FACULTY OF VETERINARY MEDICINE
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IRON OVERLOAD DISORDER IN CAPTIVE BLACK RHINOCEROS: COMPARING IRON EXCRETION WITH OTHER AFRICAN HERBIVORES

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CORONA DISCLAIMER

Due to the coronavirus pandemic, many difficulties presented during the course of this master dissertation. The objective of this thesis has changed over time due to the reasons mentioned above. Our initial objective was to compare free roaming black rhinoceroses and their counterparts under human care in terms of mineral homeostasis. Dietary and fecal samples were planned to be collected from Diergaarde Blijdorp animal facility in Rotterdam, Netherlands and OI Pejeta Conservancy in Nanyuki, Kenya whereafter mineral analysis on these samples was planned to be performed during academic year 2020-2021. The collected data would allow a comparative study between animals under human care and the free roaming animals to gain more insight on any difference between these groups that may attribute to the development of iron overload disorder under human care. However, travel restrictions limited the possibility to collect samples from wild animals abroad and because of practical reasons, cooperation with many animal facilities was not successful. This implies that the process of collecting samples was opportunistic and was prone to involuntary change. Black rhinoceros feces and diet samples could not be collected due to the reasons mentioned above and collecting bile samples failed because we could not witness any necropsies on deceased free roaming or captive animals. After many difficulties in obtaining black rhinoceros samples, we decided to change our path and rather focus on the mineral homeostasis of African herbivores in general. By great efforts of Prof. Dr. Ir. Geert Janssens, we were able to obtain remnants of dried and milled feces samples of previously conducted studies by Prof. Dr. Marcus Clauss. In addition, after many efforts zoological institutes Planckendael and Pairi Daiza were able to grant us samples of a number of their animals.

The literature study of the original objective mainly focused on iron overload disorder in the black rhinoceros. However, as a result of many uncertainties and changes, the course of this dissertation changed greatly and additional literature study was needed to correlate with the new objective. This has been performed up until a certain point, but the greatest part of the literature study remains the same due to a lack of time to restart the entire project.

PREFACE

Due to the coronavirus pandemic, the course of this dissertation changed enormously. Together with Laura, we had the dream of doing research on wildlife in Africa and get a taste of the feeling of working in a zoo. As a result of the pandemic, our visit to Kenya and Diergaarde Blijdorp got cancelled, which left us with a disappointed feeling. Nevertheless, the past year has been an incredible journey for me. My supervisor Prof. Dr. Janssens has been a kind and helpful guide throughout the entire process. I would like to thank him and Dr. Ir. An Cools for granting me the wonderful opportunity to work on such interesting matters and challenging me to explore many wonderful aspects of research. I also would like to thank Dr. Donna Vanhauteghem, who patiently and kindly helped me through difficult parts of this dissertation. My parents, my brother Armin and my wonderful partner Laura supported me through the entire process and I am genuinely grateful. Lastly, I would like to mention my beloved cousin Alireza, who passed away too soon, on the age of 24. In this way, I hope to eternalize his name in my work and thank him for everything he has done for me.

I hope that the great things that I have learned during my academic education and during the course of my master dissertation at the University of Ghent, will help me to reach my goals as a good veterinarian in the future.

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ABREVIATIONS

%Sol. Fe: percentage acid-soluble iron
AIF Fe: acid-insoluble fraction of Fe
ART: assisted reproduction techniques
ASF Fe: acid-soluble fraction of Fe
BMP6: bone morphogenic protein 6
Ca: calcium
Cu: copper
DCT1: divalent cation transporter
Dcytb: duodenal cytochrome b
DMT1: divalent metal transporter 1
et al.: et alii *(Latin: "and others")*
Fe: iron
Fe^{3+}/Fe^{2+}: ferric iron, ferrous iron
Fpn1: ferroportin 1
GIT: gastrointestinal tract
Hb: hemoglobin
HJV: hemojuvelin
i.e.: example given
ICP-OES: inductively coupled plasma - optical emission spectrometry
IOD: iron overload disorder
IUCN: The International Union for Conservation of Nature
K: potassium
kg, g, mg, ng, µg: kilogram, gram, milligram, nanogram, microgram
l, dl, ml: liter, deciliter, milliliter
m: meter
MC: mixing chamber
MCV: mean-cell-volume
Mg: magnesium
Mn: manganese
MRT: mean retention time
MRT_{fluid}: fluid mean retention time
MRT_{MC}: mean retention time mixing chamber
$MRT_{particle}$: particle mean retention time
Na: sodium
NTBI: non-transferrin bound iron
NWR: Northern white rhinoceros
PCV: packed-cell-volume
RBC: red blood cell
SWR: Southern white rhinoceros
TF Fe: total fraction of Fe
Tf, TfR1, TfR2: transferrin, transferrin receptor 1 and 2
UHC: under human care
VFA: volatile fatty acids
vs.: versus
Zn: zinc

1. Summary (English and Dutch)

Animals of various species have been kept under human care for a long period of time, mainly for conservation purposes. Although conservation of these animals is well-intended, the insufficient knowledge on animals' physiology and dietary preferences has led to the occurrence of captivity-induced disorders, one of which is iron overload disorder (IOD). When the browsing black rhinoceros (*Diceros bicornis*) is placed under human care, these animals often develop IOD, which involves the storage of excessive iron in various organs and tissues throughout the body. This disorder has been connected with various diseases that occur in this animal species when kept under human care and a connection with mortality is even speculated. Until now, the etiology of IOD has not been determined but it is believed that the difference between the wild diet of these browsing animals and the diet presented under human care could play an important role in the development of this disorder. The grazing white rhinoceros and the free roaming black rhinoceros are insusceptible to this disorder. The fact that browsing species under human care seem to be distinctively more susceptible raises the question whether evolutionary adaptations of browsing animal species in terms of mineral homeostasis could also be of great importance in the pathogenesis of this disorder, in addition to dietary differences between captivity versus the wild. The results of this study revealed distinctive differences between grazing and browsing animal species in fecal mineral concentrations as well as significant differences between fecal mineral profiles of wild animals as opposed to the ones under human care. Based on the results, it is hypothesized that mineral imbalances in diets under human care, combined with specific adaptations of browsing animals, may play an important part in the pathogenesis of IOD. The role of bile salt metabolism as a specific adaptation in the black rhinoceros is therefore potentially of great importance and further research on this matter is warranted.

(Dutch) Verscheidene diersoorten zijn gedurende een lange periode onder humane zorgen gehouden met als hoofdzakelijk doel het in stand houden van de diersoorten in kwestie. Hoewel het beschermen van deze diersoorten met goede intenties gebeurt, heeft een gebrekkige kennis over fysiologie en diëtaire behoeften van de dieren geleid tot gevangenschap-geïnduceerde aandoeningen, waaronder ijzerstapelingsziekte. Wanneer de bladetende zwarte neushoorn (*Diceros bicornis*) in gevangenschap wordt gehouden, ontwikkelen deze dieren frequent ijzerstapelingsziekte. Dit is een aandoening waarbij overmatige hoeveelheden ijzer gestapeld worden in allerlei organen en weefsels in het lichaam. Deze aandoening is vermoedelijk verbonden met een hele reeks aandoeningen en ziektes die voorkomen bij het houden van deze dieren gevangenschap. Een mogelijke link met mortaliteit wordt niet uitgesloten. Tot op heden is de etiologie van deze aandoening niet gekend maar men vermoedt dat een verschil tussen het dieet in het wild en het dieet in gevangenschap een grote rol zou kunnen spelen in het ontstaan van deze aandoening. De grazende witte neushoorn en zwarte neushoorns in het wild worden niet als gevoelig beschouwd voor ijzerstapelingsziekte. Het feit dat bladetende diersoorten in gevangenschap een veel hogere vatbaarheid kennen voor deze aandoening doet de vraag rijzen of een evolutionaire aanpassing van bladetende diersoorten inzake mineraalhuishouding ook geen significante rol zou kunnen spelen in de pathogenese van deze aandoening. De resultaten van deze studie presenteerden duidelijke verschillen inzake fecale mineralenprofielen tussen grazers en bladeters en daarnaast ook tussen wilde dieren en deze die in gevangenschap worden gehouden. Gebaseerd op deze verschillen wordt geponeerd dat een mineralendisbalans in diëten in gevangenschap, gecombineerd met specifieke aanpassingen van bladetende diersoorten aan hun voeding, een belangrijke rol kunnen spelen in de pathogenese van ijzerstapelingsziekte. De rol van het galzoutenmetabolisme is hierbij mogelijks van groot belang en verder onderzoek hieromtrent is vereist.

2. Introductory literature study

2.1 Conservation of threatened wildlife species

Our planets' enormous variety of life is currently heavily threatened. Although climate change has been a thoroughly discussed subject in recent years, it is currently believed that the loss of biodiversity has similar or even worse consequences for all life on earth (Tollefson, 2019). Human actions have led to a disastrous decrease in wildlife population numbers and led many animal species to the brink of extinction (fig. 1). The most important reasons for this loss are habitat loss and fragmentation, poaching, introduction of invasive species and pollution (Hohenlohe and Rajora, 2021). These actions have altered the environment in which animals live and reduced both quantity and quality of their feed and thus led to a great impact on their health, reproduction and survival (Birnie-Gauvin et al., 2017). Therefore, immediate conservation measures must be taken in order to preserve the enormous variety of animal species on earth.

Figure 1: The International Union for Conservation of Nature (IUCN) assesses the risk for extinction of various animal species on our planet. [1]

Preserving animal species in their natural environments with limited human influence has been defined as in situ conservation. Conversely, when animal species are placed under human care in i.e. zoos and sanctuaries and breeding of species generally occurs under human control, this is referred to as ex situ conservation (Birnie-Gauvin et al., 2017; Lueders and Allen, 2020). In situ conservation logically has been the preferred way of animal species preservation. However, due to the earlier mentioned reasons, animal population numbers are declining, and the remaining small populations often become fragmentated over smaller habitats. This eventually results in inbreeding and genetic homogenization and thus an increased vulnerability of the remaining animals (Shivaji et al., 2003). Genetic diversity is of critical value due to the significant negative consequences of inbreeding which include reduced longevity, organ malformations and morphological deformities among negative effects (Ballou et al., 2010). To maintain genetic heterozygosity and allow in situ conservation, habitat preservation is necessary and a decrease in population numbers should be limited. Unfortunately, latter measures have generally appeared to be unsuccessful and habitat loss and rising extinction rates seem to continue (Birnie-Gauvin et al., 2017). In addition, these measures require human involvement and present the question whether the natural environment of these animals is still considered to be truly wild (Lueders and Allen, 2020). These findings have led to the conclusion that although in situ conservation remains the main objective, current strategies appear to be too unsuccessful to be accepted as the sole conservation strategy and confirm the need of ex situ conservation as well.

[1] https://www.iucnredlist.org/ (last visited on 12/05/2021)

Animals are kept under human care in sanctuaries, zoological gardens, aquaria and even in facilities of private breeders (Birnie-Gauvin et al., 2017). These facilities have the aim of conserving animal species in common and one of the most effective ways to do this is via controlled breeding programs. Zoological gardens have developed a system where animals of each species found in zoos are considered to be of one population. Data on animals under human care are kept in studbooks, which contain a compilation of vital information on each individual's sex, parentage, birth and death dates and many other forms of information (Ballou et al., 2010). By using mathematical models and the collected data, controlled breeding programs can be developed which take place in all the participating zoos to increase the population numbers of animal species and maintaining the genetic diversity of each population. This implies that the population of a species under human care can also be beneficial for increasing the genetic heterozygosity in the wild via reintroduction of animals or assisted reproduction techniques (Balog et al., 2015; Lueders and Allen, 2020). Latter emphasizes the need for a general approach of conservation, because when population numbers decrease, each individuals' genetic information becomes valuable for maintaining genetic diversity and preventing inbreeding. The collaboration between in situ and ex situ conservation is currently believed to be of critical value and is referred to as the 'One Plan Approach' (Lueders and Allen, 2020).

As mentioned above, ex situ conservation is an essential strategy in preserving animal diversity on our planet. One of the reasons for this are the controlled breeding programs where to utilize assisted reproduction techniques (ART) such as embryo transfer, in vitro fertilization and artificial insemination, sufficient knowledge on animal physiology and morphology is needed. Such information is more easily gathered when animals are kept under human care and thus can be observed continuously and sample collection is possible (Lueders and Allen, 2020). However, transferring wild animals from their natural habitat to an artificial environment which differs significantly from the wild induces many challenges. Keeping animals under human care involves high costs, the need for great amounts of space and resources (Lueders and Allen, 2020). In addition, management of populations under human care is costly, demands great amounts of time in order to organize the breeding of animals of the same or different facilities and some breeding programs lack sufficient genetic diversity to meet the ultimate goal of increasing genetic heterozygosity (Ballou et al., 2010). As a result of these hurdles, animal health, welfare and reproduction may be impaired when kept under human care (Shivaji et al., 2003).

One major other concern is the lack of knowledge on animal physiology and nutrition. This information is not only essential for breeding purposes, but also for general husbandry and in order to provide a diet that will meet the nutritional needs of animals under human care (Dierenfeld, 1997). An adequate diet is essential to maintain general health of animals, prevent the occurrence of diseases and to improve growth and reproduction (Birnie-Gauvin et al., 2017; Dierenfeld, 1997). However, the search for an adequate dietary regime has been mostly focused on providing the correct quantity and thus meeting the calorific needs of the animal and less on the quality of the diet. The macro and micronutrients in the diet, such as amino acids, minerals, proteins and vitamins are essential for many functions of the body. Examples are the important role of vitamin C in iron absorption (Fuqua et al., 2012) and the antioxidative capacities of vitamin E and selenium (Sullivan, 2016). The inability of providing a diet under human care that is equivalent to the wild diet or composing a novel diet which at least meets the nutritional needs may lead to health impairment and even death (Birnie-Gauvin et al., 2017). Dietary problems are currently seen in many wildlife species under human care, among which the pangolin species (Lin et al., 2015) and the black rhinoceros.

The black rhinoceros suffers from iron overload disorder (IOD), which means that there is excessive iron storage in various tissues in the body (Paglia and Dennis, 1999). This disorder is believed to relate to many other disorders that this rhinoceros species is facing under human care which cause high

morbidity and even mortality. The high prevalence of IOD and the general finding that this disorder only is presented when these animals are kept under human care presents the question whether ex situ conservation is justified, even when the intentions mainly involve the preservation of this endangered species (Paglia and Tsu, 2012). Nevertheless, it can be concluded that in situ and ex situ conservation and especially the 'One Plan Approach' are well-intended measures that currently are the sole answer to the drastic decline in animal diversity on our planet. In addition to these conservation efforts, further research is needed to understand each animal species' to keep them in good health under human care and to guarantee reproduction successes.

2.2 Rhinoceros

2.2.1 The Rhinocerotidae family

Rhinoceros are part of the order Perissodactyla or odd-toed ungulates, a name deriving from the fact that the animals of this order bear their weight on the third toe. The Perissodactyla order contains three families, namely the Equidae, Tapiridae and Rhinocerotidae family. The Rhinocerotidae family consists of five species divided over four genera. These five species can be found in different geographical regions, namely two species in the African continent and three species in Asia.

2.2.1.1 The African rhinoceros species

The white rhinoceros (*Cerathoterium simum*) of the genus Cerathoterium is a grazer that mostly inhabits grasslands in bushveld savannas (Emslie, 2020a). This species is one of the largest mammal herbivores that roams the earth (Steuer et al., 2010). The white rhinoceros possibly gained its name through a mistranslation of the Dutch word 'wijd' which stands for wide in English. The word 'wijd' would refer to the width of the mouth of the white rhinoceros. However, this theory is currently assumed to be incorrect because of the occurrence of the name 'white rhino' in documents that were written before the infamous mistranslation took place. There are many theories about how this species derived its name, among which the idea that wallowing in calcareous soil of this animal led to the finding that early tissue samples of this species appeared to be white thus leading to their current name (Owen-Smith, 1988). Currently white rhinoceros is the most common naming for the *Ceratotherium simum*, followed by the lesser-used but more accurate square-lipped rhinoceros (Rookmaaker, 2003).

The black rhinoceroses (*Diceros bicornis*) from the genus Diceros is the other rhinoceros species that roams in Africa. Currently, three subspecies of the black rhinoceros are extant, namely *D. bicornis michaeli*, *D. bicornis minor* and *D. bicornis bicornis* (Emslie, 2020b). This species has gained its name as a distinction to the white rhinoceros accompanied by the finding that their skin will color black after wallowing in the mud. Black rhinoceros have a more accurate name as well, namely hook-lipped rhinoceros which refers to the prehensile upper-lip of this species that is used for their specialized diet[2]. Habitats of the black rhinoceros include open plains, savannas and dry forests. These animals are strict browsers and feed in these habitats on browse, which includes twigs, leaves and shrubs of trees[3].

[2] https://rhinos.org/about-rhinos/rhino-species/black-rhino/ (last consulted in 14/05/2021)
[3] https://www.britannica.com/animal/black-rhinoceros (last consulted in 14/05/2021)

2.2.1.2 The Asian rhinoceros species

The Javan rhinoceros (*Rhinoceros sondaicus*) from the genus Rhinoceros is mostly found in lowland tropical rainforest areas. This species' population numbers have decreased dramatically and the remaining animals can be found as a single population within one national park, being the Ujung Kulon NP in Indonesia (Ellis, 2020). The Javan rhinoceros has a diet that mostly exists of browse material and is therefore considered a browser (Owen-Smith, 1988; Sullivan and Valdes, 2019).

The Sumatran rhinoceros (*Dicerorhinus sumatrensis*) from the genus Dicerorhinus is also considered a co-browser and inhabits hilly areas of Indonesia. The habitat of this species mostly exists of tropical rainforests and montane moss forests. The Sumatran and Javan rhinoceros may be the most threatened large mammals on earth. Population numbers of the Sumatran rhinoceros have decreased dramatically and continue to decrease even further (Ellis and Talukadar, 2020).

The Indian (*Rhinoceros unicornis*), also from the genus Rhinoceros is also referred to as the greater one-horned rhinoceros. The current populations can be found in India and Nepal and are increasing as a result of intensive protection and habitat management. As an intermediate feeder with a focus on grasses, this species prefers plain grasslands, but is also found in swamps and forests where it feeds on browse and fruits. The decrease in population numbers of the Indian rhinoceros, cannot be explained solely by poaching. Other threats facing this species are the increase of alien plants in their grasslands and habitat occupation by humans (Ellis and Talukadar, 2019).

2.2.2 A species under threat

All five rhinoceros species are threatened and are at risk of extinction (fig. 2), mostly due to human actions. The biggest threat to all rhinoceros species is illegal hunting or poaching. Rhino horn has been used for millennia in Chinese traditional medicine for a wide range of health issues, as a form of decoration and for ceremonial daggers in the Middle East including Yemen (Biggs et al., 2013). Rhino horn is made of keratin, which is the same material that can be found in fingernails and hair and therefore makes its use or consumption not beneficial of any kind for human health. The extensive hunting of these animals for reasons mentioned above has led to a drastic decrease of rhino populations in the past decades. The black rhinoceros population was estimated to be 100,000 at the end of 1960 and dropped to less than 5000 today (Emslie, 2020b). The significant increase of rhino poaching is mainly driven by the increasing retail price of rhino horn which is estimated to be $65.000 per kilogram which makes rhino horn more valuable than gold and diamonds (Biggs et al., 2013). The dehorning strategy as a measure to reduce poaching has appeared unsuccessful because even the stubs of horn that remain on the animal are worth great amounts of money which still allures poachers. Hence, many conversationalists advance the idea of legalizing the rhino trade in order to stop illegal killing of these animals and to regain control (Biggs et al., 2013).

One other major threat to the rhinoceros species is habitat destruction for human purposes such as feeding livestock, agriculture and road construction. As a result, the already small populations get fragmentated and become even smaller hence resulting in a decrease of natural breeding and an increased risk for inbreeding depression (Ellis and Talukadar, 2020). As a result of the high poaching risk and the low population numbers, many remaining rhinoceros are transferred to fenced sanctuaries, conservation areas and private reserves where it is easier to enforce security measures and protect the animals (Emslie, 2020b). Currently there are more white rhinoceros on private land in South Africa than in all the other lands of the African continent (Emslie, 2020a). These measures have led to an increase in white rhinoceros and black rhinoceros populations in recent years. However, the

increasing interest in rhino horn and poaching has led to significantly increased security costs and limited income for conservation purposes. Therefore, currently there is less interest in rhinoceros conservation, especially for private land owners (Emslie, 2020a).

Figure 2: five rhinoceros species presented with their current estimated population numbers of mature individuals and the status received from IUCN in relation to their risk for extinction. Adapted figure from https://rhinos.org/about-rhinos/state-of-the-rhino/ with data gathered from IUCN website.[4]

Decreasing population numbers create great challenges for species preservation, as can be seen for the Northern white rhinoceros. The white rhinoceros can be divided into two subspecies, namely the northern white rhinoceros (NWR) and the southern white rhinoceros (SWR). Currently, the NWR is presumed to be extinct. In 2014, the last four potential breeding NWR were transferred from a zoo in Czech Republic to OI Pejeta Conservancy in Kenya, which is a private conservation area with the aim of increasing the breeding of these animals[5] (Emslie, 2020a). Since their arrival, two of these rhinoceros have passed away and there is little hope that the remaining two individuals would form a viable population in the long term. This can be attributed to low population size and the negative effects of inbreeding and the possibility of significant reduction of this slow growing population due to chance demographic events (Ballou et al., 2010). To create a viable population of this species, advanced reproduction techniques are needed that allow a rapid growth of rhinoceros population and enhance genetic diversity (Emslie, 2020a). To conclude, it is certain that joint efforts are needed to stop illegal rhino poaching and the trade in rhino horn with simultaneous efforts to increase their population numbers again without loss of genetic heterozygosity.

[4] Last consulted on 14/05/2021

[5] https://www.olpejetaconservancy.org/wildlife/rhinos/northern-white-rhinos/ (last consulted on 14/05/2021)

2.3 Browser versus grazer dichotomy

2.3.1 Defining grazer and browser

Dietary choices of animals have determined and shaped their physiology, anatomy and even some behavioral characteristics throughout evolution. Simultaneously, selection of food based on the specific characteristics of a particular food source relies on the animals' physiology and anatomy (Codron et al., 2019; Shipley, 1999). Dietary composition can thus be essential in defining an animal in many ways. A great example is the difference between carnivores and herbivores where carnivores have optimized characteristics of hunting prey, fighting and fleeing and spend less time consuming their diet. Conversely, herbivores spend great amounts of time consuming diets that are relatively lower in energy density due to their diet often being plentiful and stationary (Codron et al., 2019; Shipley, 1999).

An important reason for developing dietary preferences and morphological adaptations is resource partitioning. Animal species often have the need for the same habitat and dietary components. Because these resources are not unlimited, they have developed ways of coexisting without the need for competition. The latter can be explained by conducted experiments and mathematical models that indicate that if the same resources are used in exactly the same manner, superior species will always win the competition. Hence, animal species tend to specialize in different dietary elements and select certain habitats for living and obtaining water in order to avoid competition while meeting their needs (Griffin and Silliman, 2012).

Mammalian herbivores have been classified in different taxonomic groups based on their focus on either grasses or browse (Shipley, 1999; Steuer et al., 2010). Hoffman and Stewart, (1972) defined the term grazers as animals selecting a diet containing <25% browse, browsers as animals with diets containing >75% fruits, tree and shrub stems, dicot foliage and foliage, and intermediate or mixed feeders that have a diet consisting of both grasses and browse. The percentages that Hoffman and Stewart described have been matter for debate and have been altered throughout the years, with no strict percentages defined as completely correct (Codron et al., 2019). Defining browsers, grazers and intermediate feeders as such, is a categorical approach of distinction. Another way of defining dietary preferences is the continuous classification of diets where there are no strict boundaries between categories and animal species' dietary preferences are expressed by the average percentage of grass in the natural diet (Codron et al., 2019).

2.3.2 Differences between grass and browse

Dietary preferences have led to specific morphological and physiological adaptations which can be devoted to differences between herbaceous forages defined as grasses and browse.

Grasses have a thick cell wall that mainly consists of plant fibers that are slowly digestible such as cellulose which makes grasses more difficult to bite and chew (Shipley, 1999; Steuer et al., 2010). Browse, which is defined as herbs, forbs, leaves and some woody stems, have cell contents that exist of rapidly fermentable and completely digestible compounds such as sugars, lipids and proteins (Clauss et al., 2008; Hofmann and Stewart, 1972). They have a thinner cell wall with more indigestible fibers such as lignin leading to leaves that fracture more easily. Plants have secondary compounds that can influence the quality of the forage. Grasses contain high concentrations of the secondary chemical silica that reduces fiber digestibility and increases tooth wear (Clauss et al., 2008; Clauss and Hatt, 2006). However, tooth wear in grazing species might be devoted to the presence of grit and dust as

well, as grasses are often found close to the ground and the uptake of these abrasives is inevitable (Codron et al., 2019). Browse tends to have more phenolic compounds such as tannins, alkaloids and terpenes which can reduce protein digestibility, can have toxic effects, or decrease dry matter digestibility. Furthermore, plants can differ in their architectural assembly with grasses being a more homogenous source of food compared to the often heterogenous structure and nutritional value of the different browse elements (Shipley, 1999).

2.3.3 Morphophysiological adaptations of grazers and browsers

Grazing and browsing species have made adaptations to various body elements such as mouth, teeth, salivary glands, liver and gastrointestinal anatomy and physiology in order to maximize the nutritional uptake from their selected forage (Clauss et al., 2008; Shipley, 1999; Steuer et al., 2010). A specific differentiation between grazers and browsers consists of an ethmoid area that is negatively linked with percentage grass in the diet hence leaving less space for nerves to connect the brain with the nose in grazers. It is suggested that this can be devoted to the browsers' need to rely more on their smell as opposed to grazers, due to many anti-digestive and toxic characteristics of browse elements (Codron et al., 2019).

The presence of a cell wall in plants, has led to the need of a digestive measure in all herbivores because mammalian enzymes are not capable of degrading compounds such as cellulose and lignin. This indicates that herbivores rely on symbiotic microbial fermentation of their forage for the greatest part of their energy requirements which primarily consists of volatile fatty acids (VFA) (Shipley, 1999). Ruminants mainly house these microbes in the rumen, which is a large pouch where the major fraction of fermentation occurs. Fiber elements of the ingested diet are regurgitated from the rumen to the mouth until they are small enough to pass through a small opening leading to the omasum. The longer plant fiber stays in the rumen, the more complete the digestion of the fiber will be. To retain fiber particles for a longer period of time, the rumen has adaptations such as a large size, muscular walls and a small opening between rumen and omasum (Shipley, 1999; Steuer et al., 2010). Non-ruminant herbivores, which are generally browsers, mostly rely on the hindgut for microbial fermentation, including the caecum and colon. Therefore these animals are often referred to as hindgut fermenters. Because their diets contain less cellulose and more indigestible compounds like lignin, browsers have developed a rapid flowing digestive tract in order to increase food uptake (Steuer et al., 2010). Adaptations in browsers, with or without rumen, involve mainly a larger caecum and colon and a smaller abomasum or rumen.

One would expect a longer duration of food in the digestive tract of grazers which would translate in a higher mean retention time (MRT) of both particles and fluid in grazers as opposed to browsers. However, data presented in several studies have not been able to confirm this theory (Codron et al., 2019). Steuer et al., (2010) suggested that measuring MRT for the entire GIT would be less accurate and retention time should be focused on the main fermentation chamber of animals which is referred to as the mixing chamber (MC). Applying this strategy, they noticed that there was a significant difference between the MRT_{MC} of the grazing white rhinoceros and the browsing black rhinoceros hence confirming the theory that slower passage leads to higher energy extraction in grazers.

Additionally, the selectivity factor, which is the ratio of MRT of particles compared to MRT of fluids $\left(\frac{MRTparticle}{MRTfluid}\right)$, has appeared to be significantly increasing with the percentage grass in the diet. This would imply a longer particle retention in grazers as opposed to browsers (both ruminating and non-ruminating) and thus confirm that the difference in digestive physiology is correlated to percentage

grass in the diet (Steuer et al., 2010). This finding also explains the lower $MRT_{particle}$ time in browsers which need a faster passage of dietary content due to its low digestibility and additionally want a faster passage of dietary content to the main fermentation compartment of the digestive tract, being the large intestine (Codron et al., 2019). The difference in MRT_{fluid} is currently hypothesized to be connected to the level of stratification of the rumen and thus differs between browsing and grazing ruminants. However, discussing this would lead us too much off topic (for more information see (Codron et al., 2019 and Steuer et al., 2010)).

Browsers also developed specific adaptations for their high tannin diets, namely parotid salivary glands that are averagely four times larger when compared to grazers. Also the liver of browsers is about two times larger when compared to grazers (Shipley, 1999). The parotid salivary glands are significantly larger due to the high protein character of the saliva making the saliva very viscous. These glands produce tannin binding proteins that can limit the reduced protein digestibility that tannins cause (Codron et al., 2019). The increased liver tissue is meant for increased detoxification capacity which means that both adaptations have developed to limit the negative aspects of tannins (Clauss et al., 2008).

The large metabolic needs of herbivores are met by spending tremendous amounts of time, sometimes up to 10 hours a day, consuming their relatively low energy containing diets. This implies that efficient food consumption is essential to benefit the remaining hours of the day for other activities such as reproduction and avoiding predators (Shipley, 1999). An adaptation thereto is the structural difference in mouth form and teeth (Clauss et al., 2008). In general, herbivores are hypsodont which allows a longer duration of teeth wearing. This is particularly useful when diets contain abrasive foods and thus lead to high tooth-to-tooth contact, which would be expected the most in the grazing diet (Codron et al., 2019; Shipley, 1999; Steuer et al., 2010). However, it is currently believed that the hypsodont teeth are not a strict characteristic of grazers and that the benefits of this adaptation have been found in a broader range of herbivores (Codron et al., 2019).

Furthermore, because grazer diets consist mostly of homogenous grasses that are often present in large amounts in the same area, they have wide mouths with similar sized, low incisors that are pointed forward in order to maximize their bite size (Clauss et al., 2008; Shipley, 1999). Conversely, browsers carefully select the plant particles that they need and therefore have a small mouth, a relatively large mouth opening and more pronounced difference in length of the individual incisor teeth and the canine teeth (Clauss et al., 2008). Some browsers have additional adaptations such as the long tongue of the giraffe and the prehensile upper lip of the black rhinoceros (Clauss et al., 2008; Shipley, 1999).

2.4 Rhinoceros under human care

Members of the Rhinocerotidae family can be divided into forage classes based on their food preferences in the wild when using the categorical distinction of grazers and browsers. As mentioned before, black rhinoceroses are considered strict browsers with a wild diet consisting of mainly leaves and stems of trees, shrubs and herbs (Clauss et al., 2006), Javan and Sumatran rhinoceroses are considered (co-)browsers (Owen-Smith, 1988), white rhinoceroses are defined as grazers and Indian rhinoceroses are considered intermediate feeders (Clauss et al., 2006).

Wildlife dietary requirements are often insufficiently understood to provide an adequate diet under human care. As a result, animals under human care receive a diet based on a 'model species', which is an animal species for which there is already understanding of their nutritional needs (Cabana et al., 2017). For rhinoceroses, the domestic horse (*Equus caballus*) has currently been considered the best

model (Clauss et al., 2006; Dierenfeld et al., 1995). Horses are grazing hindgut fermenters with similar digestive tract anatomy and physiology as other hindgut fermenters such as elephants and black rhinoceroses (fig. 3). However, as described in the previous chapter, significant differences can be seen between grazers vs. browsers and ruminants vs. hindgut fermenters. The horse has appeared to be an appropriate model for the grazing and intermediate feeder rhinoceros species (Clauss et al., 2006). Conversely, this has not been the case for the browsing black rhinoceros (Clauss et al., 2006). The major differences between horses and black rhinoceroses will be described hereafter.

2.5 Comparing morphology and physiology of the rhinoceros to other species

The domestic horse is a monogastric grazer which has a large caecum that is used as the main fermentation chamber and where the most significant part of plant digestion occurs. The colon is also well developed but is considered to provide only secondary supportive fermentation in addition to the caecum. The digestive tract of the white rhinoceros resembles the horse the strongest. The main differences include a reduced caecum size combined with an increase in colon length to make

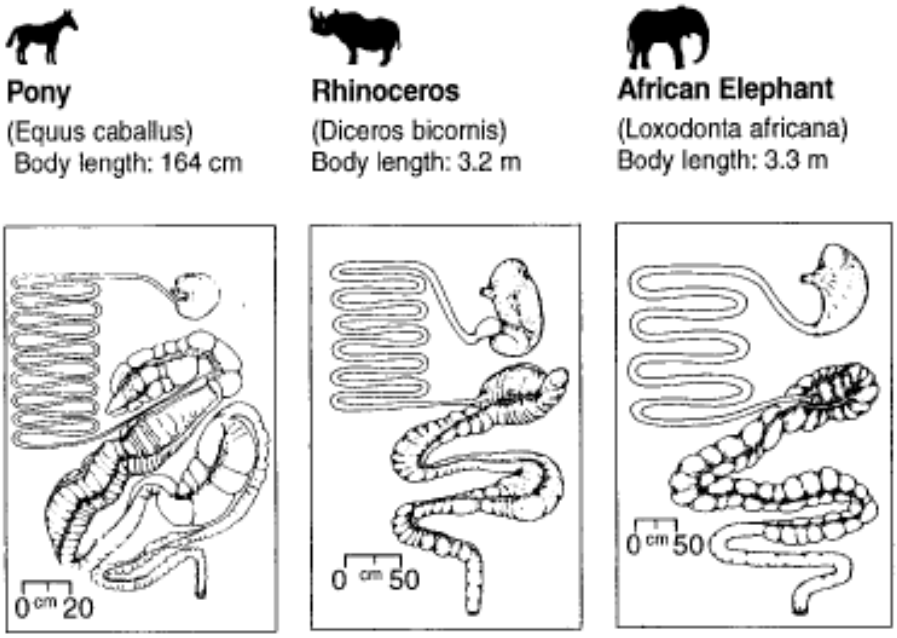

Figure 3: drawing of the gastrointestinal tract of three hindgut fermenting animal species including the domestic horse, black rhinoceros and African elephant. Figure from Stevens and Hume, (1995)

a shift from caecum to colon as primary fermentation chamber (Endo et al., 1999). The long small intestine in horses has supposedly developed as a result of the high intake of concentrate food during its domestication (Clauss et al., 2003). Browsing monogastric herbivores such as the African elephant *(Loxodonta africana)* and black rhinoceros also have a reduced caecum size and rather use their well-developed colon as the main fermentation chamber which provides them a large amount of energy. It is obvious that the elephant has a relatively shorter digestive tract as compared to the other species which is presumably to obtain a faster passage rate as discussed before. Although the digestive system of these four species is comparable, the main difference in digestion between the grazing and the browsing species is the mean retention time (Clauss et al., 2005; Steuer et al., 2010).

Table 1: length of the different segments of the gastrointestinal tract of four herbivore species with their average body weights. Ratios of each segment of the intestine to the total GIT length are presented as percentages in parentheses. Numbers from Clauss et al., (2003)

	Domestic horse	**White rhinoceros**	**Black rhinoceros**	**African elephant**
Small intestine (in m)	22.5 (72%)	13.8 (61%)	12 (65%)	13.8 (57%)
Caecum (in m)	1 (3%)	0.8 (4%)	0.7 (4%)	0.8 (3%)
Colon (in m)	7.5 (24%)	7.2 (32%)	4.9 (26%	8.5 (35%)
Total length GIT (in m)	31.3	22.8	18.5	24.2
Average body weight (in kg)	500	1900	1000	3900
GIT length/body mass ($m/kg^{0.33}$)	4.03	1.89	1.89	1.58

Clauss et al., (2006) confirmed this theory and illustrated that the black rhinoceros had both shorter retention times and a lower digestibility coefficient and suggested that these evolutionary characteristics developed in both foregut and hindgut fermenting browsers. As mentioned before, browsers have a diet which contains a great percentage of undigestible compounds such as lignin which ferments rather fast. This indicates that browse will deliver its energy quite rapidly which implies that black rhinoceroses and African elephants can benefit from a fast clearance of food particles from their digestive tract (Clauss et al., 2006; Steuer et al., 2010). By contrast, grass is fermented much slower, implying that it can maintain its fermentation energy for a longer period in time. These dietary characteristics have thus led to a longer mean retention time of dietary particles and a higher digestibility coefficient in both horses and white rhinoceroses (Clauss et al., 2006).

To conclude, these findings illustrate that species-specific digestive morphology and physiology need consideration to provide a well-suited diet for animals under human care. Comparative data of the gastrointestinal tract of the horse, white and black rhinoceros and the African elephant are presented in table 1 to illustrate differences in gastrointestinal morphology. In addition to these considerations, it is necessary to obtain sufficient knowledge on the nutrient composition and the mineral content of diets of free roaming animals, which will be discussed hereafter.

2.6 Diet of the black rhinoceros

The black rhinoceros diet consists of an enormous variety of plants, all with their specific physical and chemical characteristics. With the aim of composing a diet under human care that resembles the natural situation as much as possible, numerous studies have been conducted on the nutrient composition of the browse elements in the black rhinoceros diet (fig. 4) (Dierenfeld et al., 1995; Ghebremeskel et al., 1991). However, it should be noted that studies that take geographical variety, seasonal changes in nutritional composition, bite size, and many other variances into consideration are rare. This can be explained due to economical and practical reasons and may result in studies that only present a rough estimate of the dietary composition and thus may not be completely representative for the natural diet (Dierenfeld et al., 1995; Sullivan, 2016).

Analyzing wild diets has created a better understanding of rhinoceroses needs under human care and has led to certain dietary recommendations for these species. As mentioned before, the similar anatomy of the digestive tract of horses and rhinoceroses makes the horse the current best model for dietary recommendations. The differences in feeding physiology between the grazing, browsing and intermediate feeding rhinoceroses, however, require certain adaptations to the standard horse diet (Clauss and Hatt, 2006).

Acacia brevispica (Mimosaceae)
Acacia Hockii (Mimosaceae)
Carissa edulis (Apocynaceae)
Ecbolium revolutum (Acanthaceae)
Euclea divinorum (Ebenaceae)
Ferula communis (Umbelliferae)
Lippia javanica (Verbenaceae)
Phyllantus fischeri (Euphorbiaceae)
Rhus natalensis (Anacardiaceae)
Tinnea aethiopica (Lamiaceae)

Figure 4: Ghebremeskel et al., (1991) conducted a study on nutrient composition of browse in Kenya and presented the ten most favored plants of the black rhinoceros which are believed to form 70% of the year-round diet. Plant species names are presented on the left side and correlate with the pictures from left-above to right-down. It is believed that black rhinoceroses consume over 210 different plant species one a year-round.

2.6.1 Roughage

Black rhinoceroses have a high fiber and a low to moderate protein diet in the wild. Therefore it is advised to provide a 1:1 ratio of grass and legume hay as roughage under human care. Adding legume hay, such as lucerne or timothy hay, can provide the additional protein that this species requires as opposed to the grazing rhinoceros species for which grass hay combined with 20% of the roughage content as legume hay suffices (Clauss and Hatt, 2006; Sullivan, 2016). However, lucerne contains great amounts of protein, iron and calcium and the diet is considered excessively digestible for rhinoceroses and may lead to colic and diarrhea. Therefore, it is important to ensure that the proposed quantities are not exceeded (Dierenfeld, 1995; Sullivan and Valdes, 2019). It is needless to say that the offered hay should be of high nutritional and hygienic quality (Clauss and Hatt, 2006). The provided diet should

not exceed the energy requirement of 0.6 MJ digestible energy per $kg^{0,75}$ metabolic body mass (Clauss et al., 2005). This implies that roughage can be presented *ad libitum*, however, regular weighing of the animals combined with assessment of the body condition score is needed to prevent obesity under human care, to which all rhinoceros species appear to be prone (Clauss et al., 2012; Clauss and Hatt, 2006). Due to the presence of silica in grass, dental wear can occur in the black rhinoceros (as discussed before) and it is, therefore, advised to include lucerne hay into the diet as well (Clauss and Hatt, 2006).

Although browse should be provided as much as possible, this is often not possible due to many reasons. The most important one is the fact that free ranging animals consume 2,5% of their body weight each day which is estimated to be 30 kg of browse, and that 240 different plant species have been considered part of the wild diet hence making it practically and economically not possible to provide great quantities of browse with enormous plant species variety to animals under human care (Dierenfeld, 1995). However, attempts should be made to provide some browse under human care and following plant species have been suggested by Clauss and Hatt, (2006) *apple Malus spp., ash Fraxinus, birch Betula spp., beech Fagus spp., cherry and prune Prunus spp., hazel Corylus spp. and willow Salix spp.* among others.

2.6.2 Supplementation of pelleted feed

To meet the metabolic energy requirements and fiber needs of these animals, concentrate food, often as pelleted compound food, are provided under human care (Sullivan, 2016). The provided amount of this supplement should not exceed one-third of the overall caloric value and it is advised to aim to provide less without jeopardizing the protein and energy requirements of the animals (Clauss and Hatt, 2006). The main part of the diet should exist of roughage. Pelleted food should only be considered as a supplemental source of energy, vitamins and minerals. The general recommendation for pellet formulation involves the presence of 40-60% neutral-detergent fiber (NDF) and low amounts of starch and soluble sugars. Due to its supplemental role in the black rhinoceros diet, pelleted food have the potential to both prevent the occurrence of deficiencies but on the contrary can also lead to excesses of minerals and vitamins. Therefore, the mineral requirements and the current mineral deficiencies and excesses that black rhinoceros encounter will be discussed. Feeding advice will be presented simultaneously in the following segment.

2.6.3 Mineral requirements

Minerals are essential in animal nutrition as these elements fulfill many functions in the body. A distinction can be made between macro and microminerals based on the amount needed on a daily basis. Macrominerals are required between 1-10 grams/kg DM each day and microminerals, which are also referred to as trace elements, are required less than 1 g/kg DM each day (fig. 5) (Dermauw, 2013; Goff, 2018).

After digestion, microminerals, e.g. iron, get transported to the liver via the portal bloodstream whereafter they are either stored in the liver or transported to other tissues through the systemic bloodstream. Minerals can get secreted in milk, sweat and digestive juices such as bile. The latter will flow back into the gut and can be recycled almost entirely. Excretion of trace elements happens through feces and urine (Dermauw, 2013).

Mineral functions in the body can be divided into four groups, namely catalytic, physiological, structural and regulatory (Dermauw, 2013). Microminerals mainly have a catalytic function, which means that these elements play an important role in enzyme functioning. Subsequently, because of their involvement as cofactors for enzymes that control free radicals in the body, some microminerals play an important role in the bodies' antioxidant capabilities. However, these essential elements can also become pro-oxidants that lead to free radical formation when they are consumed in excess (Goff,

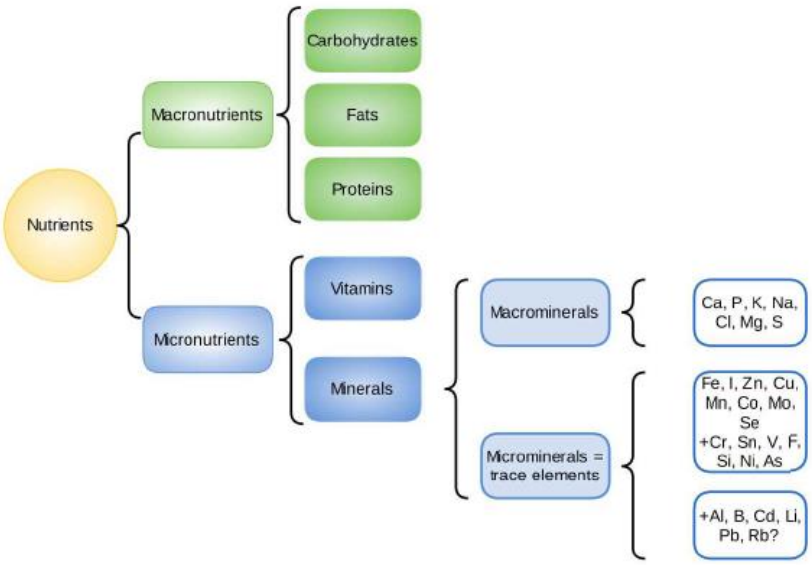

Figure 5: Different elements in nutrition including macro and microminerals. Figure from Dermauw, (2013)

2018). Free radicals include the superoxide anion (O_2^-), hydroxyl radicals ($OH^\cdot$) and free ionized metals in body fluids such as Fe and Cu. All free radicals, but free ionized metals in particular, are very destructive because they contain one or more unbound electrons that makes them very unstable. These radicals become very reactive and try to receive or donate an electron from or to any neighboring targets. One of the main targets of these free radicals is the lipid bilayer of cell membranes. As a result, the once stable compound in the cell membrane now becomes a free radical itself. This implies that free radicals generate a chain reaction of free radical formation and stabilization that will affect many more molecules in the body, and can only be stopped by antioxidant counteraction. To prevent free ionized metals such as iron from becoming free radicals, the body develops proteins that form reversible bonds with these trace elements. However, when trace elements are ingested excessively, there will not be enough proteins to bind all absorbed metals. This leads to radicalization of the metals, hence leading to cell membranes and DNA being damaged (Goff, 2018).

Mineral and vitamin requirements of black rhinoceroses have been determined by several studies in free roaming animals with the aim of maintaining the correct mineral balance and preventing excesses and deficiencies in animals under human care (table 2) (Dierenfeld et al., 2005; Ghebremeskel et al., 1991; Molenaar et al., 2008). It is believed that many disorders that black rhinoceroses are facing under human care have mineral imbalances as an underlying factor (Dierenfeld et al., 2005). Therefore, it is crucial to perform ration calculation in diets to monitor mineral and vitamin content and mimic the natural situation as much as possible (Clauss and Hatt, 2006).

Due to similar anatomy and physiology, as mentioned before, the horse is considered to be the current most appropriate model for all four rhinoceros species in terms of mineral and vitamin values.

Although similarities can be found, many differences have been discovered in terms of mineral homeostasis which can be devoted to the difference in dietary preferences of the browsing rhinoceros species and the grazing horse (Clauss et al., 2007b). However, grazing white rhinoceros species also differed in some mineral values when compared with the horse (Dierenfeld et al., 2005). It is therefore important to understand and address the difference in mineral homeostasis between all rhinoceros species and horses when keeping these species under human care (Clauss et al., 2007b; Dierenfeld et al., 2005).

In the discussion that follows hereafter, mineral levels in the liver and circulating mineral levels will be compared between reference values that have been established for domestic horses and free roaming and captive black and white rhinoceroses based on studies conducted by Dierenfeld et al., (2005) and Clauss et al., (2007). Focus will lie on minerals that need monitoring when preparing diets under human care or are significant in another matter.

Table 2: serum mineral values of black rhinoceros both free (F BR) and under human care (C BR), white rhinoceros both free (F WR) and under human care (C WR), Indian rhinoceros under human care (C IR) and domestic horse reference values (C H). F BR data was collected from four different regions in Zimbabwe, the data presented here is only from Matusadona. Horse reference values are presented as the average value of the data in the study. All data is derived from Dierenfeld et al., (2005).

	Ca (µg/ml)	**P** (µg/ml)	**Na** (mEq/l)	**K** (mEq/l)	**Mg** (µg/ml)	**Cu** (µg/ml)	**Fe** (µg/ml)	**Mn** (g/ml)	**Zn** (µg/ml)
F BR	135	43.6	142.6	5.29	24	1.5	2.71	6.52	1.49
C BR	127	40.9	131	5	21.4	2.06	2.7	1.36	1.59
F WR	105.6	35.5	123.8	4.15	20.6	1.16	1.77	2.47	1.39
C WR	139	39.7	146.7	4.83	23.7	2.53	1.66	1.65	1.47
C IR	120.7	40.7	133	4.14	17.3	2	1.3	1.89	2.35
C H	115	38.5	139	4	26.5	1.43	1.65	6	1.15

2.6.3.1 Calcium and phosphorus

Black rhinoceros' liver and circulating calcium levels were considered high when compared to horse reference values in both black and white rhinoceroses under human care. It is hypothesized that equids, rhinoceroses and tapirs have no regulation of calcium absorption and thus absorb calcium (Ca) in great amounts and excrete the excess in the urine (Dierenfeld et al., 2005). However, Clauss et al., (2007) added the following hypothesis to this matter. Black rhinoceroses have a significantly higher calcium to phosphorus ratio (P) in their natural diet ($\frac{Ca}{P}$-ratio of 14:1), which is tremendously higher than the horse diets ($\frac{Ca}{P}$-ratio of 1.4:1). Phosphorus is essential for the growth of bacteria in the digestive tract that will deliver energy by fermentation of herbaceous content in the intestines. The high Ca content may form insoluble Ca-P complexes in the intestine lumen, hence reducing the availability of P. When P is limited, less energy will be produced by the microbiota for the animals. Following this theory, it may be possible that black rhinoceroses absorb high amounts of Ca in the small intestine with the aim of rendering P available in both small intestine and hindgut (Clauss et al., 2007b).

The reason for the high calcium content in the black rhinoceros' liver and blood is presumably an evolutionary adaptation to a high Ca and low P natural diet where calcium absorption efficiency is higher than P. This implies that there may be an adaptation of this species to lower P content in the diet as well (Clauss et al., 2007b). Diets under human care including lucerne hay and pelleted food have high Ca contents. Due to their natural adaptations to Ca, excesses should not be developed as a result of these compounds alone given that pelleted food do not contain excessive Ca and the mentioned hays are presented within limits (Dierenfeld et al., 2005; Sullivan and Valdes, 2019). However, mineral supplements comprising Ca are currently often presented under human care, which is not suitable to do in either rhinoceros species (Clauss et al., 2007b; Dierenfeld et al., 2005). The dietary differences between the grazing horses and white rhinoceroses and the browsing black rhinoceroses could explain the excessive Ca storage in white rhinoceros given they are presented the same diet under human care, which includes alfalfa as well (Dierenfeld et al., 2005).

Hypophosphatemia has been connected with the occurrence of hemolytic episodes in black rhinoceroses (discussed in the section of iron overload disorder). However, no deficiency has been reported in black rhinoceroses under human care (Dierenfeld et al., 2005). Combined with the theory of evolutionary adaptations to low P diets and the current dietary regimes under human care containing high amounts of P, it is unlikely that hemolytic episodes are of dietary origin. Therefore, P supplementation is only advised for animals which show clinical signs of hemolytic anemia as it has been an effective way of preventing the occurrence of such episodes (Clauss et al., 2007b; Sullivan and Valdes, 2019).

2.6.3.2 Sodium, potassium and magnesium

Sodium (Na) has been considered a limiting factor for herbivores in general, as opposed to potassium (K) which is often present in great quantities in all forage types fed under human care. On the contrary, due to the poor availability of K in African soils, diets of free roaming animals often contain distinctively lower potassium concentrations when compared to diets under human care. Low sodium levels have been observed in free roaming black rhinoceroses which could be an evolutionary adaptation to low amounts of Na in their natural diet and the use of natural Na licks has been observed in the natural environment (Clauss et al., 2007b; Dierenfeld et al., 2005). Clauss et al., (2007) observed that the major difference in terms of Na and K homeostasis was a significantly higher endogenous fecal loss of Na and K of black rhinoceroses as opposed to horses. It was hypothesized that this may be due to the browser digestive physiology. This can be explained by a constant relation between Na and the amount of fecal bulk and fecal water that is produced.

As mentioned before, browsers tend to have faster passage of dietary compounds in the digestive tract and therefore produce more feces resulting in increased Na and K loss. Additionally, it is also possible that certain species-specific characteristics play a role in these minerals' homeostasis. Low Na have been confirmed in the browsing black rhinoceros and African elephant as well when they were kept under human care (Clauss et al., 2007b; Dierenfeld et al., 2005). It is, therefore, advised to provide salt licks under human care (Clauss and Hatt, 2006). Another finding was the accumulation of K and Mg in the liver of captive black rhinoceroses, which increased with age. Both minerals are considered to be adequately present when kept under human care and no pathologies have been correlated with the storage of these minerals in the liver (Dierenfeld et al., 2005).

2.6.3.3 Iron

Excessive iron storage has been reported numerous times in captive black rhinoceroses and has received a lot of attention due to its potential clinical consequences in these animals. Iron overload will be thoroughly discussed in a different segment of this master dissertation.

2.7 Iron homeostasis in mammals

To understand the pathophysiology of IOD, it is essential to understand the functioning of iron metabolism in the healthy body. Because information on animal physiology is scarce, and especially in the case of wildlife, human physiology will be used as a model and when species-specific information is available, the differences in physiologies will be explained.

2.7.1 An essential element

Throughout evolution organisms have opted to mainly use iron as a transition element in biological redox reactions instead of elements like manganese and copper for following reasons: 1) Presence of iron on earth is plentiful, in fact it is the second most abundant metal after aluminum; 2) Iron will be used for electron transfer and binding to biological ligands, iron's capacity of existence in multiple oxidation states is very useful; 3) Iron's redox potential range is wider than that of other transition elements. These capacities made iron the most interesting element because organisms can adapt the reactivity of iron to fulfil the physiological needs (Pantopoulos et al., 2012).

Iron is essential in mammalian physiology because it functions as a cofactor for non-heme iron binding proteins and hemoproteins. These proteins have a significant role in biological processes such as: oxygen binding, transport and metabolism (hemoglobin, peroxidases and catalases), cellular respiration and electron transport, DNA-synthesis, cell proliferation and drug metabolism among many other functions (Ems and Huecker, 2020; Pantopoulos et al., 2012).

2.7.2 Iron absorption

Iron uptake can be facilitated by passing through the placenta during the fetal life or by passage through the small intestine after feeding (Fuqua et al., 2012). Iron can be found in an absorbable state in the diet in the form of two types: heme and non-heme iron (Fuqua et al., 2012). Heme iron is the most easy absorbable form and can be derived from hemoglobin and myoglobin of animal food sources, such as meat and poultry. Non-heme iron can be found in plants and in foods to which iron is supplemented. Non-heme iron is not as easily absorbed (Ems and Huecker, 2020; Pantopoulos et al., 2012). Free ranging African herbivores such as the black rhinoceros depend solely on plants for their daily iron uptake while their counterparts under human care also receive iron-fortified dietary elements such as pelleted food.

After feeding, iron containing dietary elements pass the stomach to reach the small intestine, more specifically the duodenum and jejunum, which is where most of the dietary iron will be absorbed. At physiological pH iron exists in the ferric (Fe^{3+}) state, which is insoluble and thus unabsorbable. The ferric state (Fe^{3+}) can be reduced to a ferrous state (Fe^{2+}) to make the iron absorbable. This reduction can be obtained in two ways: either by ascorbic acid or with the aid of the ferric reductase enzyme duodenal cytochrome B which gets activated by the low pH of the gastric acid in the duodenum (fig. 6). Once soluble, the ferrous iron will be transported from the intestinal lumen into the enterocyte by divalent metal transporter 1 (DMT1) or divalent cation transporter (DCT1) (Ems and Huecker, 2020; Pantopoulos et al., 2012). Although currently less understood, it is believed that heme iron absorption happens in another way, namely via receptor-mediated endocytosis and ferritin absorption is not fully understood (Fuqua et al., 2012).

After absorption, iron can be stored in the enterocyte or can be transported into the bloodstream. Iron can be stored in cells as ferritin: inside the enterocyte there is a protein by the name of apoferritin which can oxidize ferrous iron (Fe^{2+}) to ferric iron (Fe^{3+}). After oxidation, many ferric iron will bind with apoferritin which will form a hollow shell in which iron can be stored. After binding, this molecule will be referred to as ferritin. Heme iron will be broken down by heme oxygenase and ferric iron will be released which can be stored in the same way as described above.

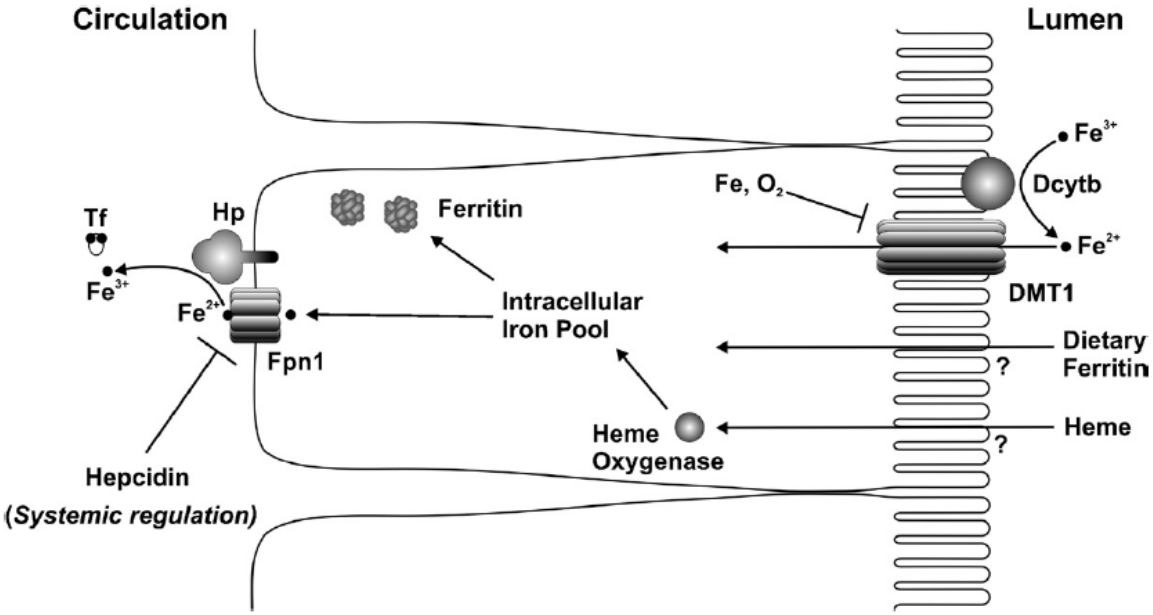

Figure 6: iron homeostasis in the mammalian body. Ferric iron (Fe^{3+}) is reduced to ferrous iron (Fe^{2+}) by ascorbic acid or duodenal cytochrome B (Dcytb) to allow iron absorption. Ferrous iron will be transported from the intestinal lumen into the enterocyte by divalent metal transporter 1 (DMT1) or divalent cation transporter (DCT1). Heme iron and dietary ferritin absorption is currently less understood. Iron can be stored in cells as ferritin after ferrous iron is oxidized to ferric iron by apoferritin. Heme iron will be broken down by heme oxygenase and ferric iron will be released which can be stored the same way as described above. If cellular iron storage is not needed, iron will pass through the enterocyte to enter the bloodstream through the basolateral membrane of the cell after reduction to ferrous iron by ferritin. Ferrous iron will be escorted out of the cell by ferroportin (Fpn1) which is a transmembrane protein. Iron will then bind to transferrin (Tf) in plasma, after oxidation to ferric iron by ceruloplasmin in the plasma and hephaestin (Hp) in the enterocyte. Hepcidin is the main molecule for systemic iron regulation in the body. Figure from Fuqua et al., (2012)

If storage in the enterocyte is not needed, iron will pass through the enterocyte to enter the bloodstream through the basolateral membrane of the cell. For this process, a reduction of ferric (Fe^{3+}) iron to ferrous (Fe^{2+}) iron is also needed, and this will be facilitated by the ferritin molecule. Ferrous iron will be escorted out of the cell by ferroportin which is a transmembrane protein (Ems and Huecker, 2020) and the only iron export protein in mammalian cells. A deletion of ferroportin would lead to accumulation of iron in the enterocytes without passage to the circulation (Fuqua et al., 2012). Subsequently, iron will bind to transferrin in the plasma, which is a carrier protein for iron. In order to bind with transferrin, ferrous iron (Fe^{2+}) will be oxidized to ferric iron (Fe^{3+}) by ferroxidases ceruloplasmin (in the plasma) and hephaestin (in the enterocyte) (Ems and Huecker, 2020; Fuqua et

al., 2012). There are two binding sites for ferric iron on apo-transferrin, which after binding will be referred to as holo-transferrin, and will transport iron through plasma to all cells in need of iron. Transferrin in plasma is only saturated for approximately 30% as a precautionary measure to create a buffering capacity to bind excessive amounts of non-transferrin bound iron (NTBI) and prevent oxidative damage to cells. Holo-transferrin binds with transferrin receptor 1 (TfR1), which most nucleated mammalian cells are thought to contain, leading to clathrin-mediated endocytosis. Iron can be released from transferrin by acidification of the endosome and is then transferred from the endosome into cell cytosol by DMT1 after reduction to the ferrous state (Fe^{2+}) (Pantopoulos et al., 2012).

2.7.3 Iron regulation

An average and healthy human being has approximately 4 g of iron in its body most of which as heme in erythrocytes and about 1 g stored in the liver and the spleen. Considering iron as an essential element, the body aims to maintain most of its iron and only loses <0.1 % of the total body iron amount each day. Iron excretion is an unregulated process and happens mostly through passive processes such as shedding of skin and loss of intestinal cells containing iron (Fuqua et al., 2012; Ganz and Nemeth, 2019). This indicates that maintaining an adequate amount of iron in the body needs to be regulated at the iron absorption level through diet (Fuqua et al., 2012). Even when significant iron overload occurs, iron excretion does not increase accordingly and thus dietary iron uptake has to be adjusted (Ganz and Nemeth, 2019). However, it should be mentioned that some studies revealed that iron excretion through binding with bile salts could also play an important role in iron excretion (see biliary excretion of minerals). Most of the iron in the body gets recycled by reticuloendothelial macrophages which take up senescent red blood cells (RBC), remove iron from the RBC and release the iron when needed. However, iron uptake through diet is necessary to make up for iron losses such as menstruation, shedding of epithelial cells (which contain iron), and during pregnancy (Ems and Huecker, 2020; Fuqua et al., 2012).

Hepcidin is a liver-synthetized protein that is essential for iron homeostasis. This protein binds with ferroportin on enterocytes, macrophages and other cells leading to internalization and degradation of ferroportin. As a result, the only cellular exporter of iron in mammalian cells is not able to fulfill its function and iron is not able to be transferred into the bloodstream (Fuqua et al., 2012; Pantopoulos et al., 2012). Hepcidin levels will increase when sufficient iron is present in the body and during infection and inflammation, hence leading to limitation of iron uptake. On the other hand, iron uptake will increase if hepcidin levels are low, during hypoxia, when the need for erythropoiesis increases and when iron deficiency occurs (Fuqua et al., 2012).

Hepcidin expression is believed to be regulated by two major pathways: the BMP6/SMAD pathway and the degree of saturation of transferrin with iron (Fuqua et al., 2012). Bone morphogenic protein 6 (BMP6) is a protein produced by hepatocytes in proportion to their iron load. BMP6 will bind to the BMP I/II receptor complex which will lead to phosphorylation of the SMAD 1, 5 and 8 proteins which on their turn will interact with another protein, being SMAD4, and form complexes. Latter will stimulate the transcription of hepcidin by the HAMP gene in the nucleus. This pathway needs the hemojuvelin (HJV) co-receptor in order to function properly. Although not fully understood, it is believed that both the hemochromatosis (HFE) protein and the transferrin receptor 2 (TfR2) protein are able to sense the degree of saturation of transferrin in plasma. The binding of HFE with TfR2 presumably upregulates the production of hepcidin (Fuqua et al., 2012; Pantopoulos et al., 2012). When plasma iron levels are low (and thus transferrin saturation is low), binding of HFE with TfR2 gets

inhibited by the sequestration of HFE by the TfR1 protein. On the contrary, when transferrin saturation levels increase, holo-transferrin inhibits the sequestration of HFE, allowing it to bind with TfR2 and activate signaling to increase hepcidin synthesis (Pantopoulos et al., 2012).

2.8 Biliary excretion of minerals

The liver plays an important role in mineral homeostasis. It can function as a storage pool for minerals and is able to secrete these accumulated elements in the blood or in bile when minerals are needed or are present in excess (Dijkstra et al., 1991). Bile is a fluid that is produced by the liver and is often stored in the gallbladder whereafter it is transported through the bile canaliculi to the small intestine. Among other components, bile contains bile salts which are components that play an important role in the absorption of fat and fat-soluble vitamins from the ingested diet (Sharma and Hiller, 2018). In addition, bile functions as a major excretion pathway for several minerals including Ca, K, Mn but also Fe, although Fe is mainly excreted through other ways (Dijkstra et al., 1991).

Bile acids are produced by the hepatocytes by degrading cholesterol through hydroxylase enzymes to primary bile acids, being cholic acid and chenodeoxycholic acid. Bile salts or conjugated primary bile acids are formed in the next step of the process by conjugation of primary bile acids to glycine, taurine, glucuronic acid or sulfates (Macierzanka et al., 2019). Bile salts will then be transferred to the intestine where they are responsible for the solubilization and transport of fatty acids and lipid-soluble nutrients (Li et al., 2019). After fulfilling their function, bile will be almost entirely absorbed and the greatest part will be transferred to the liver via the portal vein. The interaction between the intestines and liver where bile is secreted and reabsorbed in an efficient way is referred to as the enterohepatic circulation (Hofmann, 2007).

When bile salts are transferred to the small intestine, they can bind with minerals such as iron and potassium and transfer these elements with them whereafter these minerals can either be reabsorbed or are excreted with feces. This form of excretion is referred to as the hepatobiliary excretion pathway (Dijkstra et al., 1991; Mercadante et al., 2019). Although this form of excretion for Fe has never received much attention, it is currently believed that this pathway could play an important role in iron excretion in addition to other excretion routes. Multiple studies in mice have revealed that iron levels in bile fluctuate according to the amount of iron in the body. Iron levels in bile rise when iron is excessively stored and decrease in situations of deficiency (Mercadante et al., 2019). In addition, for several minerals such as Ca, K, and Mn, the hepatobiliary excretion route is considered the most important way of excretion (Dijkstra et al., 1991). Although the role of bile in iron homeostasis has received more attention recently, the exact regulation of this process is not fully understood (Dijkstra et al., 1991; Mercadante et al., 2019). These studies emphasized the potential importance of this pathway for iron excretion but further research is needed to confirm these findings in other mammals.

2.9 Iron overload disorder

Habitat loss and poaching have led to a decrease in population numbers of many animal species. Human efforts of saving species from going extinct include the transfer of animals at risk to sanctuaries and zoos to protect them from poaching and aim to increase their population numbers once again by participating them in breeding programs. However, these objectives are accompanied by many issues affecting the health status of animal species when placed under human care (Pouillevet et al., 2020). Many wildlife species, with the black and Sumatran rhinoceros in particular, have been suffering from iron overload disorder (IOD) which is a condition where excessive amounts of iron can be found in the circulation and in various tissues and organs throughout the body (Clauss and Paglia, 2012; Paglia and Tsu, 2012).

The presence of hemosiderin has been a common finding at necropsies of black and Sumatran rhinoceroses when they were kept under human care. However, necropsy reports often reported these findings as a result of past hemolytic events, being incidental or as being characteristic to rhinoceros species without laying a connection with any form of pathology (Paglia and Tsu, 2012). Two pivotal studies established the presence of a true and acquired iron overload disorder that occurs in black rhinoceroses when they are kept under human care. Following findings certified this. Firstly, blood analysis showed that serum iron, transferrin and ferritin were significantly higher in black rhinoceroses as opposed to white rhinoceros when these species were held for a relatively long time under human care. Necropsies revealed an even more significant finding, which was the five times higher amount of non-heme iron in black rhinoceroses liver when compared to white rhinoceroses under human care (Smith et al., 1995). Secondly, other necropsies revealed that hemosiderosis only occurs in black rhinoceroses when they are kept under human care, as free roaming animals did not show any sign of this finding (Kock et al., 1992). Quantitative tissue assays were performed and demonstrated a logarithmic increase of iron load in the liver in function of age or time in captivity. These findings have led to the well-established result that IOD is a condition that only occurs in black rhinoceroses when they are kept under human care and progression occurs with time in captivity (Paglia and Tsu, 2012).

2.9.1 Etiology

Although a significant amount of research has been conducted on the cause of IOD, until now none of the suspected etiologies have been able to fully explain the development of iron overload. However, a brief review will be given on the hypotheses of IOD etiology of the past decades to be able to comprehend this complex disorder.

2.9.1.1 Hemolytic anemia

Black rhinoceroses under human care have been suffering from episodes of hemolysis for decades. Due to its high prevalence and a mortality rate of 75%, hemolytic anemia has been considered one of the most leading causes of death in black rhinoceroses under human care (Dennis et al., 2018). Researchers discovered that all rhinoceros species had erythrocytes that were significantly different from red blood cells of other mammalian species (Paglia and Tsu, 2012). Red blood cell abnormalities such as an adenosine triphosphate (ATP) concentrations that were only 2-5% of the values of other mammalian red blood cells, and low or absent activity of glutathione-S-transferase and catalase enzymes among others (Paglia and Tsu, 2012). These deficiencies were recognized to cause hemolytic anemia in humans. However, these characteristics were present in all four rhinoceros species and no differences were found between the animals suffering from hemolytic anemia and the animals that do

not. As a result, this led to the acceptance of these findings as a normal characteristic of erythrocytes of the Rhinocerotidae family. As a consequence, rhinoceros erythrocytes have a significantly diminished capacity to neutralize both endogenous and exogenous oxidants (Dennis et al., 2018; Paglia and Tsu, 2012; Sullivan, 2016). Although it has not been confirmed that low ATP levels are the direct cause of hemolytic anemia, dietary phosphate supplementation has been used as successful preventive and therapeutic measure for this condition due to increasing ATP levels in erythrocytes (Dennis et al., 2018; Sullivan, 2016). These measures have led to a significant decrease in morbidity and mortality in rhinoceroses under human care in current days (Dennis et al., 2018; Sullivan, 2016).

Hemolysis will lead to hemoglobin (Hb) release whereafter Hb will be degraded to billiverdin and iron. Subsequently, iron will be stored in the body in the form of the iron-storage complex hemosiderin. Many of the necropsies performed on black and Sumatran rhinoceroses under human care throughout the years contained hemosiderin deposition in various tissues (Kock et al., 1992; Smith et al., 1995). Because of the significance of hemolytic anemia in the past, hemosiderosis was mistakenly regarded as a consequence of hemolytic episodes even though very few animals had a history of such event (Paglia and Dennis, 1999; Paglia and Tsu, 2012). Smith et al., (1995) remarked that hemosiderosis and hemolytic anemia are less likely to be correlated due to comparable haptoglobin levels between white and black rhinoceroses. This can be explained by the role of haptoglobin as a marker of hemolysis. Haptoglobin binds free hemoglobin and thus decreases when hemolysis occurs (Shih et al., 2014). One would expect lower haptoglobin levels in animals that have suffered from hemolytic episodes, which was generally not the case.

2.9.1.2 Genetic etiology

Browsing mammals, such as the black and Sumatran rhinoceros, emerged during the Oligocene epoch which was a time period wherein the bioavailability of many metals, including iron, was low (Paglia and Tsu, 2012). It is hypothesized that browsing rhinoceros species have adapted themselves to low iron diets by selecting one or multiple mutations in genes that play a part in iron homeostasis, resulting in higher iron uptake when this element was available. This adaptation would allow them to store excessive iron to benefit in times of scarcity (Ganz and Nemeth, 2019). In addition to this hypothesis, human forms of iron overload are often of genetic origin and because it is believed that iron regulation functions the same way in rhinoceros species as in humans, genetic deficiencies should be considered a possible cause for IOD.

As mentioned before, iron homeostasis is almost exclusively regulated at the iron uptake level due to passive processes of iron excretion. Dietary iron uptake is therefore strictly regulated, mostly by the hepatocyte produced hepcidin and ferroportin proteins (Fuqua et al., 2012; Ganz and Nemeth, 2019). A deficiency in genes necessary for iron regulation could thus lead to iron overload disorder.

In humans, iron overload has been well-studies and can function as a model for IOD in rhinoceroses. However, until now, not all the relevant genes of iron homeostasis have been sequenced in rhinoceroses and molecular studies are yet to be performed (Ganz and Nemeth, 2019). Human iron overload exists of multiple forms that are presented hereafter.

Iron overload can occur as a result of genetic hepcidin deficiency and is referred to as hemochromatosis. It is the most common form of iron overload in humans (Paglia, 2004) and is caused by mutations in one or more genes synthesizing HFE, HJV, hepcidin (HAMP gene), TRF2 and ferroportin (SLC40A1 gene) (Brissot et al., 2019). These mutations will lead to a varying degree of the same phenotype which consists of excessive uptake of iron, independent of dietary iron levels, due to hepcidin deficiency. As a result, hypersideremia (abnormally high serum iron levels), increased saturation of transferrin (Brissot et al., 2019) and excessive storage of iron in parenchymal cells of

mainly liver, heart and endocrine organs will occur (Ganz and Nemeth, 2019; Sullivan, 2016). In addition, it is known that reticuloendothelial macrophages are also regulated by hepcidin and low hepcidin levels increase the release of iron out of these cells through ferroportin. Due to the high amount of macrophages present in the spleen as opposed to the liver, iron will mostly accumulate in liver hepatocytes and less in spleen tissue (Ganz and Nemeth, 2019).

Acquired hepcidin deficiency is also referred to as dyserythropoiesis-related iron overload which can occur as a result of thalassemias (with or without the occurrence of transfusions) (Brissot et al., 2019). Here, erythrocyte precursors fail to mature which leads to apoptosis in the bone marrow and the emergence of anemia. The body responds by stimulating a significant increase in the erythrocyte expansion pool through increased erythropoietin production. Although not fully understood, it is believed that this process results in a decrease of hepcidin production leading to the same iron overload phenotype as mentioned above (Ganz and Nemeth, 2019).

Acquired iron overload due to excessive parenteral iron entry is the result of repeated transfusions. Each blood transfusion contributes between 200-250 g of iron to the human body and the lack of mechanisms to increase iron export lead to iron accumulation. The resulting phenotype differs from iron overload caused by hepcidin deficiency due to initial distribution of iron to the spleen. This is because the excessive iron is entering the body in erythrocytes and macrophages will perform erythrophagocytosis as usual, transferring most of the iron to the spleen. However, iron will gradually be redistributed to the hepatocytes as well (Brissot et al., 2019; Ganz and Nemeth, 2019).

Ferroportin disease presents itself with a phenotype that differs from hemochromatosis as it mainly involves iron accumulation in the spleen and shows no elevation of serum iron levels and serum transferrin saturation. This can be explained by a mutation in the ferroportin gene (SLC40A1) which results in a decrease in cellular iron export and thus iron retention. Macrophages in particular show ferroportin activity which explains the mainly splenic involvement. This mutation differs from the other mutation in the ferroportin gene mentioned before as it does not involve a reduced sensitivity to hepcidin (Brissot et al., 2019; Sullivan, 2016).

Genetic abnormalities in iron metabolism can also lead to iron overload with anemia. Mutations can be found in genes that are involved in iron homeostasis which lead to either dysregulations in cellular iron uptake (DMT1 gene), plasma iron transport (transferrin gene) or cellular iron export (ceruloplasmin gene) among other genes (Brissot et al., 2019). These disorders are however extremely rare in humans and result in a phenotype that combines iron overload and anemia (Ganz and Nemeth, 2019).

In 2001, a polymorphism was detected in the HFE gene of the black rhinoceros leading to a change in the S88T amino acid. This polymorphism was not detected in the white, Sumatran and Indian rhinoceros. It was hypothesized that this gene alteration could be an evolutionary adaptation to a low iron diet and thus can act as a potential contributor to the development of IOD (Beutler et al., 2001). However, experimental confirmation is lacking and there are arguments that question this hypothesis. Firstly, there was no evidence of S88T in the Sumatran rhinoceros that also has a history of IOD when kept under human care (Beutler et al., 2001). Secondly, recent necropsy reports of the Indian rhinoceros suggest that this species is also prone to IOD although genetic evidence is lacking in this species as well (Olias et al., 2012). Thirdly, because it is believed that the HFE gene plays an important role in hepcidin regulation, an altered HFE gene would lead to reduced hepcidin levels. Although there is a lack on data on rhinoceros hepcidin levels, histopathological evidence of macrophages being iron loaded before hepatocytes shows functioning of hepcidin at least on a certain level. This can be explained by the finding that in humans, contrariwise, macrophages are not loaded due to hepcidin

deficiency (Sullivan, 2016). The role of ferroportin disease in IOD was questioned as well due to studies emphasizing the functioning of ferroportin at least at some level (Olias et al., 2012; Sullivan, 2016).

Further research is needed to address the possibility of genetic causes as a contributor to IOD. This can happen by sequencing the relevant genes in iron homeostasis, measuring hepcidin levels and comparing results between rhinoceros species that are susceptible to IOD and the species that appear to be insusceptible (Ganz and Nemeth, 2019).

2.9.1.3 Dietary etiology

Many wildlife species under human care suffer from or are considered susceptible to IOD. Currently it is strongly believed that the difference between the wild diet and the diet presented under human care could play a major role in the etiopathology of IOD in many animal species (Clauss et al., 2002; Lavin, 2012; Smith et al., 1995). Reasons for this theory are presented hereafter.

Animal species that appear to be the most susceptible to excessive iron storage under human care are browsing and frugivorous animal species such as the black rhinoceros, Sumatran rhinoceros, tapirs, lemurs, gorillas, siamangs and marmosets among other species (Clauss et al., 2002; Clauss and Paglia, 2012). These animal species often have a diet in the wild that contains low amounts of iron or high amounts of natural chelators (Clauss et al., 2002; Clauss and Paglia, 2012). Natural chelators are secondary plant compounds such a as tannins and phytates which bind iron in the diet, leading to the formation of unabsorbable complexes and thus a reduced bioavailability of iron (Delimont et al., 2017; Lavin, 2012).

As mentioned before, it is hypothesized that animal species such as the black rhinoceros have adapted themselves throughout evolution to a low iron diet by developing highly effective iron absorption mechanisms or, on the contrary, have lost certain iron regulation mechanisms due to the lack of situations of iron excess in their natural environment (Clauss et al., 2002; Lavin, 2012; Paglia and Tsu, 2012). However, an increase in iron availability in the times that followed led these species to discovering ways of inhibiting excessive iron uptake (Paglia and Tsu, 2012). Smith et al., (1995) hypothesized that black rhinoceroses dealt with this problem by carefully selecting dietary elements which contained natural chelators to decrease iron uptake and storage. This theory can also be applied to other browsing and frugivorous animal species. The grazing white rhinoceros presumably developed protection mechanisms to prevent excessive iron uptake from its specialized grass diet that generally contains iron containing soil (Clauss and Paglia, 2012). It is believed that due to the absence of iron chelating components outside their natural environment, browsing and frugivorous animal species in particular have become prone to IOD as opposed to grazing animal species. This can be explained by the finding that grazing animals species often receive a diet under human care that resembles the diet in the wild (Clauss and Paglia, 2012; Paglia and Tsu, 2012). Providing an adequate diet under human care that sufficiently resembles the wild diet is truly challenging due to factors such as wide ranges of foraging territory, geographical differences in plant species and nutrient content, and the cost of analyzing the different dietary elements. This problem also presents itself in terms of identification and quantification of chelators in the wild diet (Sullivan, 2016). Even when there is sufficient knowledge on species-specific wild diets, providing these diets under human care is often practically and economically impossible. For instance providing the wide variety of leaves and shrubs of trees for the black rhinoceros under human care has not been manageable until now (Sullivan, 2016).

Adding chelators to diets under human care has been considered a viable solution to reduce the occurrence of IOD and positive effects have been seen in animal species such as toco toucans (*Ramphastos toco*) (Drews et al., 2004) and lemurs (*Varecia variegata*) (Wood et al., 2003). Efforts have been made to reduce iron overload by adding chelators to black rhinoceros diet under human care.

However, these attempts have appeared to be rather unsuccessful due to ineffectiveness of the therapy to reduce iron absorption or because of increased health concerns for the treated animals (Clauss et al., 2007a; Sullivan, 2016; Sullivan et al., 2015). Desferrioxamine is an example of an oral pharmacological chelator that has been successfully used in black rhinoceroses, however, due to the high cost of using this therapy on large animals it cannot be considered a routine option (Paglia, 2004). Nevertheless, chelators appear to be essential as a treatment for iron overload under human care for many animal species (Clauss and Paglia, 2012). Further research is needed to determine which chelators appear to be effective and how they can be combined to gain the best results in each species (Lavin, 2012). In addition, chelator safety should be determined in each species due to their potential negative side effects such as toxicity and possible inhibition of digestion (Clauss et al., 2007a; Clauss and Paglia, 2012; Lavin, 2012).

Other than the lack of chelating compounds, zoo diets often contain manufactured complete food such as pellets which contain high amounts of iron, mostly because of the use of high iron sources and small particles of the producing machinery that inevitably end up in the food (Clauss and Paglia, 2012). Miller et al. (2016) investigated wild diets of black rhinoceroses in different habitats and discovered that the highest amounts that could be found in the wild were still significantly lower than diets fed under human care. Although this finding has been reported in several studies (i.e. Paglia and Dennis, 1999), it should be mentioned that there are other studies that mention high serum iron levels in free roaming black rhinoceroses as well (Dierenfeld et al., 2005). Dietary iron content presumably differs geographically and presents the question whether diets in the wild are truly as low in iron as suggested.

Although it seems logical that providing a low-iron diet under human care would at least partially solve the iron overload problem, the high cost and difficulty of producing such diets have led to the inability of widespread use of this solution (Clauss et al., 2002; Miller et al., 2016). However, attempts should be made to lower iron content in diets under human care and aim to reach the current recommended dietary concentration of 50–100 mg Fe/kg diet (dry-matter basis) (Miller et al., 2016). Sullivan and Valdes, (2019) suggested that higher dietary iron concentration are also acceptable based on the practical limitations of producing low-iron pelleted food and thus their low availability. In addition, it remains very difficult to provide enough browse under human care. It is suggested to not exceed a dietary iron concentration of 300 mg/kg dry matter. There are examples of this strategy being successful, i.e. decreasing the amount of iron in the pelleted food with simultaneous reduction of the amount of pellets given to black rhinoceroses under human care has appeared to reduce serum ferritin values significantly (Mylniczenko et al., 2012).

Although the theory of an evolutionary adaptation to low iron diets has been generally accepted, some questions remain unanswered. Firstly, there are many browsing and frugivorous species that currently appear to be unsusceptible to IOD under human care such as the sloth and howler monkeys (Clauss and Paglia, 2012). Secondly, mainly hindgut fermenting species seem to be susceptible, even when diets appear similar (Clauss and Paglia, 2012; Lavin, 2012). Although there are some theories that could explain this, confirmation is still needed. Thirdly, diets containing high amounts of iron often lead to a reduced availability of other minerals such as cupper (Cu), cobalt (Co), zinc (Zn) and phosphor (P). Dierenfeld et al., (2005) could not confirm this in their research and suggested that minerals in diets under human care are relatively in proportion and that the development of IOD could not solely be of dietary origin. Generally, it is believed that in addition to a dietary cause for IOD, genetics, certain species-specific characteristics or currently unknown causes could play a role in the development of IOD under human care as well. This implies that further research is needed to be able to understand the etiology of IOD (Clauss and Paglia, 2012; Sullivan, 2016).

2.9.2 Histopathological lesions

Over the past decades, many necropsies have been performed on all four extant rhinoceros species. A general finding in all the performed necropsies was the presence of hemosiderosis in various organs in the browsing rhinoceros species, being the black and the Sumatran rhinoceros (Paglia and Dennis, 1999). Recently performed necropsies indicate the presence of hemosiderosis in organs of Indian rhinoceroses suggesting a possible susceptibility to IOD (Olias et al., 2012). The white rhinoceros showed no sign or insignificant amounts of hemosiderosis indicating insusceptibility of this species to IOD (Smith et al., 1995).

When discussing IOD two terms can be used to describe the organ lesions, first of which is hemosiderosis meaning that there is a generalized or local accumulation of the iron-storage complex hemosiderin in body tissues when no specific connection can be made to any pathology. Often hemosiderosis is the result of hemolysis. The second term is hemochromatosis which is used in specific iron overload syndromes and in conditions where there is a connection between a certain pathology (such as necrosis) and hemosiderosis (Paglia and Tsu, 2012).

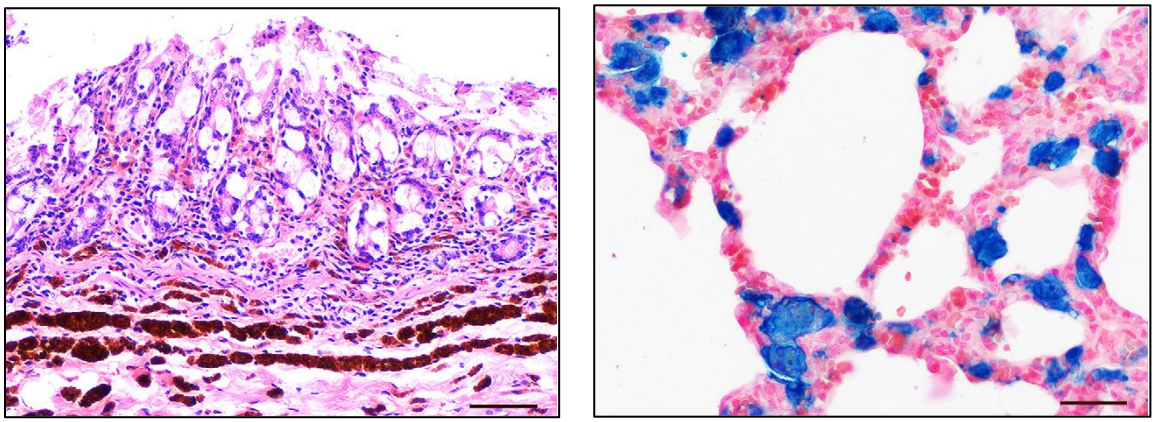

Figure 7 and 8 : histology results of adult black rhinoceros under human care. (Left) Hematoxyline-eosine stain shows iron accumulation as brown clumps in the lamina propria and submucosa of the small intestine. (Right) Turnbull blue stain used to demonstrate iron accumulation as blue spots in the interalveolar and peribronchiolar interstitium of the lungs. Figures from Olias et al., (2012).

Necropsy results of deceased black rhinoceroses under human care do not show any gross lesions, meaning that microscopic evaluation of tissues and organs is necessary to diagnose IOD (Klopfleisch and Olias, 2012; Kock et al., 1992; Smith et al., 1995). Organs most affected by hemosiderosis are spleen, liver, bone marrow and lungs (fig. 8) (Kock et al., 1992; Olias et al., 2012; Paglia and Dennis, 1999). Other organs that also appear to be affected, but less prominent, are heart, endocrine organs, intestines (fig. 7) and lymph nodes (Paglia and Dennis, 1999). It appears that initially, deposits of ferric iron can be found in the reticuloendothelial system but eventually parenchymal cells will be affected as well. Macrophages become hyperplastic and severely loaded with hemosiderin followed by iron loaded parenchymal cells with hepatocytes in particular. In severe cases even fibrosis, cirrhosis and hepatocarcinoma have been seen in histopathology (Paglia and Tsu, 2012; Sullivan, 2016). The major differences between the black rhinoceros and humans in terms of histopathological changes of IOD are the following: firstly, hemosiderosis was also present in the black rhinoceros lungs and in the lamina propria of the small and large intestine which is not the case in human hereditary hemochromatosis (HH) (Sullivan, 2016). The presence of hemosiderin in the intestines can be the result of increased iron uptake as can be seen in other animal species. Secondly, the presence of severe hemosiderin accumulation in the spleen is also a characteristic that is not seen in HH. Ultimately the

result is the same in both humans and rhinoceroses, severe iron accumulation will lead to organ malfunction, severe morbidity and even death (Klopfleisch and Olias, 2012).

2.9.3 Connection of IOD with other disorders under human care

Black rhinoceroses under human care suffer from a wide range of disorders including a high susceptibility to infections associated with high morbidity and even mortality such as Leptospira, Salmonella, mycobacteria and fungal pneumonias, skin and mucous membrane ulcerations, hepatic failure, leukoencephalomalacia and many more (Paglia and Dennis, 1999; Paglia and Tsu, 2012). A seemingly endless list of pathologies only affecting black rhinoceros under human care while other rhinoceros species appear unaffected and the absence of these disorders in free ranging black rhinoceros suggests a connection with the presence of iron overload in this species (Molenaar et al., 2008; Paglia and Tsu, 2012). It is believed that IOD can both occur as a secondary consequence of disorders such as infections and prolonged fasting. On the contrary, IOD can be the primary problem originating from a species-specific susceptibility and rather be the leading cause to secondary infections or wasting (Clauss and Paglia, 2012). In addition, because inflammation can both cause and be the result of IOD, it is possible that this disorder is self-sustaining if no anti-inflammatory or anti-iron overload treatment is undertaken (Pouillevet et al., 2020). This implies that the search for a direct connection between these pathologies and iron overload is difficult to find and has yet to be discovered. However, it is firmly believed that IOD contributes in a certain way to at least some of these conditions (Paglia and Tsu, 2012).

How exactly IOD can affect various organs has yet to be confirmed but current hypotheses are the following: firstly, as mentioned before, iron can catalyze superoxide and hydroxyl free radicals or can function as radical itself inducing injury at a cellular and subcellular level. Furthermore, if iron reacts with proteins, carbohydrates, lipids or nucleic acids, new radicals can be formed and the forthcoming chain reaction can amplify their destructive effects (Paglia and Tsu, 2012; Sullivan, 2016). Secondly, iron overload interferes with the nutritional immunity. A healthy body sequestrates iron with the aim of limiting the use of this essential element for pathogens. During excessive iron storage the latter will happen insufficiently leading to microorganisms becoming more virulent and the host becoming more susceptible to infections (Paglia and Dennis, 1999). Finally, in situations of iron excess the phagocytic and bactericidal abilities of mononuclear and polymorphonuclear leucocytes are less successful which also will increase the susceptibility to infections (Paglia and Tsu, 2012).

2.9.4 Diagnosis

In humans, IOD can remain asymptomatic but often leads to clinical symptoms such as arthritis, heart failure, liver cirrhosis, liver cancer and diabetes mellitus (Palmer et al., 2018). Initial diagnosis of IOD in humans is difficult due to non-specific symptoms at the presentation of iron overload such as fatigue and arthralgia, which cannot be linked directly to IOD. More obvious clinical signs only develop when progression of this disorder occurs and various organs become damaged (Pietrangelo, 2006). When the presence of hemochromatosis is suspected, initial diagnosis is made by genetic screening and serum analysis for elevated ferritin levels, transferrin saturation, total iron-binding capacity (TIBC), iron levels and liver enzymes such as alanine aminotransferase (ALT), aspartate aminotransferase (AST) and gamma-glutamyl transferase (GGT) (Molenaar et al., 2008; Palmer et al., 2018; Pietrangelo, 2006). Liver enzyme levels will rise due to liver damage as a result of increased iron storage (Molenaar et al., 2008). An increase of transferrin saturation above 50% in men and above 35-40% in woman can

suggest the presence of iron overload because transferrin saturations correlate with total body iron levels. Serum ferritin can be used as an indicator of excessive iron storage in body tissues, however, ferritin is an acute phase reactant and, therefore, is considered rather unspecific. Serum ferritin will also rise as a result of non-iron overload related causes such as inflammation, cytolysis or infection (Palmer et al., 2018; Pietrangelo, 2006). Normal values in men and woman are <300 µg/L and <200 µg/L respectively (Mylniczenko et al., 2012). Furthermore, normal serum ferritin levels do not exclude the presence of iron overload simply because organ iron accumulation may not have developed yet (Palmer et al., 2018). The golden standard for diagnosing hemochromatosis still remains liver biopsy with histological examination and quantification of iron accumulation (Pietrangelo, 2006).

Similarly to human hemochromatosis, the lack of obvious clinical signs that can confirm the presence of IOD in black rhinoceroses point out the need for other measures such as regular health screening of susceptible animals and prophylactic therapy in order to detect the development of iron overload in an early stage and aim to prevent an increase in severity of this disorder (Clauss and Hatt, 2006; Clauss and Paglia, 2012). Iron storage in organs can lead to irreversible damage and therefore it is believed that therapeutic measures for IOD are only successful if this disorder is detected early and treatment is started immediately (Molenaar et al., 2008; Pietrangelo, 2006).

Smith et al., (1995) developed an enzyme linked immunosorbent assay (ELISA) to measure serum ferritin levels in rhinoceros. They presented data that showed significant higher serum ferritin levels in black rhinoceros under human care as opposed to white rhinoceroses in the same conditions. Throughout the years many other studies have confirmed these findings in black rhinoceroses but also in Sumatran rhinoceroses, lemurs and other susceptible species under human care (Clauss and Paglia, 2012; Paglia and Dennis, 1999). In addition, serum transferrin saturation and serum iron levels where measured and compared and the general findings were elevated serum ferritin, transferrin and iron levels when these species were kept under human care (Mylniczenko et al., 2012; Sullivan, 2016). Multiple studies showed that transferrin saturations of black rhinoceroses under human care can vary between 40 – 100%, implying that all of these animals suffer from iron overload and need therapy when compared with human values (Mylniczenko et al., 2012; Paglia and Tsu, 2012; Sullivan, 2016). One other general finding was the increase of serum ferritin levels with age under human care as opposed to free roaming black rhinoceroses (Dierenfeld et al., 2005; Mylniczenko et al., 2012).

Current strategies of screening and monitoring IOD in susceptible animals under human care involve serum ferritin levels, serum transferrin saturation and serum iron levels. The reliability of some of the current methods has been questioned recently for reasons described hereafter. Firstly, to conduct regular health monitoring, reliable reference ranges for serum analysis are needed. A number of attempts have been made and reference ranges have been established for free roaming black, white, Sumatran and Indian rhinoceroses, as for the animals under human care (Dierenfeld et al., 2005; Molenaar et al., 2008). However, due to geographical differences in diet and soil, animal age variability, differences in used techniques, different sample size and other differences in conducted studies, great variabilities have been discovered among the established reference ranges leading to comparisons made between studies considered less reliable (Molenaar et al., 2008; Sullivan, 2016). Molenaar et al. (2008) suggested standardization of the used methods for monitoring and added that analyzing of collected samples from both free roaming as well as animals under human care should happen in the same laboratories and conducted research should use the same statistical methods. The adjustments could possibly lead to an increased reliability of reference ranges.

Secondly, the accuracy of using serum ferritin levels as a reliable diagnostic method is questioned. As mentioned before, ferritin can estimate total body iron levels because it correlates with the amount of iron stored in various organs throughout the body. However, ferritin is also an acute phase protein

which will also rise when infections, inflammation, dietary changes and many other events occur in the body making it a less specific diagnostic method for excessive iron storage (Miller et al., 2016; Wojtusik and Roth, 2018). Some studies even demonstrated that several animals with serum ferritin levels that are currently considered to be normal appeared to be severely iron overloaded during necropsy. Conversely animals with significantly increased serum ferritin levels showed no signs of clinical illness (Miller et al., 2016; Wojtusik and Roth, 2018). Although ferritin has been considered the best representation of body iron levels (Clauss and Paglia, 2012; Sullivan, 2016), the findings mentioned before have led to the consideration that serum ferritin levels still may be connected to IOD but are not considered very reliable (Wojtusik and Roth, 2018).

Other biomarkers have also been questioned to be reliable for diagnosing IOD. Serum iron does not correlate with body iron levels and is also not reliable as a biomarker (Sullivan, 2016). This was also confirmed by later studies demonstrating that although other biomarkers increased with age, serum iron levels remained the same (Dierenfeld et al., 2005). Serum transferrin saturations (serum iron concentration divided by total iron binding capacity (Paglia, 2004)) are generally accepted as a reliable biomarker for iron overload in black rhinoceroses and should be used for screening and monitoring of IOD in black rhinoceroses the same way as it is being used in humans (Molenaar et al., 2008). Liver enzymes have been described to increase in iron overloaded animals under human care but a direct connection and reference values have yet to be found and determined (Miller et al., 2016; Molenaar et al., 2008). The golden standard for IOD diagnosis remains the quantification of iron accumulation in different organs but most importantly in the liver (Sullivan, 2016).

Further research in this area is crucial, not only to prevent excessive iron accumulation leading to morbidity and mortality, but also to gain better insight in how IOD exactly functions and how it develops. In the future, comparative studies between susceptible and insusceptible animals should be conducted and a full panel of iron metabolites should be collected using standardized methods to increase reliability. In the meantime, the use of biomarkers for iron overload diagnosis and monitoring is strongly advised event though their reliability is questioned (Clauss and Paglia, 2012).

2.9.5 Treatment

In humans, the main treatment for hemochromatosis is phlebotomy which basically involves the removal of a portion of the patients' blood. In addition, oral chelating compounds such as desferrioxamine are prescribed and chelating dietary substances such as black tea and coffee are added to the diet to reduce iron uptake from the diet. Dietary iron content and vitamin C (ascorbic acid) are limited for the same reasons (Palmer et al., 2018). Latter dietary adaptations can be useful to reduce the frequency of phlebotomies, however, even if these measures would be 100% effective, they would not be able to correct the existing pathological iron overload and thus cannot replace the main therapy of phlebotomy (Paglia, 2004; Palmer et al., 2018). In severe cases of iron overload, the liver is irreversibly damaged which leaves the only remaining option of liver transplantation (Palmer et al., 2018).

In wildlife species susceptible to IOD such as the black and Sumatran rhinoceros, therapeutic measures are extrapolated from the therapy used in humans, with the necessary adaptations made for wildlife under human care. This implies that in these animals the therapy exists of the same three aspects, being, reduction of dietary iron uptake, addition of chelating compounds to their diets and performing repetitive phlebotomies (Mylniczenko et al., 2012; Paglia, 2004). The therapies based on dietary measures have been discussed at the etiology segment and focus will lie on phlebotomies in this section.

Phlebotomies consist of removing blood from patients suffering from iron overload to eliminate excessive iron. Hemoglobin, which is found in red blood cells, contains a fixed amount iron being 0.34% of its weight. By removing one liter of blood containing 15g/dl hemoglobin, exactly 0.5 g of iron will be eliminated (Paglia, 2004; Paglia and Dennis, 1999). The aim of performing phlebotomies is inducing a slight blood loss anemia in order to induce mobilization of stored iron in the body and can be achieved by reaching packed-cell-volume (PCV) levels of 5% below the normal baseline (Paglia, 2004).

Candidates for phlebotomy are selected based on the presence of clinical signs that can be associated with excessive iron storage and based on their serum transferrin saturation and serum ferritin values (Mylniczenko et al., 2012; Paglia, 2004). Paglia (2004) suggests that black rhinoceroses with transferrin saturation values of 65-70 % and serum ferritin levels of 500 ng/ml are candidates for which therapeutic interventions are justified. These values are based on the reference values of latter iron biomarkers in free roaming rhinoceroses and are two or threefold of their mean values (transferrin saturation of 34 % and ferritin values of 180 ng/ml) (Paglia, 2004). It is additionally important that animals are able to be trained to participate sufficiently to undergo phlebotomy (Mylniczenko et al., 2012). Candidates should be trained for blood collection in the fore legs and to be comfortable in a restraint chute before phlebotomies are performed on a regular basis (Mylniczenko et al., 2012). The medial radial vein of the foreleg is considered the best option to withdraw large volumes of blood rapidly with lesser risk for thrombosis as a result of the trauma caused by frequent venipuncture as opposed to the ear and the interdigital vein of the forefoot (which are generally considered the easiest option). A 17G or 18G needle is used with a vacutainer collection system. To stimulate the bone marrow to maximize the production of new red blood cells, the frequency of performing phlebotomies is increased slowly from every two weeks during the first two months to twice weekly. The amount of blood that is collected during each session is estimated to be between 1,5 – 3 liters (Mylniczenko et al., 2012; Paglia, 2004). The result of phlebotomies is the mobilization of stored iron leading to a relatively rapid decrease in serum ferritin and transferrin levels. Latter can thus be used to monitor treated animals on a regular basis (Crawford et al., 2005; Paglia, 1984). It is important to mention that rhinoceros red blood cells are maturated before they leave the bone marrow which is why mean cell volume (MCV) is assessed rather than reticulocyte count. Stored iron will gradually be mobilized in order to make up for the hemoglobin lost as a result of the performed phlebotomy leading to a slow decrease in serum ferritin levels (Mylniczenko et al., 2012; Paglia, 2004).

Iron overload disorder often does not display any obvious clinical signs until organ damage has occurred, implying that screening susceptible animals, performing repetitive phlebotomies and monitoring are crucial for their health. In addition, this therapeutic measure can be beneficial for many reasons. Preventing excessive iron storage and thus organ damage by performing phlebotomies requires a much lower cost than treating chronically ill animals for long periods in time (Paglia, 2004). Furthermore, phlebotomies performed on calves born under human care and recently translocated animals can act as a prophylaxis to avoid excessive iron storage that will eventually occur if untreated under human care (Paglia, 2004). Finally, the collected blood can be fractionated and stored in order to benefit from red blood cells, leukocytes, platelets and plasma in the future when morbidity occurs without the need for additional donors (Sullivan et al., 2015).

Negative aspects of phlebotomy include the intense training programs of all susceptible animals and keeping the animals calm and comfortable in a restraint chute for the long duration of the phlebotomy procedure. In addition, collecting large amounts of blood with proper equipment needs further improvement in the future. Currently, a 17G needle is the widest bore which rhinoceroses tolerate but leads to low blood flow and thus longer duration of the procedure which than leads to vacutainers losing pressure during the procedure and blood flow arrests due to movement of the animal as a result

of animal boredom and discomfort (Mylniczenko et al., 2012). An ethical and financial consideration should be made on prophylactic screening and treatment of susceptible animals that show no clinical signs of iron overload (Clauss and Paglia, 2012). Nevertheless, phlebotomies have appeared to be successful because serum ferritin levels have generally declined in treated patients and there were obvious signs of clinical improvement. Furthermore, the procedure is considered relatively non-invasive and has very little side effects (Crawford et al., 2005; Paglia and Dennis, 1999).

3. Problem description

In order to preserve biodiversity, human conservation efforts are needed. Conservation of wildlife in their natural surroundings is referred to as in situ conservation. This form of conservation is becoming more difficult every day due to human actions such as habitat destruction and poaching. Ex situ conservation, where animals are transferred outside their natural environment and where human influence is generally more pronounced is gaining importance every day. Reasons for this are the controlled breeding programs they often organize to increase species population numbers and their essential role in maintaining genetic heterozygosity. However, to allow longevity and good health of wild animals under human care, species specific information is needed. Currently there is generally very limited information available on animal reproduction and diet. The latter is of utmost importance to maintain good health, prevent the occurrence of diseases and to improve growth and reproduction. Although much research is conducted to determine the nutritional needs of various animal species, current lack of sufficient knowledge on this matter has led to the development of captivity-induced disorders such as iron overload disorder (IOD).

Many animal species, with the black rhinoceros in particular, suffer from IOD under human care. This disorder involves excessive storage of iron in various organs and tissues in the body and has been connected with morbidity and mortality of susceptible animals under human care. The cause of this disorder has not been determined, but it is believed that the difference between diets under human care versus the wild diets could play an important role. However, researchers have been unable to devote this disorders' etiology solely to a difference in diet. It is believed that certain species-specific characteristics must play a role in the pathogenesis of this disorder as well.

4. Plan of approach

Providing a correct diet under human care requires sufficient knowledge on animal physiology and dietary preferences in the wild. A lack of sufficient knowledge on these matters had led to dietary imbalances for animals under human care. An important part of the diet consists of minerals. These elements play a crucial role in various functions of the body. Therefore, in this study we will examine similarities and differences of fecal mineral excretion between wild animals, animals under human care, grazers and browsers. Fecal mineral profiles grant us information on differences in mineral uptake, absorption and excretion of these four groups. The aim of this study is to determine whether browsers and grazers have developed certain adaptations in terms of mineral homeostasis and which alterations occur when animals are placed under human care. By collecting such information, we aim to provide better insight on mineral imbalances that are potentially contributing to captivity-induced disorders and try to reduce the occurrence of such disorders.

5. Materials and methods

5.1 Collection of fecal and dietary samples

Feces samples were obtained from four sources. The first source was Prof. Dr. Marcus Clauss from the university of Zurich who sent us two boxes with banked samples from free roaming African animals. The samples were derived from a wide variety of animal species from which following animal species were selected for further analysis of the feces: African elephant *(Loxodonta africana),* black rhinoceros *(Diceros bicornis),* common warthog *(Phacochoerus africanus),* giraffe *(Giraffa Camelopardalis),* impala *(Aepyceros melampus),* roan antilope *(Hippotragus equinus),* waterbuck *(Kobus ellipsiprymnus)* and plains zebra *(Equus quagga).* These samples were dried and milled and were transported in plastic tubes to the Department of Nutrition, Genetics and Ethology at the Faculty of Veterinary Medicine, Ghent University in Merelbeke, Belgium.

The second source was Dr. Tim Bouts of Pairi Daiza animal facility located in Brugelette, Belgium who was kind enough to grant us feces samples of five white rhinoceroses *(Ceratotherium simum)* and one South-American tapir *(Tapirus terrestris).* Fresh feces were collected the evening before or on the morning of the day of transport and was deposited in a separate plastic bag for each animal. In addition, one pooled sample containing a fraction of the complete daily food intake of each animal species was collected and deposited in a plastic bag. Access to mineral licks has been taken into account for further steps of analyzing mineral content. Feces samples and diets were transported to the Department of Nutrition, Genetics and Ethology at the Faculty of Veterinary Medicine in Merelbeke.

The third source was White & Moore Stables in Waregem, Belgium which allowed us to take feed and feces samples of six horses *(Equus caballus)* which were kept in stables. Each feces sample was deposited in a separate plastic bag. All the horses at this facility received a different combination of dietary elements according to their needs. Each dietary element has been sampled and kept in a separate plastic bag.

The fourth source was Dr. Francis Vercammen of Planckendael animal facility located in Muizen, Belgium who granted feces samples of 3 Indian rhinoceroses *(Rhinoceros unicornis)* and a pooled feces sample of ring-tailed lemurs *(Lemur catta).* Fresh feces were collected the evening before or on the morning of the day of transport and were deposited in a separate plastic bag for each Indian rhinoceros. Fresh feces were also collected from a number of ring-tailed lemurs and the feces were pooled and deposited in a plastic bag. Four feces samples and different elements of the diet of the Indian rhinoceros were transported by car to the Department of Nutrition, Genetics and Ethology at the Faculty of Veterinary Medicine in Merelbeke. Feces and diet samples were stored in a freezer (-20°C) until analysis.

Amber Deschoemaker collected data on a pooled feces sample of free roaming black rhinoceros (OI Pejeta National Park, Kenya) and on individual feces samples of three black rhinoceroses under human care (Diergaarde Blijdorp animal facility, Rotterdam, The Netherlands) for her master dissertation in 2019-2020. Due to the inability to collect black rhinoceros fecal samples ourselves, these data were made available to use in this master dissertation as well.

5.2 Analyzing the collected feces

5.2.1 Drying and milling of feces

All feces samples were prepared for analysis using the same method that will be described below. Feces were removed from the freezer and thawed at room temperature. The feces were placed in an aluminum container after the weight of this container was determined using a Kern PCB 3500-2 scale (fig. 9). The weight of feces was determined before (wet) and after drying (dry) by subtracting the weight of the container from the total wet and dry feces weight. All weights were accurate to 0.01 g.

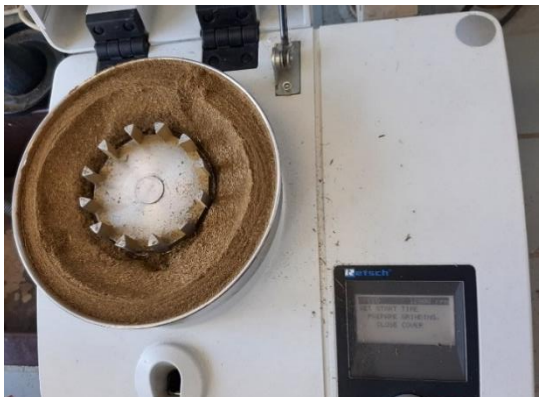

Figure 9 and 10 : Kern PCB 3500-2 scale (left) and Retsch ZM200 centrifugal mill (right) (own pictures)

After drying, the containers with feces were weighed and placed in a drying oven in separate loads to dry for 48 hours at a temperature of 103°C. Drying is needed to work with the absolute weight of the dry matter in the feces, in addition to the wet feces weight. Subsequently, each dried sample was ground over a 1 mm sieve in a centrifugal mill (Retsch ZM200; Gmbh) (fig. 10) at a speed of 18,000 rpm in separate loads (feces of each animal of each species were processed separately). Between each load, the grinding mill was cleaned with a brush, dry paper towels and compressed air to remove remnants of previous loads of dry feces in the machine. Each dried and milled feces sample was then collected in a plastic bag (separate plastic bag per load). These steps were not necessary for the samples of the first source because those samples were already dried, milled and stored in separate plastic tubes per animal of each species (fig. 11).

Figure 11: Dried and milled feces of each animal species of source 1 collected in separate plastic tubes and marked with a unique number which could be linked to an excel-file database (own picture).

5.2.2 Extraction

5.2.2.1 Preparation

An extraction protocol for feces has been developed at the department of Nutrition, Genetics and Ethology in the past. This protocol was used for extraction of all feces samples in this study (sources 1, 2, 3 and 4) and is described hereafter. Different pH-values were used to mimic the pH-levels of the stomach/intestines. Preparation involved the transfer of 2.50 g of dried and milled feces to a 50 ml Falcon tube. A Kern PCB 3500-2 scale was used to achieve a weight of 2.50 g (acceptable range 2.45 – 2.55 g) in the Falcon tubes.

5.2.2.2 Altering pH-values

The objective was to discover whether different mineral availability would be documented when using different pH-values. In the digestive tract, pH values range between 4 and 7 and it is believed that a pH-value of 4 leads to the highest availability of minerals in the digestive tract as opposed to the higher pH-values. This implies that the greatest fraction of absorption of minerals occurs at pH 4 and that absorption decreases with increasing pH-value.

Extraction was performed on feces in Falcon tubes which were buffered to an alternate pH-value. Feces obtained from animals of sources 2, 3 and 4 (white rhinoceroses, South-American tapirs, horses, Indian rhinoceroses and Ring-tailed lemurs – animals under human care) were prepared for extraction in duplo for pH values ranging between 4 -7. Additionally, extraction was also performed *in duplo* on feces which were not altered in pH with the aim of measuring the pH values of feces in their current state. Feces of three individual animals of each species obtained from animals of source 1 (African free ranging animals) were extracted for a sole pH-value of 4. The difference in extraction between the four sources can be devoted to a lack of sufficient fecal material from source 1 hence limiting the ability to perform extraction on a wider pH range.

The different pH-values could be obtained by adding 25 ml of buffer to the feces in the falcon tubes. Depending on the predetermined pH-value, a different buffer composition could be obtained by adding different amounts of demineralized water, phosphate buffer and citrate buffer (table 3). Samples were first manually shaken to obtain a homogeneous mixture.

Table 3: composition of buffer to achieve each desired pH-value. This buffer will be added to the feces in the falcon tubes.

	Phosphate buffer (ml)	**Citrate buffer (ml)**	**dH2O (ml)**
Blanco	0	0	25
pH 4	4.82	7.68	12.50
pH 5	6.45	6.06	12.49
pH 6	8.03	3.00	13.97
pH 7	10.90	1.50	12.60

Immediately after this, the pH in each falcon was determined using a Hanna PI 9125 pH-meter. If the desired pH-value was not reached, additional drops of HCl (or NaOH) were added using a 1 ml Pasteur pipette. After adding extra HCl or NaOH, the falcon tubes were manually shaken to evenly spread the added liquid and after once again the pH-value was measured. This process was repeated until the desired pH-value was reached. The pH-value could differ from the desired value with 0.1 on both ends (e.g. acceptable pH could be 3.9 – 4.1 with desired pH-level 4).

5.2.2.3 Incubation

Once all the desired pH-values in all falcon tubes had been reached, the falcon tubes were placed horizontally with the tubes facing each other in an IKA KS 4000 incubator shaker at a temperature of 37°C for 15 minutes at a shaking power of 150.

5.2.2.4 Centrifugation

After incubation and shaking of the falcon tubes, 15 ml of each tube was transferred to an ultracentrifuge tube (Thermo Scientific). Then the ultracentrifugation tubes were placed in a Sorvall RC-5B refrigerated superspeed centrifuge for a duration of 15 minutes at a speed of 10,000 g. Subsequently, 5 ml of the supernatant of each ultracentrifugation tube was transferred to a new 10 ml sample tube and these were stored in a freezer (-20°C) until further analysis (fig. 12).

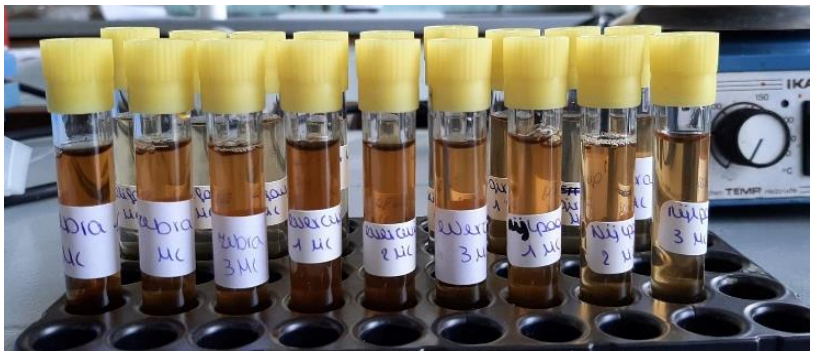

Figure 12: Sample tube containing 5 ml of supernatant after centrifugation

5.3 Inductively Coupled Plasma - Optical Emission Spectrometry (ICP- OES)[6]

ICP-OES is an analytical technique that can be used to detect and quantify the different elements that are present in a sample. Argon gas is used as a source of plasma energy and is transformed to plasma by passing a high frequency electromagnetic field. The obtained plasma has high electron density and high temperature (10,000 K) and this high energy can be transferred to the elements present in the sample. The atoms of these elements will absorb this energy and become excited. When these atoms return to their ground state, light will be emitted at a specific wavelength that is characteristic for the analyzed element. The amount of light emitted at this specific wavelength determines the quantity of this element present in the sample. By comparing these results to a calibration curve that is developed for the analyzed element, each absorption value can be correlated with a certain concentration. The great benefits of this technique are the possibility to detect multiple elements simultaneously with a high level of sensitivity.

[6] https://www.hitachi-hightech.com/global/products/science/tech/ana/icp/descriptions/icp-oes.html (last consulted on 13/05/2021)

In our fecal samples, mineral analysis was desired. Therefore, dried and milled feces from each source were transferred to a 1.5 ml plastic recipient (Eppendorf cup). Each Eppendorf cup was filled with 300 µg of feces using a Kern PCB 3500-2 scale. The Eppendorf cups were sent to LCA (laboratory of chemical analysis) of Ghent University to perform ICP-OES. First, combustion of fecal material was achieved. Then, the obtained ash was dissolved in an acid solution to perform analysis conform ISO 11885 in the following step.

5.4 Iron measurement by RANDOX SI257 serum iron kit on 96 well-plate

For the analysis of Fe in the fecal extracts, a modified version of the commercially available spectrophotometrical RANDOX serum iron kit (SI257, Randox Laboratories, London, UK) was used. The objective of this analysis is to determine the acid-soluble fraction of the iron content in the fecal extract. During this analysis, ferric iron is reduced to the ferrous form in an acid medium. The ferrous iron is then complexed with ferene (a sensitive iron indicator), to produce a blue complex that absorbs at 595 nm.

5.4.1 Procedure described for RANDOX serum iron kit

The RANDOX serum iron kit contains following reagents: R1 chromogen (Ferene), R2 reductant (ascorbic acid), R3 buffer and CAL (calibration standard).

- Step 1. Add 200 µl of R3 buffer to the wells
- Step 2. Add 10 µl of R2 reductant to the wells
- Step 3. Add 50 µl of standard/sample/blank to the wells
- Step 4. Mix (gentle tap/pipet up and down)
- Step 5. Measure the absorbance (A1) at 595 nm (590-610)
- Step 6. Add 10 µl of R1 chromogen to the wells
- Step 7. Mix (gentle tap / pipet up and down)
- Step 8. Incubate for at least 15 min at 20-25°C
- Step 9. Measure the absorbance (A2) at 595 nm (590-610)
- Step 10. Creating a calibration curve

5.4.2 Calibration curve

The same reagents as described for the samples were used for the preparation of the calibration standard. The concentration of Fe in the calibration standard is known, which implies that when the absorbance of different concentrations of calibration standard is measured, these values can relate to the Fe concentration in the form of a linear function on an XY-axis (table 5 and fig. 13). By using this linear function, measured absorbance values of the analyzed sample can be calculated. To minimize variations between different samples that were analyzed on different days, the calibration process was repeated each day before sample analysis commenced. The different steps of the process are described hereafter for one animal species, namely the horses.

- Different amounts of CAL and deionized water were added to a well (table 4).
- Difference in absorbance was calculated by using the following formula $\Delta A = A2 - A1$. All calibration standards were analyzed *in duplo* resulting in A1 and A2 being the average of the initial absorbance (A1 values) and the average of the final absorbance (A2 values) (table 5).

Table 4: combinations of CAL and deionized water added to a well to create a calibration curve.

	Amount of CAL	Amount of deionized water
2 * CAL	10 µl	0 µl
1 * CAL	5 µl	0 µl
½ * CAL	50 µl	50 µl
¼ * CAL	25 µl	75 µl

Table 5: absorbance values of calibration standard in duplo with their respective known Fe concentrations. Δ A is the difference between the average of the initial and final absorbance of Fe.

	Absorbance A1		Average A1	Absorbance A2		Average A2	Δ A	Concentration Fe (µg/dl)
blank	0.037	0.037	0.037	0.056	0.056	0.056	0.019	0
¼ CAL	0.033	0.035	0.034	0.093	0.09	0.0915	0.0575	48.75
½ CAL	0.035	0.034	0.0345	0.132	0.131	0.1315	0.097	97.5
CAL	0.035	0.035	0.035	0.212	0.214	0.213	0.178	195
CAL*2	0.036	0.036	0.036	0.373	0.377	0.375	0.339	390

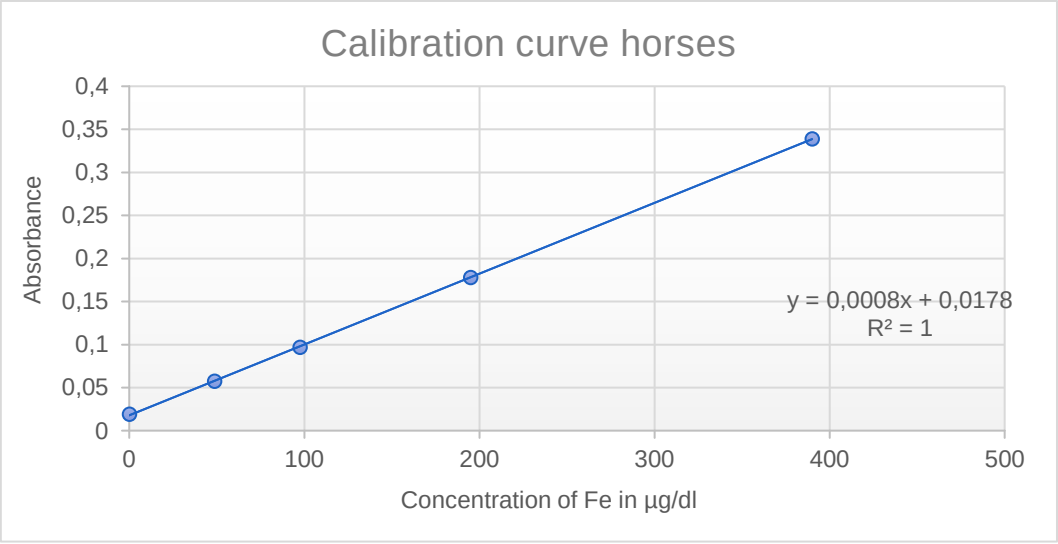

Figure 13: calibration curve for horse feces samples. A linear function is created by correlating the delta A values with their respective known Fe concentration values. This linear function can now be used to obtain the concentration of Fe in horse feces samples after absorbance values have been obtained.

- Step 11. Calculation of Fe concentration in supernatant based on the linear function obtained by the calibration curve. Following formula will be used: $x = \frac{(y-0.0178)}{0.0008}$ (with x as concentration of soluble iron and y as measured absorbance).
- Step 12. Calculation of Fe concentration in dried sample material (in mg/g) derived from the concentration of Fe in the supernatant (in µg/dl) by using following formula:

$$\frac{concentration\ of\ Fe\ \left(\frac{\mu g}{dl}\right) x\ volume\ of\ buffer\ (0{,}25\ dl)}{1000\ x\ sample\ weight\ (g)} = \frac{mg}{g} Fe\ in\ dried\ sample\ material$$

5.5 Mineral concentrations

The results of the total amount of minerals in both dried and milled feces and pH 4 supernatant after centrifugation were obtained by ICP-OES analysis. Following minerals were analyzed: iron (Fe), potassium (K), zinc (Zn), phosphorus (P), calcium (Ca), magnesium (Mg), cupper (Cu), sodium (Na), and manganese (Mn). In addition, only for iron RANDOX serum iron kit was used to determine the soluble iron concentration after centrifugation. The averages of the results of each animal species are summarized in in the supplement section.

5.6 Statistical methods

Principle component analyses (PCA) were performed to explore the associations between animal features (% grazer, body mass, captivity) and fecal mineral concentrations. Boxplots were made to visually evaluate the normal distribution of data. Pearson correlations were calculated to investigate the one-to-one relationships between variables. In addition, a two-way variance analysis (ANOVA) was performed, with captivity status (captive versus free) and feeder type (grazer versus browser) as main factors. Normality of the residuals and homogeneity of variance of each ANOVA model was verified by means of Shapiro-Wilk test in combination with the QQ-plot and by means of Levene test in combination with plotting fitted values vs. the residuals. When the interaction term was significant, a post hoc Tukey test was performed to discriminate which interaction terms were significantly different from each other. All analyses were done in SPSS 27.

6. Results

Fecal mineral concentration data was combined with animal features including % grazer, which represents the percentage of grass in the animals' diet, estimated body mass and the captive status (free roaming or under human care) (table 6). Blue table edges represent the results of the African herbivores from source 1 and green table edges represent the results of the animals under human care from sources 2,3 and 4. In addition, the results from the thesis dissertation of Deschoemaeker, (2020) are added to the results of this study as mentioned before. Black rhinoceros data is divided into free roaming black rhinoceros and black rhinoceros under human care (UHC).

Table 6: each animals species included in this study together with the % grass in their diet and estimated body mass in kg.

Common name of animal species	% Grazer	Estimated body mass in kg
African elephant	54	3900
Black rhinoceros	5	1017
Common warthog	/	56.5
Giraffe	0	1012
Impala	45	42.3
Roan Antilope	75	261
Waterbuck	95	215
Zebra	95	280
Black rhinoceros UHC	5	1017
Domestic horse	90	400
Indian rhinoceros	88	1850
Ring-tailed lemur	0	2.25
Tapir	0	147
White rhinoceros	95	1900

6.1 Comparing fecal mineral concentrations: similarities and differences

Fecal mineral concentrations of animals included in this study were presented by box-plots. The comparison of these box-plots confirms that there are major differences between mineral profiles of animals in the wild and the ones under human care. These differences were also confirmed by the following statistical analyses.

Wild animals have higher means of mineral concentrations for Ca, Mg, Mn, Fe and ASF Fe. The latter two are distinctively higher in wild grazers. However, these findings can most likely be explained by particularly high concentrations of the grazing common warthog as opposed to other wild animals (0.363 mg/g versus an overall average of 0.06 mg/g for other wild animals). Both Ca concentrations and the Ca to P ratio ($\frac{Ca}{P}$) are higher in wild animals, and especially in the browsing animal species. Animals under human care show higher concentrations of K, Zn, P and proportion of ASF Fe. In the cases of P the high concentrations are particularly present in browsing animals species.

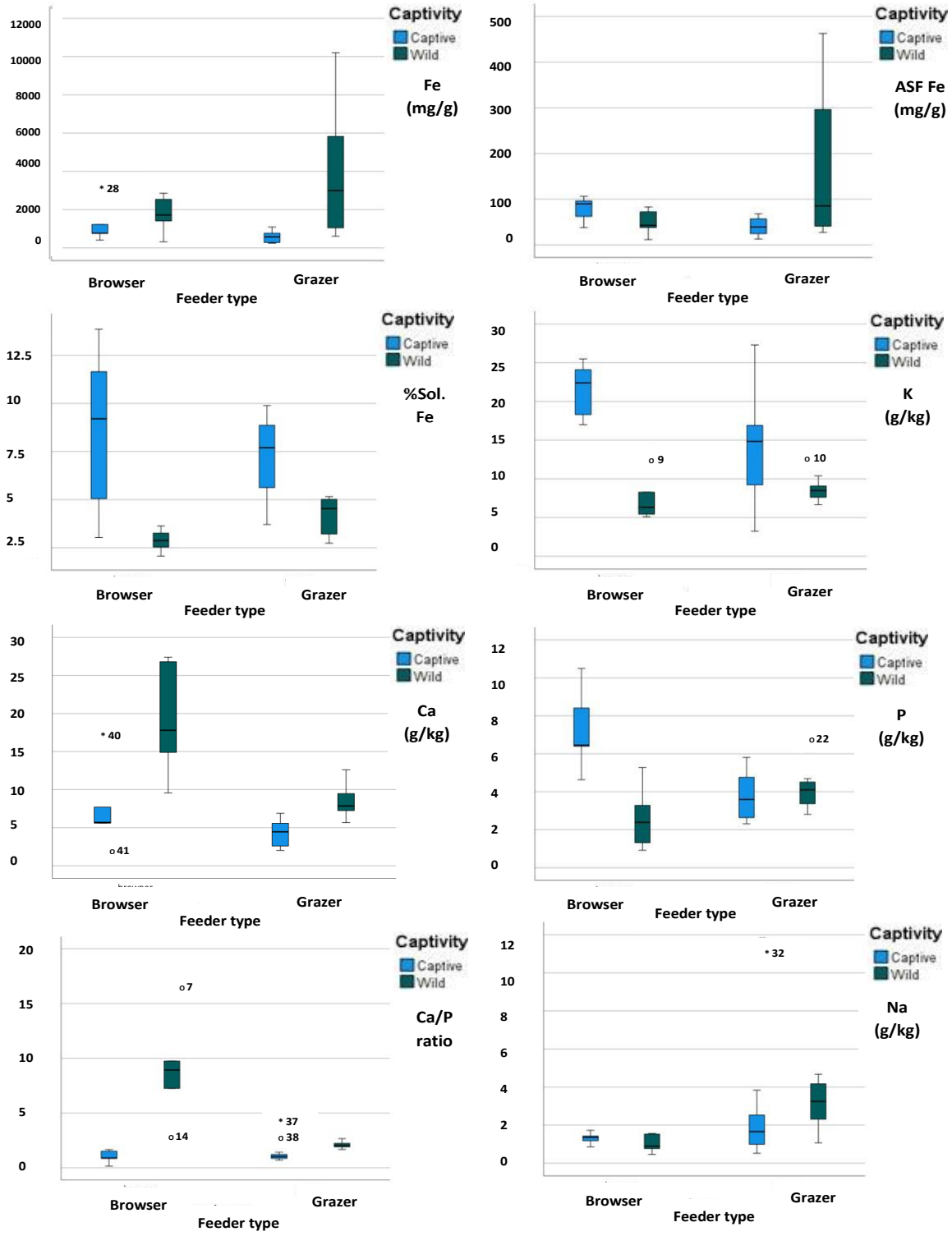

Figure 14: Box plots of fecal mineral concentrations in wild and captive browsing and grazing herbivores. The center line in the box indicates the median; the top and bottom of the box, quartile boundaries; whiskers, minimum and maximum values within 1.5 times the interquartile range of the quartile boundary; circles are outliers; and asterisks are extreme values.

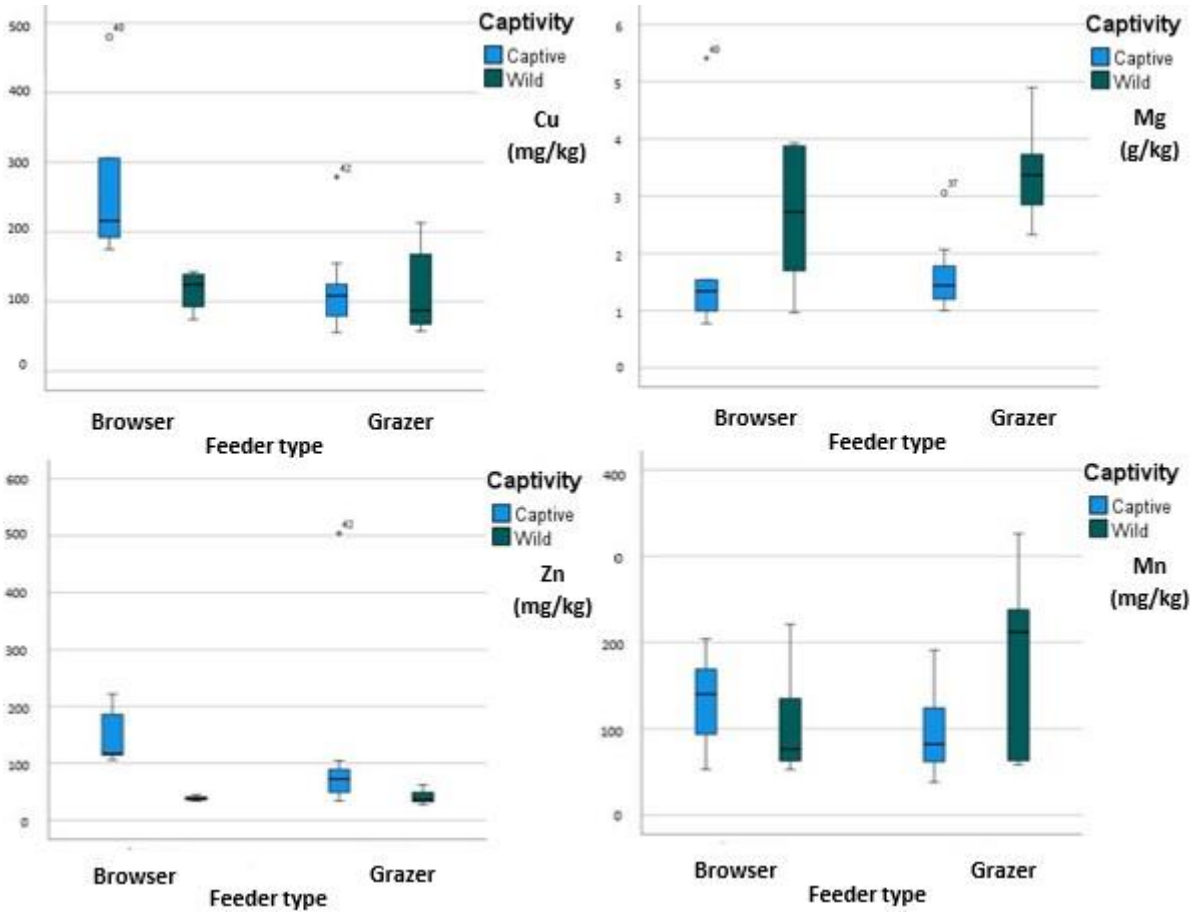

Figure 15: Box plots of fecal mineral concentrations in wild and captive browsing and grazing herbivores. The center line in the box indicates the median; the top and bottom of the box, quartile boundaries; whiskers, minimum and maximum values within 1.5 times the interquartile range of the quartile boundary; circles are outliers; and asterisks are extreme values.

6.2 Associations between fecal mineral concentrations and species characteristics

Principal component analysis (PCA) was performed on all data and the results are presented in figure 16. Additionally, PCA was performed on separate data from free ranging animals (fig. 17), animals under human care (fig. 18) and black rhinoceros data (fig. 19). PCA of all data reveals that there is a positive association between captivity and higher percentages of fecal acid-soluble Fe within the total Fe concentration ($\frac{\text{ASF Fe}}{\text{TF Fe}}$) (fig. 16). Conversely, acid-soluble and total iron concentrations are negatively associated with captivity. Other minerals that are negatively associated with captivity are Mg, Mn and Ca, whereas K and Zn are positively associated with captivity. The percentage grazing has little impact in the overall comparison because these values are close to the origin.

When comparing the PCA's of free roaming animals and the ones under human care (fig. 17 and 18), it can be noticed for the free roaming animals that percentage grazer is often positively associated with most minerals except the negative association with Ca. Conversely, for animals under human care percentage grazer is negatively associated with most minerals, including Ca. Generally, there are stronger associations between minerals for the captive data as opposed to the wild data. In particular, K and P have weak associations with other factors in the wild data (including percentage grazer), but

move to a stronger association (moving to the right) in the captive data, including a stronger negative association with percentage grazer.

PCA performed on black rhinoceros data requires careful interpretation due to limited data being available in this study (fig. 19). Yet, the analysis shows a strong positive association between captivity and K, Zn, P, Cu and proportion of acid-soluble Fe ($\frac{\text{ASF Fe}}{\text{TF Fe}}$), versus a negative association with Fe, acid-soluble Fe, Mn, Ca and Mg.

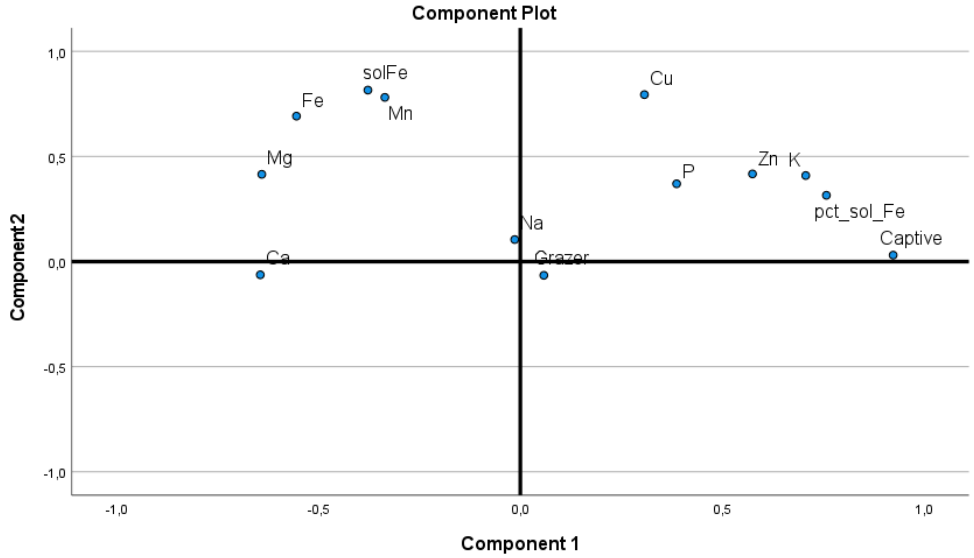

Figure 16: principle component analysis (PCA) of all data collected in this study with principal components of the different analyzed minerals (Ca, Cu, K, Mg, Mn, Na, P, Zn), solFe as the PC of acid-soluble iron fraction, Fe as PC of the total iron fraction and pct_sol_Fe as the PC of the percentage of acid-soluble iron within the total iron fraction.

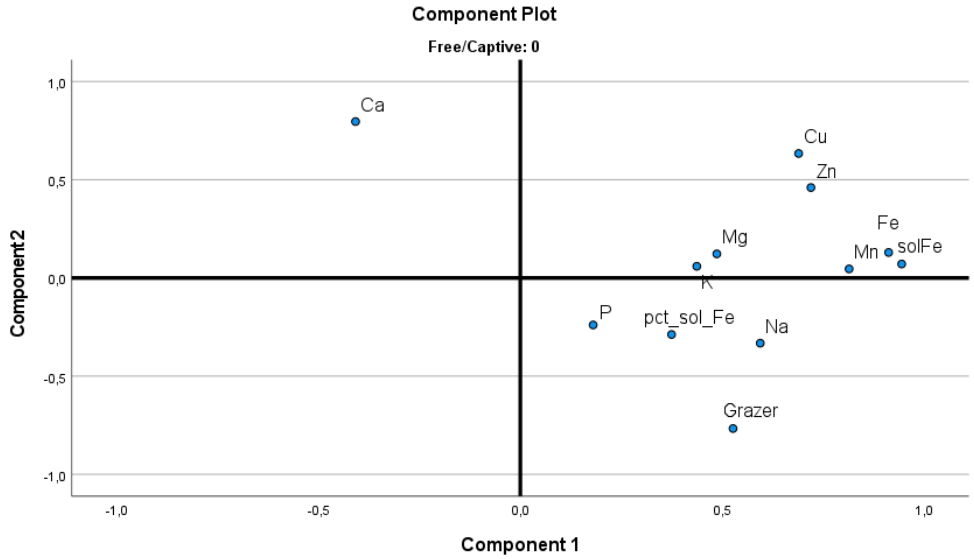

Figure 17: PCA of free roaming animals. Principal components are the same as mentioned at figure 16. In addition, 'Grazer' represents the PC of percentage grazer.

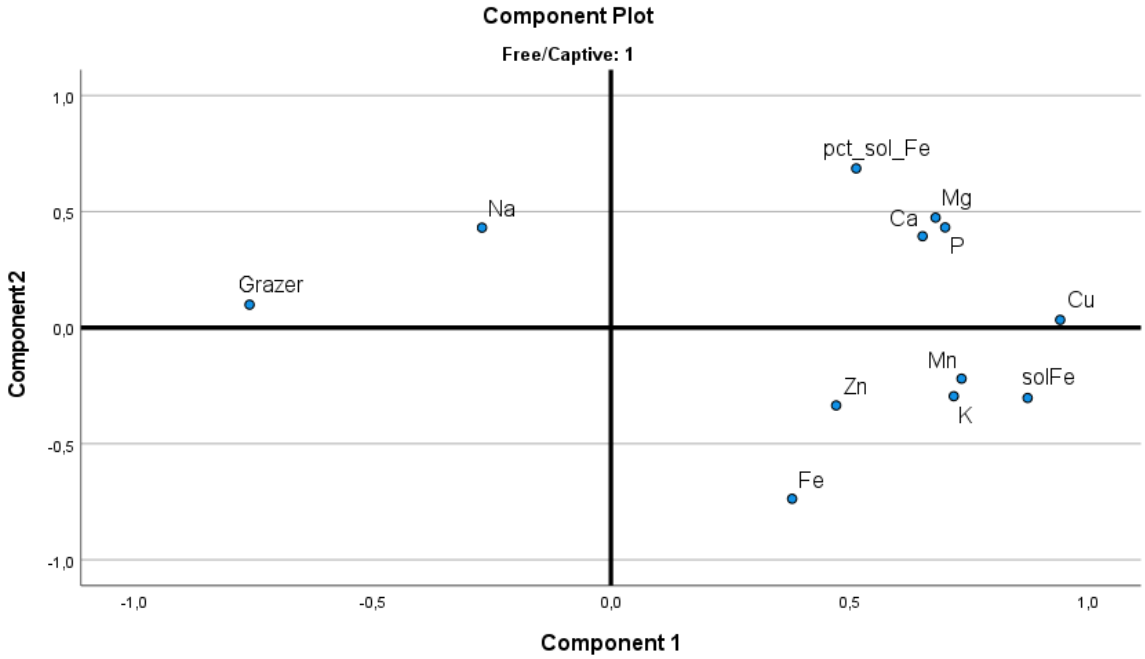

Figure 18: PCA of animals under human care. Principal components are the same as mentioned at figure 16. In addition, 'Grazer' represents the PC of percentage grazer.

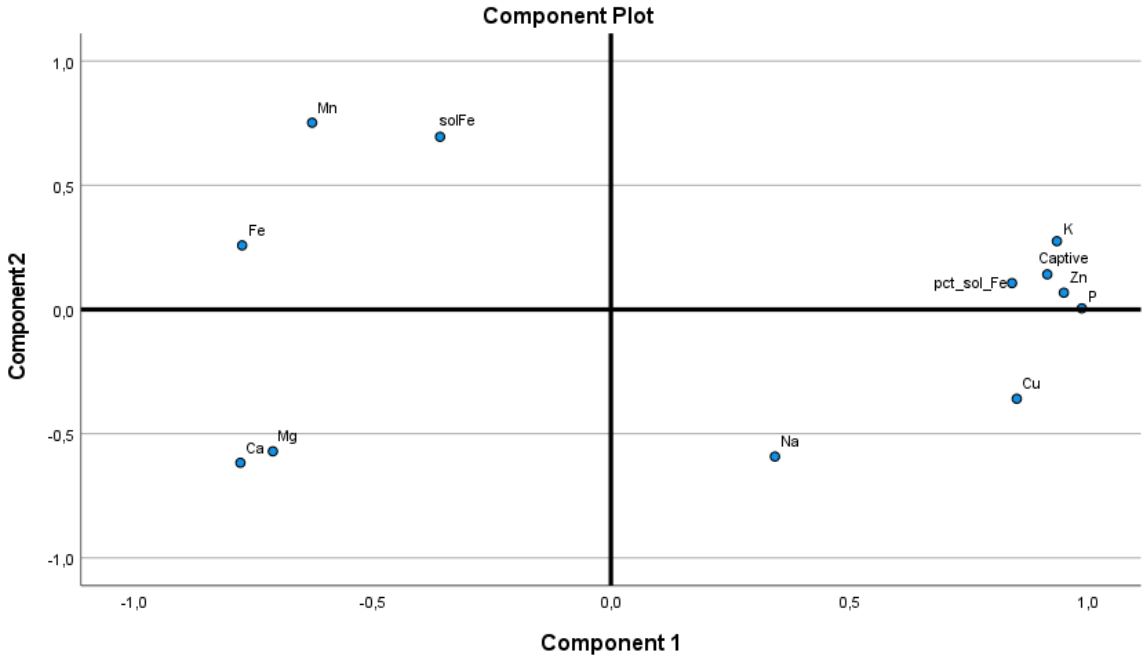

Figure 19: PCA of black rhinoceroses. Principal components are the same as mentioned at figure 16.

6.3 Searching for interactions between feeder type and captive status

The two-way variance analysis (ANOVA) was performed with feeder type (grazer vs. browser) and captive status (wild vs. captive) as main factors to determine whether these factors or their interaction affected the fecal mineral concentrations (table 8). The results revealed that significant interactions could be observed between captivity and feeder type for several minerals.

Table 7: two way ANOVA analysis. P-values of feeder type (browser versus grazer), captive status (wild versus free) and their interaction are presented. Standard error of the mean (S.E.M.) of each variable is added. If the interaction term is significant, a post hoc Tukey test was performed and different superscripts in a row indicate significant differences. ASF Fe represents the acid-soluble iron fraction of iron.

	Free roaming mean		Captive mean		S.E.M.	P-value feeder type	P-value captive status	P-value interaction
	Browser	Grazer	Browser	Grazer				
ASF Fe (mg/kg)	54.8 ^{b}	173.3 ^{a}	78.4 ^{ab}	40.2 ^{b}	13.0	0.020	0.059	0.003
% Sol. Fe	2.9	4.2	8.6	7.3	0.5	0.995	<0.001	0.104
Fe (mg/kg)	1556 ^{b}	3884 ^{a}	1269 ^{b}	568 ^{b}	289	0.030	0.002	0.007
Ca (g/kg)	15.7	8.5	7.3	4.3	0.9	< 0.001	< 0.001	0.111
Cu (mg/kg)	12.4 ^{b}	11.9 ^{b}	27.4 ^{a}	11.6 ^{b}	1.1	0.003	0.066	< 0.001
K (g/kg)	7.2 ^{c}	8.7 ^{c}	21.5 a	13.5 ^{b}	0.9	0.108	< 0.001	0.001
Mg (g/kg)	3.1	3.5	2.0	1.6	0.2	0.931	< 0.001	0.262
Mn (mg/kg)	120.7 ^{b}	180.9 ^{a}	131.9 ^{ab}	94.9 ^{ab}	9.9	0.255	0.061	0.024
Na (g/kg)	1.3	3.1	1.3	2.4	0.3	0.008	0.667	0.522
P (g/kg)	3.2 ^{b}	4.1 ^{b}	7.3 ^{a}	3.7 ^{b}	0.3 ^{b}	0.082	0.013	< 0.001
Zn (mg/kg)	40.8	41.7	149.2	98.2	11.3	0.396	0.001	0.260

6.4 Correlations between minerals and animal characteristics

The Pearson correlations confirm the distinctive differences for several minerals between wild and captive data, including percentage grazer (table 7).

A positive correlation can be seen between acid-soluble iron and % grazer for wild animals as opposed to the negative correlations for the animals under human care. This finding also applies to P.

In the wild, the proportion of acid-soluble Fe does not correlate with other minerals, whereas in captivity, positive correlations are seen with Ca, Mn and P. In addition, K only correlates with Mn in the wild animals, whereas in captivity, there is a positive correlation with acid-soluble Fe, Cu and a negative correlation with Na. Furthermore, wild data reveals a positive correlation with acid-soluble Fe whereas a negative correlation can be seen with total Fe.

*Table 8: Pearson correlations of percentage grazer with several analyzed minerals. Correlations are significant at the 0.05 level *, at the 0.01 level **and at the 0.001 level *** (two-tailed). Not significant correlations are presented with 'ns' superscript. Negative indices point to a negative association between the factors.*

		ASF Fe	TF Fe	Ca	Cu	K	P	Na	Mn
Free roaming	**% Grazer**	0.392*	0.317ns	-0.752***	-0.06ns	0.164ns	0.400*	0.561**	0,344ns
Captive		-0.642**	-0.465*	-0.45ns	-0.694**	-0.694*	-0.565***	0.196 ns	-0,33ns
Free roaming	**ASF Fe**			-0.35ns	0.627***	0.376ns	0.088ns	0.468*	0.769***
Captive				0.379ns	0.773***	0.660**	0.447ns	-0.200ns	0.805***
Free roaming	**TF Fe**			-0.32ns	0.619***	0.34ns	0.034ns	0.474*	0.760***
Captive				0.15ns	0.25ns	0.327ns	0.007ns	-0.17ns	0.374ns

7. Discussion

7.1 Remarks on the study

As previously mentioned, due to the coronavirus pandemic, the sample collection in this study was opportunistic. As a result, we were not able to collect samples from both free roaming animals and their counterparts under human care from each animal species. This means that the comparison of feeder type and captive status includes differences in animal species and number of samples. In addition, the number of samples of each animal species is limited. Hence, interpretation of results on species level warrants caution. Furthermore, it should be mentioned that some variables included in the study (such as body mass and percentage grazer) are collected from different sources which may have used different methods to obtain these values. Finally, it should be remarked that our dataset is extremely limited so only preliminary conclusions can be made.

The dietary samples collected in this study and the data on ICP-OES performed on the pH 4 supernatant of feces samples could not be analyzed in due time. These data will be used in future studies to complete the hypotheses and conclusions that were formed in this study.

7.2 Differentiating the acid-soluble and acid-insoluble fraction of minerals

Separating the absorbed from the unabsorbed mineral fraction in feces would provide an interesting proxy for mineral bioavailability. We assumed that a distinction can be made through acidification of the feces to a typical duodenal pH followed by ultracentrifugation. This fecal analysis method provides three fractions, the acid-soluble fraction of minerals (ASF), acid-insoluble fraction of minerals (AIF) and the total fraction of minerals in the feces (TF). These fractions can be expressed by the following formula $ASF + AIF = TF$.

Assuming that absorbable minerals are acid-soluble, fecal minerals that are soluble after acidification can be considered the sum of (1) the fraction of minerals that were absorbed but have flowed back into the intestine, and (2) the fraction that was available for absorption but has not been absorbed. The sum of these two fractions forms the acid-soluble fraction of minerals (ASF). Assuming that the typical pH-value found in the stomach and the small intestine (pH 4) represents the highest solubility of minerals, this would imply that the fraction that is insoluble after this process represents the fraction

that would not be absorbed at any stage of the digestion process. This can be explained due to a gradually increasing pH in the segments of the digestive tract that follow the stomach and duodenum.

Acknowledging that the soluble and the insoluble fractions may not completely reflect the absorbed and unabsorbed fraction respectively, we postulated that this non-invasive straightforward method could identify at least relative differences in mineral bio-availability between diets for a particular animal species, but may also be of use to study mineral metabolism between species on similar diets.

ICP-OES allows us to perform an exact quantitative analysis of each mineral of interest, both in the dried feces samples and the supernatant after centrifugation. The RANDOX serum iron kit determines the amount of iron present in the supernatant after centrifugation and a similar result as ICP-OES of the supernatant is expected (Serhan et al., 2020). However, the negative aspect of RANDOX is that it only represents the available mineral fraction and may not detect soluble strong complexes between iron and other molecules in the supernatant (such as proteins) and thus may be different from the ICP-OES result on the supernatant. Ferene is a chelator that will be measured spectrophotometrically after binding with ferrous iron. If the affinity of ferene for iron is lower than iron chelates in the supernatant, ferene will not be able to bind and that iron fraction will thus not be measured. Nevertheless, it is assumed that ferene's strength as a chelator is high enough to still be able to remove bound iron from most protein complexes.

Despite these downsides, RANDOX serum iron kit is still believed to be a usable, less expensive and less time-consuming alternative for ICP-OES. The obtained data are, therefore, used to reveal the acid-soluble iron fraction in the supernatant of the samples after centrifugation. The results still represent the acid-soluble fraction of iron, but the measured concentration may be not entirely accurate. In the future, further refinement of this technique is required to validate this method as an equivalent alternative to ICP-OES analysis on supernatant.

7.3 Mechanisms behind the obtained associations through principal component analysis (PCA)

When large amounts of data are collected, it becomes more difficult to interpret the results and to form hypotheses or conclusions. In this study, the objective was to discover similarities and differences in mineral homeostasis of grazers and browsers, both free roaming as the ones under human care. Comparing mineral excretion in these four groups and discovering correlations is time consuming and ineffective due to the high amount of animal species and variables. In addition, univariate analysis, which means that two variables are compared without the inclusion of covariation of other variables, can be dangerous because valuable information on variable correlation can be neglected (Bro and Smilde, 2014). Principle component analysis (PCA) is a statistical method that can offer solace to this problem. PCA reduces the dimensionality of large datasets while preserving as much statistical information or variability as possible. Variability can be maintained by developing new variables, the principal components, that have following characteristics. 1) the PCA are linear functions of the original variable values of the dataset, 2) they maximize variance, and 3) they have no correlation with each other (Jollife and Cadima, 2016).

By using this technique, we aimed to explore the data and to see whether the different variables of our study are related to each other. The loading plots presented before (fig. 16 - fig. 19) contained principal components (PCs) of different variables (fig. 16). Each axis on the plot represents the grade of association between the PCs. The greater the distance between two PCs along one axis, the less these two are associated with each other. Conversely, the closer two PCs are to each other along an

axis, the higher the grade of association can be considered for these two. Finally, the closer one principal component is to the origin, the less impact it has on the overall comparison. This technique allows to demonstrate that certain connections between variables are either present or are lacking. However, it does not define the type of connection. This implies that by using this technique, mainly hypotheses can be generated that need to be more thoroughly investigated in future studies to draw actual conclusions.

Associations between PC's could be investigated along more than two components. In this study, however, we limited the PCA to only two components for the purpose of clarity and visual simplicity. We concluded that the observed associations can be appointed to be relevant when principal components reach further than -0.5 or 0.5 on one component axis. A positive association between factors in such analysis indicates that the factors behave in the same way. This can be illustrated by following example. The observed positive association between potassium and acid-soluble iron in animals under human care indicates that a rise in one of these minerals' concentrations is likely to be accompanied by a rise in the other. On the contrary, the negative association between percentage grazer and acid-soluble iron in animals under human care could indicate that animals with less amounts of grass in the diet (i.e. browsers) are likely to have higher concentrations of acid-soluble iron when placed under human care.

7.4 Identified clues for future research

The general conclusion of all results is that the mineral profiles of wild animals versus the ones under human care differ distinctively. All analyses presented that not only captive status but also feeder type are of great importance in mineral profiles of animal species. The significant interactions between feeder type and captive status that were observed for almost all minerals could be interpreted as follows. Either there is a difference in fecal excretion of minerals between grazers and browsers under human care as opposed to the wild, or alternatively, the difference in fecal mineral excretion between captive and wild depends on the feeder type. Nevertheless, the results of this study suggest that mineral nutrition of captive herbivores is most challenging in browsers. These results strongly suggest that the mineral profiles of wild and captive diets are likely very different, as already published by for instance Claus et al. (2007b).

Correlations of factors often resemble the either positive or negative associations that are seen in PCA. This is also generally true in this study. A browser-grazer dichotomy on fecal mineral excretion was apparent from the correlations but the impact of captivity on these correlations was remarkable, again demonstrating that captive diets and potentially other captivity-related factors have a profound impact on the mineral metabolism of these herbivores. The aspect of dietary mineral availability in captive-bred wild herbivores has been neglected. Given the known disorders related to minerals in captive animals, including iron overload disease, research on mineral nutrition of wild herbivores requires more attention.

7.5 Associations between minerals and animal physiology

7.5.1 The role of bile salts in mineral homeostasis

Bile salts play an important role in the excretion of several minerals, such as Ca, K and Mn (Dijkstra et al., 1991). Although the hepatobiliary excretion pathway for iron was ignored in the past, currently it is considered to play an important role for iron excretion as well (Mercadante et al., 2019). For the black rhinoceros in particular, we hypothesize that bile salt metabolism could be an important factor in iron homeostasis and thus play a significant role in the pathogenesis of IOD.

Browser diets generally contain higher concentrations of tannins which have several antinutritional properties. To reduce the negative effects of these tannins, browsing animals species have developed specific adaptations throughout evolutions such as the tannin binding proteins in their saliva (Codron et al., 2019). The production of bile salts by herbivores in general is an adaptation that serves the same purpose. A great part of the produced bile salts will bind tannins in the intestines and form unabsorbable complexes that will be excreted with the feces and thus serve a detoxifying function (McArthur et al., 1991). We hypothesize that due to the presence of greater proportions of secondary plant compounds in browser diets, the detoxifying function of bile salts is even more pronounced in browsers when compared with grazers. A higher production of bile salts in therefore expected as an evolutionary adaptation to high-tannin diets. A smaller fraction of these bile salts will bind other components in the intestine, such as minerals. These bile salt-bound minerals will be absorbed but can later be secreted into bile again and be eliminated through feces because of the enterohepatic circulation (Mercadante et al., 2019). The major differences in fecal mineral profiles between browsers and grazers presented in this study are assumed to be the result of the specific adaptations of these groups to their specialized diet and the distinctive differences between their wild diets and their diets under human care.

7.5.2 Contribution of bile salt metabolism to IOD in the black rhinoceros

Black rhinoceros diets in the wild, as browsing animals, contain high amounts of tannins. These tannins limit the bioavailability of iron by forming unabsorbable complexes. Studies in humans have shown that complexes formed between condensed tannins and bile salts lead to higher fecal excretion of bile salts. This results in a reduced functionality of bile salts and therefore absorption of lipids becomes limited (Li et al., 2019). Subsequently, it is believed that adding tannins to the diet could prevent and ameliorate metabolic syndrome in humans (Matsumoto et al., 2011). In the case of black rhinoceroses we hypothesize that to be able to take up sufficient amounts of iron from the diet, great amounts of bile salts are produced by these animals to enhance complex formation with tannins and allow an increased absorption of iron. This hypothesis implies that the lack of sufficient amounts of browse, and thus of tannins, in diets under human care leads to two things. Firstly, generally high dietary iron content combined with less amounts of tannins allows the absorption of great amounts of iron. Secondly, because the produced bile salts cannot bind tannins under human care, they are able to bind other minerals in excess and enhance their absorption. Studies have shown that bile salts will indeed lead to increased iron absorption (Sanyal et al., 1994). Because Fe absorption in the black rhinoceros, as opposed to other minerals, depends on bile salt metabolism, this may lead to excessive iron uptake and storage. Why the incidence of IOD is that high in black rhinoceros compared with other browsing herbivores, remains to be elucidated. Yet, other browsing species have been reported to develop excessive hepatic iron accumulation such as tapirs and lemurs (M. Clauss et al., 2009; Clauss and Paglia, 2012; Wood et al., 2003). Possible similarities between diet and physiology of these species is of particular interest and needs further exploration.

7.5.3 Competition for binding with bile salts: the role of potassium and phosphorus

Dierenfeld et al., (2005) observed that potassium accumulation in the black rhinoceros liver correlates with age when kept under human care. It is most likely that this finding is the result of high potassium concentrations in the forage fed in zoos, compared with the low potassium concentrations in wild browse (Clauss et al., 2007b). This accumulation was not suspected to be problematic. However, we hypothesize that mainly potassium, but also phosphorus, could play an important role in iron overload disorder in the black rhinoceros.

Potassium absorption happens distinctively more efficient and in greater concentrations than iron. This can be explained due to the fact that K is present in high concentrations and is readily solubilized in feed and mineral supplements. Furthermore, K is almost entirely absorbed by paracellular absorption (Goff, 2018) and thus has no need for bile salts to be absorbed. The same principle applies to phosphorus. Black rhinoceros diets under human care often contain P supplements in order to reduce the occurrence of hemolytic episodes but even without adding supplements, deficiencies are very rarely reported. On the contrary, P concentrations are often limited in wild browse (Clauss et al., 2007b). Phosphorus absorption presumably occurs via passive paracellular absorption when normal to high amounts of P are available but P can also be absorbed via active transport when there is marginal supply. Potassium excretions depends mostly on complex formation with bile salts whereas P is almost entirely reabsorbed and only small amounts are excreted, mainly in the feces (Goff, 2018).

Due to the differences in homeostasis between potassium, phosphorus and iron, we hypothesize that a competition exists between K and Fe in the liver for binding with bile salts in order to be excreted. We believe that potassium's high affinity for bile salts leads to more binding in the liver as opposed to iron which results in the accumulation of Fe in the liver. Although P excretion is relatively low, it is possible that when this mineral is present in abundance in diets under human care, the same competition can occur between P and Fe. Following this hypothesis, one could expect a decrease of iron excretion of minerals with age under human care, whereas excretion of other minerals will increase. Several findings in this study support this hypothesis. Positive associations are seen between all animals under human care and K. The same image is seen for the black rhinoceros, combined with a positive association for P. On the contrary, there are generally weak associations between these minerals and other factors for wild animals. Animals under human care also have higher fecal concentrations of both K and P.

7.5.4 Hypotheses-generating findings: food for thought

The results in this study have presented the opportunity of seeing connections between several minerals which could be of greater importance than earlier assumed. The possible competition between Fe, P and K illustrates that interactions of minerals may be of greater importance for understanding the mineral homeostasis and their beneficial and disadvantageous effects than assumed in the past. Several findings in the results of this study could also be involved in the mineral-related health concerns of animals of different feeder-type and captive status. Therefore, relevant observations will be discussed hereafter.

7.5.4.1 An iron curtain: major differences between captive vs. wild and grazer vs. browser

Captivity is positively associated with a higher percentage of fecal acid-soluble Fe within the total Fe ($\frac{\text{ASF Fe}}{\text{TF Fe}}$), whereas the acid-soluble and total Fe concentrations are negatively associated. The same image is seen for black rhinoceros data. This confirms that with time in captivity, iron excretion will decrease hence invigorating the hypotheses mentioned before.

As expected, the total concentration of Fe in feces is lower for animals under human care when compared with free roaming animals. African soil generally contains great amounts of Fe and, therefore, the same is expected in the forage in those regions. Studies have shown that grazing cattle in such locations often have excessive iron storage in the liver (Dermauw, 2013). Assuming high iron availability in the forages of wild animals, this would explain the observed higher fecal iron concentrations in wild animals as opposed to animals under human care.

The difference in fecal iron excretion between wild grazers and browsers can partly be explained by the particularly high fecal Fe concentration of the grazing common warthog as opposed to other wild animals. The ingestion of great amounts of soil due this animals' rooting behavior would be a possible explanation for this finding (Treydte et al., 2006). This theory could be applied to all grazing animals as grasses are generally found close to the ground, which inevitably leads to ingestion of iron-rich soil (Codron et al., 2019). This is also supported by the finding that the fecal Fe concentration of the common warthog is not an outlier and, therefore, we assume that a similar result would be obtained if this species would be left out of the analysis. Higher fecal Fe concentrations of free roaming animals may also be due to combinations of lower Fe absorption and increased Fe secretion through bile as underlying reasons. As mentioned before, it is possible that lower absorption of iron is facilitated by some evolutionary adaptation of browsing animals. All these reasons suggest that the wild diet does not necessarily has to contain lower iron concentrations, although this has been a recurring claim in multiple studies. The comparable fecal iron concentration between wild browsers and grazers supports this assumption.

The negative correlation between ASF Fe and percentage grazer under human care as opposed to the positive correlation in the wild indicates that for browsers, the diet presented under human care has a higher availability of iron as opposed to the wild. The opposite is true for grazers. Higher concentration of fecal ASF Fe in captive browsers as opposed to the wild browsers confirms this finding. In addition, higher proportions of acid-soluble Fe within the total Fe in animals under human care indicate that from the relatively low total concentration of excreted Fe in these animals, the greatest part was not complexed in any kind and thus was available for absorption. These findings would make sense as diets under human care lack chelating compounds that lead to precipitation, hence maximizing iron bioavailability, and generally contain high concentrations of iron (Clauss and Paglia, 2012). This theory is also emphasized by the finding that the proportion of acid-soluble Fe within the total Fe is higher in browsing animals where dietary iron is less available due to the presence of tannins. Hence, based on the combination of higher iron availability under human care, particularly for browsers, and the lower iron excretion we assume that iron could accumulate in the body. Furthermore, these findings suggest that the development of excessive iron storage under human care is not solely a dietary issue and that it is possible that certain iron regulation mechanisms that occur in the wild fade out when placed under human care.

When percentage acid-soluble iron is compared between the animals in this study, it is clear that animals under human care indeed have higher values as opposed to the wild animals (fig. 20). Especially browsing animal species under human care, such as the lemur and the tapir, show distinctively higher percentages. For the black rhinoceros, it is visible that the percentage acid-soluble

Fe of captive animals is higher than the free roaming animals. In our hypothesis, the reason why some grazers under human care still show higher percentages than the black rhinoceros can be devoted to the difference in bile salt metabolism in these animals. As mentioned before, when competition between Fe, K and P occurs in terms of binding with bile salts for excretion, iron excretion may be reduced. This assumption is supported by the findings of Deschoemaeker, (2020) where the proportion acid-soluble iron within the total iron distinctively increased with age in black rhinoceroses under human care. Following this hypotheses we assume that in black rhinoceroses under human care the major problem lies within the decreased iron excretion with age. Many studies presented the recurring claim of the progression of IOD with age in animals under human care (Clauss and Paglia, 2012; Dierenfeld et al., 2005; Miller et al., 2016). This finding may be explained by the potentially important role of bile salt metabolism in Fe excretion as well. Although speculative, it is possible that excessive iron storage gradually will lead to a less functioning liver, hence leading to decreased bile salt production and even further decrease in iron excretion. Following this hypotheses, the higher percentages seen in grazers can potentially be devoted to a difference in bile salt metabolism between these animals, as mentioned before, because it is assumed that iron absorption in grazers does not depend on bile salt metabolism in particular.

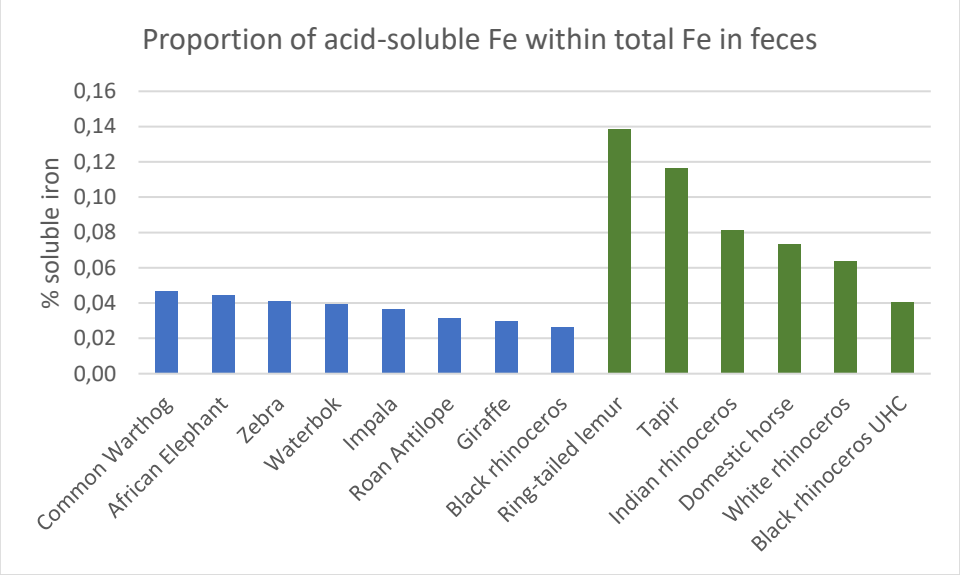

Figure 20: bar chart of proportion of acid-soluble iron within the total iron Fe in feces of animals included in this study. Blue bars represent the wild animals; green bars represent the animals kept under human care.

The high percentages seen in the tapir and lemurs could be appointed to the same assumptions made for black rhinoceroses as these animals are browsing animals that are susceptible to IOD under human care as well. However, it is clearly visible that the results differ distinctively between these animals. Our study only included one tapir and one pooled sample of lemurs. Because these samples were solely obtained from animals kept under human care, we are unable to compare the results with free roaming animals. In addition, the low sample size of this study and the lack of knowledge on the diets of these animals in their facilities limit our ability to draw conclusions. Clauss et al., (2009) suggested that Fe concentrations may be different in diets presented to tapirs under human care depending on the location of the animal facility. Diets presented to tapirs in Europe generally contain lower amounts of pelleted food, which is rich in iron. Conversely, North-American zoo diets generally contain greater proportions of pellet in the diet. It is therefore assumed that tapirs that are held in North-American

animal facilities may be more susceptible to iron overload. The occurrence of IOD in captive lemurs has been appointed to be of dietary origin as well, but as opposed to tapirs and other lemur species, the ring-tailed lemur is considered less susceptible to IOD (Clauss and Paglia, 2012; Wood et al., 2003). Both animal facilities reported that no signs of iron overload were witnessed in animals of these species (personal communication). However, based on the limited results of this study, it can be seen that a great fraction of the excreted iron was available for absorption. Further conclusions can only be drawn when more data is available hence emphasizing the need for further research.

7.5.4.2 Calcium, phosphorus and magnesium: impact of dietary differences in mineral content

Previously conducted studies showed that generally all hindgut-fermenting animal species, including the black rhinoceros, tapir and domestic horse, have higher apparent absorption coefficients for Ca and that this coefficient increases with an increasing dietary $\frac{Ca}{P}$-ratio as opposed to ruminants (Clauss et al., 2007b; Marcus Clauss et al., 2009). It has been speculated that this is an evolutionary adaptation to render more P available in the hindgut (Clauss et al., 2007b).

The negative association between percentage grazer and Ca for wild animals suggest that there are distinctive differences in Ca concentrations of browser versus grazer diets as well. Browsers, such as the black rhinoceros and tapir, have adapted to a high calcium and low phosphorus diet whereas grazer diets generally are not rich in Ca (Marcus Clauss et al., 2009; Dierenfeld et al., 2005). Grazers and browsers under human care often receive diets that are similar in consistency. We hypothesize that lower concentrations of this mineral under human care could explain the decrease in fecal excretion. This is more pronounced in browsers as there is even a larger difference between Ca concentrations between wild and captive diets. Relatively lower Ca concentrations combined with higher P concentrations in captive diets result in a decrease in $\frac{Ca}{P}$-ratio. It is possible that browsers, including the black rhinoceros, cannot cope with this decreased ratio due to evolutionary adaptations to their diet.

Although some studies suggest to not supplement Ca to browsers under human care, these results reveal that it may be beneficial to supplement Ca for both browsers and grazers to meet the concentrations that are absorbed in the wild. Subsequently, reducing P concentrations for browsers under human care could also be beneficial. However, great caution is needed to not increase the occurrence of hemolytic episodes in black rhinoceroses. The benefit of these mineral alterations in the diet is a possible reduction in competition between P and Fe for binding bile salts which in turn may lead to lower storage of iron in the liver.

The absorption mechanism for Mg is similar to that of Ca in hindgut fermenters and ruminants (Marcus Clauss et al., 2009). In addition, wild diets are generally high in Mg (Dermauw, 2013). Therefore, the high fecal Mg concentration in wild animals as opposed to animals under human care that is seen in this study's results may also be explained by lower Mg concentrations in diets under human care. A strong positive association between Ca and Mg under human care whereas a weak association is seen in the wild supports this conclusion.

7.5.4.3 Salt and copper: possible associations of Na and Cu with iron overload

High fecal concentrations of cupper in captive browsers can be explained by two hypotheses. This may originate either from Cu-rich diets presented under human care but also from a lower bioavailability due to for instance higher levels of antagonists such as Zn, Fe, S and Mo. High dietary iron is an important antagonist for Cu absorption. Currently it is believed that Fe does not compete with Cu for

absorption at the intestinal level, but rather at the level of tissues that want to take up Cu, such as the liver (Goff, 2018). Because captive browsers are considered most susceptible to iron overload, the high copper excretion that is seen in browsers under human care may be explained by the iron accumulation of these animals (Dermauw, 2013). The acid-soluble fraction of Cu could clarify which of these two hypotheses is more likely. In the case of high levels of antagonists, the latter would form complexes with Cu and reduce its ability to be absorbed. Hence, Cu will remain in the acid-insoluble fraction (AIF Cu). On the contrary, if Cu is just present in excess in diets under human care, Cu will be absorbed in great numbers but will flow back into the intestine thereafter. This implies that a greater concentration of acid-soluble Cu will be seen (ASF Cu).
A comparable image is seen for Zn and P in browsers under human care, whereas the three other groups showed distinctively lower concentrations. Diets containing high amounts of iron often lead to a reduced availability of other minerals such as cupper (Cu), zinc (Zn) and phosphor (P). Dierenfeld et al., (2005) could not confirm this theory in their research and suggested that minerals in diets under human care are relatively in proportion. Our results, however, illustrate the theory that is explained for Cu could be applied for these minerals as well. Following this theory, we may assume that captive diets are indeed higher in iron content.

Sodium has been considered a limiting factor for African herbivores in general as African soils generally have lower sodium concentrations and the use of natural Na licks has been observed in the natural environment of these animals (Birhanu et al., 2018; Dermauw, 2013). Browsing animal species such as the black rhinoceros and African elephant have lower apparent dry matter digestion coefficients which implies that a higher intestinal passage rate can be expected and thus more feces production (Codron et al., 2019). Clauss et al., (2007b) observed a lower Na absorption coefficient in black rhinoceroses as opposed to horses and hypothesized that the excretion of great amounts of Na in these animals could be appointed to the high endogenous fecal loss of this mineral in browsing animal species as opposed to grazing species. Because fecal Na excretion can be considered an effect of both fecal bulk and fecal water content, a constant relation between Na excretion and fecal water is expected. Furthermore, the presence of secondary plant compounds in browse could additionally lead to increased Na excretion in free roaming browsing animals (Robbins, 1993). Because browsing animals produce relatively more feces, the absolute amount of Na is expected to be higher, but a similar or lower concentration of fecal Na concentration in the browsing animal species involved in this study would be expected. Our results support this theory by illustrating that both captive as free roaming grazers showed higher fecal concentrations of Na. More specifically, the same finding has been seen when comparing the results of the domestic horses with black rhinoceroses where horses showed higher fecal Na concentrations.

One other study that involved the browsing tapir showed that similar Na absorption coefficients could be observed between horses and tapirs. This study assumed that the particularity that was observed in black rhinoceroses is not a characteristic of all browsing species per se (Marcus Clauss et al., 2009). However, generally lower fecal Na concentrations of all browsing animals as opposed to the grazing animals included in this study indicate that Na homeostasis may in fact be a specific characteristic of browsing animals (fig. 21). It should once again be mentioned that for the interpretation of these results caution is warranted due to the small sample size of this study. Further research is needed with more comparative data and exact fecal amounts to confirm our findings and to draw conclusions.

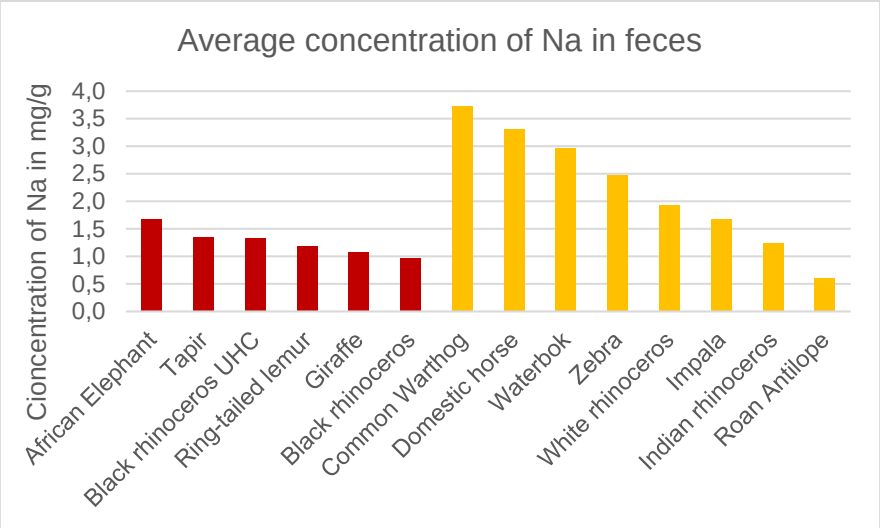

Figure 21: bar chart of average fecal Na concentration of animals included in this study. Red bars represent the browsing animals; orange bars represent the grazing animals.

The results of this study also revealed that in the wild, sodium has several significant correlations with other minerals such as ASF Fe, K and Mn whereas under human care, a negative correlation with K is the only correlation with significance. In addition, wild animals show a positive correlation between acid-soluble Fe and Na whereas a negative correlation is seen between Na and total Fe. A possible hypotheses for these findings could be that some minerals that are present in high concentrations under human care, such as K and P, may interfere with Fe metabolism in captivity. Potassium and phosphorus may act as antagonists on Na digestion. As a result, a potential regulating role of Na on Fe metabolism in the wild may be suppressed when kept under human care.

8. Conclusion

The results of this study clearly demonstrated that fecal mineral profiles differ significantly between free roaming animals and the ones kept under human care. As most likely cause, the diets presented under human care are likely to have different compositions when compared to the wild diets. Also, distinctive differences in fecal mineral excretions were observed between browsing and grazing animal species which may be appointed to species-specific adaptations to the diet. Study of the mineral nutrition of captive versus wild herbivores may reveal the problems that mostly browsers are facing in captivity. Disorders such as IOD that mainly occur in browsing animal species when placed under human care are assumed to be of dietary origin. Although the importance of the diet on mineral homeostasis has been supported by this study, the results also emphasize that morphophysiological differences between browsers and grazers will determine how animals deal with changes in dietary mineral supply.

To reduce captivity-induced problems in the future, the focus of research should lie on comparing the entire physiology of animals and considering their specialized evolutionary adaptations when composing their diet under human care. In the case of black rhinoceros, the role of bile salt metabolism on iron overload should be subject to more research. Differences in mineral profiles between animals of different feeder type and captive status emphasize the need to consider the role of all minerals in the diet of these animals. It is highly likely that captivity-induced problems such as IOD are not the result of one mineral imbalance but are rather of multifactorial nature.

9. References

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10. Supplements

Averages of soluble and total iron concentrations in mg/g for each animal species

Common name of animal species	RANDOX ASF Fe average (mg/g)	ICP-OES TF Fe average (mg/g)
African elephant	0.0629	1.4062
Black rhinoceros	0.0657	2.4733
Common warthog	0.3629	7.7233
Giraffe	0.0311	1.0530
Impala	0.0801	2.1800
Roan Antilope	0.0258	0.8197
Waterbuck	0.1230	3.1033
Zebra	0.0340	0.8260
Black rhinoceros UHC	0.0654	1.6037
Domestic horse	0.0220	0.2988
Indian rhinoceros	0.0598	0.7330
Ring-tailed lemur	0.1061	0.7660
Tapir	0.0897	0.7700
White rhinoceros	0.0503	0.7920

Averages of total K. P and Zn concentrations in mg/g for each animal species

Common name of animal species	ICP-OES K average (mg/g)	ICP-OES P average (mg/g)	ICP-OES Zn average (mg/g)
African elephant	9.9967	2.2333	0.0400
Black rhinoceros	8.8967	1.4030	0.0416
Common warthog	9.8567	3.5667	0.0550
Giraffe	6.0400	3.7933	0.0355
Impala	3.0033	4.5967	0.0480
Roan Antilope	5.5467	5.0867	0.0399
Waterbuck	8.2267	4.7600	0.0365
Zebra	7.9467	3.9033	0.0335
Black rhinoceros UHC	21.1667	5.8267	0.1127
Domestic horse	8.3217	4.6467	0.0511
Indian rhinoceros	19.8333	3.2367	0.0711
Ring-tailed lemur	25.5000	0.1061	0.1061
Tapir	18.3000	0.0897	0.0897
White rhinoceros	15.7800	2.8460	0.1709

Averages of total Na. Ca and Mg concentrations in mg/g for each animal species

Common name of animal species	ICP-OES Na average (mg/g)	ICP-OES Ca average (mg/g)	ICP-OES Mg average (mg/g)
African elephant	1.6798	14.998	2.9967
Black rhinoceros	0.9653	14.557	1.4623
Common warthog	3.7367	8.027	4.2400
Giraffe	1.0757	23.533	3.8467
Impala	1.6800	15.533	4.4600
Roan Antilope	0.6010	10.533	3.1333
Waterbuck	2.9600	9.553	3.5767
Zebra	2.4733	7.977	2.5933
Black rhinoceros UHC	1.3400	6.337	1.0367
Domestic horse	3.3038	5.102	1.5950
Indian rhinoceros	1.2397	5.530	2.1733
Ring-tailed lemur	1.1800	16.000	5.4100
Tapir	1.3600	1.250	1.5400
White rhinoceros	1.9248	2.644	1.1620

Averages of total B. Cu and Mn concentrations in mg/g for each animal species

Common name of animal species	ICP-OES Na average (mg/g)	ICP-OES Ca average (mg/g)	ICP-OES Mg average (mg/g)
African elephant	0.0186	0.0148	0.1355
Black rhinoceros	0.0240	0.0116	0.1456
Common warthog	0.0117	0.0182	0.2840
Giraffe	0.0599	0.0117	0.0626
Impala	0.0225	0.0139	0.1477
Roan Antilope	0.0069	0.0075	0.0972
Waterbuck	0.0233	0.0114	0.1980
Zebra	0.0034	0.0062	0.0606
Black rhinoceros UHC	0.0331	0.0194	0.0955
Domestic horse	0.0002	0.0088	0.0606
Indian rhinoceros	0.0153	0.0124	0.1010
Ring-tailed lemur	0.0154	0.0480	0.2040
Tapir	0.0001	0.0306	0.1690
White rhinoceros	0.0002	0.0146	0.1324