaz-Portero, R. Cadenas, H. Lutz, A. Vargas and M. A. Simón (2009). "Management measures to control a feline leukemia virus outbreak in the endangered Iberian lynx." Anim Conserv 12: 173-182.

Ludwig, C., B. Wachter, S. Silinski-Mehr, A. Ganswindt, H. Bertschinger, H. Hofer and M. Dehnhard (2013). "Characterisation and validation of an enzyme-immunoassay for the non-invasive assessment of faecal glucocorticoid metabolites in cheetahs (*Acinonyx jubatus*)." Gen Comp Endocrinol 180: 15-23.

Maas, M., D. F. Keet, V. P. M. G. Rutten, J. A. P. Heesterbeek and M. Nielen (2012). "Assessing the impact of feline immunodeficiency virus and bovine tuberculosis co-infection in African lions." Proc R Soc Lond *B* Biol Sci 279: 4206-4214.

Mansfield, K., L. McElhinney, O. Hübschle, F. Mettler, C. Sabeta, L. H. Nel and A. R. Fooks (2006). "A molecular epidemiological study of rabies epizootics in kudu (*Tragelaphus strepsiceros*) in Namibia." BMC Vet Res 2: 2-2.

Manyarara, R., U. Tubbesing, M. Soni and B. H. Noden (2015). "Serodetection of *Ehrlichia canis* amongst dogs in central Namibia." J S Afr Vet Assoc 86: 1272.

Marker-Kraus, L., D. Kraus, D. Barnett and S. Hurlbut (1996). “Cheetah survival on Namibian farmlands”. Windhoek, Cheetah Conservation Fund.

Marker, L., L. Munson, P. A. Basson and S. Quackenbush (2003). "Multicentric T-cell lymphoma associated with feline leukemia virus infection in a captive Namibian cheetah (*Acinonyx jubatus*)." J Wildl Dis 39: 690-695.

McCormick, A. E. (1983). "Canine distemper in African cape hunting dogs (*Lycaon pictus*): possibly vaccine induced." J Zoo Anim Med 14: 66-71.

Meli, M. L., V. Cattori, F. Martinez, G. Lopez, A. Vargas, F. Palomares, J. V. Lopez-Bao, R. Hofmann-Lehmann and H. Lutz (2010). "Feline leukemia virus infection: A threat for the survival of the critically endangered Iberian lynx (*Lynx pardinus*)." Vet Immunol Immunopathol 134: 61-67.

Meli, M. L., V. Cattori, F. Martinez, G. Lopez, A. Vargas, M. A. Simon, I. Zorrilla, A. Munoz, F. Palomares, J. V. Lopez-Bao, J. Pastor, R. Tandon, B. Willi, R. Hofmann-Lehmann and H. Lutz (2009). "Feline leukemia virus and other pathogens as important threats to the survival of the critically endangered Iberian lynx (*Lynx pardinus*)." PLoS ONE 4: e4744.

Meny, M., A. Schmidt-Kuntzel and L. L. Marker (2012). "Diagnosis-based treatment of helminths in captive and wild cheetahs (*Acinonyx jubatus*)." J Zoo Wildl Med 43: 934-938.

Millward, I. R. and M. C. Williams (2005). "*Cryptococcus neoformans* granuloma in the lung and spinal cord of a free-ranging cheetah (*Acinonyx jubatus*). A clinical report and literature review." J S Afr Vet Assoc 76: 228-232.

Molia, S., B. B. Chomel, R. W. Kasten, C. M. Leutenegger, B. R. Steele, L. Marker, J. S. Martenson, D. F. Keet, R. G. Bengis, R. P. Peterson, L. Munson and S. J. O'Brien (2004). "Prevalence of *Bartonella* infection in wild African lions (*Panthera leo*) and cheetahs (*Acinonyx jubatus*)." Vet Microbiol 100: 31-41.

Mora, M., C. Napolitano, R. Ortega, E. Poulin and J. Pizarro-Lucero (2015). "Feline immunodeficiency virus and feline leukemia virus infection in free-ranging guignas (*Leopardus guigna*) and sympatric domestic cats in human pertubed landscapes on Chiloé island, Chile." J Wildl Dis 51: 199-208.

Müller, T. F., R. Schröder, P. Wysocki, T. C. Mettenleiter and C. M. Freuling (2015). "Spatio-temporal use of oral rabies vaccines in fox rabies elimination programmes in Europe." PLoS Negl Tropic Dis 9: e0003953.

Munson, L., L. Marker, E. Dubovi, J. A. Spencer, J. F. Evermann and S. J. O'Brien (2004a). "Serosurvey of viral infections in free-ranging Namibian cheetahs (*Acinonyx jubatus*)." J Wildl Dis 40: 23-31.

Munson, L., K. A. Terio, R. Kock, T. Mlengeya, M. E. Roelke, E. Dubovi, B. Summers, A. R. Sinclair and C. Packer (2008). "Climate extremes promote fatal co-infections during canine distemper epidemics in African lions." PLoS ONE 3: e2545.

Munson, L., K. A. Terio, M. Worley, M. Jago, A. Bagot-Smith and L. Marker (2005). "Extrinsic factors significantly affect patterns of disease in free-ranging and captive cheetah (*Aciynoyx jubatus*) populations." J Wildl Dis 41: 542-548.

Munson, L., R. Wack, M. Duncan, R. J. Montali, D. Boon, I. Stalis, G. J. Crawshaw, K. N. Cameron, J. Mortenson, S. Citino, J. Zuba and R. E. Junge (2004b). "Chronic eosinophilic dermatitis associated with persistent feline herpes virus infection in cheetahs (*Acinonyx jubatus*)." Vet Pathol 41: 170-176.

Murray, D. L., C. A. Kapke, J. F. Evermann and T. K. Fuller (1999). "Infectious disease and the conservation of free-ranging large carnivores." Anim Conserv 2: 241-254.

Neil, J. C., R. Fulton, M. Rigby and M. Stewart (1991). Feline leukaemia virus: generation of pathogenic and oncogenic variants. In: Retroviral insertion and oncogene activation. H.-J. Kung and P. K. Vogt (eds.): 67-93. Berlin, Heidelberg, Springer.

Nikolin V. M., X. A. Olarte-Castillo, N. Osterrieder, H. Hofer, E. Dubovi, C. J. Mazzoni, E. Brunner, K.V. Goller, R. D. Fyumagwa, P. D. Moehlman, D. Thierer and M. L. East (2016, *revision submitted*). “Canine distemper virus in the Serengeti ecosystem: molecular adaptation to different carnivore species.” Mol Ecol.

Nikolin, V. M., K. Osterrieder, V. von Messling, H. Hofer, D. Anderson, E. Dubovi, E. Brunner and M. L. East (2012a). "Antagonistic pleiotropy and fitness trade-offs reveal specialist and generalist traits in strains of canine distemper virus." PLoS ONE 7: e50955.

Nikolin, V. M., G. Wibbelt, F.-U. F. Michler, P. Wolf and M. L. East (2012b). "Susceptibility of carnivore hosts to strains of canine distemper virus from distinct genetic lineages." Vet Microbiol 156: 45-53.

Noden, B. H. and M. Soni (2015). Vector-borne diseases of small companion animals in Namibia: Literature review, knowledge gaps and opportunity for a One Health approach. J S Afr Vet Assoc 86: 1307.

Noden, B. H. and B. E. van der Colf (2013). "Neglected tropical diseases of Namibia: Unsolved mysteries." Acta Tropica 125: 1-17.

O’Brien, S. J., J. L. Troyer, M. A. Brown, W. E. Johnson, A. Antunes, M. E. Roelke and J. Pecon-Slattery (2012). "Emerging viruses in the felidae: Shifting paradigms." Viruses 4: 236-257.

Olarte-Castillo, X. A., F. Heeger, C. J. Mazzoni, A. D. Greenwood, R. Fyumagwa, P. D. Moehlman, H. Hofer and M. L. East (2015). "Molecular characterization of canine kobuvirus in wild carnivores and the domestic dog in Africa." Virology 477: 89-97.

Overbaugh, J. and C. R. M. Bangham (2001). "Selection forces and constraints on retroviral sequence variation." Science 292: 1106-1109.

Pecon-Slattery, J., J. L. Troyer, W. E. Johnson and S. J. O'Brien (2008). "Evolution of feline immunodeficiency virus in Felidae: Implications for human health and wildlife ecology." Vet Immunol Immunopathol 123: 32-44.

Pedersen, A. B., K. E. Jones, C. L. Nunn and S. Altizer (2007). "Infectious diseases and extinction risk in wild mammals." Conserv Biol 21: 1269-1279.

Penzhorn, B. L. (2006). "Babesiosis of wild carnivores and ungulates." Vet Parasitol 138: 11-21.

Philippa, J., A. Visee, M. W. G. van de Bildt, M. Hoyer and A. Osterhaus (2006). "Humoral immune response of African wild dogs (*Lycaon pictus*) to novel canine distemper vaccines." Proc. Am. Assoc. Zoo Vet: 254-255.

Radford, A. D., D. Addie, S. Belak, C. Boucraut-Baralon, H. Egberink, T. Frymus, T. Gruffydd-Jones, K. Hartmann, M. J. Hosie, A. Lloret, H. Lutz, F. Marsilio, M. G. Pennisi, E. Thiry, U. Truyen and M. C. Horzinek (2009). "Feline calicivirus infection. ABCD guidelines on prevention and management." J Feline Med Surg 11: 556-564.

Ramírez, F. (1998). "Glucocorticoids induce a Th2 response in vitro." J Immunol Res 6: 233-243.

Ramsauer, S., G. Bay, M. Meli, R. Hofmann-Lehmann and H. Lutz (2007). "Seroprevalence of selected infectious agents in a free-ranging, low-density lion population in the Central Kalahari Game Reserves in Botswana." Clin Vaccine Immunol 14: 808-810.

Randall, D. A., S. D. Williams, I. V. Kuzmin, C. E. Rupprecht, L. A. Tallents, Z. Tefera, K. Argaw, F. Shiferaw, D. L. Knobel, C. Sillero-Zubiri and M. K. Laurenson (2004). "Rabies in endangered Ethiopian wolves." Emerg Inf Dis 10: 2214-2217.

Risi, E., A. Agoulon, F. Allaire, S. Le Dréan-Quénec'hdu, V. Martin and P. Mahl (2012). "Antibody response to vaccines for rhinotracheitis, caliciviral disease, panleukopenia, feline leukemia, and rabies in tigers (*Panthera tigris*) and lions (*Panthera leo*)." J Zoo Wildl Med 43: 248-255.

Roelke-Parker, M. E., L. Munson, C. Packer, R. Kock, S. Cleaveland, M. Carpenter, S. J. O'Brien, A. Pospischil, R. Hofmann-Lehmann, H. Lutz, G. L. Mwamengele, M. N. Mgasa, G. A. Machange, B. A. Summers and M. J. Appel (1996). "A canine distemper virus epidemic in Serengeti lions (*Panthera leo*)." Nature 379: 441-445.

Roelke, M. E., M. A. Brown, J. L. Troyer, H. Winterbach, C. Winterbach, G. Hemson, D. Smith, R. C. Johnson, J. Pecon-Slattery, A. L. Roca, K. A. Alexander, L. Klein, P. Martelli, K. Krishnasamy and S. J. O'Brien (2009). "Pathological manifestations of feline immunodeficiency virus (FIV) infection in wild African lions." Virology 390: 1-12.

Roelke, M. E., D. J. Forrester, E. R. Jacobson, G. V. Kollias, F. W. Scott, M. C. Barr, J. F. Evermann and E. C. Pirtle (1993). "Seroprevalence of infectious disease agents in free-ranging Florida panthers (*Felis concolor coryi*)." J Wildl Dis 29: 36-49.

Rogers, D. J. and S. E. Randolph (2006). "Climate change and vector-borne diseases." Adv Parasitol 62: 345-381.

Rust, N. A. and L. L. Marker (2013). "Attitudes toward predators and conservancies among Namibian farmers." Hum Dimens Wildl 18: 463-468.

Sakuma, T., J. M. Tonne, J. A. Malcolm, T. Thatava, S. Ohmine, K. W. Peng and Y. Ikeda (2012). "Long-term infection and vertical transmission of a gammaretrovirus in a foreign host species." PLoS ONE 7: e29682.

Sakuma, T., J. M. Tonne, K. A. Squillace, S. Ohmine, T. Thatava, K. W. Peng, M. A. Barry and Y. Ikeda (2011). "Early events in retrovirus XMRV infection of the wild-derived mouse *Mus pahari*." J Virol 85: 1205-1213.

Scheepers, J. L. and K. A. E. Venzke (1995). "Attempts to reintroduce African wild dogs *Lycaon pictus* into Etosha National Park, Namibia." S Afr J Wildl Res 25: 138-140.

Schneider, H. P. (1994). Animal health and veterinary medicine in Namibia. Agrivet. Windhoek, Namibia.

Seimon, T. A., D. G. Miquelle, T. Y. Chang, A. L. Newton, I. Korotkova, G. Ivanchuk, E. Lyubchenko, A. Tupikov, E. Slabe and D. McAloose (2013). "Canine distemper virus: an emerging disease in wild endangered Amur tigers (*Panthera tigris altaica*)." mBio 4: e00410-00413.

Semenza, J. C. and B. Menne (2009). "Climate change and infectious diseases in Europe." Lancet Infect Dis 9: 365-375.

Silva, C. P. A. e., S. S. M. Onuma, D. M. de Aguiar, V. Dutra and L. Nakazato (2016). "Molecular detection of feline leukemia virus in free-ranging jaguars (*Panthera onca*) in the Pantanal region of Mato Grosso, Brazil." Braz J Inf Dis 20: 316-317.

Spencer, J. A. and P. Morkel (1993). "Serological survey of sera from lions in Etosha national park." S Afr J Wildl Res 23: 60-61.

Stuke, K., V. King, K. Southwick, M. I. Stoeva, A. Thomas and M. T. Winkler (2014). "Efficacy of an inactivated FeLV vaccine compared to a recombinant FeLV vaccine in minimum age cats following virulent FeLV challenge." Vaccine 32: 2599-2603.

Tasker, S., I. R. Peters, A. D. Mumford, M. J. Day, T. J. Gruffydd-Jones, S. Day, A.-M. Pretorius, R. J. Birtles, C. R. Helps and H. Neimark (2010). "Investigation of human haemotropic *Mycoplasma* infections using a novel generic *haemoplasma* qPCR assay on blood samples and blood smears." J Med Microbiol 59: 1285-1292.

Terio, K. A., L. Marker and L. Munson (2004). "Evidence for chronic stress in captive but not free-ranging cheetahs (*Acinonyx jubatus*) based on adrenal morphology and function." J Wildl Dis 40: 259-266.

Thalwitzer, S. (2007). Reproductive activity in cheetah females, cub survival and health of male and female cheetahs on Namibian farmland. Berlin, Freie Universität, Berlin, Germany; Mensch & Buch Verlag.

Thalwitzer, S., B. Wachter, N. Robert, G. Wibbelt, T. Muller, J. Lonzer, M. L. Meli, G. Bay, H. Hofer and H. Lutz (2010). "Seroprevalences to viral pathogens in free-ranging and captive cheetahs (*Acinonyx jubatus*) on Namibian farmland." Clin Vaccine Immunol 17: 232-238.

Thomas, L. H., I. V. Seryodkin, J. M. Goodrich, D. G. Miquelle, R. J. Birtles and J. C. M. Lewis (2016). "Detection of *Hepatozoon felis* in ticks collected from free-ranging Amur tigers (*Panthera tigris altaica*), Russian Far East, 2002–2012." J Wildl Dis 52: 674-676.

Troyer, J. L., J. Pecon-Slattery, M. E. Roelke, W. Johnson, S. VandeWoude, N. Vazquez-Salat, M. Brown, L. Frank, R. Woodroffe, C. Winterbach, H. Winterbach, G. Hemson, M. Bush, K. A. Alexander, E. Revilla and S. J. O'Brien (2005). "Seroprevalence and genomic divergence of circulating strains of feline immunodeficiency virus among Felidae and Hyaenidae species." J Virol 79: 8282-8294.

Troyer, J. L., M. E. Roelke, J. M. Jespersen, N. Baggett, V. Buckley-Beason, D. MacNulty, M. Craft, C. Packer, J. Pecon-Slattery and S. J. O'Brien (2011). "FIV diversity: FIV(Ple) subtype composition may influence disease outcome in African lions." Vet Immunol Immunopathol 143: 338-346.

Turnbull, P. C. B., B. W. Tindall, J. D. Coetzee, C. M. Conradie, R. L. Bull, P. M. Lindeque and O. J. B. Huebschle (2004). "Vaccine-induced protection against anthrax in cheetah (*Acinonyx jubatus*) and black rhinoceros (*Diceros bicornis*)." Vaccine 22: 3340-3347.

Uhart, M. M., M. V. Rago, C. A. Marull, H. d. V. Ferreyra and J. A. Pereira (2012). "Exposure to selected pathogens in Geoffroy's cats and domestic carnivores from central Argentina." J Wildl Dis 48: 899-909.

van Heerden, J., J. Bingham, M. van Vuuren, R. E. Burroughs and E. Stylianides (2002). "Clinical and serological response of wild dogs (*Lycaon pictus*) to vaccination against canine distemper, canine parvovirus infection and rabies." J S Afr Vet Assoc 73: 8-12.

Viana, M., S. Cleaveland, J. Matthiopoulos, J. Halliday, C. Packer, M. E. Craft, K. Hampson, A. Czupryna, A. P. Dobson, E. J. Dubovi, E. Ernest, R. Fyumagwa, R. Hoare, J. G. C. Hopcraft, D. L. Horton, M. T. Kaare, T. Kanellos, F. Lankester, C. Mentzel, T. Mlengeya, I. Mzimbiri, E. Takahashi, B. Willett, D. T. Haydon and T. Lembo (2015). "Dynamics of a morbillivirus at the domestic–wildlife interface: Canine distemper virus in domestic dogs and lions." Proc. Natl. Acad. Sci. USA 112: 1464-1469.

Wack, R. F., L. W. Kramer, W. L. Cupps, S. Clawson and D. R. Hustead (1993). "The response of cheetahs (*Acinonyx jubatus*) to routine vaccination." J Zoo Wildl Med 24: 109-117.

Walston, J., J. G. Robinson, E. L. Bennett, U. Breitenmoser, G. A. B. da Fonseca, J. Goodrich, M. Gumal, L. Hunter, A. Johnson, K. U. Karanth, N. Leader-Williams, K. MacKinnon, D. Miquelle, A. Pattanavibool, C. Poole, A. Rabinowitz, J. L. D. Smith, E. J. Stokes, S. N. Stuart, C. Vongkhamheng and H. Wibisono (2010). "Bringing the tiger back from the brink—The six percent solution." PLoS Biol 8: e1000485.

Watson, F. G. R., M. S. Becker, J. Milanzi and M. Nyirenda (2015). "Human encroachment into protected area networks in Zambia: implications for large carnivore conservation." Reg Environ Change 15: 415-429.

Weise, F. J., J. R. Lemeris, S. J. Munro, A. Bowden, C. Venter, M. van Vuuren and R. J. van Vuuren (2015). "Cheetahs (*Acinonyx jubatus*) running the gauntlet: an evaluation of translocations into free-range environments in Namibia." PeerJ 3: e1346.

Weise, F. J., K. J. Stratford and R. J. van Vuuren (2014). "Financial costs of large carnivore translocations--accounting for conservation." PLoS ONE 9: e105042.

Willett, B. J., J. N. Flynn and M. J. Hosie (1997). "FIV infection of the domestic cat: an animal model for AIDS." Immunol Today 18: 182-189.

Willi, B., C. Filoni, J. L. Catao-Dias, V. Cattori, M. L. Meli, A. Vargas, F. Martinez, M. E. Roelke, M. P. Ryser-Degiorgis, C. M. Leutenegger, H. Lutz and R. Hofmann-Lehmann (2007). "Worldwide Occurrence of Feline Hemoplasma Infections in Wild Felid Species." J Clin Microbiol 45: 1159-1166.

Williams, B. M., A. Berentsen, B. C. Shock, M. Teixiera, M. R. Dunbar, M. S. Becker and M. J. Yabsley (2014). "Prevalence and diversity of *Babesia*, *Hepatozoon*, *Ehrlichia*, and *Bartonella* in wild and domestic carnivores from Zambia, Africa." Parasitol Res 113: 911-918.

Woodroffe, R. (2001). "Assessing the risks of intervention: immobilization, radio-collaring and vaccination of African wild dogs." Oryx 35: 234-244.

5. Appendix

5.1 Measuring hormone levels for health assessment

For a comprehensive assessment of the health status of an individual, not only pathogen screening is important but also the measurements of glucocorticoids and sex steroid hormones and their metabolites (Munck et al. 1984; Hau and Goymann 2015; Kalbitzer et al. 2015; Duque and Munhoz 2016). Glucocorticoids consist mainly of corticosterone and cortisol and their metabolites and can be used to assess the allostatic load ("stress") of an individual, whereas androgens such as testosterone can be used to assess reproductive function (Preston et al. 2012; Benhaiem et al. 2013).

Handling of free-ranging or captive animals often induces stress and glucocorticoid concentrations increase within a few minutes (Romero and Reed 2005). Thus, measurement of glucocorticoids in the blood after handling is misleading. Similarly, measuring testosterone concentrations in the blood might be inaccurate because this hormone is excreted in pulses over the course of a day (Ludwig et al. 2013). Thus in both cases, measurement of the hormone metabolites in faeces is a good alternative, because it is a non-invasive method and provides an integrated measurement of the hormone status over the time period between two defecations, which in the case of cheetahs approximates to 24 hours (Palme et al. 2005; Pribbenow et al. 2015).

To determine the concentration of hormone metabolites in faeces, species-specific immunoassays need to be developed. For cheetahs, several radio-immunoassays (RIA) and one enzyme-immunoassay (EIA) were available to measure faecal glucocorticoid metabolites (fGCM; Jurke et al. 1997; Terio et al. 1999; Wasser et al. 2000; Young et al. 2004), but no characterisation of the fGCM or exact affinity to the immunoassays were available. Therefore, the CRP tested and compared several non-radioactive immunoassays and identified the most reliable antibody for accurately assessing metabolite levels (Ludwig et al. 2013).

For faecal testosterone metabolites (fTM), no EIA was available, thus the evaluation and verification of an EIA was conducted from scratch and involved more comprehensive tests and measurements than the EIA for fGCMs (Pribbenow et al. 2016). The development of an EIA for fTM requires several steps, including (1) the collection of basic information on testosterone metabolism using a ^{3}H-testosterone radiometabolism study, (2) the characterisation of fTMs by high-performance liquid chromatography (HPLC) analyses and (3) the physiological validation of an EIA to measure fTM by injecting exogenous testosterone and by conducting a GnRH challenge, which induces an increase in testosterone measurable as fTM after approximately 24 hours (Pribbenow et al. 2016).

Because the biochemical structure of androgen metabolites and glucocorticoid metabolites are similar (Ganswindt et al. 2003; Pribbenow et al. 2015), cross-reactions of antibodies used in the EIAs might occur, as was demonstrated in African elephants (*Loxodonta africana*) and spotted hyenas (Ganswindt et al. 2003; Pribbenow et al. 2015). Thus, a fourth aim of the fTM study was to conduct a cross-validation experiment to exclude cross-reactivity with the EIA developed for fGCMs (Ludwig et al. 2013; Pribbenow et al. 2016).

The EIA for fTMs is supposed to work with faeces from free-ranging cheetahs in their natural habitat in Africa. For this purpose, my team colleagues and I collected faeces from immobilised cheetahs as test samples. If faeces are collected in the field, they often are exposed to the local weather conditions for several hours before they are collected, thus we investigated as the fifth aim of the study the duration of stability of fTMs. For this purpose, I collected a sub-sample from fresh faeces of two captive males in Namibia every second hour for the first 12 hours and then at 24 hours, 36 hours, 48 hours and 72 hours. FTM concentrations did fluctuate within the first 24 hours after sub-sample collection but did not vary thereafter (Pribbenow et al. 2016).

5.2 Validation of an enzyme-immunoassay for the non-invasive monitoring of faecal testosterone metabolites in male cheetahs (*Acinonyx jubatus*)

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Validation of an enzyme-immunoassay for the non-invasive monitoring of faecal testosterone metabolites in male cheetahs (*Acinonyx jubatus*)

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ABSTRACT

In mammals, the sex hormone testosterone is the major endocrine variable to objectify testicular activity and thus reproductive function in males. Testosterone is involved in the development and function of male reproductive physiology and sex-related behaviour. The development of a reliable androgen enzyme-immunoassay (EIA) to monitor faecal testosterone metabolites (fTM) is a powerful tool to non-invasively assess the gonadal status of males. We validated an epiandrosterone EIA for male cheetahs by performing a testosterone radiometabolism study followed by high-performance liquid chromatography (HPLC) analyses and excluding possible cross-reactivities with androgenic metabolites not derived from testosterone metabolism. The physiological and biological relevance of the epiandrosterone EIA was validated by demonstrating (1) a significant increase in fTM concentrations within one day in response to a testosterone injection, (2) a significant increase in fTM concentrations within one day in response to a gonadotropin-releasing hormone (GnRH) injection, which failed following a placebo injection, and (3) significant differences in fTM concentrations between adult male and adult female cheetahs and between adult and juvenile male cheetahs of a free-ranging population. Finally, we demonstrated stability of fTM concentrations measured in faecal samples exposed to ambient temperatures up to 72 h. Our results clearly demonstrate that the epiandrosterone EIA is a reliable non-invasive method to monitor testicular activity in male cheetahs.

1. Introduction

Cheetahs (*Acinonyx jubatus*) have undergone a substantial decline in population size due to habitat loss, degradation, fragmentation, and conflicts with humans attempting to decrease the threat to their livestock and game species (Marker et al., 2007; Purchase et al., 2007). Today, Namibia is considered to host the largest free-ranging cheetah population, with approximately 3100–5800 individuals (Hanssen and Stander, 2004). However, this population lives primarily outside protected areas on land used for livestock and game species (Marker et al., 2007). As the cheetah population is declining, captive breeding becomes important for species management and conservation. However, contrary to free-ranging cheetahs, which exhibit high reproductive performance, low cub mortality and low disease susceptibility (Thalwitzer et al., 2010; Wachter et al., 2011), captive individuals have low reproductive performance, high cub mortality and high disease predisposition (Lindburg et al., 1993; Marker-Kraus and Grisham, 1993). Marked differences in breeding success among institutions suggested that husbandry conditions and animal management may be crucial, because captive but not free-ranging cheetahs suffered from chronic stress (Terio et al., 2004). Chronic stress, characterised by long-term hyperadrenal activity, may have adverse effects on reproductive performance and testicular activity (Caro and Laurenson, 1994; Wildt et al., 1993, 1988). For male cheetahs in captivity, permanent visual or olfactory contact with females may be detrimental for promoting natural interest in females and courtship behaviour (Bertschinger et al., 2008; Brown et al., 1996b)

Comparative analyses of adrenocortical and testicular steroid hormone excretion would improve our understanding of reproductive biology in male cheetahs and thus provide essential information for husbandry management to facilitate successful breeding in captivity. However, taking blood samples to objectify gonadal activity is often limited by restricted accessibility to study animals,

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particularly in wildlife species (Goymann, 2005; Palme et al., 2005). In contrast, monitoring faecal testosterone metabolites (fTM) using enzyme immunoassays (EIAs) provides a valuable method to evaluate the hormonal status of an animal (Jewgenow et al., 2006; Pribbenow et al., 2015). Compared to snap-shots in blood plasma based on single samples, hormone metabolite concentrations in faeces represent averaged values of excreted hormones over a species-specific time period, and thus are less affected by the pulsatory hormone secretion pattern than blood hormone levels (Goymann, 2005; Palme et al., 2005). Furthermore, due to the non-invasive character of this method fTM concentrations are less affected by handling procedures such as capture, restraint and anaesthesia, which may alter circulating testosterone concentrations (Möstl and Palme, 2002; Palme et al., 2005; Sapolsky, 1982). The selection of an appropriate assay plays an important role in fTM analysis including physiological and biological validations to confirm that changes in testicular activity are reflected in fTM concentrations measured by the respective assay. The most widely used experiments to stimulate testicular activity and thus to physiologically validate an assay are challenge tests to provoke an increase in peripheral testosterone levels and to detect an elevation in faecal androgen metabolites following the injection of (1) exogenous testosterone (Pribbenow et al., 2015) or (2) a gonadotropin-releasing hormone (GnRH) analogue (Dloniak et al., 2004; Hogan et al., 2010; Wildt et al., 1984). For the development of a reliable androgen EIA it is also necessary to exclude possible cross-reactivities with structurally related androgenic hormone metabolites derived from adrenal glucocorticoid metabolism (Ganswindt et al., 2003; Palme, 2005; Young et al., 2004; Pribbenow et al., 2015).

The overall goal of our study was to develop and validate a reliable non-invasive method using an EIA to monitor testicular activity in male cheetahs. The first aim was to provide basic information on testosterone metabolism and excretion using a ^{3}H-testosterone radiometabolism study and to characterise fTM by HPLC analyses. The second aim was to physiologically validate the suitability of an epiandrosterone (epi-A) EIA to measure fTM by injecting exogenous testosterone and conducting a GnRH challenge. The third aim was to exclude cross-reactivities with androgenic glucocorticoid metabolites; samples from an adrenocorticotropic hormone (ACTH) challenge test (Ludwig et al., 2013) were used to investigate whether faecal glucocorticoid metabolites (fGM) cross-react with the epi-A antibody. The fourth aim was to biologically validate the epi-A EIA by measuring fTM concentrations of free-ranging adult male, adult female and juvenile male cheetahs on farmland in Namibia, with the expectation that the fTM concentration in adult males is higher than in adult females and juvenile males. Finally, we investigated the effect of time on the stability of fTM concentrations when faeces are exposed to natural environmental conditions at ambient temperatures prior to their collection.

2. Materials and methods

2.1. Study animals and sampling procedures

2.1.1. Cheetahs kept in German zoos

Two adult males (M1 from Zoo Wuppertal, M2 from Zoo Muenster) and one juvenile male (M3 from Zoo Muenster) were involved in the experimental part of this study between January 2010 and July 2012. The injections of radiolabelled ^{3}H-testosterone, placebo (NaCl), GnRH analogue and testosterone were conducted with M1 with time periods of 11, 1 and 17 months between injections, respectively. The adult animals were individually housed in one enclosure each. The juvenile male was kept with two females in one enclosure. Faecal samples were collected twice per day, at approximately 08:45 am and 03:45 pm and stored at −20° until transported to the Leibniz Institute for Zoo and Wildlife Research (IZW). All procedures were performed in accordance with the requirements of the Ethics Committee on Animal Welfare of the IZW (permission numbers: 2009-10-02).

2.1.2. Captive cheetahs in Namibia

Faecal samples from two adult males (M4, M5) individually housed in large enclosures (1 ha) on a private farm in Namibia were used to test whether concentrations of fTM changed with exposure time to natural environmental conditions. Fresh faecal samples estimated to be not older than two hours were collected in the morning at approximately 06:00 am.

2.1.3. Free-ranging cheetahs in Namibia

A total of 76 faecal samples from 46 adult males, 18 adult females and 12 juvenile males were collected in the area of east-central Namibia (−21°45′S to −22°45′S and 16°30′E to 18°30′E). The animals were captured in box traps, immobilised and handled as described in Thalwitzer et al. (2010). Age was assessed following the age class categories described in Caro and Laurenson (1994). Faeces were taken manually from the rectum of the animals using a medical glove and either kept in a cool box with ice blocks until they were frozen in liquid nitrogen at the research station or frozen directly in liquid nitrogen when a dissection was being conducted at the research station (mean ± sd: 5.6 ± 6.6 hours later, range: 0.0–26.75 h).

After transport to the IZW, all faecal samples were thawed, mixed well and subsamples of 1 g were divided into two aliquots of 0.5 g.

2.1.4. Post-defaecation metabolism (stability of fTM concentrations)

The concentrations of fTM can change over time when faecal samples cannot be frozen immediately at −20 °C after defaecation (Ludwig et al., 2013). Three fresh samples of M1 and one fresh sample from each of M4 and M5 were analysed to examine whether fTM concentrations were affected by time at ambient temperatures.

The three faecal samples from M1 (Wuppertal, Germany) were collected and stored outdoors at ambient temperatures of 0–4 °C (during winter time) and sub-sampled every hour for the first 12 h and then every 1 or 2 h until 48 h following defaecation, to simulate sample storage in a cool box, and then stored at −20 °C until assayed. Faecal samples from M4 and M5 (Namibia) were sub-sampled every second hour for the first 12 h and then at 24 h, 36 h, 48 h and 72 h following defaecation. Ambient temperatures ranged from approximately 15 °C during the night to approximately 30 °C during the day. Faecal samples were stored at −20° in a freezer on site and transported on dry ice to the IZW for analyses.

2.2. Experimental studies

2.2.1. Radiometabolism study

To characterise excreted fTM, a radiometabolism study was conducted with M1. The cheetah was immobilised with a mixture of 10% ketamine (CP-Pharma, Burgdorf, Germany, 4.5 mg/kg) and medetomidine (Janssen Animal Health, Neuss, Germany, 0.04 mg/kg). A radiolabelled solution containing 0.25 ml of ~250 μCi 1,2,6,7-^{3}H testosterone (TRK407, Amersham Bioscience, UK) in ethanol was mixed with 2.25 ml sterile 0.9% NaCl solution and injected into the cephalic vein. Faecal samples were collected from 3 days before until 4 days after injection and stored at −20 °C until analyses. Aliquots of each sample (n = 12) were extracted for fTM determination and radioactivity counting. The sample collected

21 h after injection contained the highest percentage of radioactivity (82%) and therefore was used for HPLC analyses (see Section 2.3). Radioactive counting was conducted in a MicroBeta TriLux 1450 liquid scintillation counter (Perkin Elmer, Waltham, Massachusetts, USA).

Because androgens may be converted into polar water soluble conjugates prior to excretion via faeces (Jewgenow et al., 2006; Möhle et al., 2002; Pribbenow et al., 2015; Velloso et al., 1998), the faecal extract was subjected to enzyme hydrolysis (see Section 2.4.2) before HPLC separation.

2.2.2. Testosterone challenge test

The testosterone challenge was conducted with M1 by injecting 20 mg of long-acting testosterone intramuscularly (for immobilisation protocol see Section 2.2.1). Faecal samples were collected 7 days before and 10 days after injection. The enclosure was checked for faeces once per day. FTM concentrations were examined in non-hydrolysed and hydrolysed extracts, respectively, which produced similar results (see Section 3.2.1). Therefore, all further analyses were performed using non-hydrolysed faecal extracts. As a control, faecal samples of M2 and M3 were collected twice per day over a period of 14 days and stored at −20 °C until analysis.

2.2.3. GnRH challenge test and placebo administration

The GnRH challenge test was conducted with M1 by injecting 1 ml of GnRH analogue (Receptal (Buserelin 0.04 mg) Intervet, Unterschleissheim, Germany) intramuscularly with a blow pipe to stimulate testosterone secretion. As a control, M1 received a placebo (saline) one month prior to the GnRH challenge consisting of 3 ml of 0.9% NaCl injected intramuscularly with a blow pipe. Faecal samples were collected 5 days before and 6 days following injection, and 4 days before and 5 days following injection, respectively. In both experiments the enclosure was checked for faeces twice per day and samples were stored at −20 °C until analysis.

2.3. HPLC analyses of fTM

Aliquots of 150 µl of the faecal extract with the highest percentage of radioactivity from M1 and one fresh sample each from M4 and M5 (see Section 2.1.2) containing high concentrations of fTM were selected for reversed phase HPLC analyses, with and without enzymatic hydrolysis, respectively. Sample preparation on C_{18} columns and chromatographic separation using an Ultra Sep ES-RP 18/6 µm HPLC column (250 × 4 mm, Sepserv, Berlin, Germany) were conducted as described previously (Pribbenow et al., 2014). The elution positions of native cortisol, corticosterone, testosterone, epi-A and dihydrotestosterone (DHT) on this column had been determined previously in separate HPLC runs.

2.4. Processing of faecal samples

2.4.1. Extraction of wet faeces

Masses of 0.5 g of wet faeces were extracted for 30 min with 4.5 ml of 90% methanol and gently shaken on a horizontal shaker (SM-30, Edmund Buhler, Hechingen, Germany) to allow extensive mixing. After centrifugation (15 min, 1000 g) the supernatant was transferred to a new tube. Aliquots of these extracts were used for HPLC analyses. For EIA analyses aliquots were diluted 1:2 with water and 20 µl were subjected to the epi-A EIA.

2.4.2. Hydrolysis with β-glucuronidase from Helix pomatia

Enzymatic hydrolysis was performed as previously described (Pribbenow et al., 2015). Briefly, 0.1 ml of faecal extracts in 90% methanol were hydrolysed in 900 µl of 0.05 M acetate buffer (pH 4.8) containing 4 µl β-glucuronidase and arylsulfatase from *Helix pomatia* (Roche Diagnostics GmbH). After triple extraction of faecal steroids with 2.5 ml of tert methylbutylether/petroleum ether (30:70, v/v), phase separation was achieved by freezing for 15 min at −80 °C. The extracts were combined, evaporated under nitrogen in a sample concentrator (Dri Block DB3, Techne, Staffordshire, UK) at 55 °C, dissolved in 1 ml of 40% methanol and stored at −20 °C until HPLC analyses.

2.5. Enzyme immunoassay

Aliquots (20 µl) from methanolic faecal extracts were analysed with an "in-house" epi-A EIA as previously described for spotted hyenas (Pribbenow et al., 2015). The antibody was polyclonal and raised in rabbits against the 3-hemisuccinat (HS)-steroid coupled with BSA. The corresponding epiandrosterone-3-HS-peroxidase was used as label. Cross-reactivities of the antibody were determined as follows: 100% epiandrosterone (5α-androstan-3β-ol-17-one), 88% epiandrosterone glucuronide (5α-androstan-3β-ol-17-one glucosiduronate), 68% epiandrosterone sulphate, 60% androsterone (5α-androstan-3α-ol-17-one), 60% androstenedione (4-androsten-3, 17-dione), 24% androsterone sulphate (5α-androstan-3α-ol-17-one sulphate), 19% dehydroandrosterone (5-androsten-3α-ol-17-one), 17% dehydroepiandrosterone (5-androsten-3β-ol-17-one), and 0% for testosterone, cortisol and corticosterone. The assay was validated by demonstrating parallelism of faecal extracts to the epiandrosterone calibration curve (S1 Fig.). The intra-assay and inter-assay coefficients of variation were determined using faecal extracts containing known concentrations of epi-A. The inter-assay coefficients were 6.3% ($n = 6$) and 11.2% ($n = 6$) for extracts containing low and high concentrations of endogenous epi-A, respectively. The corresponding intra-assay coefficients were 8.3% ($n = 6$) and 11.6% ($n = 6$), respectively.

2.6. Cross-reactivity of epi-A EIA with faecal glucocorticoid metabolites

To ensure that measurements of fTM based on the epi-A EIA were not biased by fGM that may cross-react with the epi-A antibody, samples from an ACTH challenge conducted previously with M1 and one adult female (F1) (Ludwig et al., 2013) were analysed with the epi-A EIA expecting no ACTH dependant increase in fTM. Detailed description of treatment and sample collection procedure has been described in Ludwig et al. (2013).

2.7. Data analysis

Calculation of baseline values was performed using an iterative process excluding all values greater than the mean + 2SD (Brown et al., 1996a). Baseline values of testosterone concentrations are quoted as mean ± SD. Significant increases were defined as peaks with values exceeding the baseline + 2SD. Statistical analyses were performed with Systat 13 (Systat Software, Inc., San Jose, California, USA). The level of significance was set at 5% and all tests were two-tailed. A Wilcoxon signed rank test was performed to examine whether fTM concentrations of non-hydrolysed and hydrolysed extracts differed. To ensure both the biological validity of the epi-A EIA we compared fTM concentrations between free-ranging adult males, adult females and juvenile males by applying a Kruskal–Wallis test *post hoc* Dwass–Steel–Chritchlow–Fligner test. FTM concentrations are quoted as mean ± SD.

3. Results

3.1. Analytical validation

3.1.1. HPLC analysis of radiolabelled testosterone metabolites

To characterise the radiolabelled fTM in male M1, a non-hydrolysed and hydrolysed faecal extract was analysed by reversed phase HPLC (Fig. 1A). Thereby, fTM were separated according to their polarity with the more polar metabolites being eluted first. Arrows depict the elution positions of steroid standards of cortisol, corticosterone, testosterone, epi-A and DHT when applying the corresponding steroid hormone specific assays. In the non-hydrolysed faecal extract, only one major polar radiolabelled peak in fractions 1–5 was detected, indicating a cluster of conjugated metabolites. Enzymatic hydrolysis of the faecal extract distinctly changed the elution patterns of radiolabelled fTM. Polar radiolabelled conjugates disappeared and were substituted by a major radiolabelled peak in fractions 39–42, co-eluting with the epi-A standard. Minor radioactive peaks were detected in fractions 34–36 and 36–39. No radiolabelled fTM were detected at the elution position of native testosterone, indicating that the circulating hormone itself is not present in faeces. Additionally, no radiolabelled fTM were detected at elution positions of native cortisol, corticosterone and DHT.

3.1.2. HPLC analyses of immunoreactive testosterone metabolites

Our epi-A EIA evaluation of a faecal sample from male M1 revealed HPLC immunograms (Fig. 1B) which coincided with the profiles of radiolabelled fTM. Prior to enzymatic hydrolysis the epi-A EIA detected one polar immunoreactive peak, eluting infractions 1–5. Similar to radiolabelled fTM, hydrolysis of the faecal extract affected the elution pattern of immunoreactive fTM. Deconjugation of steroid conjugates led to the appearance of one distinct immunoreactive peak in fractions 39–42, corresponding to the elution position of native epi-A and radiolabelled fTM. HPLC immunograms of faecal samples from two non-stimulated adult males (M4 and M5) yielded similar profiles (S2 Fig.). Again, enzymatic hydrolysis led to the appearance of one major immunoreactive peak, corresponding with the elution position of native epi-A. In addition, similar to the HPLC profile of radiolabelled fTM, no immunoreactivities were detectable at the elution positions of any of the native steroid standards.

3.2. Physiological validation

3.2.1. Testosterone challenge test

Faecal samples from the testosterone challenge of male M1 were analysed with the epi-A EIA, each with and without enzymatic hydrolysis (Fig. 2). In the non-hydrolysed extracts a significant increase in fTM was detected one day after the injection of testosterone, with a peak concentration of 9.2 µg/g, exceeding baseline levels (2.1 ± 1.0 µg/g) by approximately 4-fold (Fig. 2). FTM concentrations returned to baseline within 4 days after injection. Hydrolysis of faecal samples revealed a similar fTM profile with a peak concentration of 4.2 µg/g, also approximately 4-fold above baseline concentrations of 0.8 ± 0.4 µg/g (Fig. 2), indicating that course and amount of increase of fTM concentrations do not differ between both extraction procedures ($Z = -5.086$, $P < 0.0001$, Wilcoxon signed rank test). Therefore, all further analyses in this study were conducted without hydrolysis of faecal samples. As a control, fTM concentrations of non-hydrolysed faecal samples of two non-stimulated adult (M2) and juvenile males (M3) were measured, resulting in 0.6 ± 0.4 µg/g for M2 and 0.7 ± 0.4 µg/g for M3. In both males, concentrations of fTM remained stable over the entire period of sample collection (Fig. 2).

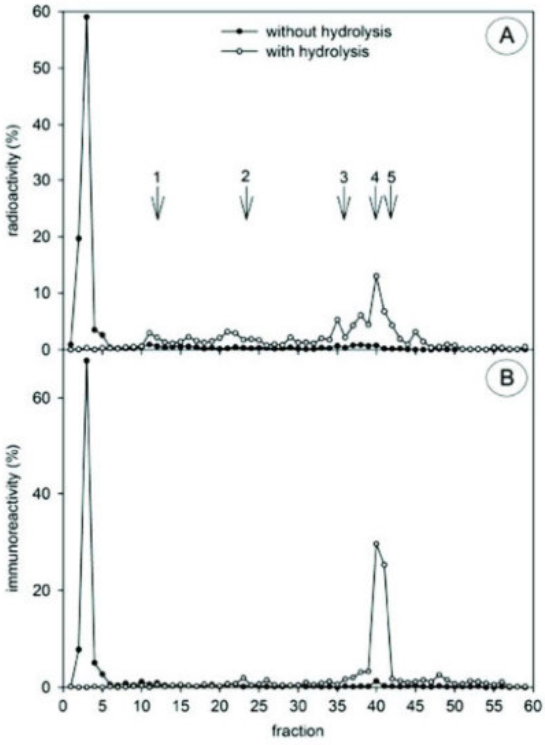

Fig. 1. HPLC profile of (A) radiolabelled and (B) immunoreactive faecal testosterone metabolites (fTM). FTM were analysed with the epi-A EIA in a non-hydrolysed (black circles) and hydrolysed (white circles) faecal extract of a male (M1) that received an injection of radiolabelled ^{3}H testosterone. Radiolabelled testosterone and immunoreactive metabolites are presented as percentage of overall eluted activity. Arrows indicate the elution positions of steroid standards: (1) cortisol, (2) corticosterone, (3) testosterone, (4) epi-A, and (5) DHT.

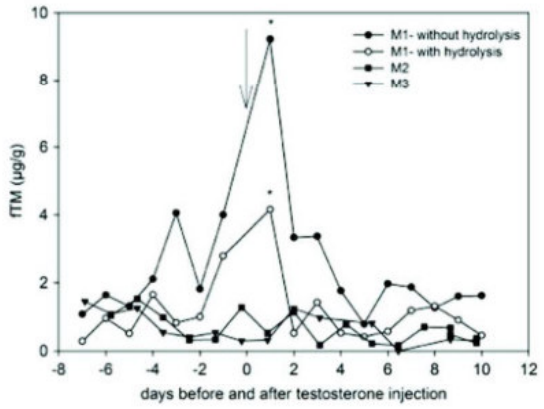

Fig. 2. Changes in fTM concentrations in response to a testosterone challenge in an adult male (M1) in comparison to fTM concentrations in a non-stimulated adult male (M2) and juvenile male (M3). Concentrations of fTM were measured with the epi-A EIA after a testosterone injection, as indicated by the arrow, in non-hydrolysed (black circles) and hydrolysed (white circles) faecal extracts of M1 and in non-hydrolysed faecal extracts of M2 (black squares) and M3 (black triangles). Faecal samples were collected 7 days prior to the injection until 10 days after injection. The asterisks indicate fTM elevations exceeding baseline concentrations + 2SD.

3.2.2. GnRH challenge test and placebo administration

Administration of GnRH analogue resulted in a significant increase in fTM concentrations in male M1 (Fig. 3). Peak excretion of 17.6 µg/g occurred in the sample collected 21.5 h following injection, which is 16-fold higher compared to baseline concentrations of 1.1 ± 0.5 µg/g. FTM concentrations returned to pre-treatment levels within 50 h following injection. Both the GnRH and testosterone challenges revealed similar results regarding latency until peak fTM concentrations were reached, however, in the case of GnRH, the peak levels were approximately twice as high (compare Figs. 2 and 3). In contrast, administration of the placebo NaCl induced no increase in fTM concentrations, thus values remained constant over the entire period of sample collection (Fig. 3).

3.2.3. Exclusion of the epi-A EIA from cross-reactivities with faecal glucocorticoid metabolites

As validated by Ludwig et al. (2013), fGM in cheetahs can be measured non-invasively using an antibody directed against corticosterone-3-CMO detecting significant increases in fGM concentrations following ACTH injection. To exclude the possibility that the epi-A EIA detects androgenic steroids derived from cortisol metabolism, we analysed faecal samples from the above mentioned ACTH challenges in one captive adult male (Fig. 4A) and adult female (Fig. 4B) cheetah. In contrast to the fGM, fTM concentrations measured with the epi-A EIA did not show any ACTH induced changes (Fig. 4A, B). These results indicate that our epi-A exclusively detects faecal testosterone metabolites but does not recognize androgenic glucocorticoid metabolites that might falsify fTM measurements in cheetahs.

3.3. Biological validation – comparison of fTM concentrations between free-ranging cheetahs

There were significant differences in fTM concentrations between free-ranging adult male, adult female and juvenile male cheetahs (Fig. 5, KW = 18.586, df = 2, $P < 0.0001$). In adult males, fTM concentrations were 2.0 ± 1.5 µg/g, ranging from 0.32 to 6.90 ng/g, in adult females 0.7 ± 0.6 µg/g, ranging from 0.16 to 2.09 ng/g and in juvenile males 0.9 ± 0.5 µg/g, ranging from 0.14 to 1.79 ng/g, indicating high inter-individual variation. *Post hoc* pairwise comparisons revealed that adult males had higher fTM concentrations than adult females ($P < 0.0001$) or juvenile males ($P < 0.0001$), whereas juvenile males had significantly higher fTM concentrations than adult females ($P = 0.01$).

3.4. Stability of fTM concentrations under field conditions

FTM concentrations in samples from the captive males M1, M4 and M5 did not significantly change within 48 h and 72 h at ambient temperature, respectively, neither in a German zoo in winter conditions nor in Namibian farmland conditions in summer (Fig. 6, German zoo: $R^2 = 0.05$, $F = 2.613$, df = 1,9, $n = 31$, $P = 0.12$, regression equation: $y = 80.7 - 0.33x$); Namibian farmland: $R^2 = 0.002$, $F = 1.016$, df = 1, 8, $n = 10$, $P = 0.34$, regression equation: $y = 113.95 - 0.22x$). Noticeably, in both time series fTM fluctuations occurred during the first 24 h, but remained more stable thereafter.

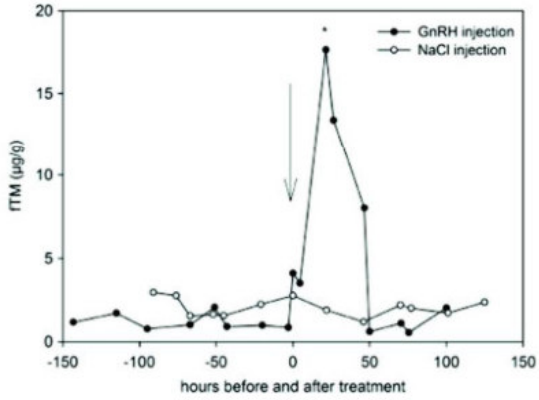

Fig. 3. Changes in fTM concentrations in response to a GnRH challenge and placebo injection (NaCl) in the adult male M1. The arrow indicates the time of GnRH and NaCl administration, respectively. The asterisk indicates fTM elevations exceeding baseline concentrations + 2SD.

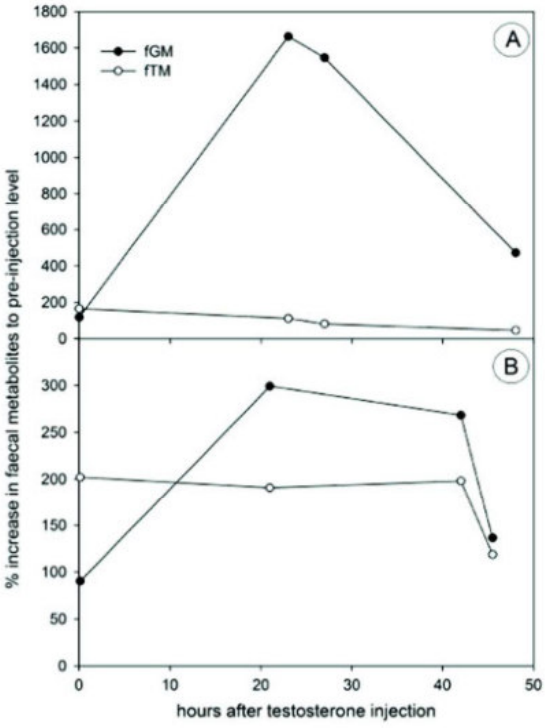

Fig. 4. Percent increase in fTM and fGM concentrations compared to pre-injection levels in response to administration of synthetic ACTH in the (A) captive male M1 and (B) captive female cheetah F1. Faecal samples collected from 76 h before until 45 h after injection were analysed with the epi-A EIA and compared to fGM concentrations previously determined with the corticosterone-3-CMO EIA (Ludwig et al., 2013).

4. Discussion

In this study we demonstrated that variations in fTM concentrations in male cheetahs can be reliably monitored non-invasively using the epi-A EIA because conjugated epi-A represents the major faecal testosterone metabolite in cheetahs. Our results showed the predicted peaks in faecal hormone excretion following testosterone injection and GnRH stimulation, indicating that faecal epi-A measured by this assay reflects biologically meaningful testicular activity.

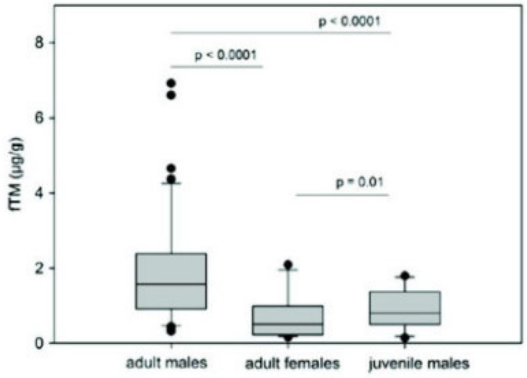

Fig. 5. FTM concentrations of 46 adult male, 18 adult female and 12 juvenile male cheetahs determined with the epi-A EIA.

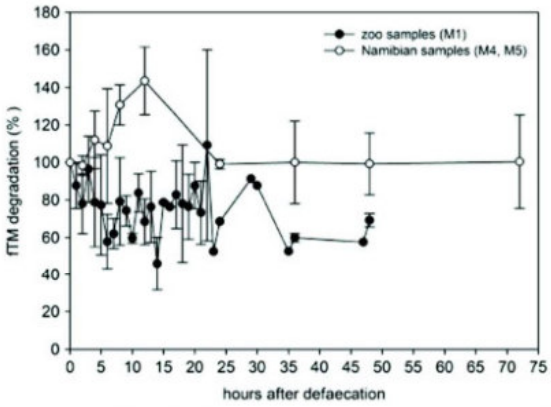

Fig. 6. Relative degradation of fTM (%) in three samples of M1 over a period of 48 h and one faecal sample each from M4 and M5 over a period of 72 h, given as mean values ± SEM, respectively. Relative fTM concentrations were calculated in relation to the reference concentration of the sample frozen immediately after defaecation, representing 100%.

In felids, previous studies demonstrated that gonadal steroids are mainly excreted via faeces as non-hydrolysable polar conjugates, steroid sulphates or glucuronides (Brown et al., 1996a; Brown et al., 1994; Jewgenow et al., 2006). In the male cheetah, our radiometabolism study confirmed that radiolabelled fTM are mainly excreted as polar conjugates. However, these conjugates were hydrolysable with enzymes from *Helix pomatia* liberating epi-A from its conjugates. The HPLC immunograms of a male from Zoo Muenster and two males from Namibia showed that the epi-A antibody detects both polar epi-A conjugates and free epi-A following hydrolysis. Moreover, the almost identical HPLC profiles of radiolabelled and immunoreactive metabolites in a male cheetah demonstrate that our epi-A EIA detects faecal metabolites derived from testosterone metabolism. We assume that epi-A-glucuronide is the major conjugate as its cross-reactivity towards the epi-A antibody (directed against the 3-HS-steroid coupled with BSA) was 88%. Thus, pre-treatment enzymatic hydrolysis seems non-essential given that analyses with and without hydrolysis resulted in similar biological response profiles following testosterone injection.

Based on structural similarities of androgen and glucocorticoid metabolites (Ganswindt et al., 2003; Pribbenow et al., 2015), antibodies directed against androgen metabolites might cross-react with glucocorticoid metabolites of androgenic structure. Thus, we examined whether the results of our epi-A EIA were biased by such cross-reactivities using faecal extracts from an ACTH challenge experiment (Ludwig et al., 2013). We demonstrate in both sexes that an increase in fGM did not influence the measurements of fTM. This is consistent with our HPLC immunograms of fTM demonstrating that the epi-A EIA only detects metabolites derived from testosterone degradation, mainly consisting of epi-A and/or epi-A-conjugates. Epiandrosterone (5α androstane-3β-ol-17-one), one of the major excreted 17-oxoandrogen metabolites, is derived from testosterone by oxidoreductive reactions at the A- and D-rings (Schänzer, 1996). To specify, the initial step in testosterone metabolism is the reduction of the C-4,5 double bond by 5α-reductase, followed by a hydroxy-reduction of the C–3-keto group by 3β-hydroxysteroid dehydrogenase (HSD). The final step is the enzymatic oxidation by 17β-HSD to form the 17-keto steroid (reviewed by Schänzer, 1996). Therefore, the conjugation of epi-A takes place at the position C-3, as this is the only hydroxy group. Epiandrosterone has also been reported as a major testosterone metabolite in faeces of macaques (Girard-Buttoz et al., 2009; Möhle et al., 2002) and spotted hyenas (Pribbenow et al., 2015). EIAs using antibodies directed against epiandrosterone have also been validated for gibbons (Barelli and Heistermann, 2012), spiny mice (de Bruin et al., 2014) and elephants (Ganswindt et al., 2002; Ghosal et al., 2013).

To evaluate the ability of our epi-A EIA to monitor testicular activity in the cheetah, a physiological validation had to be conducted. Therefore, we increased fTM concentrations with an injection of native testosterone and stimulated gonadal testosterone secretion by applying a GnRH analogue. FTM concentrations increased significantly within 1 day following both testosterone and GnRH injection, corresponding to the time course of excreted radiolabelled ^{3}H-testosterone metabolites. Our results are in line with previously reported excretion patterns of faecal steroid metabolites in felids (Brown et al., 1996a; Ludwig et al., 2013). In contrast, a placebo injection of NaCl did not result in elevated fTM concentrations, confirming that our epi-A EIA is an appropriate indicator of testicular activity.

The biological validity of the epi-A EIA was further demonstrated by the ability to distinguish between free-ranging adult male, adult female and juvenile male cheetahs. FTM concentrations were higher in free-ranging adult males than in adult females and juvenile males as expected. We did not find a difference between the adult (M2) and juvenile (M3) male from the zoo in Muenster. This may have been due to the small sample size or potentially to chronic stress in captive cheetahs. Terio et al. (2004) demonstrated significantly enlarged cortices, a measurement for chronic stress, in captive cheetahs housed in North American zoos, and higher fGM but lower fTM concentrations compared to free-ranging individuals from Namibia. This is consistent with our study as we found that free-ranging adult males had higher fTM concentrations (2.0 ± 1.5 µg/g) than the captive adult male M2 from Zoo Muenster (0.6 ± 0.4 µg/g).

In the last two decades, a number of EIAs have been developed and applied in a variety of species to evaluate endocrine function non-invasively. Various studies demonstrated that faecal hormone metabolites may differ considerably between individuals and sexes due to variations in steroid metabolism, excretion and composition of gut bacteria (Palme et al., 1996, 2005). However, faecal steroid metabolite concentrations may also be affected by faecal sampling regime and storage under field conditions. Because it is often difficult to collect fresh faecal samples from free-ranging animals, samples are exposed to various environmental conditions before being frozen. This might promote bacterial degradation and thus may alter faecal metabolite composition and affect measurements

(Abáigar et al., 2010; Hodges and Heistermann, 2011; Terio et al., 2002; Wasser et al., 1988). In a given species, stability may differ between metabolites of different steroids (Terio et al., 2002) and may also affect different assays in different ways (Morrow et al., 2002). Thus, the effect of storage mimicking field conditions should be performed in each species for the steroid metabolite of interest. Similarly to fGM concentrations in cheetahs (Ludwig et al., 2013), we obtained no evidence for changes in fTM concentrations over time, suggesting that fTM concentrations in male cheetahs were unaffected by environmental conditions. Despite exposure to low (samples from Zoo Wuppertal) or high ambient temperatures and direct sunlight (samples from Namibia), fTM concentrations remained stable up to 3 days.

In conclusion, we demonstrated that the epi-A EIA provides a beneficial and practicable tool to evaluate testicular activity in male cheetahs. This non-invasive method allows the study of the impact of life history parameters, husbandry conditions, nutrition and environment in this species. Thus, comparative analyses of fTM and fGM can contribute to the improvement of male reproductive physiology and mating success in cheetahs.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ygcen.2016.01.015.

References

Abáigar, T., Domené, M.A., Palomares, F., 2010. Effects of fecal age and seasonality on steroid hormone concentration as a reproductive parameter in field studies. Eur. J. Wildl. Res. 56, 781–787.

Barelli, C., Heistermann, M., 2012. Sociodemographic correlates of fecal androgen levels in wild male white-handed gibbons (*Hylobates lar*). Int. J. Primatol. 33, 784–798.

Bertschinger, H.J., Meltzer, D.G.A., van Dyk, A., 2008. Captive breeding of cheetahs in South Africa – 30 years of data from the de Wildt Cheetah and Wildlife Centre. Reprod. Domest. Anim. 43, 66–73.

Brown, J.L., Wasser, S.K., Wildt, D.E., Graham, L.H., 1994. Comparative aspects of steroid hormone metabolism and ovarian activity in felids, measured noninvasively in feces. Biol. Reprod. 51, 776–786.

Brown, J.L., Terio, K.A., Graham, L.H., 1996a. Fecal androgen metabolite analysis for non invasive monitoring of testicular steroidogenic activity in felids. Zoo Biol. 15, 425–434.

Brown, J.L., Wildt, D.E., Wielebnowski, N., Goodrowe, K.L., Graham, L.H., Wells, S., Howard, J.G., 1996b. Reproductive activity in captive female cheetahs (*Acinonyx jubatus*) assessed by faecal steroids. J. Reprod. Fertil. 106, 337–346.

Caro, T.M., Laurenson, M.K., 1994. Ecological and genetic factors in conservation – a cautionary tale. Science 263, 485–486.

de Bruin, P.R., Medger, K., Bennett, N.C., Ganswindt, A., 2014. Assessment of reproductive function in southern African spiny mice (*Acomys spinosissimus*) using faeces as hormone matrix. Afr. Zool. 49, 44–53.

Dloniak, S.M., French, J.A., Place, N.J., Weldele, M.L., Glickman, S.E., Holekamp, K.E., 2004. Non-invasive monitoring of fecal androgens in spotted hyenas (*Crocuta crocuta*). Gen. Comp. Endocrinol. 135, 51–61.

Ganswindt, A., Heistermann, M., Borragan, S., Hodges, J.K., 2002. Assessment of testicular endocrine function in captive African elephants by measurement of urinary and fecal androgens. Zoo Biol. 21, 27–36.

Ganswindt, A., Palme, R., Heistermann, M., Borragan, S., Hodges, J.K., 2003. Non-invasive assessment of adrenocortical function in the male African elephant (*Loxodonta africana*) and its relation to musth. Gen. Comp. Endocrinol. 134, 156–166.

Ghosal, R., Ganswindt, A., Seshagiri, P.B., Sukumar, R., 2013. Endocrine correlates of musth in free-ranging Asian elephants (*Elephas maximus*) determined by non-invasive faecal steroid hormone metabolite measurements. PLoS One 8, e84787.

Girard-Buttoz, C., Heistermann, M., Krummel, S., Engelhardt, A., 2009. Seasonal and social influences on fecal androgen and glucocorticoid excretion in wild male long-tailed macaques (*Macaca fascicularis*). Physiol. Behav. 98, 168–175.

Goymann, W., 2005. Noninvasive monitoring of hormones in bird droppings: physiological validation, sampling, extraction, sex differences, and the influence of diet on hormone metabolite levels. Ann. N. Y. Acad. Sci. 1046, 35–53.

Hanssen, L., Stander, P., 2004. Namibia Large Carnivore Atlas. Predator Conservation Trust, Windhoek, Namibia.

Hodges, J.K., Heistermann, M., 2011. Field Endocrinology: Monitoring Hormonal Changes in Free-Ranging Primates. Cambridge University Press.

Hogan, L.A., Phillips, C.J., Horsup, A.B., Keeley, T., Nicolson, V., Janssen, T., Lisle, A., Johnston, S.D., 2010. Monitoring male southern hairy-nosed wombat (*Lasiorhinus latifrons*) reproductive function and seasonality in a captive population. Anim. Reprod. Sci. 118, 377–387.

Jewgenow, K., Naidenko, S.V., Goeritz, F., Vargas, A., Dehnhard, M., 2006. Monitoring testicular activity of male Eurasian (*Lynx lynx*) and Iberian (*Lynx pardinus*) lynx by fecal testosterone metabolite measurement. Gen. Comp. Endocrinol. 149, 151–158.

Lindburg, D.G., Durrant, B.S., Millard, S.E., Oosterhuis, J.E., 1993. Fertility assessment of cheetah males with poor quality semen. Zoo Biol. 12, 97–103.

Ludwig, C., Wachter, B., Silinski-Mehr, S., Ganswindt, A., Bertschinger, H., Hofer, H., Dehnhard, M., 2013. Characterisation and validation of an enzyme-immunoassay for the non-invasive assessment of faecal glucocorticoid metabolites in cheetahs (*Acinonyx jubatus*). Gen. Comp. Endocrinol. 180, 15–23.

Marker, L., Dickman, A., Wilkinson, C., Schumann, B., Fabiano, E., 2007. The Namibian cheetah: status report. Cat News 3, 4–13.

Marker-Kraus, L., Grisham, J., 1993. Captive breeding of cheetahs in North American Zoos: 1987–1991. Zoo Biol. 12, 5–18.

Möhle, U., Heistermann, M., Palme, R., Hodges, J.K., 2002. Characterization of urinary and fecal metabolites of testosterone and their measurement for assessing gonadal endocrine function in male nonhuman primates. Gen. Comp. Endocrinol. 129, 135–145.

Morrow, C.J., Kolver, E.S., Verkerk, G.A., Matthews, L.R., 2002. Fecal glucocorticoid metabolites as a measure of adrenal activity in dairy cattle. Gen. Comp. Endocrinol. 126, 229–241.

Möstl, E., Palme, R., 2002. Hormones as indicators of stress. Domest. Anim. Endocrinol. 23, 67–74.

Palme, R., 2005. Measuring fecal steroids: guidelines for practical application. Ann. N. Y. Acad. Sci. 1046, 75–80.

Palme, R., Fischer, P., Schildorfer, H., Ismail, M.N., 1996. Excretion of infused C-14-steroid hormones via faeces and urine in domestic livestock. Anim. Reprod. Sci. 43, 43–63.

Palme, R., Rettenbacher, S., Touma, C., El-Bahr, S.M., Mostl, E., 2005. Stress hormones in mammals and birds: comparative aspects regarding metabolism, excretion, and noninvasive measurement in fecal samples. Ann. N. Y. Acad. Sci. 1040, 162–171.

Pribbenow, S., Jewgenow, K., Vargas, A., Serra, R., Naidenko, S., Dehnhard, M., 2014. Validation of an enzyme immunoassay for the measurement of faecal glucocorticoid metabolites in Eurasian (*Lynx lynx*) and Iberian lynx (*Lynx pardinus*). Gen. Comp. Endocrinol. 206C, 166–177.

Pribbenow, S., East, M.L., Ganswindt, A., Tordiffe, A.S., Hofer, H., Dehnhard, M., 2015. Measuring faecal epi-androsterone as an indicator of gonadal activity in spotted hyenas (*Crocuta crocuta*). PLoS One 10, e0128706.

Purchase, G., Mateke, C., Purchase, D., 2007. A review of the status and distribution of carnivores, and levels of human-carnivore conflict, in the protected areas and surrounds of the Zambezi Basin, Zambezi Society.

Sapolsky, R.M., 1982. The endocrine stress-response and social status in the wild baboon. Horm. Behav. 16, 279–292.

Schänzer, W., 1996. Metabolism of anabolic androgenic steroids. Clin. Chem. 42, 1001–1020.

Terio, K.A., Brown, J.L., Moreland, R., Munson, L., 2002. Comparison of different drying and storage methods on quantifiable concentrations of fecal steroids in the cheetah. Zoo Biol. 21, 215–222.

Terio, K.A., Marker, L., Munson, L., 2004. Evidence for chronic stress in captive but not free-ranging cheetahs (*Acinonyx jubatus*) based on adrenal morphology and function. J. Wildl. Dis. 40, 259–266.

Thalwitzer, S., Wachter, B., Robert, N., Wibbelt, G., Muller, T., Lonzer, J., Meli, M.L., Bay, G., Hofer, H., Lutz, H., 2010. Seroprevalences to viral pathogens in free-ranging and captive cheetahs (*Acinonyx jubatus*) on Namibian Farmland. Clin. Vaccine Immunol. CVI 17, 232–238.

Velloso, A.L., Wasser, S.K., Monfort, S.L., Dietz, J.M., 1998. Longitudinal fecal steroid excretion in maned wolves (*Chrysocyon brachyurus*). Gen. Comp. Endocrinol. 112, 96–107.

Wachter, B., Thalwitzer, S., Hofer, H., Lonzer, J., Hildebrandt, T.B., Hermes, R., 2011. Reproductive history and absence of predators are important determinants of reproductive fitness: the cheetah controversy revisited. Conserv. Lett. 4, 47–54.

Wasser, S.K., Risler, L., Steiner, R.A., 1988. Excreted steroids in primate feces over the menstrual-cycle and pregnancy. Biol. Reprod. 39, 862–872.
Wildt, D.E., Chakraborty, P.K., Meltzer, D., Bush, M., 1984. Pituitary and gonadal response to Lh releasing hormone administration in the female and male cheetah. J. Endocrinol. 101, 51–56.
Wildt, D.E., Phillips, L.G., Simmons, L.G., Chakraborty, P.K., Brown, J.L., Howard, J.G., Teare, A., Bush, M., 1988. A comparative analysis of ejaculate and hormonal characteristics of the captive male cheetah, tiger, leopard, and puma. Biol. Reprod. 38, 245–255.
Wildt, D.E., Brown, J.L., Bush, M., Barone, M.A., Cooper, K.A., Grisham, J., Howard, J.G., 1993. Reproductive status of cheetahs (*Acinonyx jubatus*) in North American Zoos: the benefits of physiological surveys for strategic planning. Zoo Biol. 12, 45–80.
Young, K.M., Walker, S.L., Lanthier, C., Waddell, W.T., Monfort, S.L., Brown, J.L., 2004. Noninvasive monitoring of adrenocortical activity in carnivores by fecal glucocorticoid analyses. Gen. Comp. Endocrinol. 137, 148–165.

5.3 References

Benhaiem, S., H. Hofer, M. Dehnhard, J. Helms and M. L. East (2013). "Sibling competition and hunger increase allostatic load in spotted hyaenas." Biol Lett 9: 20130040.

Duque, E. d. A. and C. D. Munhoz (2016). "The pro-inflammatory effects of glucocorticoids in the brain." Front Endocrinol 7: 78.

Ganswindt, A., R. Palme, M. Heistermann, S. Borragan and J. K. Hodges (2003). "Non-invasive assessment of adrenocortical function in the male African elephant (*Loxodonta africana*) and its relation to musth." Gen Comp Endocrinol 134: 156-166.

Hau, M. and W. Goymann (2015). "Endocrine mechanisms, behavioral phenotypes and plasticity: known relationships and open questions." Front Zool 12: S7-S7.

Jurke, M., N. Czekala, D. Lindburg and S. Millard (1997). "Fecal corticoid metabolite measurement in the cheetah (*Acinonyx jubatus*)." Zoo Biol 16: 133-147.

Kalbitzer, U., M. Heistermann, D. Cheney, R. Seyfarth and J. Fischer (2015). "Social behavior and patterns of testosterone and glucocorticoid levels differ between male chacma and Guinea baboons." Horm Behav 75: 100-110.

Ludwig, C., B. Wachter, S. Silinski-Mehr, A. Ganswindt, H. Bertschinger, H. Hofer and M. Dehnhard (2013). "Characterisation and validation of an enzyme-immunoassay for the non-invasive assessment of faecal glucocorticoid metabolites in cheetahs (*Acinonyx jubatus*)." Gen Comp Endocrinol 180: 15-23.

Munck, A., P. M. Guyre and N. J. Holbrook (1984). "Physiological functions of glucocorticoids in stress and their relation to pharmacological actions." Endocr Rev 5: 25-44.

Palme, R., S. Rettenbacher, C. Touma, S. El-Bahr and E. Möstl (2005). "Stress hormones in mammals and birds: comparative aspects regarding metabolism, excretion, and noninvasive measurement in fecal samples." Ann. N. Y. Acad. Sci 1040: 162-171.

Preston, B. T., I. R. Stevenson, G. A. Lincoln, S. L. Monfort, J. G. Pilkington and K. Wilson (2012). "Testes size, testosterone production and reproductive behaviour in a natural mammalian mating system." J Anim Ecol 81: 296-305.

Pribbenow, S., M. L. East, A. Ganswindt, A. S. W. Tordiffe, H. Hofer and M. Dehnhard (2015). "Measuring faecal epi-androsterone as an indicator of gonadal activity in spotted hyenas (*Crocuta crocuta*)." PLoS ONE 10: e0128706.

Pribbenow, S., B. Wachter, C. Ludwig, A. Weigold and M. Dehnhard (2016). "Validation of an enzyme-immunoassay for the non-invasive monitoring of faecal testosterone metabolites in male cheetahs (*Acinonyx jubatus*)." Gen Comp Endocrinol 228: 40-47.

Romero, L. M. and J. M. Reed (2005). "Collecting baseline corticosterone samples in the field: is under 3 min good enough?" Comp Biochem Physiol A Mol Integr Physiol 140: 73-79.

Terio, K. A., S. B. Citino and J. L. Brown (1999). "Fecal cortisol metabolite analysis for noninvasive monitoring of adrenocortical function in the cheetah (*Acinonyx jubatus*)." J Zoo Wildl Med 30: 484-491.

Wasser, S. K., K. E. Hunt, J. L. Brown, K. Cooper, C. M. Crockett, U. Bechert, J. J. Millspaugh, S. Larson and S. L. Monfort (2000). "A generalized fecal glucocorticoid assay for use in a diverse array of nondomestic mammalian and avian species." Gen Comp Endocrinol 120: 260-275.

Young, K., S. Walker, C. Lanthier, W. Waddell, S. Monfort and J. Brown (2004). "Noninvasive monitoring of adrenocortical activity in carnivores by fecal glucocorticoid analyses." Gen Comp Endocrinol 137: 148-165.

6. Summary

Serological and microbiological evaluations of the health status of free ranging and captive cheetahs (*Acinonyx jubatus*) on Namibian farmland

The largest free-ranging population of cheetahs, classified as vulnerable, currently lives on Namibian farmland. For this thesis, free-ranging cheetahs were captured, examined, sampled and radio-collared, and captive cheetahs held in large enclosures were examined and sampled. With these collected samples and data, as well as previously collected ones, I performed the tests and analyses included in this thesis.

The aims of this thesis were to examine the health status of the Namibian cheetahs and to analyse blood samples serologically and microbiologically. Firstly, the serological tests followed on from a previous study that detected the first seropositive results for antibodies against feline leukemia virus (FeLV), a gammaretrovirus, in free-ranging cheetahs. For this I used various molecular tests as well as cell cultures. Secondly, I evaluated the immune response to FeLV vaccinations in captive cheetahs. Finally, I describe the first evidence of a hitherto unrecognized species of hemoplasma in cheetahs. In the appendix, I present a study in which I collaborated on the development and validation of an enzyme immunoassay to measure the metabolites of testosterone in faecal samples of cheetahs.

Viral infections pose worldwide a significant threat to free-ranging and captive held wildlife species. Prominent examples are the fatal outbreaks of FeLV infections reported in free-ranging Iberian lynxes (*Lynx pardinus*) and in captive cheetahs. Thus, it was of high importance to follow up on a previous study providing the first evidence that Namibian free-ranging cheetahs came into contact with gammaretroviruses. In my study, I conducted numerous analyses and measured, depending on the test used, in up to 19% of free-ranging cheetahs antibodies against FeLV or a closely related gammaretrovirus. Seropositive animals were also seropositive for the Rauscher murine leukemia virus. Yet, no proviral DNA was detected. The seropositive, clinically healthy cheetahs might have been infected with a weakly pathogenic retrovirus or turned seropositive due to the expression of an endogenous retroviral sequence. Thus, the gammaretrovirus does not seem to pose currently a risk to the health of the cheetah population.

The cheetahs held in enclosures had been vaccinated yearly for several years with a FeLV vaccine developed for the use in domestic cats. Antibody formation was measured in 86% of the captive cheetahs and no animal developed any notable adverse reactions. Thus, this vaccine might be a useful protection for cheetahs. However, vaccinations in free-ranging animals should only be used after very careful risk-benefit analyses and after extensive laboratory tests under controlled conditions.

Infections with hemoplasmas, cell wall-free bacteria that parasitize red blood cells, have been described in many free-ranging and domestic animals and can cause feline infectious anemia in cats. Particularly in immunocompromised animals the effects of a hemoplasma infection can be life threatening. Until now there was no description of hemoplasma in any free-ranging feline species in southern Africa. My case study thus represents a first description. This is, however, not only the first report of an infection of a free-ranging Namibian cheetah with hemoplasma, but also a newly described isolate assigned to the *Mycoplasma haemofelis/haemocanis* group after the sequencing of the 16S rRNA and the RNAse P genes. The clinical implications and the mode of transmission of this disease remain unknown, but the infected cheetah did not show any signs of health impairment upon examination and was localised alive via radio collar for another 48 months.

Hence, studies on serological and microbiological surveys are important for the assessment of the health status of a free-ranging population and are also relevant in the development of management to reduce human-animal conflicts. Concerning the latter, translocations of carnivores are performed regularly, yet without the evaluation of risks of infections in the new or also the old habitats. It would be advisable to routinely perform such surveys to eliminate the risk of disseminating pathogens during translocations. Comparative serological studies of free-ranging populations and captive held animals are very promising and should be continued. Only with sufficient science based information can we plan the urgently needed long-term strategies for further management of endangered carnivores and react on potential future epidemics.

7. Zusammenfassung

Serologische und mikrobiologische Untersuchungen zum Gesundheitsstatus von freilebenden und in menschlicher Obhut gehaltenen Geparden (*Acinonyx jubatus*) auf namibischem Farmland

Derzeit lebt der größte freilebende Bestand des als gefährdet eingestuften Gepards auf Farmland in Namibia. Im Rahmen dieser Dissertation wurden sowohl frei lebende Geparde gefangen, untersucht, beprobt und besendert, als auch in großen Gehegen gehaltene Geparde untersucht und beprobt. Anhand dieser neu gewonnenen, und auch bereits vorhandener, Proben wurden die Untersuchungen für diese Dissertation durchgeführt.

Ziel der Arbeit war es, den Gesundheitsstatus der namibischen Geparde zu untersuchen und Blutproben serologisch und mikrobiologisch zu analysieren. Erstens bauen die serologischen Tests auf eine vorangehende Studie auf, die erste seropositive Ergebnisse von Antikörpern gegen das feline Leukämievirus (FeLV), einen Gammaretrovirus, bei frei lebenden Geparden nachwies. Hierfür nutzte ich verschiedene molekulare Tests, sowie Zellkulturen. Zweitens habe ich die Immunantwort auf FeLV Impfungen bei in Gehegen gehaltenen Geparden untersucht. Drittens beschreibe ich den ersten Nachweis von einer bisher unbeschriebenen Hemoplasmenart bei Geparden. Im Appendix wird eine weitere Studie über die Entwicklung und Validierung eines Enzymimmunoassays zur Messung von Testosteronmetaboliten in Kotproben von Geparden vorgestellt, an der ich mitbeteiligt war.

Virale Erkrankungen stellen für frei lebende und in menschlicher Obhut gehaltene Wildtierarten weltweit eine große Bedrohung dar. Prominente Beispiele sind fatale FeLV-Erkrankungen bei frei lebenden Iberischen Luchsen (*Lynx pardinus*) und in menschlicher Obhut gehaltenen Geparden. Es ist daher von großer Bedeutung, den ersten Hinweisen einer vorangehenden Studie auf einen Kontakt der frei lebenden namibischen Gepardenpopulation mit Gammaretroviren zu folgen. In meiner Studie wurden mehrere Analysen durchgeführt und je nach Testmethode in bis zu 19% der frei lebenden Tiere Antikörper gegen FeLV oder einen nah verwandten Gammaretrovirus nachgewiesen. Seropositive Tiere waren auch seropositiv für das Rauscher murine Leukämievirus. Es konnte aber keine provirale DNA nachgewiesen werden. Die seropositiven, klinisch gesunden Geparde könnten sich entweder mit einem schwach pathogenen Retrovirus infiziert haben oder sie wurden durch die Expression einer endogenen retroviralen Sequenz seropositiv. Der Gammaretrovirus scheint daher keinen gesundheitsgefährdenden Einfluss auf die Gepardenpopulation zu haben.

Die in Gehegen gehaltenen Geparde wurden über mehreren Jahre hinweg jährlich mit einem für Hauskatzen entwickelten FeLV Impfstoff geimpft. In 86% der Geparde wurden Antikörper

gemessen und keines der Tiere entwickelte erkennbare Nebenwirkungen. Dieser Impfstoff könnte daher einen geeigneten Schutz für Geparde darstellen. Allerdings sollten Impfungen bei frei lebenden Wildtieren nur nach einer gezielten Nutzen-Risiko-Abwägung und erst nach ausführlichen Tests unter kontrollierten Bedingungen durchgeführt werden.

Infektionen mit Hemoplasmen, die als zellwandfreie Bakterien Blutzellen parasitieren, wurden bei diversen frei lebenden und domestizierten Tierarten beschrieben und können bei Katzen zur felinen infektiösen Anämie führen. Besonders bei immunkomprimierten Tieren können die Folgen einer Hemoplasmeninfektion lebensbedrohlich sein. Bisher gab es keine Beschreibungen von Hemoplasmen bei frei lebenden Katzenartigen im südlichen Afrika. Meine Studie stellt daher eine Erstbeschreibung dar. Es wurde nicht nur die erste Infektion eines frei lebenden namibischen Geparden mit Hemoplasmen nachgewiesen, sondern mithilfe von Sequenzierungen der 16S rRNA und RNAse P Gene ein neues Isolat in der *Mycoplasma haemofelis/haemocanis* Gruppe beschrieben. Die klinische Bedeutung dieser Erkrankung, sowie deren Übertragungsweg, sind weiterhin ungeklärt, aber das infizierte Tier zeigte bei der Untersuchung keine Anzeichen eines verminderten Allgemeinbefindens und konnte per Senderhalsband noch weitere 48 Monate lebend geortet werden.

Studien zu serologischen und mikrobiologischen Untersuchungen sind nicht nur für die Abschätzung des Gesundheitsstatus einer Population relevant, sondern auch für Management-Strategien bei der Reduzierung von Mensch-Tier-Konflikten. Diesbezüglich werden immer wieder Translokationen von Raubtieren durchgeführt, allerdings ohne vorherige Abklärung von Infektionsrisiken in den neuen und alten Streifgebieten. Es wäre empfehlenswert, solche Untersuchungen routinemäßig durchzuführen, um einer Verschleppung von Erregern bei Translokationen vorzubeugen. Vergleichende serologische Arbeiten von frei lebenden Populationen mit in Gehegen gehaltenen Tieren sind vielversprechend und sollten weitergeführt werden. Nur mit umfassenden wissenschaftsbasierten Informationen können die dringend benötigten langfristigen Pläne für das weitere Management von bedrohten Raubtierarten gemacht und auf eventuelle weitere Seuchenausbrüche reagiert werden.

8. Publication list

2016

14. Pribbenow, S., B. Wachter, C. Ludwig, **A. Weigold** and M. Dehnhard (2016). "Validation of an enzyme-immunoassay for the non-invasive monitoring of faecal testosterone metabolites in male cheetahs (*Acinonyx jubatus*)." Gen Comp Endocrinol 228: 40-47.

2015

13. Aqrawi, T., A. C. Stohr, T. Knauf-Witzens, **A. Krengel,** K. O. Heckers and R. E. Marschang (2015). "Identification of snake arenaviruses in live boas and pythons in a zoo in Germany." Tierarztl Prax Ausg K Kleintiere Heimtiere 43: 239-247.

12. Knauf-Witzens, T., C. Spahr and **A. Weigold** (2015). “Erfahrungen mit zwei geriatrischen Elefanten“. 35. Arbeitstagung d ZooTÄ i dtschspr Raum, Rapperswil/CH. In press.

11. **Krengel, A.,** V. Cattori, M. L. Meli, B. Wachter, J. Boni, L. R. Bisset, S. Thalwitzer, J. Melzheimer, M. Jago, R. Hofmann-Lehmann, H. Hofer and H. Lutz (2015). "Gammaretrovirus-specific antibodies in free-ranging and captive namibian cheetahs." Clin Vaccine Immunol 22: 611-617.

2014

10. **Krengel, A.,** T. Knauf-Witzens, C. Süß-Dombrowski, I. Schwabe and G. Schares (2015). ”Fatal toxoplasmosis in two juvenile slender–tailed meerkats (*Suricata suricata*) in a German zoo –clinical, pathological and serological findings.” Proc Int Conf Dis Zoo Wild Anim, p 18. Warsaw (PL).

9. Knauf-Witzens, T. and **A. Krengel** (2014). ”Ape moves – translocation of the Wilhelma bonobos and gorillas into the new MENSCHEN.AFFEN.HAUS!” Proc Int Conf Dis Zoo Wild Anim, p 43-54. Warsaw (PL).

8. **Krengel, A.,** F. Göritz, T. B. Hildebrandt and T. Knauf-Witzens (2014). “Apathie und Adipsie bei einer 48-jährigen asiatischen Elefantenkuh.“ 34. Arbeitstagung d ZooTÄ i dtschspr Raum, Kronberg: 54-64.

2013

7. **Krengel, A.** and T. Knauf- Witzens (2013). “Umzug ins neue Heim- die afrikanischen Menschenaffen der Wilhelma ziehen in das neue Haus.“ 33. Arbeitstagung d ZooTÄ i dtschspr Raum, Görlitz: 107-112.

6. **Krengel, A.,** M. L. Meli, V. Cattori, B. Wachter, B. Willi, S. Thalwitzer, J. Melzheimer, H. Hofer, H. Lutz and R. Hofmann-Lehmann (2013). "First evidence of hemoplasma

infection in free-ranging Namibian cheetahs (*Acinonyx jubatus*)." Vet Microbiol 162: 972-976.

2012

5. **Krengel, A.,** W. Rietschel, U. Walliser and T. Knauf-Witzens (2012). “Wundertüte Okapi ein Krankheitsbericht.“ 32. Arbeitstagung d ZooTÄ i dtschspr Raum, Landau: 119-123.

4. **Krengel, A.,** T. Löhrich and T. Knauf-Witzens (2012). “Auswertung der letzten 17 Jahre Känguruhaltung in der Wilhelma Erkrankungen, Therapien, Sektionen.“ 11. DVG-ZWE Tagungsband, Görlitz, CD, Session 5 on the 1.12.12, 9 pages.

2009

3. Melzheimer, J., **A. Krengel,** H. Hofer and B. Wachter (2009) “Knowledge on spatial movements of Namibian cheetahs (*Acinonyx jubatus*) might decrease the human-cheetah conflict.” Proc Int Conf on Behaviour, Physiology and Genetics of Wildlife, p126. Berlin (GER).

2006

2. Kleinschmidt, S., Beckold, E., **Krengel, A.,** Brettschneider, J.& Hewicker-Trautwein, M., 2006; ‚Primäres nicht-epitheliotropes gastrointestinales T-Zell-Lymphom und lymphoplasmazelluläre Gastrokolitis bei einem Hund‘. Kleintierpraxis 51(3): 143-148.

1. **Krengel, A.,** Klein, S., Hettling, P., Pfeffer, M., Baumgärtner, W., 2006; ‚Leishmaniose als Ursache für eine Gonarthritis bei einer Huskyhündin‘. Kleintierpraxis 51(7-8): 402-405.

Poster

Krengel A. and T. Knauf-Witzens (2015). ”Raising Nyota - experiences of semi hand rearing an okapi (*Okapia johnstoni*)”. Proc Int Conf Dis Zoo Wild Anim, p 145. Barcelona (Poster).

Krengel, A., M. L. Meli, V. Cattori, B. Wachter, B. Willi, H. Hofer, H. Lutz and R. Hofmann-Lehmann (2010). ”First evidence of hemoplasma infection in free-ranging Namibian cheetah (*Acinonyx jubatus*).” Proc Int Conf Dis Zoo Wild Anim, p 206. Madrid (ES)

Krengel, A., M. L. Meli, V. Cattori, B. Wachter, B. Willi, H. Hofer, H. Lutz and R. Hofmann-Lehmann (2010). ”First evidence of hemoplasma infection in free-ranging Namibian cheetah (*Acinonyx jubatus*).” EWDA Conference, Healthy wildlife, healthy people, p 64. Vlieland (NL)

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10. Selbständigkeitserklärung/Declaration of originality

Selbständigkeitserklärung:

Hiermit bestätige ich, dass ich die vorliegende Arbeit selbständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen in Anspruch genommen habe.

Declaration of originality:

I herewith confirm that the present work was autonomously prepared. I assure that I only used cited sources or acknowledged help.

Stuttgart, den 22.08.2016

Annika Weigold