

Equid herpesvirus infections in zebras (*Equus* sp.):
host physiology and non-invasive detection of
virus shedding

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List of abbreviations

Virus species

AsHV-3	<i>Asinine Herpesvirus 3</i>
AsHV-4	<i>Asinine Herpesvirus 4</i>
AsHV-5	<i>Asinine Herpesvirus 5</i>
AsHV-6	<i>Asinine Herpesvirus 6</i>
EHV-1	<i>Equine Herpesvirus 1</i>
EHV-2	<i>Equine Herpesvirus 2</i>
EHV-3	<i>Equine Herpesvirus 3</i>
EHV-4	<i>Equine Herpesvirus 4</i>
EHV-5	<i>Equine Herpesvirus 5</i>
EHV-6	<i>Equine Herpesvirus 6</i>
EHV-7	<i>Equine Herpesvirus 7</i>
EHV-8	<i>Equine Herpesvirus 8</i>
EHV-9	<i>Equine Herpesvirus 9</i>
WAH	Wild Ass Herpesvirus (tentative species)
ZHV	Equus Zebra Herpesvirus (tentative species)

others

ACTH	adrenocorticotrophic hormone
APP	acute phase proteins
APR	acute phase response
bp	base pairs
DNA	deoxyribonucleic acid
DOI	digital object identifier
DPOL	DNA polymerase
eDNA	environmental DNA
EHV	equid herpesvirus
ELISA	enzyme-linked immunosorbent assay
EIA	enzyme immunoassay
fGCM	faecal glucocorticoid metabolites
gB	glycoprotein B
GC	glucocorticoids
gD	glycoprotein D
gG	glycoprotein G
HA	hemagglutination
HV	herpesvirus
IgG	immune globulin G
kb	kilobase
MHC-I	major histocompatibility complex I
NP	national park
SAA	serum amyloid A
SD	standard deviation

CHAPTER 1: General Introduction

1.1 Pathogen transmission in wildlife

Patterns of pathogen transmission in wildlife among and between species (or populations) are not well understood. In order to elucidate these mechanisms and to make predictions of the emergence of infectious diseases and population viability, it is important a) to identify the underlying mechanisms of pathogen transmission and b) to assess host susceptibility, which depends on individual immunocompetence.

Infectious diseases have become an increasingly important factor to consider regarding wildlife health and management as well as species conservation (Collinge and Ray, 2006; Spalding and Forrester, 1993). Free-ranging wildlife is faced with shrinking habitats due to anthropogenic land use and associated conversion of pristine natural habitats to agricultural land. These landscape changes produce an increasing livestock-wildlife interface, which leads to higher contact rates and increases the risk of pathogen transmission from and to wildlife (Aguirre and Tabor, 2008; Collinge and Ray, 2006). Moreover, conservation efforts can have adverse consequences on wildlife populations when potential pathogen transmission is not considered: in the last two decades, multiple translocation projects for local population re-stocking resulted in the introduction and spread of novel pathogens (reviewed in Artois et al., 2009; Daszak, 2000, and Deem et al., 2008). Taken together, many wildlife populations experience an increase of pathogen pressure (in terms of diversity and abundance of pathogens), mostly due to direct or indirect anthropogenic impacts (Daszak, 2000; Horrocks et al., 2011).

To make predictions on pathogen dynamics, it is necessary to understand aspects such as host physiology, pathogen transmission, and host-pathogen interactions, all of which depend on the acquisition of adequate primary data on specific pathogen prevalence, effective pathogen transmission, and factors potentially promoting transmission.

Moreover, diagnostic testing is important for monitoring the health of captive and free-ranging wildlife populations (Moehlman, 2002). In order to collect primary data on the occurrence of pathogens and diseases, it is crucial to sample a sufficient number of individuals of a population by means such as a) remote observation of clinical manifestation, b) direct invasive sampling, or c) indirect non-invasive sampling. Research on pathogen transmission and host susceptibility in wildlife *in situ* is often hampered by the difficulty of obtaining direct (invasive) samples (at an adequate sample size). In order to facilitate effective sampling of wildlife for pathogen screening, novel sampling techniques (potentially for large-scale application) need to be developed and evaluated.

1.1.1 Pathogen detection by invasive sampling

Obtaining biological sample material directly from the organism in question is likely the most accurate approach in order to detect the presence of pathogens (whether using direct or indirect assays), or to measure various physiological functions related to diseases (e.g. immunological or endocrine assessments). Invasively obtained substrates for pathogen screening or antibody assays include blood, serum, tissue (biopsy), and others, which can typically not be collected from free-ranging wildlife without prior immobilization. Although rodents or some small carnivores may simply be trapped for manipulation and sampling, larger mammals are usually chemically immobilized by remote injection ('darting'), which can be associated with substantial risks for the health and survival of the darted animal (Walzer et al., 2006). The time span from darting to complete anaesthesia can be considerably stressful for wild animals due to a state of disorientation, and anaesthetic drugs can lead to cardiorespiratory depression, the combination of which may cause severe complications (Arnemo et al., 2006; Dickens et al., 2010). Furthermore, the stress of capture and restraint may have immunosuppressive effects, which could give rise to primary infections or reactivation of latent infections and subsequent pathogen spreading (Dickens et al., 2010; Fischer-Tenhagen et al., 2000).

Certain sampling methods which are not invasive *per se* may also require invasive procedures to facilitate sampling, such as, for instance, the collection of mucosal swabs,

which in most free-ranging wildlife species is typically performed upon chemical immobilization. Here, I therefore use the term 'invasive sampling' to define all sampling methods which involve any procedures (including chemical anaesthesia) at any stage of sampling to directly obtain sample material from the target organism. Invasive sampling is common practice for certain purposes (e.g. circulating plasma concentrations of specific immune markers); however, alternative approaches have been developed for other measurements in some species. Particularly for animals which are at a higher risk of suffering from potential adverse consequences of immobilization (e.g. aged or pregnant individuals), non-invasive procedures should be considered as an alternative in order to avoid endangering the life of individuals for the benefit of obtaining sample material.

1.1.2 Pathogen detection by non-invasive sampling

Non-invasive sampling methods are by definition methods that do not involve direct handling of the target organism. By employing non-invasive sampling methods, various risks associated with invasive procedures can be circumvented, e.g. health and survival risks for the target animal or health and safety risks for people involved in the procedure. A further advantage of non-invasive sampling is the avoidance of considerable logistic and financial efforts associated with the immobilization of large animals.

Sample materials which can be obtained non-invasively include faeces, urine, hair, saliva, breath condensate, or any other substrate which is shed from an animal's body into its environment (e.g. Acevedo-Whitehouse, Rocha-Gosselin, & Gendron, 2010; East et al., 2001; Ezenwa & Jolles, 2011; Grueter, Deschner, Behringer, Fawcett, & Robbins, 2014; Romain-Bondi et al., 2004; Schwarzenberger, 2007). Furthermore, blood-sucking invertebrates have been used as a source of obtaining sample material from target organisms, although typically on a small scale (Schnell et al., 2015; Vos et al., 2010). The applications of non-invasively obtained samples include parasitological, bacterial, and virological surveys, and immunological, endocrine, genetic, and toxicological analyses.

Pathogens can be traced through detecting their genetic material, their products (such as eggs or oocysts, in the case of parasites), or the responses of the hosts. Using non-invasive sampling approaches, pathogens are usually detected directly (as opposed to indirect detection which is more common using invasively collected samples, e.g. serum antibodies; e.g. Guevara et al., 2018; Kuzmina et al., 2012). However, for direct pathogen detection it must be considered that certain pathogens may not be traceable in every substrate due to specific shedding routes (e.g. pathogens shed by nasal excreta may not appear in faeces). Furthermore, detection efficiency in sample types which are not the main pathogen shedding route also strongly depends on the sensitivity of the employed detection method. Recently developed high-sensitivity methods for pathogen detection, such as hybridization enrichment capture or digital droplet PCR (Briese et al., 2015; Periyannan Rajeswari et al., 2017) facilitate tracing of pathogens of which only infinitesimal amounts of DNA are present in the sample. These approaches open up novel possibilities for high-sensitivity detection of multiple pathogens in parallel in untypical sample types, and may therefore substantially reduce the effort of collecting samples for pathogen screening in wildlife. However, the high financial costs associated with these novel screening methods compared to traditional approaches may hamper their broad use in field research in the near future.

1.2 Infectious diseases in zebras

An infection is defined as the invasion of an organism's tissues by pathogens. If an infection gives rise to symptoms which have adverse consequences for the host, this infection by definition becomes a disease (Begon et al., 2006). Infectious diseases and pathogens in horses have been comparably well studied, and although many viral, bacterial, protozoal, and fungal diseases are known to affect wild equids (Moehlman, 2002), little is known about the respective pathogen transmission dynamics. Although comparably few of the pathogens of equids are relevant for zoonotic diseases (e.g. the equine morbilliviruses; Barclay and Paton, 2000; reviewed in Dwyer, 2015), equids can carry various pathogens which also spread to other wildlife taxa where they may be substantially more harmful than in their equid hosts, e.g. *Streptococcus equi* (Höner et al., 2012; Speck et al., 2008), *Bacillus anthracis* (Dwyer, 2015), *Brucella sp.* (Assenga et al., 2015), and equid herpesviruses (Greenwood et al., 2012; Ma et al., 2013), among others. Various pathogens carried by equids can infect other hoofed animals (ungulates), but also pathogen transmission among equid species may have severe consequences, such as the African horse sickness virus, which is a common pathogen in zebras and African wild asses where it is mostly subclinical, whereas transmission to horses is usually fatal (Rodriguez et al., 1992).

Almost 100 extant ungulate species are known on the African continent (Kingdon and Hoffmann, 2013), many of which are threatened by extinction (IUCN, 2018; Toit, 1999). Within the order of perissodactyla, the family of equids is represented by four wild species in Africa: the wild ass (*E. africanus somaliensis*), the mountain zebra (*E. zebra*), Grévy's zebra (*E. grevyi*), and the plains zebra (*E. quagga*) (Ransom and Kaczensky, 2016). Also domesticated horses (*E. ferus*) and donkeys (*E. africanus asinus*) are common in many African countries. As classified by the International Union for the Conservation of Nature, *E. africanus somaliensis* is 'critically endangered', *E. zebra* and *E. grevyi* are threatened ('vulnerable', and 'endangered', respectively), and *E. quagga* is 'near threatened' (IUCN, 2018). The African equids typically inhabit savannahs and arid ecosystems. Habitat loss and fragmentation, poaching, competition with livestock for

forage and water, and genetic bottlenecks are considered the main threats for African wild equids.

In the conservation and population management of endangered ungulate species, disease transmission is an important but often neglected factor (Gippoliti et al., 2018). For small wildlife populations, outbreaks of severe diseases (e.g. anthrax) may have grave consequences for the total population count (Moehlman, 2002; Muoria et al., 2007). Locally shrinking populations and loss of genetic diversity gave rise to translocation programs in Grévy's zebras (Franceschini et al., 2008). In this regard, the potential adverse consequences of animal relocations in terms of pathogen introduction and transmission should be considered, which is why pathogen transmission patterns should be investigated in order to understand the underlying mechanisms.

Before control of disease outbreaks in wildlife is possible, there is a need for increased understanding of the dynamics of infections in wild and domestic animal populations (Moehlman, 2002). Although disease-related mechanisms such as immune defence or pathogen transmission are often highly host-specific (Horrocks et al., 2011), the principles found in zebras may to some degree also apply to other perissodactyl species. Furthermore, besides pathogen transmission among the perissodactyls, also transmission of equid pathogens to more distant taxa such as ruminants or even carnivores can occur (Greenwood et al., 2012; Höner et al., 2012; Schrenzel et al., 2008); therefore insights on the respective transmission patterns may be useful for the understanding of pathogen dynamics in other, more distant taxa, such as ruminants that inhabit similar ecological niches. I focused this thesis on three zebra species (*E. quagga*, *E. zebra*, and *E. grevyi*), which under natural conditions inhabit different ecosystems of sub-Saharan Africa. I examined plains zebras and mountain zebras *in situ* (in the Serengeti National Park, Tanzania, and in Etosha National Park and Khomas region, Namibia), and extended my research to individuals of all three zebra species in captivity (zoological gardens in central Europe).

1.3 Equid herpesviruses

Zebras can be naturally infected by pathogens of varying virulence, some of which lead to fatal diseases (e.g. anthrax; Muoria et al., 2007; Turner et al., 2013). The equid herpesviruses (EHV) typically exhibit moderate virulence in their original hosts, but high prevalence in all equid populations worldwide (up to 100%; Ehlers et al. 1999; Ataseven et al. 2009; Hartley et al., 2013; Dunowska, 2014a; Marenzoni et al. 2015). One of the most intriguing aspects of EHV is their ability to establish life-long infections during which the virus is mostly dormant, but can reactivate (through stressors or immunosuppression) to enter a phase of lytic replication. Furthermore, the EHV have been shown to readily infect novel hosts (Azab et al., 2018; Greenwood et al., 2012; Schrenzel et al., 2008). These traits make EHV interesting models to investigate pathogen transmission and hosts infection physiology. In this thesis, I investigated reactivation and transmission patterns of EHV, which stand out among equid pathogens due to their high prevalence, moderate virulence, and their ability to infect novel hosts.

Herpesviruses (HV) are among the largest and most complex of viruses. Their virions are 200 - 250 nm in diameter, and consist of a linear double-stranded DNA genome of 125 - 245 kb (encoding 100 – 200 genes) within an icosahedral capsid (approximately 125 nm in diameter), wrapped in a lipid membrane which contains several viral glycoproteins (Davison, 2002). The space between the envelope and the capsid is lined by the viral tegument, which consists of a cluster of proteins that are important for viral DNA replication and evading the host's immune response (Kapoor et al., 2014). Within the family of *Herpesviridae*, the EHV belong to two of three subfamilies, *Alphaherpesvirinae* and *Gammapherpesvirinae*, and are mostly assigned to the two genera *Percavirus* and *Varicellovirus* (Table 1, according to Davison, 2010). The two subfamilies mainly differ by their host cell entry pathway, mediated by the glycoproteins B (gB), D (gD), and G (gG) (Dunowska, 2014a; Ma et al., 2013). Equid HVs comprise various virus species, such as equine herpesviruses (EHV-1 to EHV-9), asinine herpesviruses (AsHV-1 to AsHV-6), Wild Ass Herpesvirus (WAH), and Equus Zebra Herpesvirus (ZHV) (table 1). The latter two are regarded as 'tentative species' and have no taxonomic standing so far. Currently, some 14

different species of HVs infecting equids are recognized, although consistent classification, nomenclature, and phylogeny of are still missing (e.g. EHV-1 and EHV-8 both share the synonym 'AsHV-3'). New virus species or strains are often named after the host in which they were first discovered, rather than by their actual original host or their true phylogenetic relationships, as is the case with Gazelle Herpesvirus-1 (GHV-1), WAH, ZHV, or the clade of AsHVs. Genetic recombination of different EHV species producing new strains or strain variants adds to the complexity of classification - for instance, different loci within one single virus genome may seem to belong to different virus species (Greenwood et al., 2012).

Table 1. Classification of equid herpesviruses (EHV), comprising *Equine Herpesvirus-1* to *Equine Herpesvirus-9*, *Asinine Herpesvirus-1* to *Asinine Herpesvirus-6*, and the tentative species Wild Ass Herpesvirus and Equus Zebra Herpesvirus, according to the genus classification revised by Davison, 2010; Osterrieder and Van de Walle, 2010; Ostlund, 1993, and species denotation by the respective reference.

Species	Synonym	sub-family	Genus	Reference
EHV-1	equine abortion virus; AsHV-3	α	<i>Varicellovirus</i>	Telford et al., 1992
EHV-2	equine cyto-megalovirus	γ	<i>Percavirus</i>	Telford et al. 1995
EHV-3	equine coital-exanthema virus	α	<i>Varicellovirus</i>	Sijmons et al., 2014
EHV-4	equine rhino-pneumonitis virus	α	<i>Varicellovirus</i>	Izume et al., 2017
EHV-5	<i>none</i>	γ	<i>Percavirus</i>	Wilkie et al., 2015
EHV-6	AsHV-1	α	<i>unassigned</i>	Ostlund, 1993
EHV-7	AsHV-2	γ	<i>unassigned</i>	Bell et al., 2008
EHV-8	AsHV-3	α	<i>Varicellovirus</i>	Garvey et al., 2018
EHV-9	gazelle herpesvirus 1	α	<i>Varicellovirus</i>	Fukushi et al., 1997
AsHV-4	<i>none</i>	γ	<i>unassigned</i>	Kleiboeker et al., 2002
AsHV-5	<i>none</i>	γ	<i>unassigned</i>	Rushton et al., 2014
AsHV-6	<i>none</i>	γ	<i>unassigned</i>	Kleiboeker et al., 2004
ZHV	<i>none</i>	γ	<i>unassigned</i>	Ehlers et al., 1999
WAH	<i>none</i>	γ	<i>unassigned</i>	Ehlers et al., 1999

1.3.1 Equid herpesvirus-associated pathogenesis

EHVs are responsible for considerable economic losses in the equine industry (Lunn et al. 2009; Dunowska 2014a). Most common among domestic and wild equids are the *Alphaherpesvirinae* EHV-1 and EHV-4, and *Gammaherpesvirinae* such as EHV-2, EHV-5, and EHV-7, with prevalences of up to 100%, depending on the species (Ataseven et al., 2009; Ehlers et al., 1999; Marenzoni et al., 2015). All EHVs can cause clinical symptoms in the respiratory tract, apart from EHV-3 and EHV-6 which cause coital exanthema (Osterrieder and Van de Walle, 2010). Virus species belonging to the alpha subfamily are more often the cause of clinical symptoms than gamma HVs. However, the role of gamma-EHVs as symptom-causing pathogens in equids is not entirely clear (Fortier et al., 2010; Kleiboeker et al., 2002). Initial EHV infections in equids typically cause diseases of the respiratory tract (rhinopneumonitis), and symptoms such as fever, anorexia, nasal and ocular discharge, cough, and progressive lymphadenopathy. Furthermore, also abortion, neonatal mortality, and neurotropic diseases can occur (Dunowska, 2014b; Fortier et al., 2010). During pregnancy, initial infection with a new EHV species or strain often results in an abortion, or can lead to fatal neurological diseases and perinatal death of the mare (Chowdhury et al. 1986; Fritsche and Borchers, 2011). In zebras, clinical symptoms after EHV infections include necrotizing rhinitis, hydrothorax, abortion, and pulmonary oedema (Blunden et al., 1998). Neurological diseases are typically referred to as Equid Herpesvirus Myeloencephalopathy with symptoms such as paresis or paralysis, as well as neurotropic diseases such as encephalitis (Goehring et al., 2006). Other symptoms include decreased coordination, general weakness of the hindquarters, sensory deficits in the perineal area as well as bladder dysfunction, lethargy, and depression (Dunowska, 2014b). The incubation period for Equine Herpesvirus Myeloencephalopathy is difficult to define, as it can either cover the period since endogenous re-activation of a latent virus, or start with a novel infection from an exogenous source (Dunowska, 2014a). Once the nervous system is affected, encephalitis can lead to ataxia, paralysis and death, although neurological symptoms of EHV infection are comparably rare in horses (Goehring et al., 2006). In wild equids, however, not much is known about EHV-associated neurological pathogenesis.

EHVs readily adapt to infect new (non-equid) host species. In these novel hosts, EHV infections are typically more severe than in equids, and are often fatal. Besides myeloencephalopathy, symptoms in novel host species commonly include respiratory disease, retinitis, abortion, and neonatal death (Abdelgawad et al., 2014; Greenwood et al., 2012; Ma et al., 2013). Non-equid species which have reportedly been infected with EHV with a severe to fatal outcome include Thomson's gazelle (*Eudorcas thomsonii*; Fukushi et al., 1997), giraffe (*Giraffa camelopardalis*; Schrenzel et al., 2008), Indian rhinoceros (*Rhinoceros unicornis*; Abdelgawad et al., 2014), llamas and alpacas (*Lama glama*; *Vicugna pacos*, respectively, Rebhun et al., 1988), polar bears (*Ursus maritimus*; Greenwood et al., 2012), brown bears (*Ursus americanus*; Wohlsein et al., 2011), and guinea pigs (*Cavia porcellus*; Wohlsein et al., 2011).

The fact that EHVs readily adapt to infect new host species is particularly relevant for captive collections of allopatric species. Individuals of threatened species which are (potential) novel EHV hosts are often housed in the same zoological collections as equids. EHVs have been reported to be stable in the environment for a considerable amount of time (Dayaram et al., 2017), which facilitates indirect (e.g. anthropogenic) transmission. Potential indirect transmission is thus an important factor to consider regarding the survival (and successful reproduction) of captive animals which are novel EHV host species. Therefore, in order to implement measures for the prevention of pathogen transmission, it is necessary to gather information on various epidemiological parameters, including conditions which elicit EHV shedding in the original hosts, transmission patterns, and viral adaptation to novel hosts.

1.3.2 Equid herpesvirus latency, reactivation, and transmission

Much like herpes simplex in humans, EHVs typically remain in their hosts as a latent lifelong infection (Ostlund, 1993; Reese, 2016). After initial infection, the viruses remain in a state of latency (also referred to as non-cytopathic infection) in host neural tissue (e.g. in the trigeminal ganglion), and lymphoid cells (e.g. B cells, macrophages, dendritic cells; Reese, 2016; Slater et al., 1994; White et al., 2012). In order for HVs to remain in

their hosts as latent infections, they have evolved a number of immune evasion strategies, such as molecular mimicry by encoding homologs of cellular interleukins, chemokines, or chemokine receptors (Nicholas, 2005). These homologs can impair natural killer cell-mediated killing of infected B cells, modulate cellular cytokine responses, and interfere with T cell activity (Huang et al., 2014; Ma et al., 2013). This can be achieved, for instance, by the expression of membrane proteins that induce substantial down-regulation of cell-surface expression of the major histocompatibility complex I (MHC-I), thereby inhibiting the recognition of infected cells by T cells (Huang and Osterrieder, 2015; Ma et al., 2013). This and various other immune-evasive strategies are generally accepted to be a product of a long history of co-evolution of HVs with their hosts (Ma et al., 2013).

To establish latency, the viral genome is circularized and forms an episomal DNA complex which is packaged in histones (Grinde, 2013). During viral latency there is little (but continuous) viral replication and minimal viral gene expression, despite the presence of the viral genome in the nucleus of infected cells (Reese, 2016; White et al., 2012). When the host cell undergoes mitosis, latent viruses are copied by cellular DNA polymerases; in contrast, after reactivation viral DNA polymerases are engaged for lytic replication (i.e. viral DNA is replicated separately from the host's DNA) (Grinde, 2013).

Herpesviruses can be reactivated to enter a stage of lytic replication, which is characterized by linearization of the viral genome and expression of large amounts of viral lytic genes (Grinde, 2013; Reese, 2016). Reactivation typically results in the destruction of the infected host cell (White et al., 2012). The process of reactivation requires multiple signalling steps that may or may not be accompanied by detectable increases in lytic gene transcription (Suzich and Cliffe, 2018). The mechanisms which elicit a transition from the latent to the lytic phase are not fully understood, but HV reactivation is typically associated with acute stress or reduced immunocompetence of the host individual (e.g. through immunosuppressive processes or acute infection with another pathogen; White et al., 2012). Virus reactivation (also referred to as recrudescence) subsequently leads to viraemia and shedding of infectious virus particles into the environment.

Reactivation of latent herpesviruses can be induced by chronically elevated levels of glucocorticoids (GC) (Glaser and Kiecolt-Glaser, 2005), and experimentally administered GC can induce EHV reactivation in domestic equids (Barrandeguy et al., 2008; Edington et al., 1985). Psychological stress (which occurs when an individual perceives that the environmental demands tax or exceed its adaptive capacity; Cohen et al. 2007) is also known to be a factor causing herpesvirus reactivation, in humans and rodents (Padgett et al., 1998). Exposure to high doses of UV radiation (which is known to be a physiological or physical stressor) also seems to elicit herpesvirus reactivation, likely due to radiation-induced immune challenge (Padgett et al., 1998).

EHV reactivation may or may not be accompanied by clinical symptoms. Viral recrudescence typically leads to virus shedding and infectiousness (Dunowska, 2014b; Fortier et al., 2010). EHV-1 seems to be highly contagious with up to 100% transmission success upon direct contact (on a naso-nasal route by direct contact with nasal discharge or aerosol) with a virus shedder in horses (Allen et al., 2008). Transmission of e.g. EHV-1 occurs mainly through inhalation of the virus shed by infectious individuals as respiratory droplets, or contact with aborted fetuses or placentas (Borchers et al., 2008; Dunowska, 2014b). In general, domestic horses seem to clear EHV-1 from their respiratory systems within one to three weeks *post*-infection, although there seems to be high inter-individual variance (Dunowska, 2014b; Hartley et al., 2013).

In wildlife, anthropogenic interventions (e.g. capture, immobilization, translocation, and subsequent re-structuring of social relations) are known to induce stress (Arnemo et al., 2006; Dickens et al., 2010; Linklater et al., 2010). Capture and translocation procedures are commonly employed for breeding and re-stocking purposes of wild equids (Franceschini et al., 2008; Kaczensky et al., 2007), and although the potential risks of these procedures are known in principle, their relevance as stressors that may contribute to the reactivation and transmission of EHV-1 in captive and wild settings has been little acknowledged. It would therefore be helpful to identify potential stressors for wild equids in their natural environment. In this thesis I aimed to assess environmental factors in both, natural and captive settings, which potentially elicit a stress response in zebras, and thus may increase the probability of EHV-1 reactivation.

1.4 Immune functions in equids

The ability to resist pathogens is an important part of survival. Immunity is considered to be the state when a host has (established) sufficient defence mechanisms to clear infection with a specific pathogen (and therefore prevent disease). Two branches of the immune system can generally be distinguished by their functional mechanism and origin: innate and adaptive immunity.

The innate immune system is subdivided into constitutive and induced responses. Constitutive factors (e.g. natural antibodies and lysozyme) are present without the need for prior exposure to a pathogen, whereas the induced immune response (e.g. acute phase proteins) is only activated in response to an invading pathogen (Janeway et al., 2001). The constitutive parts of the innate immune system are typically associated with relatively low energetic costs, whereas the induced innate immunity entails high energetic costs for mounting an immune response (Lee, 2006). In addition, the induced innate immune response is also more likely to cause immune-pathological damage, e.g. during inflammatory reactions (Lochmiller and Deerenberg, 2000; Rauw, 2012; Sandland and Minchella, 2003; Svensson et al., 1998).

The innate immune response acts as a first line of defence against a broad range of pathogens. This branch of immunity is present and active from birth, but has no memory of specific pathogens. One critical function of innate immunity is to impede growth and systemic spread of pathogens upon infection, until the specific factors of adaptive immunity can effectively deactivate pathogens. The functions of the innate immune system include direct mechanisms such as phagocytosis (e.g. by neutrophils, monocytes and dendritic cells), or indirect mechanisms such as binding of free iron to prevent its usage in bacterial metabolism (Janeway et al., 2001). Upon invasion by pathogens, affected cells (e.g. epithelial cells) release chemokines and cytokines, both of which have important signalling functions to elicit processes such as the acute phase response. The acute phase response (APR) is a reaction to non-specific immune stimuli, such as trauma, inflammation, acute infection, and oxidative stress, but also by a (chronic) increase in

glucocorticoid hormones (Cray, 2012; Pepys et al., 1989; Valle et al., 2015). An APR is characterized by a substantial change in the concentration of certain circulating plasma proteins (termed acute phase proteins [APP]). The majority of known APPs in equids increase during the APR, therefore referred to as 'positive' APPs; in contrast, 'negative' APPs decrease during the APR (Cray, 2012). Positive APPs are subdivided by the magnitude of their increase: major APPs increase 10- to 1000-fold, usually within 24 to 48 hours after the stimulus. Moderate APPs increase only 5- to 10-fold with a slower response time of about three or more days, in many animals, and minor APPs increase less than 2-fold (Cray, 2012). Major, moderate, and minor APP differ between animal species, and in contrast to many other taxa, in equids, the only major APP is serum amyloid A (SAA). Moderate APPs include haptoglobin, C-reactive protein, and ceruloplasmin (Jacobsen, 2007).

Unlike the innate response, the adaptive immunity is specific to particular, previously encountered pathogens, and therefore functions based on an immunological memory (Janeway et al., 2001), which is why this branch of immunity relies on the innate immune system for antigen presentation. The cellular component of the adaptive immune response comprises B and T lymphocytes, which are responsible for antibody- and cell-mediated responses. Regarding viral infections, lysis of infected cells mediated by cytotoxic T lymphocytes (CD8⁺ T lymphocytes, in particular) is an important mechanism that depends on recognition of viral peptides presented on the cell surface by the MHC-I (Janeway et al., 2001; Ma et al., 2013). In the case of HV infections, as mentioned above, down-regulation of MHC-I expression is one of the most crucial immunomodulatory functions for HVs to maintain latency (Huang et al., 2014).

Mammalian immunity is complex and measuring any single immune marker is insufficient to draw conclusions on general immune function. Immune markers do not necessarily co-vary, therefore it is vital to measure multiple components of the immune system (Heinrich et al., 2017; Lee, 2006; Pap et al., 2010; Peck et al., 2016). In vertebrates, there is substantial variation between host species regarding the strength and functioning of the immune response and general immunocompetence (Graham et al., 2011), which is why the fitness consequences of life history trade-offs, environmental effects, and the

combination of these are becoming of more research interest in the field of ecoimmunology. Thus, it is crucial to consider the effect of intrinsic and extrinsic factors which may modulate the animals' immunocompetence.

1.4.1 Effects of environmental stressors on the immune system

Environmental stimuli which disturb homeostasis in an individual may lead to a decline in individual fitness such as reduced viability (Hofer and East, 2012). The cumulative energetic costs of maintaining homeostasis, fulfilling basic physiological costs, and costs of responding to environmental stressors are termed allostatic load (McEwen, 1998). The term stressor can be defined as an adverse environmental stimulus which potentially compromise fitness if not avoided or successfully endured (Hofer and East, 1998; Sapolsky et al., 2000). As a response to a stressor, catecholamines (epinephrine and norepinephrine) and, subsequently, GCs are released, inducing metabolic and behavioural changes (Creel et al., 2013; Hofer and East, 2012; McEwen and Wingfield, 2003). Therefore, GC concentrations are considered a useful indicator of the allostatic load (Benhaiem et al., 2013; Clinchy et al., 2013; Nelson et al., 2002). The central nervous system, the endocrine system, and the immune system are strongly interlinked, which is why exogenous factors can interfere with the immune response (Martin, 2009; Ottaviani et al., 2008) and are then recognized as stressors. Stressors that affect the organism for short periods of time (minutes to hours) can elicit enhanced activity of the immune system by increased secretion of catecholamines, thereby activating the neuroendocrine and cardiovascular systems, which may further enhance immunocompetence with regard to wound healing or fighting off infections (Hofer and East, 2012; Sapolsky et al., 2000). In contrast, stressors lasting for extended periods of time (such as several days to months, or longer) have an adverse effect on immunocompetence (Martin, 2009). In general, GCs are used to mobilise energy but may in consequence also cause a humoral immune deficiency. Why stress affects immune function is commonly attributed to three functions (Martin, 2009):

1. Energetically costly immunity is diminished during stress to redirect resources towards immediate survival.

2. Immune suppression may represent a sacrifice to provide resources to more critical processes by active catabolisation of immune cells.
3. An increased level of stress hormones (catecholamines and GCs) leads to appearance of self-antigens due to the degradation of tissue. Immune activity would then be suppressed to avoid damage caused by autoimmune mechanisms.

The effect of stressors on physiological functions in horses including immune functions has been shown in studies on competition horses, demonstrating significantly elevated levels of various immune markers and oxidative stress following competition events (Robson et al., 2003; Valle et al., 2015). Also in wild ungulates, stressors may cause increased GC concentrations (Franceschini et al., 2008; Linklater et al., 2010; Vick et al., 2012), which can be expected to affect immune functions. Therefore, it is necessary to identify environmental factors which may lead to (chronically) elevated GC levels in wild equids in their natural habitats in order to understand immune function and disease ecology in these animals.

Various environmental factors that modulate the immune function of animals have been identified, ranging from physico-chemical parameters to the social environment (Bowden, 2008; Buehler et al., 2008; Ewenson et al., 2001; Tung et al., 2012). In many regards, *ex situ* captivity is a substantial deviation from the natural environment of many wildlife species. These differences include factors such as diet, climate social factors, parasite and pathogen pressure (regarding diversity and abundance), movement space, medical treatment and prophylaxes, and direct anthropogenic disturbance. Thus, the differences between natural settings and *ex situ* captivity in general may be expected to affect immune functions in captive wildlife. Regular feeding, antiparasitic treatments, vaccinations, and medical care help to maintain individual health and to ensure longevity of captive wildlife. In contrast, factors such as persistent aversive sound, artificial lighting, unnatural temperatures and substrates, restricted movement, reduced hiding spaces, unnatural feeding schemes, unnatural social grouping, and limited behavioural opportunities may negatively affect individual health (Hofer and East, 1998; Mason, 2010; Morgan and Tromborg, 2007). Few studies have addressed the question whether captivity affects immune functions in wild animals, compared to their free-ranging counterparts: Flies et al. (2015) showed that in spotted hyenas serum antibodies were significantly

lower in captive compared to wild individuals. Similarly, a study on bottlenose dolphins (*Tursiops truncatus*) showed that various immune markers had lower expression in captive than in wild animals (Fair et al., 2017). Regarding wild equids, no comparable studies have been conducted to assess the effect of captivity on immune functions. In this thesis, I examined whether *ex situ* captivity affects a suit of immune markers in two zebra species.

1.4.2 Effects of gestation and lactation on the immune system

Life history theory predicts that when resources are limited, trade-offs between physiological functions will occur (Stearns, 1989, 1976). One of the most prominent life-history trade-off involves the cost of reproduction and the cost of survival (Stearns, 1989). Lactation is one of the most energetically costly life history stages in equids (Ofstedal et al., 1983), thus when nutrient supply is insufficient to sustain both lactation and self-maintenance, resource allocation trade-offs such as decreased investment in immune processes may be expected. This has been shown as a decreased resistance against parasites in species such as the bighorn sheep (*Ovis Canadensis*; Festa-Bianchet 1989) and the spotted hyena (*Crocuta crocuta*; East et al. 2015).

Zebras have a gestation period which is 20% longer than that of comparably sized ruminants. Gestation and subsequent lactation are a considerable energetic burden to a mare, as the foal usually doubles its birth weight within only a month (Duncan, 1992). In equids, pregnancy and lactation have a marked effect on various immune functions, although these are not necessarily immunosuppressive: pregnancy was associated with a down-regulation of T-cells in domestic horses (Noronha and Antczak, 2012), but other immune factors such as IgG, haptoglobin, and lysozyme, increased significantly during late pregnancy and early lactation (Budzynska, 2002). Equids have an epitheliochorial placenta (Samuel et al., 1974), which prevents intra-uterine passage of antibodies from mother to foetus. Thus, passive immunization of the foal by antibody transfer via the colostrum is crucial (Borghesi et al., 2014; Moffett and Loke, 2006). In equids, colostrum is produced within two weeks *pre-partum* and contains high concentrations of antibodies such as IgG and IgM. These large maternal antibodies can be absorbed through the foal's intestinal

epithelial cells exclusively within about 32 hours *post-partum* (Acworth, 2010), as the specialized antibody-absorbing epithelial cells are then replaced by mature 'closed' epithelial cells. The concentrations of these large antibodies in the mare's milk decreases substantially within 24 hours *post-partum* (Acworth, 2010; Veronesi et al., 2014).

In conclusion, it is possible that in wild equids, the energetic costs of pregnancy and lactation may be associated with adverse effects on the immune system and a down-regulation of active immunity. In contrast, passive immunization of the foal may demand an up-regulation of the respective immune factors. Regarding pathogen transmission and disease monitoring it is therefore important to determine to which extent there is a trade-off between reproduction and active immune defence, and whether pregnant or lactating mares may be more prone to novel infections and subsequent pathogen spreading. I therefore examined whether lactation in zebras affects the expression of a suite of immune markers in terms of either potential resource re-allocation at the expense of certain immune functions, or up-regulation due to passive immunization of nursing offspring.

1.5 Study aims

The aim of this thesis is to investigate the factors which may contribute to EHV transmission under natural settings and in captivity, and to evaluate novel non-invasive sampling methods for pathogen screening in wild equids. The prevalence of EHV in general ranges up to 100% in all equid populations, and clinically silent viraemia seems to be common. However, wild equids in captivity may be sources of infection which are in fact a potential threat to range of other species. Thus, I aimed to determine the ecological and intrinsic factors which can be expected to increase the probability of novel infections and subsequent pathogen shedding and transmission in captive and wild settings.

I address these aims with four studies:

1. In a methodological study I established a detection method using environmental DNA (eDNA) to assess acute EHV shedding in captive individuals of Grévy's, plains, and mountain zebras (**Chapter 2**).
2. In a study on captive Grévy's zebras, I examined the effect of environmental stressors (long-distance transport and social group re-structuring) on individual faecal glucocorticoid metabolite (fGCM) concentrations, and their potential association with EHV reactivation and virus shedding (**Chapter 3**).
3. An immunological survey of wild and captive plains and mountain zebras was conducted to investigate the effect of different environments (i.e. captivity vs. natural habitats) on immune markers of the constitutive innate, induced innate, and adaptive immune response, and on the relative incidence of EHV shedding (**Chapter 4**).
4. In a study on a large population of free-ranging plains zebras in their natural habitat I assessed the effect of environmental factors and life history stages on fGCM concentrations. This research aimed to identify extrinsic and intrinsic factors which could potentially elicit a sufficiently strong physiological stress response that may, in consequence, depress the immune system and result in increased pathogen spreading (**Chapter 5**).

1.6 References

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CHAPTER 2: Manuscript I

Equine behavioural enrichment toys as tools for non-invasive recovery of viral and host DNA.

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CHAPTER 3: Manuscript II

Environmental stressors may cause equine herpesvirus reactivation in captive Grévy's zebras (*Equus grevyi*)

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CHAPTER 4: Manuscript III

***Ex situ* captive environments and lactation influence innate immunity in zebra mares**

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Abstract

Immunity in mammals is influenced by physiological and environmental factors. Wild mammals in *ex situ* captivity experience substantially different environmental conditions from free-ranging conspecifics in terms of diet, climatic conditions, social factors, infection pressure, movement space, and direct anthropogenic disturbance. Moreover, animals in captivity experience more management interventions such as medical treatments and prophylaxes. Few studies have compared immune marker expression between captive and free-ranging wildlife, and captivity has been shown to affect immunological responses in few terrestrial and marine mammals, but it is unclear whether this can be generalized to other taxa. Furthermore, little is known about how energetically costly life history stages influence the immune system in wild mammals. We investigated the effect of a) environments (captivity in European zoos versus African national parks), and b) lactation on markers of the constitutive and induced innate, and adaptive immune system in plains and mountain zebras (*Equus quagga* and *E. zebra*). As a proxy for general immune function, we screened for equid herpesvirus (EHV) DNA in blood and nasal swabs, as these are common latent pathogens which can be reactivated in response to stress and immune challenge. We found significantly lower levels of constitutive innate immune markers in captive than in wild zebras. In contrast, markers for energetically costly induced immunity were higher in captive animals. Lactation was associated with higher titers of natural antibodies and lysozyme. Acute EHV infection was not significantly correlated with any of the measured immune markers. Our results indicate that captivity and lactation may be important immunomodulators in zebra mares.

Key words: Innate immunity, Captivity, Plains zebra, Mountain zebra, Equine herpesvirus

Introduction

Maintaining captive wildlife populations has become a crucial conservation strategy for some species (Schulte-Hostedde and Mastromonaco, 2015). Although life in captivity is beneficial for health and longevity in some species, it can have adverse effects on health and fecundity of others (Mellor et al., 2018). Negative impacts of captivity on reproduction can strongly constrain the success of captive breeding programs (Mason, 2010).

The ability to resist pathogens is one of the most important physiological functions in long-lived animals. Various environmental factors that modulate the immune function of animals have been identified, ranging from physico-chemical parameters to the social environment (Bowden, 2008; Buehler et al., 2008; Ewenson et al., 2001; Tung et al., 2012). In many regards, *ex situ* captivity reflects a substantial deviation from the environment of many free-ranging wildlife species. Key differences include factors such as diet, climatic conditions, social factors, infection pressure, movement space, and anthropogenic interventions including disturbance but also medical care and prophylaxes (Boyd, 1986; Morgan and Tromborg, 2007; Schulte-Hostedde and Mastromonaco, 2015), all of which may affect immune functions. Intestinal parasites, for instance, are typically less abundant in captive animals due to prophylaxes and treatment schemes (e.g. Wambwa et al., 2004). Taken together, the multi-factorial differences and *ex situ* captivity and more natural settings in general may be expected to alter immune functions in various ways.

Although a number of studies have been performed on captive wildlife immune function, the results are not necessarily generalizable to their free-ranging conspecifics (Pedersen and Babayan, 2011; Tian et al., 2015). Few studies have compared immune functions of captive mammals with free-ranging individuals. For example, Flies et al. (2015) showed that in spotted hyenas (*Crocuta crocuta*) the concentrations of immunoglobulins G (IgG) and M (IgM) and the bacterial killing capacity of serum were significantly lower in captive than free-ranging individuals. Similarly, a study on bottlenose dolphins (*Tursiops truncatus*) showed that various immune markers (e.g. cytokines) showed lower

expression in captive than in wild animals (Fair et al., 2017). Regarding other mammalian taxa, no such comparative studies have been conducted, to our knowledge.

Mammalian immunity is complex, and measuring any single immune marker is insufficient to draw conclusions on overall immune function (Ruoss et al., 2018). Immune markers do not necessarily co-vary, therefore it is vital to measure multiple components of the immune system (Heinrich et al., 2017; Lee, 2006; Pap et al., 2010; Peck et al., 2016). In vertebrates, immune defense consists of the innate and the adaptive immune system, both with humoral and cellular effectors. The innate immune system is the first and unspecific response to infection that serves to eliminate and prevent spreading of potential pathogens within the organism, and to initiate an inflammatory response (Janeway et al., 2001). The innate immune system is further subdivided into constitutive and induced responses. The constitutive factors such as natural antibodies and lysozyme, are present without the need for prior exposure to a pathogen, whereas the induced immune response (e.g. acute phase response) is only activated in response to an invading pathogen (Janeway et al., 2001). Adaptive immunity is characterized by highly specific pathogen recognition that induces responses to pathogens that were previously encountered (Janeway et al., 2001).

Mounting an immune response can be energetically costly (Lee, 2006) and can also involve immuno-pathological consequences, e.g. during inflammatory reactions (Lochmiller and Deerenberg, 2000; Rauw, 2012; Sandland and Minchella, 2003). Constitutive innate immunity is typically associated with relatively low energetic costs (e.g. lysozyme and natural antibodies), whereas induced innate responses (e.g. acute phase proteins) entail higher energetic costs. In addition, the induced innate immune response is more likely to cause immune-pathological damage (Lee, 2006; Rauw, 2012). There is evidence that in various animal species an increase in energetic costs elsewhere (e.g. investment in growth or reproduction) may lead to a trade-off in terms of reduced immunity (French et al., 2007; Lee, 2006; Peck et al., 2016; Sandland and Minchella, 2003). Several studies have shown the effect of increased energy expenditure on total parasite load as a proxy measure of the immune function in various mammalian species (East et al., 2015; Lloyd, 1983; Sheldon and Verhulst, 1996). In contrast to this down-

regulation of active immunity, lactation also serves passive immunization of the offspring (Borghesi et al., 2014; Moffett and Loke, 2006) and has been shown to be associated with increased expression of certain adaptive and innate immune markers such as immunoglobulin G, haptoglobin, and lysozyme (Budzyńska, 2002; Veronesi et al., 2014).

In this study, we compared the levels of several immune markers in free-ranging zebra mares in two African national parks with those in zebra mares in *ex situ* captivity (European zoos). Furthermore, we examined the effect of lactation on the tested immune markers. We measured two markers of the constitutive innate immune system (natural antibodies and lysozyme), two markers of induced innate immunity (haptoglobin and serum amyloid A [SAA]), and one marker of the adaptive immune response (total IgG). Natural antibodies are largely unspecific and their concentration can rapidly increase following infection (Boes, 2001), and the majority of natural antibodies in equids are accounted for by IgM (Borghesi et al., 2014). Lysozyme is an integral part of antibacterial constitutive immunity and is thus an important biomarker of the innate immune response. Haptoglobin and SAA are acute phase proteins (APP) and are vital components of the acute phase response in equids (Belgrave et al., 2013; Cray et al., 2009; Cray and Belgrave, 2014). The APPs are part of the early response of the innate immunity, which is elicited by inflammation, infection, stress, or trauma. IgG is the most abundant circulating antibody serotype, and the main effector of the humoral adaptive immunity and specifically binds antigens such as bacteria and viruses that are recognized upon initial exposure (Janeway et al., 2001). During immune challenge and/or immunosuppression, opportunistic viral reactivation is a common feature of otherwise latent viral infections, such as herpesviruses (Marenzoni et al., 2015; White et al., 2012). Thus, acute herpesvirus infection might be an indicator of general immunocompetence in latently infected organisms. We screened for equid herpesvirus (EHV) DNA in blood and nasal swabs as a potential proxy of general immunocompetence, and we assessed seroprevalence of specific antibodies against two clinically important equine herpesviruses (EHV-1, and EHV-9) using a peptide-based enzyme-linked immunosorbent assay (ELISA) as described by Abdelgawad et al. (2015).

Materials & Methods

Sample collection

Blood samples were collected from wild mountain and plains zebras in Etosha National Park, Namibia, in December 2015, and from plains zebras in Serengeti National Park, Tanzania, from the end of May to the end of June 2016 (table 1). This corresponded to the late dry season in Namibia, and the early dry season in Tanzania. All wild animals sampled were adult mares ($N = 29$) that were categorized by their reproductive state as either lactating (when mares had a suckling foal at foot, $N = 8$) or non-lactating ($N = 21$). Foals were estimated to be between several weeks and six months of age, based on body size. Due to the non-seasonal reproduction pattern of plains zebras, lactating as well as non-lactating mares may have been in an early to middle stage of pregnancy. No late-pregnant mares (judged by the size of the abdomen) were used in this study.

The animals were immobilized with a dart gun (as described in Costantini et al., 2018), and blood samples were drawn from the jugular vein approximately 15 - 30 min after darting and placed into serum tubes and EDTA tubes (S-Monovette Z, and S-Monovette K, respectively, Sarstedt, Hildesheim, Germany). Serum was separated from the clot after 15 - 30 min into cryotubes. In order to screen for acute EHV infections, we collected nasal swabs and blood samples for extraction of viral DNA. All samples were transported in a cooling box on ice packs, and then stored at $-20\text{ }^{\circ}\text{C}$ until transport to the laboratory facilities of the Leibniz Institute for Zoo and Wildlife Research, where they were stored at $-80\text{ }^{\circ}\text{C}$.

Blood of captive adult individuals ($N = 22$) was collected from plains and mountain zebra mares in four different zoological collections in Europe (Réserve Africaine de Sigean, France; Zoo Antwerp, Netherlands; Zoo Landau, Germany; and Givskud Zoo, Denmark). Blood, serum, and swab sampling was performed as described for wild animals. Captive zebras comprised 22 non-lactating mares. No clinical signs of acute disease were observed in any of the captive or wild zebras.

Table 1. Distribution of samples by zebra species, environment, and geographical origin (site).

Species	Environment	Site	N
Plains zebra	natural	Namibia	5
		Tanzania	17
	captive	zoos (Europe)	11
Mountain zebra	natural	Namibia	7
	captive	zoos (Europe)	11

Hemagglutination assay

Hemagglutination is characterized by the appearance of clumped red blood cells due to natural antibodies binding multiple antigens. Hemagglutination was measured in 96-well microtiter plates as described in Heinrich et al. (2017). Briefly, samples were serially diluted from 1:1 to 1:1,024 in PBS, 25 μ L of 1% chicken red blood cells suspension was added, then plates were vortexed and incubated at 37 °C for 90 min. After incubation, plates were tilted at a 45° angle to increase the visualization of agglutination and kept at room temperature for 20 min until measurement. Natural antibody titers are given as the \log_2 of the reciprocal of the highest dilution (i.e. lowest concentration) of serum showing positive hemagglutination (Matson et al. 2005).

Lysozyme assay

To measure lysozyme concentration, a lysoplate assay was used (Heinrich et al. 2017): 25 μ L plasma was inoculated in the test holes of a 1% Noble agar gel (Sigma Aldrich; # A5431-250G) containing 50 mg/100 mL lyophilized *Micrococcus lysodeikticus* (Sigma Aldrich; # M3770-5G), a bacterium that is particularly sensitive to lysozyme. Crystalline hen egg white lysozyme (Sigma Aldrich; # L6876) (dilutions: 0.75, 1, 2, 3, 5, 7.5, 10, 15 and 30 μ g/mL) was used to prepare a standard curve in each plate. Plates were incubated at room temperature (25 – 27 °C) for 20 h. During this period, as a result of bacterial lysis, a clear zone developed in the area of the gel surrounding the sample inoculation site. The diameters of the cleared zones are proportional to the log of the lysozyme concentration. This area was measured three times digitally using the software ImageJ (version 1.48, <http://imagej.nih.gov/ij/>) and the mean was converted on a semilogarithmic plot into hen

egg lysozyme equivalents (HEL equivalents, expressed in $\mu\text{g}/\text{mL}$) according to the standard curve (Rowe et al., 2013).

Haptoglobin assay

Haptoglobin concentrations were measured with a commercial kit (Tridelta Developments Ltd. sold by Biorepair GmbH; #BR220) following the instructions of the manufacturer. Haptoglobin concentrations (mg/mL) in plasma samples were calculated according to the standard curve on each plate (Matson et al. 2006).

Serum amyloid A (SAA) assay

SAA concentrations were measured using a commercial solid phase sandwich SAA multispecies ELISA kit (Tridelta Developments Ltd. sold by Biorepair GmbH; # BR230), following the instructions of the manufacturer. Serum samples were initially diluted 1:2,000 as per recommendation of the manufacturer for measuring horse samples, or higher when SAA concentrations were above the measurement range (up to 1:16,000). Absorbance of the ELISA was measured using a standard spectrophotometer (Biotek; μ Quant Microplate Spectrophotometer), and SAA concentrations were calculated according to the standard curve on each plate. Most samples ranged from 111.71 to 381,630.7 mg/L , however, in several samples ($N = 9$) the SAA concentration was too high for accurate measurement; these samples were assigned with an absolute maximum value (500,000 mg/L) for statistical analysis. Several other samples ($N = 25$) had a SAA concentration too low for accurate measurement at the initial dilution, thus an absolute minimum value (1 mg/L) was assigned to these samples for statistical analyses.

IgG assay

A commercially available highly sensitive two-site ELISA kit (Abnova, # KA2036) was used to quantify total IgG in serum. IgG concentration in serum was determined by comparing measurements to a standard curve and adjusting for the dilution factor (1:200,000).

EHV peptide-based ELISA

To investigate previous exposure to two of the most common equid alpha-herpesviruses, a peptide-based ELISA was employed for measuring EHV-1- and EHV-9-specific antibody

titers, as described by Abdelgawad et al. (2015). Each serum sample was measured four times in total to produce mean antibody titers.

EHV screening

DNA was extracted from whole blood samples and nasal swabs using a commercially available kit (NucleoSpin® Tissue, Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions for the respective substrate (blood, or buccal swab protocol, respectively). To test for EHV DNA, a nested PCR was performed as described by Kleiboeker et al. (2002) with a modified thermocycling protocol (Seeber et al., 2017). Amplified products were visualized on a 1.5% agarose gel and purified using a kit (NucleoSpin® Gel and PCR Clean-up, Macherey-Nagel, Düren, Germany) according to the manufacturer instructions. Purified PCR products were Sanger-sequenced by LGC Genomics GmbH, Berlin, Germany, and virus species were identified in GenBank (Benson et al. 2009) databases using BLAST searches.

Statistical analyses

A linear model was fitted for each immune marker as a response variable (natural antibody titer, lysozyme, and IgG concentrations, and log-transformed haptoglobin and SAA concentrations) with the following predictors: sampling site (Namibia, Tanzania, or captivity), reproductive state (lactating or non-lactating), EHV status (positive or negative by PCR detection), and species. Visual inspections of the models indicated no obvious violations of assumptions of normality and homogeneity of error variances. Inspection of variance inflation factors (all ≤ 2.6) did not indicate a problem of multicollinearity in any of the models. To assess the statistical significance of the predictor variables we used F-tests to compare the full model to reduced models in which the respective predictor was removed. Significant differences between predictor variables were analyzed *post-hoc* by a Tukey's Multiple Comparison of Means test. The difference of EHV-1 and EHV-9 antibody presence between wild and captive animals was tested using a Fisher's exact test. To exclude variation among zoos, a Kruskal-Wallis test was performed for each marker. Statistical analyses were performed in R version 3.2.5 (R Development Core Team 2016) using the "multcomp" package (Hothorn et al. 2008) for *post-hoc* tests. Statistical significance is reported at an α -level of $p < 0.05$.

Ethical standards

This study was approved by the Internal Committee for Ethics and Animal Welfare of the Leibniz-Institute for Zoo and Wildlife Research, approval number 2016-09-03. All protocols adhered to the laws and guidelines of Namibia, Tanzania, and Germany, respectively. Sampling in Namibia and Tanzania was performed with permission of the Environment and Tourism (permit No. 2094/2016), and the Tanzania Commission for Science and Technology (permit No. 2015-168-NA-90-130), respectively. Permission to export sample material from Namibia was granted by an MET export permit (No. 105336), and samples were transported to Germany in full compliance with the Convention on International Trade in Endangered Species (CITES; permit no. 108448) and in compliance with the Nagoya Protocol on Access to Genetic Resources.

Results

The three sampling site categories, Namibia, Tanzania, and *ex situ* captivity, differed significantly with regard to natural antibody titers, lysozyme, haptoglobin, and SAA concentrations (table 2). Specifically, the two constitutive innate immune markers (natural antibody titer and lysozyme concentration) were significantly lower in captive zebras than in those in each of the natural African environments, whereas the two induced innate markers (haptoglobin and SAA) were higher in captive than in wild zebra mares. SAA was significantly higher in captive animals than in Namibian zebras, whereas the difference between captive and Tanzanian zebras was not statistically significant (table 2, table 3 fig. 1). None of the markers differed significantly among zoos. For future reference purposes, the respective immune marker concentrations in captive and wild plains and mountains zebras are shown in table 4.

Both constitutive innate markers were significantly higher in lactating, than in non-lactating zebra mares (table 2, fig. 2). No significant effect of lactation on haptoglobin or SAA concentrations (table 2) was observed. No significant effect of the environment or reproductive state on IgG concentration was identified (table 2). Species was a significant predictor for SAA concentrations, with lower SAA levels in mountain zebras, than in plains zebras (table 2). IgG concentrations were not affected by any of the predictors.

The prevalence of acute EHV infections was similar in captive and wild animals (64% and 62%, respectively). Most viruses identified belonged to the gamma-herpesviruses (90%); active alpha-herpesvirus infections included EHV-9 ($N = 2$), and EHV-1 ($N = 1$). EHV status, however, was not a significant predictor of any of the immune markers tested (table 2). Applying the peptide-based antigen ELISA, EHV-1 specific antibodies were detected in 50% of captive, and 97% of wild zebras, and EHV-9 specific antibodies in 5% of captive, and 90% of wild zebras. The infection of both EHV-1 and EHV-9 prevalence differed significantly between wild and captive animals ($p < 0.001$, each).

Table 2. Results of linear models to assess the effects of sampling site (Namibia, Tanzania, or *ex situ* captivity), lactation, EHV status, and species on the immune markers. Haptoglobin and SAA concentrations were log-transformed. Asterisks indicate significant effects at $p < 0.05$.

response	predictor	df	F	p
Natural antibodies	site	2	14.8	<0.001 *
	lactation	1	5.5	0.024 *
	EHV	1	0.3	0.581
	species	1	0.2	0.694
Lysozyme	site	2	22.9	<0.001 *
	lactation	1	27.3	<0.001 *
	EHV	1	0.1	0.767
	species	1	1.89	0.176
IgG	site	2	1.5	0.228
	lactation	1	0.0	0.877
	EHV	1	0.7	0.402
	species	1	3.1	0.086
Haptoglobin	site	2	13.1	<0.001 *
	lactation	1	1.2	0.301
	EHV	1	3.4	0.071
	species	1	1.7	0.192
SAA	site	2	7.9	0.001 *
	lactation	1	0.1	0.764
	EHV	1	0.2	0.663
	species	1	10.28	0.003 *

Table 3. Results of post-hoc tests assessing the effect of sampling site on the respective immune marker concentration. Results are derived from Tukey’s multiple comparison of means. Haptoglobin and SAA concentrations were log-transformed. Asterisks indicate significant effects at $p < 0.05$.

response	site	estimate	std. error	t	p
Natural antibodies	Zoo → Namibia	1.29	0.37	3.47	0.003 *
	Zoo → Tanzania	2.20	0.44	4.96	<0.001 *
	Namibia → Tanzania	0.92	0.50	1.84	0.163
Lysozyme	Zoo → Namibia	1.48	0.31	4.73	<0.001 *
	Zoo → Tanzania	2.23	0.38	5.91	<0.001 *
	Namibia → Tanzania	0.74	0.42	1.75	0.194
Haptoglobin	Zoo → Namibia	-1.17	0.25	-4.73	<0.001 *
	Zoo → Tanzania	-0.92	0.29	-3.19	0.006 *
	Namibia → Tanzania	0.25	0.32	0.77	0.722
SAA	Zoo → Namibia	-5.16	1.31	-3.92	<0.001 *
	Zoo → Tanzania	-2.44	1.58	-1.55	0.275
	Namibia → Tanzania	2.72	1.78	1.54	0.279

Table 4. Concentrations of immune markers and their range in captive and wild plains zebras and mountain zebras. Values (mean ± standard deviation, min-max) include data from non-lactating and lactating zebras.

		Natural antibodies		Lysozyme		IgG		Haptoglobin		SAA	
		Hemagglutination titers		mg/L		mg/L		mg/L		mg/L	
		mean ±std dev	min-max	mean ±std dev	min-max	mean ±std dev	min-max	mean ±std dev	min-max	mean ±std dev	min-max
Plains zebras	captive N = 11	0.36 ±0.5	0-1	2.05 ±1.20	0.68-5.01	31.5 ±19.0	1.41-61.7	6.18 ±4.57	2.47-16.3	26,5506 ±249,271	0->500,000
	wild N = 22	2.95 ±1.43	1-6	4.73 ±1.58	1.92-8.00	30.59 ±7.00	18.8-45.7	1.92 ±0.71	0.64-3.41	2,595 ±4,946	0-23,476
Mountain zebras	captive N = 11	0.73 ±1.01	0-3	1.38 ±0.44	0.63-1.92	40.5 ±18.8	16.1-84.8	3.56 ±2.55	1.90-10.2	5,580 ±10,896	0-30,361
	wild N = 7	1.71 ±0.76	1-3	3.19 ±0.67	2.60-4.56	31.2 ±10.3	18.8-46.4	1.52 ±1.42	0.34-4.55	249 ±660	0-1,748

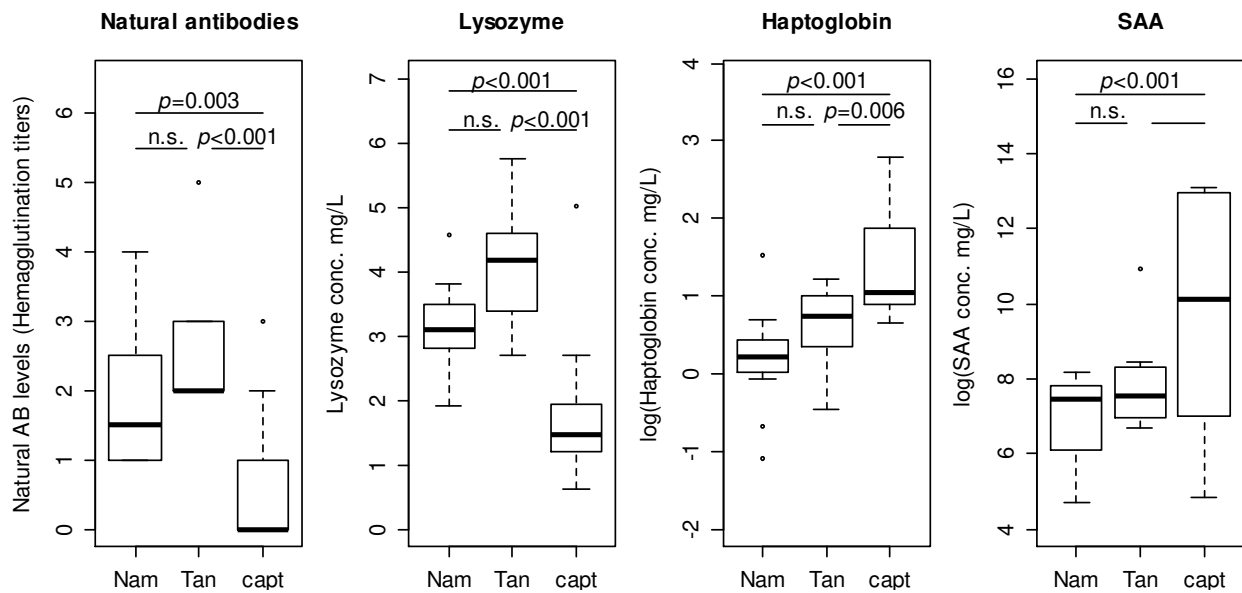


Figure 1. Effect of environment (Nam: Namibia, Tan: Tanzania; capt: *ex situ* captivity) on concentrations of the respective innate immune marker in non-lactating zebra mares. Boxes indicate 2nd and 3rd quartiles, centre lines indicate median values, upper (and lower) whiskers extend to the highest (and lowest) value that is within 1.5 times the interquartile range. Data points beyond the end of the whiskers are plotted as open dots. The p-values of the respective post-hoc tests on the complete data set are shown.

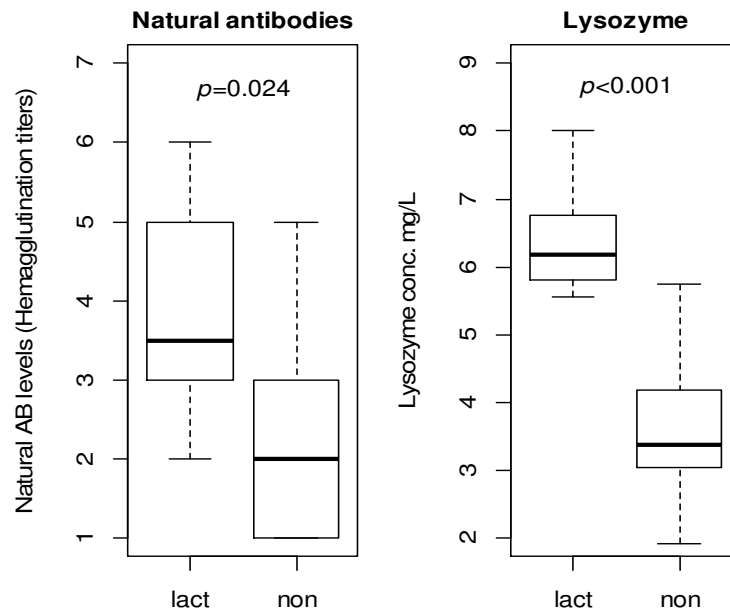


Figure 2. Effect of lactation on natural antibodies (hemagglutination titers) and lysozyme concentrations in wild zebras in their natural habitats (lact: lactating; non: non-lactating). Boxes indicate 2nd and 3rd quartiles, centre lines indicate median values, upper (and lower) whiskers extend to the highest (and lowest) value that is within 1.5 times the interquartile range. Data points beyond the end of the whiskers are plotted as open dots. The p-values of the respective post-hoc tests on the complete data set are shown.

Discussion

Both markers of the constitutive innate immunity were lower in zebra mares in *ex situ* captivity than in free-ranging mares. Various factors may be associated with changes of the immune functions of captive wildlife, including differences regarding diet, climatic conditions, social factors, infection pressure, movement space, medical treatment and prophylaxes, life span, and anthropogenic disturbance. The respective contribution of each of the multi-factorial differences between *ex situ* captivity and natural living conditions could not be identified in this study; however, infection pressure, nutrient supply, and stress are among the most prominent factors shaping immune functions (Martin et al., 2006). Disease prevention and parasite control are major management efforts in wildlife husbandry (e.g. lower intestinal parasite burden in captive than in free-ranging plains zebras; Wambwa et al. 2004), which reduces infection pressure. Furthermore, smaller population sizes and thus lower contact rates in captive than in free-ranging zebras should result in lower pathogen transmission rates, which would explain reduced investment in constitutive innate immunity.

In contrast, the serum concentrations of induced innate immune markers were higher in captive than in wild zebras (fig. 1). The energetic costs of constitutive innate immunity are considered low, whereas the two markers of the induced innate immunity that were measured are considered energetically costly and may result in immuno-pathological consequences (Lee, 2006; Rauw, 2012). Wild animals in captivity typically do not suffer from (seasonal) shortages of macronutrients, and the lack of energetic constraints would therefore not restrict the activation of the more costly immune factors (Ewenson et al., 2001). Concentrations of APPs increase rapidly during an inflammatory response, or in response to a short-term stressor (Heinrich et al., 2017). Chronic stress (due to exposure to adverse stimuli and behavioral restrictions) in captive environments puts captive animals at risk of chronic inflammation (Boyd, 1986; Mason, 2010; Schulte-Hostedde and Mastro Monaco, 2015), which may be another factor down-regulating induced innate immune functions but stimulating APP expression. Individual age is another factor that may affect immune markers such as IgG in mammals (Nagele et al., 2013). In several species, captive individuals reach a longer life span than wild conspecifics, thus age may

be a confounding factor of captivity. In this study, no data on individual age was available, however, we found no effect of captivity on IgG.

Lactation is also a modulator of innate immunity in zebras. The two constitutive immune markers (natural antibody and lysozyme concentrations) were significantly higher in lactating than in non-lactating zebra mares, whereas no difference was observed in the other immune markers. In comparison, Budzynska (2002) showed that in domestic horses immune factors such as IgG, haptoglobin, and lysozyme increase significantly during late pregnancy and lactation. The epitheliochorial placenta of equids prevents intra-uterine passage of antibodies from mother to fetus, therefore passive immunity transfer from mother to foal via the colostrum is vital (Borghesi et al., 2014; Moffett and Loke, 2006). In equids, the colostrum is produced within the last two weeks of pregnancy and contains large amounts of antibodies such as IgG and IgM, the concentrations of which decline rapidly from about 24 h post-partum (Acworth, 2010; Veronesi et al., 2014). Absorption of maternal antibodies by the foal's intestinal epithelial cells is effective exclusively within about 32 h post-partum (Acworth, 2010). As a result, it can be expected that the effect of lactation on serum antibodies is limited to a comparably narrow time window. The mares that were considered lactating in this study had foals of an estimated age of up to 6 months, which therefore likely explains the lack of an effect of lactation on serum IgG. Natural antibody concentrations were significantly higher in lactating than in non-lactating mares. It has been shown that several domestic mammals and laboratory animals are more susceptible to parasite infection during late pregnancy and lactation (Lloyd, 1983). The significantly higher natural antibody titers we found in lactating mares could reflect active immune response, rather than up-regulation of antibody expression for passive immunization of the foals. In contrast to antibodies, immune-related compounds such as lysozyme in milk can still have a protective effect during later stages of the foal's life (Veronesi et al., 2014). Thus, up-regulation of these compounds for passive immunization should be detectable longer during lactation compared to that of antibodies, which seems to be confirmed by our results.

Equid herpesviruses are endemic in equid populations throughout the world, with prevalences of over 90%, depending on the species (Ataseven et al., 2009; Barnard and

Paweska, 1993; Borchers and Frölich, 1997; Ehlers et al., 1999; Guevara et al., 2018). Contrary to our expectations, EHV status was not a significant predictor for general immune function and thus might not function as a proxy measure of general immune function in zebras. A primary herpesvirus infection would be expected to elicit increased IgG titers (Svansson et al., 2009), however, no significant effect of virus status on IgG titers was found, thus EHV-positive animals may not have been primarily infected, but instead may have undergone EHV reactivation and therefore experienced no significant activation of immunity. Consistent with previous studies such as Abdelgawad et al. (2015) significantly higher prevalence of EHV-1 and EHV-9 infection was found in wild zebras, compared to captive individuals. This result provides support for lower infection pressure in the captive environment. EHV reactivation may not trigger a strong immune reaction, and the high prevalence may indicate a long-term accommodation between virus and host that is not strongly influenced by captivity.

Taken together, our results suggest that both *ex situ* captivity and lactation affect immune functions in zebra mares. Further research is needed to identify the factors (and possible interactions between them) that cause the observed effects, and to elucidate the underlying mechanisms. A more robust understanding of environmental effects on immune function may help to ensure individual health and reproductive success of wild equids in captive environments.

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CHAPTER 5: Manuscript IV

Plains zebra (*Equus quagga*) adrenocortical activity increases during times of large aggregations in the Serengeti ecosystem

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CHAPTER 6: General Discussion

With this thesis, I aimed to examine the factors which may contribute to EHV reactivation in wild equids in their natural habitats and in *ex situ* captivity, in order to identify ecological and intrinsic factors which can be expected to increase the probability of disease transmission. For this, I established a novel non-invasive sampling method for EHV screening of wild equids in captivity which can be used on a long-term basis. Using a non-invasive sampling method for EHV screening in captive Grévy's zebras, I found that anthropogenic environmental stressors (i.e. translocation and social group re-structuring) were associated with a significant increase of both fGCM concentrations and EHV shedding. In a different study to identify conditions of lowered immunocompetence which may increase the probability of EHV reactivation, I found that *ex situ* captivity and lactation significantly affected selected immune markers in two zebra species, but did not affect EHV viraemia and shedding frequency. Lastly, in a population of free-ranging zebras in their natural habitat, the Serengeti ecosystem, I assessed several environmental and intrinsic factors which were predicted to potentially contribute to the animals' allostatic load, in order to identify the conditions under which EHV reactivation is most likely to occur. In this study I found that large aggregations of zebras were associated with a significant increase of fGCM concentrations, which may also increase the probability of reactivation of latent EHV infections.

The non-invasive method for sampling of nasal discharge (and saliva) which I established in **chapter 2** can be used to detect EHV shedding in captive wild equids for extended periods of time without the need to disturb the animals. EHV's are typically transmitted on a naso-nasal route by direct contact with nasal discharge or aerosol (apart from the sexually transmitted EHV-3 and EHV-6). Therefore the attempt of non-invasive sampling is challenging. Invasive sampling (including nasal swabs) on a regular basis is possible in domestic horses, but is considerably harder to achieve in wild equids in zoos, and typically impossible in free-ranging wild equids. The approach I tested is a promising method for collecting DNA of nasally shed pathogens and hosts in captivity. In the future, similar

approaches should be evaluated for their use on free-ranging equids in order to be able to obtain adequate sample material for EHV screens without the need for capture and restraint, as this may result in the reactivation of latent infections. As a further step towards large-scale non-invasive EHV screening of free-ranging equids, it would be of interest to explore whether the virus could be traced in faeces, given a sufficiently sensitive detection method. As acute EHV recrudescence is often associated with increased amounts of nasal discharge, it seems possible that shedding individuals might swallow considerable amounts of virus. Thus, a certain amount of virus DNA may endure the gut passage and could be traced in faeces, as is the case in experimentally infected foals of domestic horses (Patel et al., 1982). However, the viral load of infection experiments in this case substantially exceeds that of natural infections and reactivations. Furthermore, it may be possible that the strong neurotropism of EHV-1 also leads to viruses infecting neural tissues other than the olfactory nervous pathway, such as the enteric nervous system. If this is the case, reactivation may also result in a certain amount of EHV DNA shed in faeces, as virus material would be expected to be released into the intestinal content after reactivation in intestinal neurons and lytic infection of epithelial cells. It has in fact been shown that EHV DNA is traceable in faeces of experimentally infected foals of domestic horses (Patel et al., 1982). It remains to be investigated whether faecal samples of naturally infected animals may be of use for EHV screening in wild equids. PCR-based EHV detection in herbivore faecal DNA may be substantially hampered by the presumably small amount of viral DNA and exceedingly high amounts of polymerase inhibiting substances (e.g. bile salts, plant secondary metabolites, and complex sugars) which are common in herbivore faeces (Wilson, 1997).

Herpesviruses that have established a latent infection can react swiftly to an elevation of glucocorticoid concentrations (Glaser and Kiecolt-Glaser, 2005), which then induces reactivation and viral replication. Experimentally administered GCs have been shown to elicit EHV-1 and EHV-3 reactivation in domestic equids (Barrandeguy et al., 2008; Edington et al., 1985), but less is known about how physiological elevations of circulating GCs might affect reactivation of naturally infected equids. In order to assess the relationship between potential environmental stressors and EHV reactivation, I investigated in **chapter 3** how social group re-structuring and long-distance transport

might affect fGCM concentrations and the probability of EHV shedding in captive Grévy's zebras. The results indicated a significant positive association between fGCM concentrations and the probability of EHV shedding. This also indicates the sensitivity of wild equids to (anthropogenic) social perturbations which may induce a stress response and, in consequence, be immunosuppressive. Therefore, elevated stress levels due to human interference may contribute to the spreading of pathogens. This finding is particularly relevant for wild equids which are relocated in the course of restocking local populations (e.g. Franceschini et al., 2008; Kaczensky et al., 2007). Regarding management procedures such as the inherently stressful conditions of relocation, it should be considered that not only the spread of novel EHV species or strain variants, but also opportunistic superinfections with other pathogens may be a threat to population health.

In **chapter 4** I examined the effect of environmental conditions and the intrinsic factors reproductive state and acute EHV infection on selected immune markers in two zebra species. Although captivity and lactation both seemed to significantly affect some of these markers, acute EHV infection did not. The ability to persist as a latent infection, immunomodulation, and immune evasion are important traits of herpesviruses, which are the result of a long history of co-evolution with their hosts (Ma et al., 2013). The opportunistic nature of EHV recrudescence thus suggests that viral reactivation should occur during times of low immunocompetence and therefore acute infections should covary with certain immune markers. Like other herpesviruses, several EHV species have been shown to modulate both the innate and the adaptive immune system of their hosts (reviewed in Ma et al., 2013). It has been suggested that the genomes of both equid alpha- and gamma-HVs encode homologues of interleukin and G-protein receptors, which may act as chemokine receptor decoys and promote down-regulation of these factors and of MHC-1 expression (Dunowska, 2014; Hartley et al., 2013; Paillot et al., 2008). It therefore remains to be elucidated how the various factors of innate and adaptive immunity are affected during the time from reactivation of a latent EHV infection, until its return to dormancy. Furthermore, the modulation of immune functions by EHV has been associated with the facilitation of secondary infections with bacteria (Fortier et al., 2010; Marenzoni et al., 2015), e.g. *Rhodococcus equi*, which causes

pneumonia in horses. At present it is unclear whether EHV reactivation really is associated with superinfections or whether herpesvirus latency may in fact be a symbiotic relationship with immune benefits for the host, due to an up-regulation of innate immunity (Barton et al., 2007). This mechanism has been demonstrated in rodent models which showed resistance against bacterial infection in the case of latency of a murine herpesvirus. The extent to which this beneficial effect of latency might apply to wildlife and in general to animals under non-experimental conditions is unclear.

In **chapter 5** I investigated the ecological and intrinsic factors which potentially can elicit stress responses in free-ranging plains zebras, in order to identify the conditions which may affect immunity and the probability of EHV reactivation and shedding in their natural habitat. The observed pattern of higher fGCM concentrations in large zebra aggregations indicates that under these conditions an increase in the probability of EHV shedding and thus an increase in the transmission rates can be expected. Moreover, family group stallions showed higher fGCM concentrations than bachelor stallions, which may be a result of agonistic interactions or the perceived potential thereof. In contrast, lactation as an energetically demanding life history stage did not seem to affect fGCM levels. Also, the availability of only dry vegetation (and therefore lower quality diet) did not affect fGCM levels. Taken together, it seems that social stressors are more likely to affect adrenocortical activity in zebras, than energetically demanding conditions. Social interactions and changes in group structures are known to be stressors in domestic horses (Nuñez et al., 2014; York and Schulte, 2014), which also seems to be the case in plains zebras. The observed fGCM patterns may also reflect the probability of EHV reactivation. However, the degree to which the elevation in fGCM concentrations in free-ranging plains zebras in fact correlates with the probability of EHV shedding could not be determined in this study, due to the lack of an adequate non-invasive sampling method. For this purpose, the evaluation of a method to detect EHV shedding in substrates such as faeces would be a necessity. PCR-based detection in faeces of herbivores has not yet been reported. EHV probably do not occur in a substantial amount in the intestinal content and PCR amplification from faecal DNA of herbivores is often impeded by the large amount of polymerase inhibitors (Wilson, 1997).

In the context of the high contact rates in large host aggregations, the observed elevation of fGCM concentrations and the (mostly) adverse effect of stressors on the immune system (Webster Marketon and Glaser, 2008), EHV may be seen as model pathogens to study general host-pathogen interaction mechanisms. Other pathogens can be assumed to benefit similarly from potentially high transmission rates and temporarily immunocompromised hosts. Moreover, evidence suggests that after an initial increase of plasma glucocorticoids, herpesviruses themselves may in fact actively stimulate a further elevation of stress hormones in their hosts (in mice; Bonneau et al., 1993; Sapolsky, 2004). This reinforcement of a physiological stress response and the associated suppression of immune functions would thus further contribute to pathogen spreading in large aggregations of wild equids.

EHVs deserve attention due to the potentially severe symptoms they cause in domestic and wild equids. Pregnant mares are prone to abortions (and perinatal death) when they undergo an infection with an EHV species or strain variant that they have not encountered previously (Chowdhury et al., 1986; Fritsche and Borchers, 2011). Thus, although EHV in general are highly prevalent in all equid populations, the introduction of novel virus species to naïve populations by translocation of infected hosts may have severe consequences on reproduction in domestic and wild equids. Regarding translocation of equids the risk of spreading novel strains or strain variants should therefore be considered, particularly when mares in sensitive states of reproduction, neonates, or immunocompromised animals are involved.

In natural systems, EHV are pathogens with a high prevalence, moderate virulence, and opportunistic recrudescence, all of which makes them an intriguing group of pathogens for investigations on pathogen dynamics, infection biology, and disease ecology. EHV are of research interest for various reasons: besides the economic loss in the equine industry caused by EHV infections and their consequences, they are also known to infect novel hosts and this poses a serious problem for captive collections of allopatric species (Azab et al., 2018; Greenwood et al., 2012). The housing (and breeding) of individuals of threatened species which are potential novel hosts may in fact produce adverse effects on individual health when EHV infections spread e.g. by fomite transmission from equids

to other species (Abdelgawad et al., 2014). A redefinition of the host range of equid herpesviruses (and their species-specific pathogenicity) might have implications for the assembly and management of wild animals in zoos as well as for epidemiology and wildlife disease management.

This thesis sheds light on basic principles of EHV transmission dynamics, reactivation mechanisms, and related immune functions in zebras, which are likely to apply to other wild equid species as well. However, my work also emphasizes the potential and necessity for finding new methods of sampling wildlife non-invasively, in order to investigate patterns of pathogen transmission and the ecological circumstances which may facilitate the spread of pathogens. Long-term screening of EHV latency, reactivation, and transmission patterns are key for a comprehensive understanding of this pathogen's epidemiology. Therefore, novel approaches of sampling should be tested in order to evaluate their suitability for EHV detection (potentially in combination with high-sensitivity detection methods) in order to allow for large-scale EHV screening in wild and captive animals.

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ZUSAMMENFASSUNG

Die equinen Herpesviren (EHV) sind weltweit hochprävalente Pathogene aller Equiden. Die Infektionsprävalenz ist abhängig vom Virusstamm und kann bis zu 100% betragen. In der Pferdezucht-Industrie sind die EHV verantwortlich für große finanzielle Schäden, jedoch können diese Viren neben den Pferdeartigen auch neue Wirtsarten infizieren. Während Equiden eine EHV-Infektion symptomfrei überdauern können, kann eine EHV-Infektion in neuen Wirtsarten wesentlich schwerwiegender Symptome verursachen und kann tödlich enden. Herpesviren bleiben nach der Erstinfektion lebenslang als latente Infektion in ihrem Wirt, meist in Nervengewebe und lymphoiden Zellen, und können durch akuten Stress und herabgesetzte Immunkompetenz reaktiviert werden. Derzeit sind jedoch die Auslöser für Virusreaktivierung noch nicht vollständig bekannt. Nach der Reaktivierung und akuter Virämie werden die Viruspartikel meist mit dem Nasensekret ausgeschieden und übertragen, daher werden Blut- und Nasentupfer als Probenmaterial bevorzugt. Wildtiere müssen für diese invasive Beprobung jedoch meist immobilisiert werden, was nicht nur mit beträchtlichen Kosten sondern auch mit erheblichen Risiken für Tier und Mensch einhergeht. Im Gegensatz dazu wird bei non-invasiver Beprobung Analysematerial gesammelt das vom Organismus in die Umwelt abgegeben wird (z.B. Kot, Urin, Speichel oder Haare). Um non-invasiv Nasalsekret und Speichel von Zebras für EHV-Tests sammeln zu können, habe ich in **Kapitel 2** eine entsprechende Methode getestet. Diese Methode wurde an drei Zebraarten in Gefangenschaft erprobt, und es konnte erfolgreich EHV- und Zebra-DNA isoliert werden. Eine ähnliche, non-invasive Methode wurde von mir in **Kapitel 3** verwendet um Zebras in Gefangenschaft nach dem Auftreten von umweltbedingten Stressfaktoren (Umsiedelung und soziale Restrukturierung) auf EHV-Ausscheidung und Virusübertragung zu testen und dies mit den Konzentrationen von Glucocorticoidmetaboliten aus Kotproben (fGCM) zu korrelieren. Mit dieser Studie konnte ich zeigen dass sowohl fGCM-Konzentrationen als auch die Wahrscheinlichkeit der Virusreaktivierung und Übertragung unmittelbar nach dem Auftreten der Stressfaktoren signifikant anstiegen. Meine Ergebnisse weist darauf hin dass soziale Faktoren bei wilden Equiden das neuroendokrine System erheblich beeinflussen, was in weiterer Folge zu einer gesteigerten Wahrscheinlichkeit der Virusausscheidung und Übertragung führt.

Die Haltung von Wildtieren in zoologischen Gärten weicht in vielen wesentlichen Punkten von den natürlichen Lebensbedingungen ab (z.B. Unterschiede hinsichtlich Klima, Ernährung, Bewegungsfreiheit, Sozialstruktur, und veterinärmedizinischer Prophylaxe und Versorgung). In **Kapitel 4** habe ich untersucht ob sich die Immunfunktionen zweier Zebraarten zwischen Tieren in Gefangenschaft und freilebenden Artgenossen unterscheiden, und ob akute EHV-Infektionen die Konzentrationen der untersuchten Immunmarker beeinflusst. Des Weiteren habe ich den Effekt von Laktation auf ebendiese Immunmarker untersucht, da sowohl herabgesetzte aktive Immunabwehr wie auch gesteigerte Immunkörperbildung für die passive Immunisierung säugender Jungtiere bei anderen Tierarten während der Laktation beschrieben wurden. Immunmarker der konstitutiven angeborene Immunabwehr bei Tieren in ihrem natürlichen Lebensraum waren signifikant höher waren als bei Artgenossen in Gefangenschaft, während Marker der induzierten angeborenen Immunabwehr bei Zootieren signifikant höher waren als bei wildlebenden Tieren. Die Ursachen dieser Änderung der Immunfunktionen sind derzeit nicht bekannt. Laktierende Zebrastuten zeigten signifikant höhere Werte der konstitutiven angeborenen Immunabwehr, was vermutlich in der passiven Immunisierung ihrer Fohlen sowie in gesteigerten aktiven Immunfunktionen begründet ist. Keiner der gemessenen Immunmarker wurde durch akute EHV-Infektion beeinflusst. In **Kapitel 5** habe ich ermittelt, welche physiologischen und (sozio-)ökologischen Faktoren in freilebenden Steppenzebras im Serengeti-Ökosystem einen Anstieg der fGCM-Konzentrationen verursachen würden, und somit eine EHV-Reaktivierung und Ausscheidung begünstigen können. Während weder Laktation noch Trockenzeit einen Anstieg der Glucocorticoide auszulösen schienen, wurde festgestellt dass fGCM-Konzentrationen signifikant positiv mit der Herdengröße korrelierten. Des Weiteren hatten Hengste in Familiengruppen deutlich höhere fGCM-Werte als Hengste in reinen Hengstgruppen. Dies weist darauf hin dass EHV-Reaktivierung und Übertragung in großen Herden am wahrscheinlichsten ist, und häufiger in Haremshengsten als in Junggesellenhengsten auftreten sollte. Zusammengefasst zeigen meine Ergebnisse dass soziale Faktoren bei Zebras als Stressoren fungieren können und somit zu einer erhöhten Wahrscheinlichkeit der Reaktivierung und Verbreitung latenter Infektionen führen. In Zukunft sollten weitere Methoden für non-invasive EHV-Tests validiert werden, um großflächige Untersuchungen an wilden Equiden in ihren natürlichen Habitat zu

ermöglichen, und damit Faktoren identifizieren zu können die zur Übertragung latenter Infektionen beitragen.

SUMMARY

The equid herpesviruses (EHV) are highly prevalent pathogens in all global equid populations, with infection prevalence rates of up to 100%. They are responsible for considerable economic losses in the equine industry, and can also readily adapt to infecting new (non-equid) host species. In these novel hosts, EHV infections are typically more severe than in equids and often fatal. Herpesviruses typically remain in their hosts as a latent, lifelong infection in the host's neural tissue and lymphoid cells and can reactivate during times of acute stress or reduced immunocompetence. So far, the triggers causing EHV reactivation in equids are not comprehensively understood. Following viral recrudescence and viraemia, EHV are typically shed in nasal discharge and transmitted via a naso-nasal infection route. Therefore, EHV monitoring in wildlife has mostly been performed employing invasive methods, including capture and immobilization. These approaches, however, involve various risks for the target animal and for the people involved in the procedures. Furthermore, due to intense time and financial effort, invasive measures are usually limited to very few individuals. In contrast, non-invasive sampling relies on collecting sample material which is shed from the target organism into its environment, thereby circumventing the need for direct contact with the animal. In **chapter 2** I established a non-invasive, indirect sampling method to collect nasal discharge and saliva of captive zebras. This method was tested on three different zebra species, and I successfully isolated DNA of sporadically reactivated EHV as well as host DNA from an enrichment toy which was provided to the study animals for certain periods of time. A similar approach might be tested on wild equids in their natural habitats to facilitate non-invasive EHV screening *in situ* with a time- and cost-efficient approach.

In **chapter 3** I used non-invasive sampling methods to screen EHV shedding in captive zebras in order to investigate the effect of presumed environmental stressors on the probability of EHV reactivation and transmission. In addition, I measured faecal glucocorticoid metabolite (fGCM) concentrations to assess a potential physiological stress response to a translocation event and subsequent social group re-structuring. Both fGCM concentrations and EHV shedding frequencies increased significantly after the translocation event and group re-structuring, compared to control periods, in the

translocated and in the non-translocated animals. This indicates that environmental stressors, including potential social conflict, may play a crucial role for reactivation and transmission of EHV infections in wild equids.

In **chapter 4** I examined whether *ex situ* captivity and its inherent deviations from natural living conditions of zebras (e.g. differences in climate, diet, movement space, social structure, and infection pressure) and acute EHV infections would have had an effect on selected markers of the innate and adaptive immune system. Moreover, I also examined the effect of lactation on these markers, as it is known from other species that energy re-allocation may lead to a down-regulation of certain immune functions, while other immune markers are up-regulated for passive immunisation of nursing offspring. I found that constitutive innate markers were significantly higher in the natural, than in the captive environment, whereas induced immune markers were significantly higher in the wild. Lactating zebras also showed significantly higher constitutive immune functions than non-lactating mares, which may be a result of passive immunization of the offspring, but also of increased activity of this branch of immunity. Acute EHV infections, however, did not affect the measured immune markers.

In **chapter 5** I aimed to identify the factors which contribute to an increase of the allostatic load of free-ranging plains zebras in the Serengeti ecosystem. For this, I measured fGCM concentrations of zebras at different life history stages and under different (socio-)ecological conditions. I found significantly higher fGCM levels in zebras during times of large aggregations, compared to medium-sized and small aggregations. Furthermore, family group stallions produced higher fGCM concentrations than bachelors. Lactation and season, however, had no effect on fGCM concentrations. This indicates that EHV recrudescence is most likely when zebras are in large aggregations, and is presumably more frequent in family group stallions than in bachelors.

Taken together, my results indicate that social cues seem to be important factors contributing to perceived stress, and thereby, to the probability of EHV reactivation, in zebras. Non-invasive methods for VH screening should be evaluated in the future to facilitate large-scale screening of free-ranging equid populations, in order to identify the factors which promote reactivation and transmission of latent infections such as EHV.

List of publications and author contributions

- I. **P.A. Seeber**, S.E. Soilemetzidou, M.L. East, C. Walzer, A.D. Greenwood (2017). Equine behavioral enrichment toys as tools for non-invasive recovery of viral and host DNA. *Zoo Biology* 36:341–344. DOI: 10.1002/zoo.21380.

PAS, SES, CW, and ADG designed the study, PAS and SES collected samples, performed DNA extractions, and PCRs, and PAS performed vector cloning, PAS drafted the manuscript, and SES, MLE, CW, and ADG commented on and added to the manuscript.

- II. **P.A. Seeber**, B. Quintard, F. Sicks F, M. Dehnhard, A.D. Greenwood, M. Franz (2018). Environmental stressors may cause equine herpesvirus reactivation in captive Grévy's zebras (*Equus grevyi*). *PeerJ*. DOI: 10.7717/peerj.5422

PAS and MF conceived the study, PAS collected the samples and performed molecular genetic laboratory experiments, MD supervised the endocrine laboratory analyses, PAS and MF performed statistical analyses, PAS drafted the manuscript and MF, BQ, FS, MD, and ADG commented on the manuscript.

- III. **P.A. Seeber**, M. Franz, T. Morrison, A. Ortega, A.D. Greenwood, G.Á. Czirják. Innate immunity in zebras is influenced by captivity and lactation. *Manuscript in preparation*.

PAS and GAC conceived the study, PAS compiled the sample material, GAC supervised laboratory analyses, PAS and MF performed statistical analyses, PAS wrote the manuscript and GAC, MF, TM, AO, and ADG commented on and added to the manuscript.

- IV. **P.A. Seeber**, M. Franz, M. Dehnhard, A. Ganswindt, A.D. Greenwood, M.L. East (2018): Plains zebra (*Equus quagga*) adrenocortical activity increases during times of large aggregations in the Serengeti ecosystem. *Hormones and Behavior* 102: 1-9. DOI: 10.1016/j.yhbeh.2018.04.005.

ADG, MLE, and PAS conceived the study, PAS collected and processed samples, MD supervised laboratory analyses, PAS and MF performed statistical analyses, PAS drafted the manuscript and MF, MD, AG, ADG, and MLE commented on and added to the manuscript.

Curriculum Vitae

Der Lebenslauf ist in der Online-Version aus Gründen des Datenschutzes nicht enthalten.

List of peer-reviewed publications

1. Aurich C, **Seeber PA**, Müller-Schlösser F (2007): Comparison of different extenders with defined protein composition for storage of stallion spermatozoa at 5 degrees C. *Reproduction in Domestic Animals* 42 (4).
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5. **Seeber PA**, Soilemetzidou SE, East ML, Walzer C, Greenwood, AD (2017): Equine behavioral enrichment toys as tools for non-invasive recovery of viral and host DNA. *Zoo Biology* 36.
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7. Costantini D, **Seeber PA**, Soilemetzidou S-E, Azab W, Bohner J, Buuveibaatar B, Cziráj GÁ, East ML, Greunz EM, Kaczensky P, Lamglait B, Melzheimer J, Uiseb K, Ortega A, Osterrieder N, Sandgreen DM, Simon M, Walzer C, Greenwood, AD (2018): Physiological costs of infection: herpesvirus replication is linked to blood oxidative stress in equids. *Scientific Reports* 8: 10347.
8. **Seeber PA**, Quintard B, Sicks F, Dehnhard M, Greenwood AD, Franz M (2018): Environmental stressors may cause equine herpesvirus reactivation in captive Grévy's zebras (*Equus grevyi*). *PeerJ* 6: e5422.

Selbständigkeitserklärung

Hiermit erkläre ich, dass ich die vorliegende Doktorarbeit selbständig und ohne unzulässige Hilfe oder Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe.

I hereby confirm that I have made this work autonomously. I assure that I have read and only used the specified sources claimed in this work.

Berlin, September 18 2018

Peter A. Seeber