Assessing iron storage disease in eastern black rhinoceroses

(*Diceros bicornis michaeli*)

Reference ranges for iron levels and biochemistry

Fieke Molenaar

Submitted in part fulfilment of the requirements for the degree of

Master of Science in Wild Animal Health

University of London, 2005

ZSL Institute of Zoology
LIVING CONSERVATION

Table of contents

Table of contents

Haemochromatosis in black rhinoceroses (*Diceros bicornis*)

Fieke Molenaar

Literature Review submitted in part fulfilment of the requirements for the degree of Master of Science in Wild Animal Health

University of London, 2005

HAEMOCHROMATOSIS IN BLACK RHINOCEROSES (*Diceros bicornis*)

1. Biology and conservation

Taxonomy

The Family Rhinocerotidae (Order Perissodactyla) contains five extant rhinoceros species: the black rhinoceros (*Diceros bicornis*) and white rhinoceros (*Ceratotherium simum*) in Africa and the Greater One-horned Indian rhinoceros (*Rhinoceros unicornis*), Javan rhinoceros (*Rhinoceros sondaicus*) and Sumatran rhinoceros (*Dicerorhinus sumatrensis*) in Asia (Nowak, 1991).

The black rhinoceros is a medium sized rhino, 2.86-3.05 m long and weighing approximately 950-1,300 kg (Owen-Smith and Berger, 2001). It has two slowly growing horns on its head, consisting of compressed fibrous keratin (Nowak, 1991) and has a typical triangular prehensile lip (Owen-Smith and Berger, 2001).

The black rhinoceros is currently listed on the IUCN Red List as Critically Endangered.[1]

Habitat

Unlike the grazing rhinoceros species, both the black and the Sumatran rhinoceroses are browsers, living solely off woody twigs, thick leaves and legumes (Nowak, 1991). Where the Sumatran rhinoceros inhabits dense rainforests, the black rhinoceros favours open woodland (Miller, 2003) and dense thorn bushes (Jones, 1979) in the proximity of water (Nowak, 1991).

Black rhinoceros density and home range are determined by browse availability and proportional plant species composition (Adcock, 2001; Buk, 2004). Mainly a solitary animal, small groups may occasionally be seen together in mud wallows (Lent and Fike, 2003). Several individuals inhabiting a favoured range will have a dominant influence on the local ecology (Nowak, 1991).

[1] See *Appendix I: IUCN Categories*

Population decline

In 1970, the black rhinoceros population was estimated at 65,000 individuals (Miller, 2003) and, although it plummeted to approximately 2,500 individuals in 1995 (Callister and Bythewood, 1995), the current wild population is estimated at approximately 3,650 individuals (Amin, Thomas, Emslie, Foose and van Strien, 2005). Originally black rhinoceroses inhabited most of eastern and southern Africa, but the size of their range has decreased considerably (Nowak, 1991). Today, they can only be found in isolated pockets of their former range (Figure 1).

There are several reasons for this drastic population decline. Firstly, in the beginning of the 20th century, governments encouraged hunting of rhinoceroses to clear the way for human settlement (Nowak, 1991). Secondly, traditional Chinese medicine uses rhinoceros horn as an antipyretic and some Indians use it as an aphrodisiac (Leader-Williams, 1992; Callister and Bythewood, 1995). However, for traditional medicine, Asian rhinoceros horn is preferred (Leader-Williams, 1992). More importantly, black rhinoceros horn is favoured for the carved handles of traditional daggers (*jambias*) in Yemen (Leader-Williams, 1992).

The drastic decline of the black rhinoceros population called for conservation measures. The Asian rhinoceroses have been on Cites Appendix I since 1975 and in 1977 the African rhinoceroses were added, prohibiting international commercial trade in all rhinoceros species (Leader-Williams, 1992).[2] This coincided with a change in the political situation in North Yemen due to increased oil production. With the influx of wealth, the demand for black rhinoceros horn for the production of daggers increased. Combined with the new legislation, this caused a surge in poaching and flourishing of the black market trade (Nowak, 1991). Most of the smuggled raw horn originates from Kenya, Sudan and Ethiopia (Martin and Vigne, 1995). Several attempts are being made to discourage the poaching and black market trade. Traditional Chinese medicine has been used for centuries, but the antipyretic effects of rhinoceros horn have so far not been proven in clinical trials (But, Lung and Tam, 1990). However, the use of traditional medicine may not be best discouraged by trivialising it, but by educating the users about the environmental side effects of using materials from endangered species. Also, more effort may need to be put into the enforcement of laws prohibiting imports of traditional medicine containing parts of

[2] See *Appendix II: Cites*

endangered species (Callister and Bythewood, 1995). In Yemen, the use of acceptable substitutes for the production of dagger handles, such as local mined agate, is being stimulated (Martin and Vigne, 1995).

Additionally, by locating black rhinoceros subpopulations, concentrating resources in small areas, translocating individuals to unoccupied habitats in their former range and placement of microchips in their horns, several conservation successes have been achieved (Leader-Williams, 1992; Amin et al., 2005).

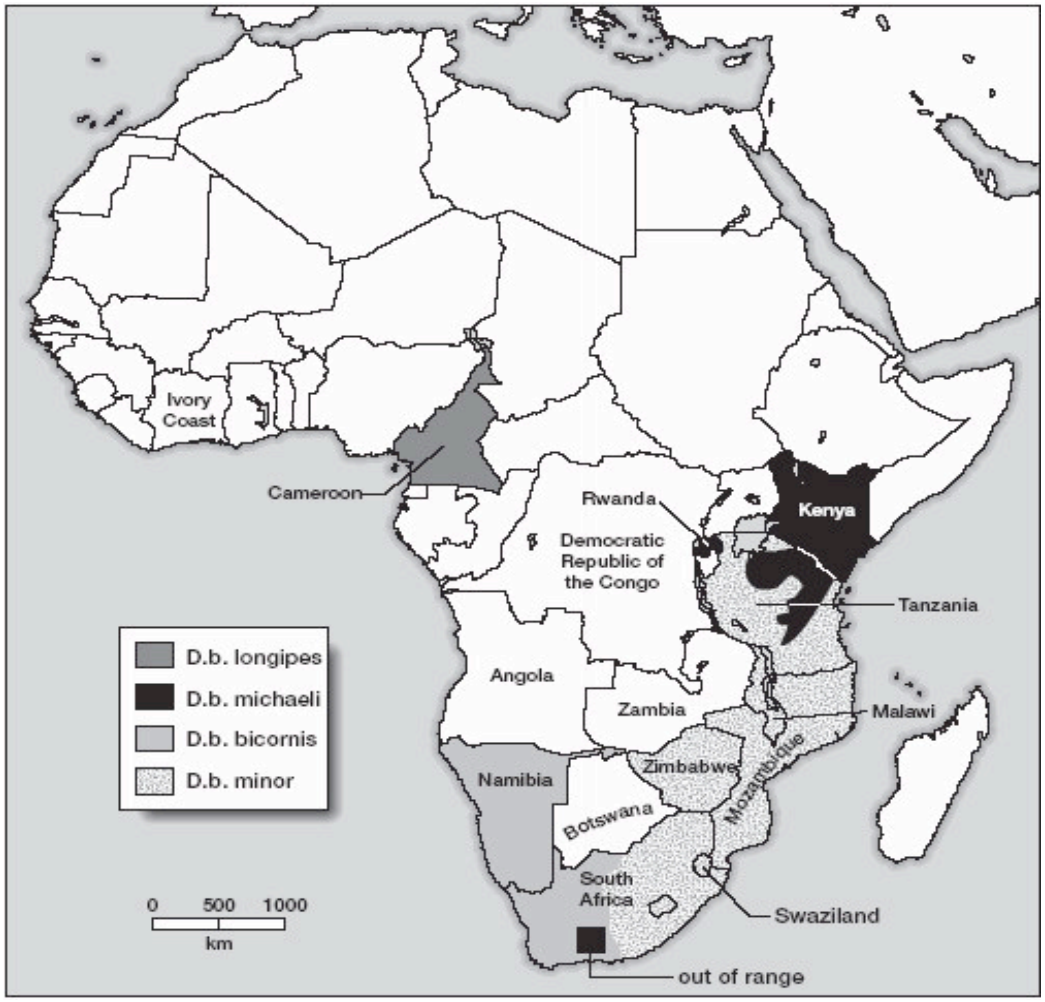


Figure 1. Distribution of the four recognised subspecies of black rhino in 2003. According to the latest data (2004-2005) the black rhinoceros population in Mozambique is now thought to have gone extinct and recent reintroductions have returned the black rhinoceros to Zambia, Botswana and Ethiopia (Amin et al., 2005).

2. Nutrition

Rhinoceroses are monogastric herbivores with a well-developed caecum, relying on hind-gut fermentation (Jones, 1979; Miller, 2003). The anatomy of their gastro-intestinal system resembles that of equids (Frost, 1998) and the domestic horse, *Equus domesticus* (Order Perissodactyla, Family Equidae), has been used frequently as a nutritional model for all rhinoceros species (Dierenfeld, 1999). This has been proven useful for diet design of grazing Indian rhinoceroses (Clauss, Polster, Kienzle, Wiesner, Baumgartner, von Houwald, Ortmann, Streich and Dierenfeld, 2005b). However, black rhinoceroses are selective browsers (Dierenfeld, 1999), feeding solely on shrubs, leaves and twigs (Miller, 2003). Although this is widely known, most zoos feed them a similar diet to grazing species, without taking the differences in digestive physiology between browsers and grazers into account (Grant, Brown and Dierenfeld, 2002).

Free-ranging black rhinoceroses appear to favour herbs, shrubs and young sprouts of several woody plant species, especially of the genus *Acacia*, *Sesbania*, (Hennig and Grindig, 2001) and *Euphorbia* (Wood, Foggin and Naude, 1997). This diet is highly lignified, difficult to digest, relatively low in protein and marginal in some minerals (Dierenfeld, 1999).

Wild browse contains high levels of the biological antioxidant vitamin E (Dierenfeld, 1999) and black rhinoceroses in captivity appear to have significantly lower levels than free-ranging individuals (Dierenfeld, du Toit and Miller, 1988), possibly due to an adaptive reduction of absorption (Lewis and Kirkwood, 1990). Since supplementation has been implemented, no difference in vitamin E levels between captive and free-ranging rhinoceroses has been found (Clauss, Jessup, Norkus, Chen, Holick, Streich and Dierenfeld, 2002). Vitamin E levels of all rhinoceros species are significantly lower than of equids, indicating a difference in vitamin metabolism between equids and rhinoceroses and therefore making the horse an unsuitable model for the vitamin metabolism of rhinoceroses (Clauss et al., 2002). Wild browse also contains high levels of natural iron-chelators, such as tannins (Clauss, Gehrke, Hatt, Dierenfeld, Flach, Hermes, Castell, Streich and Fickel, 2005a), which are secondary plant compounds in the seeds and bark that function as a herbivore feeding deterrent (Spelman, Osborn and Anderson, 1989). To counteract these, black rhinoceroses secrete tannin-binding proteins in their saliva in response to the seasonally varying

tannin content of the forage (Clauss et al., 2005a). Additionally, wild browse contains low levels of sodium (Na), phosphorus (P) and selenium (Se) (Miller, 2003).

In the late 1970s, the standard captive rhinoceros diet, whether browsing or grazing, consisted of clover or lucerne (alfalfa) hay, grass and vegetables, with occasional browse in summer (Jones, 1979). The current knowledge of black rhinoceros nutrition emphasises the inclusion of browse in the black rhinoceros' diet, either fresh or from frozen stores (Dierenfeld, 1999). Thus, today's recommended zoo diet consists of grass-legume hay, specially manufactured pellets (e.g. Mazuri® Zoo Foods, Essex) and browse (Miller, 2003). However, a survey conducted under North American zoos between 1995-1997 revealed that black rhinoceroses only receive browse as 5% of their diet (Grant et al., 2002). Local browse species fed to captive black rhinoceroses in the United Kingdom (UK) are willow (*Salix spp.*), oak (*Quercus spp.*), fruit trees such as apple (*Malus domistica*) and cherry (*Prunus axium*) and sweet chestnut (*Castanea sativa*) (Frost, 1998). Although there are significant nutritional differences between major dietary components of locally available browse and African browse, importing the necessary amounts of browse from Africa would be financially unfeasible (Grant et al., 2002), since the daily dry matter intake is 1-2% of the bodyweight (Miller, 2003).

3. Disease

Infectious disease

Free-living black rhinoceroses are susceptible to infectious diseases like other rhinoceros species. They can harbour a wide variety of endoparasites, both helminths and protozoa (Ramsay and Zainuddin, 1989), develop an ulcerative, seasonal skin condition caused by filarial nematodes (Munson and Miller, 1999) and can be host to a variety of ticks (Knapp, Krecek, Horak and Penzhorn, 1997). Antibodies against several (tick-transmitted) infectious diseases have been found in free-living black rhinoceroses (Fischer-Tenhagen, Hamblin, Quandt and Frolich, 2000). It is thought that this natural low-level exposure can induce some degree of resistance to certain infections (Jessup, Miller, Bolin, Kock and Morkel, 1992).

Compared to their free-ranging relatives, captive black rhinoceroses seem to be more susceptible to various infectious diseases, including *Salmonella* septicaemia and enteritis, mycobacteriosis, leptospirosis, tetanus and fungal pneumonia caused by *Aspergillus sp.* and *Phycomyces sp.* (Powers and Price, 1967; Ramsay and Zainuddin, 1989; Miller, 2001). Captivity-related immunosuppression appears to be the cause of this increased susceptibility (Ramsay and Zainuddin, 1989; Miller, 2001).

Non-infectious disease

Apart from the typical susceptibility to infectious disease, certain non-infectious diseases occur specifically in captive black rhinoceroses and are not seen in the captive grazing rhinoceros species, including haemolytic anaemia, mucocutaneous ulcerative disorder and congenital leucoencephalomalacia (Paglia, Miller and Renner, 1996). Several causes have been suggested, such as vitamin E deficiency and autoimmune disorders, but recently excessive iron storage with subsequent oxidative stress due to free radical formation has been postulated as the basis for all these conditions (Paglia and Dennis, 1999; Weber, Paglia and Harley, 2001).

Haemolytic anaemia is seen in 40% of all mortality cases (Chaplin, Malecek, Miller, Bell, Gray and Hunter, 1986), and 75% of all cases of haemolytic anaemia in black rhinoceroses are fatal (Miller, 1993). Haemolysis can be caused by either erythrocyte enzyme deficiency or toxic drugs, infections or immune mediated disorders (Chaplin et al., 1986). Significant polymorphism of the haemoglobin in black rhinoceroses has been found, but this appeared to be unrelated to the cases of haemolytic anaemia (Fairbanks and Miller, 1990). Initial research into enzyme abnormalities of erythrocytes was unable to determine the cause of haemolysis (Paglia, Valentine, Miller, Nakatani and Brockway, 1986), but later studies identified a link to decreased natural antioxidants and antioxidant enzyme levels (Paglia and Miller, 1993; Weber, Paglia and Harley, 2004) and a restriction in the energy concentration in the erythrocyte, making them more sensitive to oxidative stress (Paglia et al., 1996). Haemolytic anaemia has been seen in conjunction with vitamin E deficiency, haemosiderosis in parenchymal tissues, hypophosphataemia and leptospirosis (Miller, 1993).

Mucocutaneous ulcerative disorder is a superficial necrolytic dermatopathy with bilateral symmetric epidermal plaques, vesicles or pustules that erode and ulcerate

(Miller, 2001) and was initially thought to be the result of hypoalbuminaemia caused by dietary deficiencies (Grant et al., 2002).

Leucoencephalomalacia, cavitating necrosis of the white matter of the cerebrum, has been reported after post mortem examination of several female calves suffering from rapidly fatal, progressive neurological symptoms. Remarkably, all calves were born to dams with severely elevated body iron stores (Miller, 2001).

Another interesting disorder specific for black rhinoceroses is idiopathic intravascular vasculopathy, with haemorrhagic extravasation leading to marked swelling of neck and limbs (Miller, 2001). Although an immune complex disease was thought to be the cause, this has so far not been proven (Murray, Lung, Alvarado, Gamble, Miller, Paglia and Montali, 1999).

Haemosiderosis, the deposition of excess body iron into insoluble iron clusters in soft tissue (Jones, Hurt and King, 1997), appears to be a common post mortem finding in captive black rhinoceroses (Smith, Chavey and Miller, 1995). In the past, it was considered to be of no clinical significance (Jones, 1979). Some years later, haemosiderosis was thought to be secondary to haemolytic anaemia (Miller, 1993). However, it has also been seen in the Sumatran rhinoceros and in both species it progresses with time in captivity, unrelated to cases of haemolytic anaemia (Miller, 2001).

Haemochromatosis, interference of haemosiderosis with normal physiological functioning (Jones et al., 1997), is now thought to be the cause and not the result of many idiopathic conditions of captive black rhinoceroses that do not appear to affect their grazing counterparts (Paglia, Dierenfeld and Tsu, 2001a). This is the current direction of intensive study.

4. Haemochromatosis

Iron metabolism

The ‘trace elements’ are fifteen elements necessary for normal functioning of the body, making up less than 0.01% of the body mass (Schmidt-Nielsen, 1997). Iron is an essential trace element, used for red cell formation and oxygen transport to tissues as part of the haemoglobin and myoglobin molecules, and for the production of

cellular energy (adenosine-tri-phosphate, ATP) (Guyton and Hall, 2000; Seeley, Stephens and Tate, 2003). As a component of cytochromes and because of its redox potential (Fe^{2+}/Fe^{3+}), it is used in several cellular enzyme systems (Rolfs and Hediger, 1999; Martini, 2001). An increased need for iron can be seen in growing animals and in reproducing females because of foetal growth (Schmidt-Nielsen, 1997).

Absorption

Iron is available in the diet as haem-iron (Fe^{2+}) in red meat and as inorganic salts (Fe^{3+}) in vegetables (Kutchai, 1996; Mainous, Wells, Carek, Gill and Geesey, 2004). Before the inorganic form (Fe^{3+}) can be absorbed, it needs to be reduced by gastric acid to the more soluble organic form (Fe^{2+}) (Silbernagl and Despopoulos, 1996). This reduction can be enhanced by dietary ascorbic acid (vitamin C). Intestinal iron uptake can occur via two different pathways. Apotransferrin is an iron transport protein produced by the liver and either excreted in the bile or recycled from enterocytes (Kutchai, 1996). It can bind iron, forming transferrin, which can attach to the transferrin receptor on the cell surface of the enterocyte, entering via endocytosis of the transferrin-receptor complex (Guyton and Hall, 2000). Iron uptake (Fe^{2+}) can also occur through a divalent metal-ion transporter in the microvilli of the enterocytes. The iron within the enterocyte is released into the plasma at the basolateral membrane using a yet unidentified transporter (Rolfs and Hediger, 1999; Griffiths, Kelly, Smith and Cox, 2000).

The required daily iron intake for humans, *Homo sapiens* (Order Primates, Tribe Hominini) is 10-18 mg. Of this, only 0.5-1 mg (5-10%) is absorbed in the duodenum and ileum, because most iron will form insoluble salts with hydroxide, phosphates, bicarbonates, phytates, tannins and cereal fibres, subsequently excreted in the faeces (Kutchai, 1996; Pearson and Andreasen, 2001).

Foetal iron absorption

Maternal iron passes through the placenta and accumulates rapidly in haemoglobin and in the liver, to be used for several months after birth for neonatal haemoglobin production (Guyton and Hall, 2000).

Circulating iron

The liver secretes apotranferrin into the circulation, which can bind loosely to iron released by the enterocytes into the plasma, forming transferrin, from which iron can be re-released at any tissue cell with a transferrin receptor (Guyton and Hall, 2000). Eighty percent of the cellular transferrin receptors are present in the erythroid bone marrow (Brittenham, Weiss, Brissot, Laine, Guillygomarc'h, Guyader, Moirand and Deugnier, 2000). Iron recycled from old erythrocytes that have been phagocytised by macrophages in the spleen, bone marrow and liver, will be incorporated into transferrin – facilitated by ceruloplasmin – and recycled into the circulation (Brittenham et al., 2000).

Storage

Excess iron is deposited in all cells of the body, especially in the hepatocytes and in the reticuloendothelial cells in the bone marrow (Guyton and Hall, 2000). There, Fe^{2+} is bound to apoferritin and oxidised to Fe^{3+}, forming soluble ferritin, the main intracellular iron storage protein (Cavallo, Mei, Stefanini, Rosato, Finazzi-Agro and Chiancone, 1998; Brittenham et al., 2000; Hynes and Coinceanainn, 2002). Of the total iron load in the body, 65% is present in haemoglobin and 15-30% is stored as ferritin (Guyton and Hall, 2000).

Loss of iron

Normally, the daily loss is less than 0.05% of total body iron (Brittenham et al., 2000). Iron can be found in several secretions and excretions of the body. Traces can be found in the urine in daily varying concentrations (Martini, 2001; Beutler, Hoffbrand and Cook, 2003). In domestic animals, low levels of iron have been found in semen, possibly indicating physiological levels in the body (Massanyi, Trandzik, Nad, Toman, Skalicka and Korenekova, 2003). Although some iron is excreted in the bile, most of it will be reabsorbed before reaching the faeces (Pearson and Andreasen, 2001). Excess iron stored as ferritin within the enterocyte will be lost in the intestinal lumen when intestinal epithelial cells slough off (Kutchai, 1996). In humans, women have half the iron reserves of men due to loss through menstruation, foetal growth and partus (Martini, 2001). Overall, the daily loss of iron approximates the daily absorption (Hash, 2001).

Regulation

As outlined in the previous paragraph, there is no effective excretory pathway for iron in mammals and homeostasis is mainly regulated by a feedback mechanism influencing the intestinal iron uptake (Griffiths and Cox, 2000).

When all apoferritin in the tissues is saturated, the plasma transferrin cannot release any more iron. Its normal saturation of 33% will increase, reducing uptake of new iron presented by the enterocytes. The increase of intracellular iron in the enterocytes induces down-regulation of its transferrin receptors with reduction of iron absorption from the intestinal lumen. Additionally, apotransferrin production in the liver decreases, with less secretion in bile and plasma, again reducing iron absorption (Guyton and Hall, 2000). This is also influenced by an erythropoietic regulator from the bone marrow (Hash, 2001).

Role of regulation

Both the regulation of iron absorption and sequestration of excess iron in non-reactive forms such as ferritin are part of the antioxidant defence of the body (Kallianpur, Hall, Yadav, Christman, Dittus, Haines, Parl and Summar, 2004). Excess free iron will react with oxygen to produce free hydroxyl radicals that can damage lipid membranes of cells and organelles, change enzymatic proteins and damage DNA (Paglia and Dennis, 1999; Ahmad, Kitchin and Cullen, 2000). Through reduction of the concentration of free iron available to micro-organisms, iron sequestration is also an important part of the natural (innate) immunity (Paglia and Dennis, 1999).

Haemosiderosis

When the total amount of iron in the body exceeds the apoferritin capacity, it will be stored intracellularly in clusters of insoluble hemosiderin, visible as golden brown pigment granules on HE staining and reacting blue-green with Prussian blue (Guyton and Hall, 2000). Hemosiderin can be found in the macrophages of the spleen, in places where there have been haemorrhages and in the epithelium and interstitium of the kidneys after excessive haemolysis (Jones et al., 1997). It is seen after cell injury, with inflammatory processes, aplastic anaemias, ineffective erythropoiesis, transfusions, parental iron administration, haemoparasitism, starvation with muscle catabolism, ceruloplasmin deficiency due to copper deficiency and in cases of copper excess blocking iron release from the cells (Lowenstine and Munson, 1999).

Haemochromatosis

When the hemosiderin deposition is such that it causes pathological changes, it is called haemochromatosis (Beutler et al., 2003). The excess iron can be found stored in the cytoplasm of most epithelial cells, distributed in an extensive variety of tissues (Jones et al., 1997).

A distinction can be made between primary haemochromatosis and secondary haemochromatosis. The term primary haemochromatosis is used when, due to a mutation, a genetic defect is the cause of increased iron absorption (Hash, 2001; Beutler et al., 2003). The iron will be depositioned in a wide variety of tissues, especially the hepatocytes and Kupffer cells (Griffiths and Cox, 2000), interfering with normal cellular function (Jones et al., 1997).

Secondary or acquired haemochromatosis is seen in cases of dietary iron overload due to excessive iron consumption, ineffective erythropoiesis, chronic anaemias, multiple transfusions, primary liver disease, iatrogenic iron supplementation and chronic haemodialysis (Hash, 2001). In acquired haemochromatosis, iron will be mostly deposited in reticuloendothelial cells and hepatocytes (Jones et al., 1997; Beutler et al., 2003). A study into human diet-induced haemochromatosis in particular found most iron in the macrophages of the spleen and the bone marrow and in the lamina propria of the small intestine (Theron and Mekel, 1971).

Pathophysiology of haemochromatosis

Either form of haemochromatosis leads to fibrosis of the affected organs (Lowenstine and Munson, 1999). Fibrosis of the liver, with loss of original hepatic architecture and increased connective tissue, is termed cirrhosis. Cirrhosis disrupts the normal blood flow, inducing portal shunt formation with passive congestion of the spleen and digestive organs, causing ascites. Representing an end-stage of liver disease, it is progressive, non-reversible and fatal (Jones et al., 1997). In case of prior hepatic damage, caused by for example viral infections (hepadnavirus / hepatitis C) or exposure to aflatoxins, cirrhosis will occur more rapidly (Lowenstine and Munson, 1999; Hash, 2001). The intracellular pigment also causes abnormal pigmentation of the skin and discolouration of the lymph nodes. In addition, excess iron can disrupt the calcium-phosphate compounds of bone, causing osteomalacia (Jones et al., 1997).

When all iron storage facilities are saturated, free iron (Fe^{2+}) will become increasingly available (Hash, 2001). This can react with oxygen, creating free oxi-radicals (Rolfs and Hediger, 1999):

$$Fe^{2+} + O_2 \leftrightarrow Fe^{3+} + O_2^{-\cdot}$$

These free radicals impair the cellular integrity and the functions of mitochondria and lysosomes by causing oxidative damage to the phospholipids of cellular membranes. Additionally, they disrupt protein synthesis and induce gene mutations by damaging nucleic acids of DNA and RNA, which can lead to malignant transformation of the cell (Britton, Leicester and Bacon, 2002). Iron is also a growth factor for proliferating neoplastic cells and may suppress host anti-tumour immunity (Kallianpur et al., 2004). In humans, an increased prevalence of primary (liver) tumours is frequently seen (Beutler et al., 2003; Kallianpur et al., 2004). Oxidative stress in the β-cells of the pancreas can cause apoptosis and desensitisation, resulting in decreased insulin secretion, which can aggravate concurrent diabetes mellitus (Cooksey, Jouihan, Ajioka, Hazel, Jones, Kushner and McClain, 2004). Free radicals in the circulation can also worsen atherosclerosis by damaging endothelial cells of the vascular walls (Guyton and Hall, 2000; Gaenzer, Marschang, Sturm, Neumayr, Vogel, Patsch and Weiss, 2002). Mammalian hearts are usually protected from cellular injury by a defence system of natural antioxidants and antioxidant enzymes (Das and Maulik, 1994). Free radical damage is dependent on the level of antioxidants present (Turoczi, Jun, Cordis, Morris, Maulik, Stevens and Das, 2003). In patients with hereditary haemochromatosis, levels of these antioxidants are lower than usual (Livrea, Tesoriere, Pintaudi, Calabresse, Maggio, Frieslebel, D'Arpa, D'Anna and Bongiorno, 1996). Oxidative damage to the myocardium causes cardiomyopathy and arrhythmias, combined with diastolic dysfunction due to the mechanical presence of iron (Hash, 2001; Turoczi et al., 2003). Additionally, patients with haemochromatosis show an increased susceptibility to infection and death due to sepsis is not uncommon (Beutler et al., 2003).

Haemochromatosis in humans

Primary haemochromatosis is a common autosomal recessive genetic disorder, affecting 0.33% of the white population (Parkkila, Waheed, Britton, Bacon, Zhou, Tomatsu, Fleming and Sly, 1997; Brissot, Guyader, Loreal, Laine, Guillygomarc'h, Moirand and Deugnier, 2000). It has its origin in a single mutation in the gene coding for the *HFE* protein (Brittenham et al., 2000). This is a regulatory protein that decreases cellular iron uptake by stabilising the transferrin receptor (Riedel, Muckenthaler, Gehrke, Mohr, Brennan, Herrmann, Fitscher, Hentze and Stremmel, 1999) and by influencing the hepatic production of hepcidin, an iron-regulating peptide (Fleming, 2005). Mutation of the *HFE* gene results in *HFE* deficiency, leading to increased, unregulated uptake of dietary iron (Hash, 2001). Although the mutation is common, clinical disease is quite rare and will only occur in certain individuals, influenced by additional mutations in other genes, the level and type of iron intake, alcoholism and hepatitis C (Beutler, 2003).

Genetically, primary haemochromatosis can be subdivided into adult, juvenile and neonatal haemochromatosis, the mutation affecting a different part of the gene in each case (Griffiths and Cox, 2000). Adult hereditary haemochromatosis is accompanied by lethargy, impotence, chronic arthritis and elevated aminotransferases. In a later stage, the classic presentation is a middle-aged male with diffuse hyperpigmentation, hepatomegaly and diabetes mellitus (Brittenham et al., 2000). Previous alcohol abuse can exacerbate the damaging effect of excess iron on the liver (Hash, 2001). Once hepatic cirrhosis is present, the risk of developing hepatocellular carcinomas increases 200-fold (Kallianpur et al., 2004), usually affecting one in five patients. Additionally, there appears to be an association between increased prevalence of *HFE* gene mutation and primary invasive breast cancer in women (Kallianpur et al., 2004). Cardiac dysfunction is seen in 50% of the cases (Hash, 2001) and is responsible for one third of the haemochromatosis deaths (Nakao, Toyozaki, Nagakawa, Himi, Yamada, Watanabe, Masuda and Asai, 2001). Women show signs later due to loss of iron through the foetus and through menstruation (Hash, 2001; Gaenzer et al., 2002). The *HFE* protein is also thought to play a role in the transfer of iron from maternal blood to the foetus (Parkkila et al., 1997). In juvenile hereditary haemochromatosis, patients under 30 years of age show hypogonadism, growth retardation and cardiomyopathy. Neonatal hereditary haemochromatosis is characterised by

congenital cirrhosis or severe hepatitis with hepatic and extra-hepatic iron deposits (Cox and Halsall, 2002), which can be diagnosed *in utero* using Doppler ultrasonography (Oddone, Bellini, Bonacci, Barocci, Toma and Serra, 1999). This can be preceded by abnormalities of the amnion or placenta with intrauterine growth retardation or stillbirth (Kelly, Lunt, Rodrigues, Berry, Flynn, McKiernan, Kelly, Mieli-Vergani and Cox, 2001).

Patients with secondary or acquired haemochromatosis will present with similar clinical symptoms as those with primary haemochromatosis (Nakao et al., 2001). Acquired haemochromatosis is seen in cases of accidental or deliberate excessive iron ingestion (Hennigar, Greene, Walker and de Saussure, 1979; Gordeuk, 1992), chronic (hereditary) anaemias (Nakao et al., 2001), ineffective erythropoiesis, multiple transfusions, primary liver disease and chronic haemodialysis (Hash, 2001).

Diagnosis in humans

Guided by initial, non-specific clinical signs of liver disease, measurement of the serum transferrin saturation after overnight fasting is a sensitive screening tool (Hash, 2001; Limdi and Hyde, 2003). Normal transferrin saturation should be less than 40%. Elevations of more than 60% on at least two occasions are indicative of haemochromatosis (Beutler et al., 2003). Simultaneous elevation of serum ferritin, which is normally less than 300 ng/ml, can indicate hepatic cirrhosis (Hash, 2001; Beutler et al., 2003). Non-transferrin-bound iron can be found in plasma of severely iron-overloaded patients (Beutler et al., 2003). Elevated transferrin saturation can also be seen with reduced transferrin production due to hepatic failure of different origin, including excessive alcohol consumption (Brittenham et al., 2000), and elevated ferritin with inflammatory processes (Brittenham et al., 2000; Limdi and Hyde, 2003). Therefore, liver enzymes need to be analysed to establish the extent of liver damage and further confirmation of the diagnosis is required by gene testing of a blood sample for mutation of the *HFE* gene, serial phlebotomy without inducing iron-limited erythropoiesis or by determining tissue iron (Hash, 2001). The latter can be performed non-invasively using computed tomography (CT), magnetic resonance imaging (MRI) or magnetic susceptometry with a superconducting quantum interference device (SQUID) (Brittenham et al., 2000; Beutler et al., 2003). Hepatic iron concentrations of more than 15 mg/g dry weight have a poor prognosis due to the risk of cardiac disease (Beutler et al., 2003). A liver biopsy for determination of the tissue iron can be

inaccurate due to uneven iron distribution. However, it can be used to confirm iron overload, assess periportal and hepatocytic distribution and detect cirrhosis, which is important to define the prognosis (Brittenham et al., 2000; Beutler et al., 2003). Further recommended tests are glucose studies, electrocardiogram (ECG), cardiac ultrasonography and radiography of the joints. Preventatively, measurements of the serum transferrin saturation can be used as a screening tool for families at risk (Brittenham et al., 2000).

Treatment of humans

Before irreversible damage – such as cirrhosis or diabetes – has occurred, treatment will improve quality of life and return life expectancy to normal (Brissot et al., 2000; Brittenham et al., 2000; Hash, 2001). The aim of treatment is to reduce the total iron content of the body to within its normal limits (Beutler et al., 2003).

Serial phlebotomy is the treatment of choice in patients with primary (hereditary) haemochromatosis (Beutler et al., 2003). Initial aggressive twice-weekly treatment should be followed-up indefinitely with the aim of creating a mild iron deficiency anaemia (Hash, 2001; Beutler et al., 2003). Because this induces increased erythropoiesis, the diet needs to be adjusted for protein content, vitamin B_{12} and folate. Progress should be monitored by regular measurement of the haematocrit and serum ferritin (Hash, 2001). Phlebotomies will improve skin pigmentation, liver congestion and cardiomyopathy, but will not affect cirrhosis, hepatocellular carcinomas, arthritis or endocrinopathies (Brittenham et al., 2000; Hash, 2001). Phlebotomies are contraindicated in cases of mild iron overload and asymptomatic or young individuals (Brittenham et al., 2000).

Chelation therapy is the treatment of choice in patients with secondary (acquired) haemochromatosis. Deferroxamine is most commonly used (Hershko, Link, Konijn and Cabantchik, 2005). This is an iron-specific chelator that enters the hepatocytes and slowly mobilises labile iron to be excreted in the urine (Boturao-Neto, Marcopito and Zago, 2002). It is administrated subcutaneously by slow infusion over a period of 8-12 hours. To be effective, 250 infusions per year are necessary (Nakao et al., 2001). Close monitoring for side effects, including deafness, retinal damage and skeletal damage, is essential. Deferiprone is an oral iron chelator with less side effects (Beutler et al., 2003). Although compliance is more achievable, the expense prevents common use of this drug. Combination of parental deferroxamine and oral deferiprone appears

to have a synergistic effect (Alymara, Bourantas, Chaidos, Bouranta, Gouva, Vassou and Tzouvara, 2004; D'Angelo, Mirra, Rocca and Carnelli, 2004; Hershko et al., 2005). As an alternative to chelation therapy, blocking of the divalent metal-ion transporter in the microvilli of the enterocytes has been suggested. However, this could affect the uptake of other metal ions and requires further research (Rolfs and Hediger, 1999).

Additionally, the dietary iron intake of patients should be adjusted, since elevated transferrin saturation (>55%) combined with high dietary iron intake has been associated with an increased mortality risk (Mainous et al., 2004). Patients with hepatocellular carcinoma have a poor prognosis regardless of dietary adjustments, because treatment will require liver resection or transplantation (Harrison and Bacon, 2005).

Haemochromatosis in other species

Apart from the black rhinoceros, haemochromatosis has been reported in a variety of species from different taxa (Lowenstine and Munson, 1999).

Amongst the domesticated species, it has been recorded in several adult horses, occurring as a chronic disease (Olsman and Sloet van Oldruitenborgh-Oosterbaan, 2004). Biochemical evaluation revealed elevated gamma-glutamyltransferase (GGT), alkaline phosphatase (ALP), aspartate aminotransferase (AST) and bile acids, but not always the transferrin saturation seen in human haemochromatosis (Pearson, Hedstrom and Poppenga, 1994). In a trial of long-term excessive oral iron administration to healthy adult ponies, haemochromatosis could not be induced. Thus, equine haemochromatosis does not appear to be associated with excessive dietary iron intake (Pearson and Andreasen, 2001) and seems more comparable to hereditary haemochromatosis. Clinical symptoms are non-specific and do not surface until advanced stages of the disease. Horses are presented with anorexia, depression, decreased stamina and weight loss. Their skin can show seborrhoea, alopecia and pruritis. Icterus is only occasionally seen. End-stage hepatic failure will be presented with coagulation problems and depression, leading to coma (Olsman and Sloet van Oldruitenborgh-Oosterbaan, 2004). Treatment consists mainly of restricting protein intake, reducing stress, administering antioxidants and supportive therapy, but success

has been variable (Pearson et al., 1994; Olsman and Sloet van Oldruitenborgh-Oosterbaan, 2004).

In captive mynah birds (Order Passeriformes, Family Sturnidae) progressive iron accumulation in the hepatocytes is commonly found from 10 days after hatching. Most are found dead without prior clinical signs (Gosselin and Kramer, 1983). This iron accumulation does not appear to be diet induced and has a distribution comparable with that of hereditary haemochromatosis in humans (Gosselin and Kramer, 1983; Mete, Hendriks, Klaren, Dorrestein, van Dijk and Marx, 2003). Haemochromatosis has also been a post mortem finding in great bustards (*Otis tarda*) (Bailey and Flach, 2003) and has been found in toucans (Order Piciformes, Family Rhamphastidae) in captivity (Spalding, Kollias, Mays, Page and Brown, 1986; Roels, Ducatelle and Cornelissen, 1996). So far, phlebotomy, combined with a low-iron diet, has been used in several toucans and chelation therapy has been proven successful in one channel-billed toucan (*Ramphastos vitellinus*) (Lowenstine and Munson, 1999). Dietary modifications that have been suggested for birds include low iron diets (Schoemaker and Beynen, 2001), tree gums, tamarind juice, increased fibre, leafy browse and limited citrus fruit (Lowenstine and Munson, 1999).

Free-living lemurs (Suborder Prosimii, Superfamily Lemuroidea) mainly consume leaves, fruit, seeds and flowers, gums and bark, and some species can tolerate high levels of toxic plant compounds such as cyanide (Rowe, 1996). In captivity, several lemurs have developed progressive haemochromatosis with clinical signs related to hepatic failure. At post mortem examination, clusters of hemosiderin have been found in the reticuloendothelial system of the duodenum, spleen and liver. The older the lemur, the more generalised the iron storage. This progressive accumulation appeared to be diet-related (Spelman et al., 1989). Their natural diet contains high levels of tannins, oxalates and phosphates, functioning as natural iron chelators. Thus, iron bioavailability is very low. It is thought that, as an adaptation to low absorbable dietary iron, lemurs are very efficient in iron absorption and have a limited capacity to synthesize ferritin. Compared to their natural diet, the food offered in captivity, such as potatoes, tomatoes, berries and green leafy vegetables, is low in natural iron-chelators and contains high levels of vitamin C, which enhances the conversion of Fe^{3+} to the more bioavailable Fe^{2+}, thus increasing iron uptake. This may also be enhanced by inadequate dietary copper. So far, treatment of lemurs has been

attempted by adding tannin-rich tamarind pods and tamarind syrup to their diet (Spelman et al., 1989).

Haemochromatosis has also been recorded in gum-eating marmosets, folivorous gorillas, hyrax and house shrews, and less commonly in forest-dwelling bongos, duikers, dik-diks and old orang-utans in captivity. Clinical signs of haemochromatosis in most animals are chronic wasting, elevated liver enzymes, hypoproteinaemia, elevated serum iron and increased serum transferrin saturation (Lowenstine and Munson, 1999). Serial blood analysis should be performed prior to further diagnoses. Bile acids and GGT will provide the best information about hepatic function. Ultrasonography can distinguish major changes in liver and bile ducts and can be used to facilitate taking liver biopsies, especially in case of focal liver damage (Olsman and Sloet van Oldruitenborgh-Oosterbaan, 2004). Liver biopsies are necessary to provide a prognosis (Lowenstine and Munson, 1999), but only after a coagulation profile has been established (Olsman and Sloet van Oldruitenborgh-Oosterbaan, 2004).

Haemochromatosis in black rhinoceroses

In the captive black rhinoceros population, elevated serum iron concentrations and transferrin saturation during life and haemosiderosis at post mortem, have been a consistent finding (Smith et al., 1995; Paglia et al., 2001a). Neither biochemical abnormalities nor pathological changes have been found in free-living black rhinoceroses (Kock, Foggin, Kock and Kock, 1992) or in captive grazing rhinoceros species (Smith et al., 1995; Paglia and Dennis, 1999). So far, limited research has been done on the browsing Sumatran rhinoceros, but results show comparable changes (Paglia and Dennis, 1999).

The pathological storage of excess iron seems to be related to some of the clinical diseases of uncertain aetiology unique to captive black rhinoceroses: haemolytic anaemia, mucocutaneous ulcerative disorder, congenital leucoencephalomalacia and exceptional susceptibility to infections (Paglia et al., 1996). Studies on black rhinoceros erythrocytes have revealed inherently low concentration of certain antioxidant enzymes (e.g. catalase) and a restricted energy (ATP) concentration. Although this is thought to be a natural defence against haemo-parasites such as malaria, it also makes them more sensitive to oxidative stress (Paglia and Miller, 1993). Combined with the pathophysiology of haemochromatosis, with increased

levels of free radicals, this may explain why so many black rhinoceroses suffer from haemolytic anaemia (Paglia et al., 2001a). In the early 1990s, several cases have been reported of female black rhinoceros calves with rapidly fatal, progressive neurological symptoms. On post mortem examination, bilateral symmetric leucoencephalomalacia with cavitation of the white matter and minimal inflammatory response could be found (Miller, Cambre, De Lahunta, Brannian, Spraker, Johnson and Boever, 1990; Kenny, Cambre, Spraker, Stears, Park, Colter, De Lahunta and Zuba, 1996). Retrospective research revealed that the dams of these calves suffered from excessive serum ferritin concentrations and extensive iron deposition in spleen, liver, bone marrow, lungs and female reproductive organs (Paglia, Kenny, Dierenfeld and Tsu, 2001b). Concurrent copper deficiency may play a role in the pathogenesis of leucoencephalomalacia in calves of dams with iron overload, but further research into this subject is required (Paglia et al., 2001b).

Haemochromatosis is also thought to have played a role in the case of a black rhinoceros calf with unwarranted sensitivity to the cardiotoxic effects of chemotherapy. Iron positive granules were found in the myocardial fibres, indicating prior cardiac damage due to excess iron, priming the heart for extra stressors (Paglia and Radcliffe, 2000; Radcliffe, Paglia and Couto, 2000).

Several studies have shown that the amount of iron stored in black rhinoceroses increases with time kept in captivity (Smith et al., 1995; Paglia and Dennis, 1999; Paglia et al., 2001a). From as little as three weeks in boma confinement during translocations, significant increase in hemosiderin deposition has been found (Kock and Morkel, 1993). Extensive iron deposition can be found in reticuloendothelial and parenchymal cells of multiple organs, similar to other dietary overload syndromes such as the nutritional haemochromatosis earlier discussed in lemurs and humans (Paglia and Dennis, 1999). Consuming mostly twigs, bark, leaves and shrubs, the natural diet of free-ranging black rhinoceroses contains high levels of natural antioxidants and chelators like tannins (polyphenols), reducing the bioavailability of dietary iron (Clauss et al., 2002). Adaptation to natural low iron bioavailability could have led to enhanced intestinal iron absorption (Paglia et al., 2001a). Because in their captive diet both antioxidant levels and natural iron chelators are low, black rhinoceroses could be suffering from the increased bioavailability of iron. Current studies focus on the addition of iron chelators such as tannins to the diet (Clauss, Froeschle, Lechner-Doll, Hatt, Ganslosser and Dierenfeld, 2001).

Some research has been done into the genome of rhinoceroses in search of a similar mutation as seen in humans with hereditary haemochromatosis. Although data could indicate a comparable mutation, no conclusions could be drawn from these preliminary results (Beutler, West, Speir, Wilson and Worley, 2001).

Treatment is only successful at an early stage of the disease, when iron stores are not yet extensive. Being non-invasive, inexpensive and free from side effects, phlebotomy appears to be the treatment of choice. Chelation therapy with deferroxamine has been proven successful in one case, but compliance and costs pose serious problems (Paglia and Dennis, 1999).

5. Conclusion

The pathological changes seen with the various black rhinoceros-specific diseases can be explained by the combination of excessive dietary iron absorption due to natural adaptation, with inherent impairments of antioxidant activities in various tissues. These diseases may be managed by adding iron chelators to the diet, increasing dietary antioxidants and monitoring serum iron levels and biochemistry to anticipate the onset and progression of iron overload.

6. References

ADCOCK, K. 2001. User guide to Rhino Management Group black rhino carrying capacity model, version 1. SADC Regional Programme for Rhino Conservation.

AHMAD, S., K. T. KITCHIN AND W. R. CULLEN. 2000. Arsenic species that cause release of iron from ferritin and generation of activated oxygen. Archives of Biochemistry and Biophysics 382: 195-202

ALYMARA, V., D. BOURANTAS, A. CHAIDOS, P. BOURANTA, M. GOUVA, A. VASSOU AND E. TZOUVARA. 2004. Effectiveness and safety of combined iron-chelation therapy with deferroxamine and deferiprone. Hematological Journal 5: 475-479

AMIN, R., K. THOMAS, R. EMSLIE, T. FOOSE AND N. VAN STRIEN. 2005. An overview of the conservation status of, and threats to rhinoceroses in the wild. International Zoo Yearbook, accepted for publication July 2005

BAILEY, T. A. AND E. J. FLACH. 2003. Disease and mortality among great bustards (*Otis tarda*) at Whipsnade Wild Animal Park, 1989-1999. Veterinary Record 153: 397-400

BEUTLER, E., C. WEST, J. A. SPEIR, I. A. WILSON AND M. WORLEY. 2001. The *hHFE* gene of browsing and grazing rhinoceroses: a possible site of adaptation to a low-iron diet. Blood Cells, Molecules & Diseases 27: 342-350

_____. 2003. The *HFE* Cys282Tyr mutation as a necessary but not sufficient cause of clinical hereditary hemochromatosis. Blood 101: 3347-3350

_____, A. V. HOFFBRAND AND J. D. COOK. 2003. Iron deficiency and overload. Hematology (The American Society of Hematology Education Program) 2003: 40-61

BOTURAO-NETO, E., L. F. MARCOPITO AND M. A. ZAGO. 2002. Urinary iron excretion induced by intravenous infusion of deferoxamine on beta-thalassemia homozygous patients. Brazilian Journal of Medical and Biological Research 35: 1319-1328

BRISSOT, P., D. GUYADER, O. LOREAL, F. LAINE, A. GUILLYGOMARC'H, R. MOIRAND AND Y. DEUGNIER. 2000. Clinical aspects of hemochromatosis. Transfusion Science 23: 193-200

BRITTENHAM, G. M., G. WEISS, P. BRISSOT, F. LAINE, A. GUILLYGOMARC'H, D. GUYADER, R. MOIRAND AND Y. DEUGNIER. 2000. Clinical consequences of new insights in the pathophysiology of disorders of iron and heme metabolism. Hematology (The American Society of Hematology Education Program) 2000: 39-50

BRITTON, R. S., K. L. LEICESTER AND B. R. BACON. 2002. Iron toxicity and chelation therapy. International Journal of Hematology 76: 219-228

BUK, K. G. 2004. Habitat suitability for southwestern black rhino (*Diceros bicornis bicornis*). MSc thesis, University of Copenhagen, Copenhagen.

BUT, P. P.-H., L.-C. LUNG AND Y.-K. TAM. 1990. Ethnopharmacology of rhinoceros horn. I: Antipyretic effects of rhinoceros horn and other animal horns. Journal of Ethnopharmacology 30: 157-168

CALLISTER, D. J. AND T. BYTHEWOOD. 1995. Of tiger treatments & rhino remedies: Trade in endangered species medicines in Australia and New Zealand. TRAFFIC Oceania, Sydney, pp. 32

CAVALLO, S., G. MEI, S. STEFANINI, N. ROSATO, A. FINAZZI-AGRO AND E. CHIANCONE. 1998. Formation and movement of Fe(III) in horse spleen, H- and L-recombinant ferritins. A fluorescence study. Protein Science 7: 427-432

CHAPLIN, H., JR., A. C. MALECEK, R. E. MILLER, C. E. BELL, L. S. GRAY AND V. L. HUNTER. 1986. Acute intravascular hemolytic anemia in the black rhinoceros: hematologic and immunohematologic observations. American Journal of Veterinary Research 47: 1313-1320

CLAUSS, M., R. FROESCHLE, M. LECHNER-DOLL, J. M. HATT, U. GANSLOSSER AND E. S. DIERENFELD. 2001. Feeding tannins to captive black rhinoceros (*Diceros bicornis*): Results of a pilot study. *In* Proceedings of the International elephant and rhino research symposium: a research update on elephants and rhinos, Vienna, pp. 310

_____, D. A. JESSUP, E. B. NORKUS, T. C. CHEN, M. F. HOLICK, W. J. STREICH AND E. S. DIERENFELD. 2002. Fat soluble vitamins in blood and tissues of free-ranging and captive rhinoceros. Journal of Wildlife Diseases 38: 402-413

_____, J. GEHRKE, J. M. HATT, E. S. DIERENFELD, E. J. FLACH, R. HERMES, J. CASTELL, W. J. STREICH AND J. FICKEL. 2005a. Tannin-binding salivary proteins in three captive rhinoceros species. Comparative Biochemistry and Physiology, Part A 140: 67-72

_____, C. POLSTER, E. KIENZLE, H. WIESNER, K. BAUMGARTNER, F. VON HOUWALD, S. ORTMANN, W. J. STREICH AND E. S. DIERENFELD. 2005b. Studies on digestive physiology and feed digestibilities in captive Indian rhinoceros (*Rhinoceros unicornis*). Journal of Animal Physiology and Animal Nutrition (Berl) 89: 229-237

COOKSEY, R. C., H. A. JOUIHAN, R. S. AJIOKA, M. W. HAZEL, D. L. JONES, J. P. KUSHNER AND D. A. MCCLAIN. 2004. Oxidative stress, beta-cell apoptosis, and decreased insulin secretory capacity in mouse models of hemochromatosis. Endocrinology 145: 5305-5312

COX, T. M. AND D. J. HALSALL. 2002. Hemochromatosis-neonatal and young subjects. Blood Cells, Molecules, and Diseases 29: 411-417

D'ANGELO, E., N. MIRRA, A. ROCCA AND V. CARNELLI. 2004. Combined therapy with desferrioxamine and deferiprone: a new protocol for iron chelation in thalassemia. Journal of Pediatric Hematology/Oncology 26: 451-453

DAS, D. K. AND N. MAULIK. 1994. Evaluation of antioxidant effectiveness in ischemia reperfusion tissue injury methods. Methods of Enzymology 233: 601-610

DIERENFELD, E. S., R. DU TOIT AND R. E. MILLER. 1988. Vitamin E in captive and wild black rhinoceros (*Diceros bicornis*). Journal of Wildlife Diseases 24: 547-550

_____. 1999. Rhinoceros feeding and nutrition. *In* Zoo and Wild Animal Medicine, M. E. Fowler (ed.). Fourth Edition, WB Saunders, Philadelphia, pp. 568-571

FAIRBANKS, V. F. AND R. E. MILLER. 1990. Beta-globin chain hemoglobin polymorphism and hemoglobin stability in black rhinoceroses (*Diceros bicornis*). American Journal of Veterinary Research 51: 803-807

FISCHER-TENHAGEN, C., C. HAMBLIN, S. QUANDT AND K. FROLICH. 2000. Serosurvey for selected infectious disease agents in free-ranging black and white rhinoceros in Africa. Journal of Wildlife Diseases 36: 316-323

FLEMING, R. E. 2005. Advances in understanding the molecular basis for the regulation of dietary iron absorption. Current Opinion in Gastroenterology 21: 201-206

FROST, J. 1998. The rhinoceros browse survey. The North of England Zoological Society, Chester Zoo, Chester, pp. 21

GAENZER, H., P. MARSCHANG, W. STURM, G. NEUMAYR, W. VOGEL, J. PATSCH AND G. WEISS. 2002. Association between increased iron stores and impaired endothelial function in patients with hereditary hemochromatosis. Journal of the American College of Cardiology 40: 2189-2194

GORDEUK, V. R. 1992. Hereditary and nutritional iron overload. Baillieres Clinical Haematology 5: 169-186

GOSSELIN, S. J. AND L. W. KRAMER. 1983. Pathophysiology of excessive iron storage in mynah birds. Journal of the American Veterinary Medical Association 183: 1238-1240

GRANT, J. B., D. L. BROWN AND E. S. DIERENFELD. 2002. Essential fatty acid profiles differ across diets and browse of black rhinoceros. Journal of Wildlife Diseases 38: 132-142

GRIFFITHS, W. AND T. COX. 2000. Haemochromatosis: novel gene discovery and the molecular pathophysiology of iron metabolism. Human Molecular Genetics 9: 2377-2382

_____, A. L. KELLY, S. J. SMITH AND T. M. COX. 2000. Localization of iron transport and regulatory proteins in human cells. QJM 93: 575-587

GUYTON, A. C. AND J. E. HALL. 2000. Textbook of Medical Physiology. Tenth Edition, WB Saunders, Philadelphia, pp. 386-388, 761-964

HARRISON, S. A. AND B. R. BACON. 2005. Relation of hemochromatosis with hepatocellular carcinoma: epidemiology, natural history, pathophysiology, screening, treatment, and prevention. Medical Clinics of North America 89: 391-409

HASH, R. B. 2001. Hereditary hemochromatosis. Journal of the American Board of Family Practice 14: 266-273

HENNIG, J. AND T. GRINDIG. 2001. Feeding habits of desert dwelling black rhinoceros (*Diceros bicornis*), in Kunene Region northwest of Namibia. *In* Proceedings of the International elephant and rhino research symposium: a research update on elephants and rhinos, Vienna, pp. 311-314

HENNIGAR, G. R., W. B. GREENE, E. M. WALKER AND C. DE SAUSSURE. 1979. Hemochromatosis caused by excessive vitamin iron intake. American Journal of Pathology 96: 611-623

HERSHKO, C. M., G. M. LINK, A. M. KONIJN AND Z. I. CABANTCHIK. 2005. Iron chelation therapy. Current Hematology Report 4: 110-116

HYNES, M. J. AND M. O. COINCEANAINN. 2002. Investigation of the release of iron from ferritin by naturally occurring antioxidants. Journal of Inorganic Biochemistry 90: 18-21

JESSUP, D. A., R. E. MILLER, C. A. BOLIN, M. D. KOCK AND P. MORKEL. 1992. Retrospective evaluation of leptospirosis in free-ranging and captive black rhinoceroses (*Diceros bicornis*) by microscopic agglutination titers and fluorescent antibody testing. Journal of Zoo and Wildlife Medicine 23: 401-408

JONES, D. M. 1979. The husbandry and veterinary care of captive rhinoceroses. International Zoo Yearbook 19: 239-252

JONES, T. C., R. D. HURT AND N. W. KING. 1997. Veterinary Pathology. Sixth Edition, Williams & Wilkins, Baltimore, pp. 68-70, 804-805, 1027, 1098-1101

KALLIANPUR, A. R., L. D. HALL, M. YADAV, B. W. CHRISTMAN, R. S. DITTUS, J. L. HAINES, F. F. PARL AND M. L. SUMMAR. 2004. Increased prevalence of the *HFE* C282Y hemochromatosis allele in women with breast cancer. Cancer Epidemiology Biomarkers & Prevention 13: 205-212

KELLY, A. L., P. W. LUNT, F. RODRIGUES, P. J. BERRY, D. M. FLYNN, P. J. MCKIERNAN, D. A. KELLY, G. MIELI-VERGANI AND T. M. COX. 2001. Classification and genetic features of neonatal haemochromatosis: a study of 27 affected pedigrees and molecular analysis of genes implicated in iron metabolism. Journal of Medical Genetics 38: 599-610

KENNY, D. E., R. C. CAMBRE, T. R. SPRAKER, J. C. STEARS, R. D. PARK, S. B. COLTER, A. DE LAHUNTA AND J. R. ZUBA. 1996. Leukoencephalomalacia in a neonatal female black rhinoceros (*Diceros bicornis*): report of a fourth case. Journal of Zoo and Wildlife Medicine 27: 259-265

KNAPP, S. E., R. C. KRECEK, I. G. HORAK AND B. L. PENZHORN. 1997. Helminths and arthropods of black and white rhinoceroses in southern Africa. Journal of Wildlife Diseases 33: 492-502

KOCK, M. D. AND P. MORKEL. 1993. Capture and translocation of the free-ranging black rhinoceros: medical and management problems. *In* Zoo and Wild Animal Medicine, M. E. Fowler (ed.). Third Edition, WB Saunders, Philadelphia, pp. 466-475

KOCK, N., C. FOGGIN, M. D. KOCK AND R. KOCK. 1992. Hemosiderosis in the black rhinoceros (*Diceros bicornis*): a comparison of free-ranging and recently captured with translocated and captive animals. Journal of Zoo and Wildlife Medicine 23: 230-234

KUTCHAI, H. W. 1996. Gastrointestinal system: Digestion and absorption - Absorption of iron. *In* Principles of Physiology, R. M. Berne and M. N. Levy (ed.). Second Edition, Mosby-Year Book, St. Louis, pp. 491-492

LEADER-WILLIAMS, N. 1992. The world trade in rhino horn: a review. TRAFFIC International, Cambridge, pp. 40

LENT, P. C. AND B. FIKE. 2003. Home ranges, movements and spatial relationships in an expanding population of black rhinoceros in the Great Fish River Reserve, South Africa. South African Journal of Wildlife Research 33: 109-118

LEWIS, J. C. AND J. K. KIRKWOOD. 1990. Studies on vitamin E supplementation in a black rhinoceros (*Diceros bicornis*). Veterinary Record 126: 558

LIMDI, J. K. AND G. M. HYDE. 2003. Evaluation of abnormal liver function tests. Postgraduate Medical Journal 79: 307-312

LIVREA, M. A., L. TESORIERE, A. M. PINTAUDI, A. CALABRESSE, A. MAGGIO, H. J. FRIESLEBEL, D. D'ARPA, R. D'ANNA AND A. BONGIORNO. 1996. Oxidative stress and antioxidant status in beta-thalassemia major: iron overload and depletion of lipid-soluble antioxidants. Blood 88: 3608-3614

LOWENSTINE, L. J. AND L. MUNSON. 1999. Iron overload in the animal kingdom. *In* Zoo and Wild Animal Medicine, M. E. Fowler (ed.). Third Edition, WB Saunders, Philadelphia, pp. 260-268

MAINOUS, A. G., 3RD, B. WELLS, P. J. CAREK, J. M. GILL AND M. E. GEESEY. 2004. The mortality risk of elevated serum transferrin saturation and consumption of dietary iron. Annals of Family Medicine 2: 139-144

MARTIN, E. B. AND L. VIGNE. 1995. Agate replaces rhino horn in Yemen's new dagger handles. Oryx 29: 154

MARTINI, F. H. 2001. Fundamentals of Anatomy & Physiology. Fifth Edition, Prentice-Hall, Upper Saddle River, pp. 31, 633, 928-929

MASSANYI, P., J. TRANDZIK, P. NAD, R. TOMAN, M. SKALICKA AND B. KORENEKOVA. 2003. Seminal concentrations of trace elements in various animals and their correlations. Asian Journal of Andrology 5: 101-104

METE, A., H. G. HENDRIKS, P. H. KLAREN, G. M. DORRESTEIN, J. E. VAN DIJK AND J. J. MARX. 2003. Iron metabolism in mynah birds (*Gracula religiosa*) resembles human hereditary haemochromatosis. Avian Pathology 32: 625-632

MILLER, R. E., R. C. CAMBRE, A. DE LAHUNTA, R. E. BRANNIAN, T. R. SPRAKER, C. JOHNSON AND W. J. BOEVER. 1990. Encephalomalacia in three black rhinoceroses (*Diceros bicornis*). Journal of Zoo and Wildlife Medicine 21: 192-199

_____. 1993. Hemolytic anaemia in the black rhinoceros. *In* Zoo and Wild Animal Medicine, M. E. Fowler (ed.). Third Edition, WB Saunders, Philadelphia, pp. 455-458

_____. 2001. An overview of diseases of black rhinoceroses in North America 1980-2001. *In* Proceedings of the International elephant and rhino research symposium: a research update on elephants and rhinos, Vienna, pp. 210-214

_____. 2003. Rhinoceridae (Rhinoceroses). *In* Zoo and Wild Animal Medicine, M. E. Fowler and R. E. Miller (eds.). Fifth Edition, WB Saunders, Philadelphia, pp. 558-569

MUNSON, L. AND R. E. MILLER. 1999. Skin disease of black rhinoceroses. *In* Zoo and Wild Animal Medicine, M. E. Fowler (ed.). Fourth Edition, WB Saunders, Philadelphia, pp. 551-555

MURRAY, S., N. P. LUNG, T. P. ALVARADO, K. C. GAMBLE, M. A. MILLER, D. E. PAGLIA AND R. J. MONTALI. 1999. Idiopathic hemorrhagic vasculopathy syndrome in seven black rhinoceros. Journal of the American Veterinary Medical Association 216: 230-233

NAKAO, M., T. TOYOZAKI, H. NAGAKAWA, T. HIMI, K. YAMADA, S. WATANABE, Y. MASUDA AND T. ASAI. 2001. Cardiac dysfunction because of secondary hemochromatosis caused by congenital non-spherocytic hemolytic anemia. Japanese Circulation Journal 65: 126-128

NOWAK, R. M. 1991. Perissodactyla: Family Rhinocerotidae. *In* Walker's Mammals of the World, R. M. Nowak (ed.). Fifth Edition, The John Hopkins University Press, London, pp. 1322-1333

ODDONE, M., C. BELLINI, W. BONACCI, M. BAROCCI, P. TOMA AND G. SERRA. 1999. Diagnosis of neonatal hemochromatosis with MR imaging and duplex Doppler sonography. European Radiology 9: 1882-1885

OLSMAN, A. F. AND M. M. SLOET VAN OLDRUITENBORGH-OOSTERBAAN. 2004. [Primary liver disease in the horse]. Tijdschrift voor Diergeneeskunde 129: 510-522

OWEN-SMITH, N. AND J. BERGER. 2001. Rhinoceroses. *In* The New Encyclopedia of Mammals, D. MacDonald (ed.). Oxford University Press, Oxford, pp. 476-481

PAGLIA, D. E., W. N. VALENTINE, R. E. MILLER, M. NAKATANI AND R. A. BROCKWAY. 1986. Acute intravascular hemolysis in the black rhinoceros: erythrocyte enzymes and metabolic intermediates. American Journal of Veterinary Research 47: 1321-1325

_____AND R. E. MILLER. 1993. Erythrocytes of the black rhinoceros (*Diceros bicornis*): susceptibility to oxidant-induced hemolysis. International Zoo Yearbook 32: 20-27

_____, _____ AND S. W. RENNER. 1996. Is impairment of oxidant neutralization the common denominator among diverse diseases of black rhinoceroses? *In* Proceedings of the American Association of Zoo Veterinarians, pp. 37-41

_____ AND P. DENNIS. 1999. Role of chronic iron overload in multiple disorders of captive black rhinoceroses (*Diceros bicornis*). *In* Proceedings of the American Association of Zoo Veterinarians, pp. 163-171

_____ AND R. W. RADCLIFFE. 2000. Anthracycline cardiotoxicity in a black rhinoceros (*Diceros bicornis*): evidence for impaired antioxidant capacity compounded by iron overload. Veterinary Pathology 37: 86-88

_____, E. S. DIERENFELD AND I. H. TSU. 2001a. Pathological iron overloads acquired in captivity by browsing (but not by naturally grazing) rhinoceroses. *In* Proceedings of the International elephant and rhino research symposium: a research update on elephants and rhinos, Vienna, pp. 217

_____, D. E. KENNY, E. S. DIERENFELD AND I. H. TSU. 2001b. Role of excessive maternal iron in the pathogenesis of congenital leukoencephalomalacia in captive black rhinoceroses (*Diceros bicornis*). American Journal of Veterinary Research 62: 343-349

PARKKILA, S., A. WAHEED, R. S. BRITTON, B. R. BACON, X. Y. ZHOU, S. TOMATSU, R. E. FLEMING AND W. S. SLY. 1997. Association of the transferrin receptor in human placenta with *HFE*, the protein defective in hereditary hemochromatosis. *In* Proceedings of the National Academy of Sciences of the United States of America 94: 13198-13202

PEARSON, E. G., O. R. HEDSTROM AND R. H. POPPENGA. 1994. Hepatic cirrhosis and hemochromatosis in three horses. Journal of the American Veterinary Medical Association 204: 1053-1056

_____ AND C. B. ANDREASEN. 2001. Effect of oral administration of excessive iron in adult ponies. Journal of the American Veterinary Medical Association 218: 400-404

POWERS, R. D. AND R. A. PRICE. 1967. Human tuberculosis in a rhinoceros. Journal of the American Veterinary Medical Association 151: 890-892

RADCLIFFE, R. W., D. E. PAGLIA AND C. G. COUTO. 2000. Acute lymphoblastic leukemia in a juvenile southern black rhinoceros (*Diceros bicornis minor*). Journal of Zoo and Wildlife Medicine 31: 71-76

RAMSAY, E. C. AND Z. Z. ZAINUDDIN. 1989. Infectious diseases of the rhinoceros and tapir. *In* Zoo and Wild Animal Medicine, M. E. Fowler (ed.). Third Edition, WB Saunders, Philadelphia, pp. 495-465

RIEDEL, H. D., M. U. MUCKENTHALER, S. G. GEHRKE, I. MOHR, K. BRENNAN, T. HERRMANN, B. A. FITSCHER, M. W. HENTZE AND W. STREMMEL. 1999. *HFE* downregulates iron uptake from transferrin and induces iron-regulatory protein activity in stably transfected cells. Blood 94: 3915-3921

ROELS, S., R. DUCATELLE AND H. CORNELISSEN. 1996. Quantitative image analysis as an alternative to chemical analysis for follow-up of liver biopsies from a toucan with hemochromatosis. A technique with potential value for the follow-up of hemochromatosis in humans. Analytical and Quantitative Cytology and Histology 18: 221-224

ROLFS, A. AND M. A. HEDIGER. 1999. Metal ion transporters in mammals: structure, function and pathological implications. Journal of Physiology 518: 1-12

ROWE, N. 1996. The pictoral guide to the living primates. Pogonias Press, Charlestown, Rode Island, pp. 27-47

SCHMIDT-NIELSEN, K. 1997. Animal physiology: Adaptation and environment. Fifth Edition, Cambridge University Press, Cambridge, pp. 154-158

SCHOEMAKER, N. J. AND A. C. BEYNEN. 2001. [Composition of commercial feeds for mynah birds with particular attention to the iron content]. Tijdschrift voor Diergeneeskunde 126: 620-623

SEELEY, R. R., T. D. STEPHENS AND P. TATE. 2003. Anatomy & Physiology. Sixth Edition, McGraw-Hill, Boston, pp. 27, 30, 919

SILBERNAGL, S. AND A. DESPOPOULOS. 1996. Atlas of physiology. Tenth Edition, Bosch and Keuning NV, Baarn, pp. 208

SMITH, J. E., P. S. CHAVEY AND R. E. MILLER. 1995. Iron metabolism in captive black (*Diceros bicornis*) and white (*Ceratotherium simum*) rhinoceroses. Journal of Zoo and Wildlife Medicine 26: 525-531

SPALDING, M. G., G. V. KOLLIAS, M. B. MAYS, C. D. PAGE AND M. G. BROWN. 1986. Hepatic encephalopathy associated with hemochromatosis in a toco toucan. Journal of the American Veterinary Medical Association 189: 1122-1123

SPELMAN, L. H., K. G. OSBORN AND M. P. ANDERSON. 1989. Pathogenesis of hemosiderosis in lemurs: role of dietary iron, tannin and ascorbic acid. Zoo Biology 8: 239-251

THERON, J. J. AND R. C. P. M. MEKEL. 1971. Ultrastructural localization of iron in the human jejunum in iron overload (Bantu siderosis). British Journal of Haematology 21: 165-171

TUROCZI, T., L. JUN, G. CORDIS, J. E. MORRIS, N. MAULIK, R. G. STEVENS AND D. K. DAS. 2003. *HFE* mutation and dietary iron content interact to increase ischemia/reperfusion injury of the heart in mice. Circulation Research 92: 1240-1246

WEBER, B., D. E. PAGLIA AND E. H. HARLEY. 2001. Red cell metabolism in the black rhinoceros: relevance to haemolytic disease. *In* Proceedings of the International elephant and rhino research symposium: a research update on elephants and rhinos, Vienna, pp. 181

_____, _____AND _____. 2004. Elevated free tyrosine in rhinoceros erythrocytes. Comparative Biochemistry and Physiology, Part A 138: 105-109

WOOD, P. A., D. C. FOGGIN AND T. W. NAUDE. 1997. Suspected calcium oxalate raphide irritation in a black rhinoceros (*Diceros bicornis*) due to ingestion of *Xanthosoma mafaffa*. Journal of the South African Veterinary Association 68: 2

Assessing iron storage disease in eastern black rhinoceroses

(*Diceros bicornis michaeli*)

Reference ranges for iron levels and biochemistry

Fieke Molenaar
MSc Wild Animal Health
Institute of Zoology (IoZ)
Regents Park
London NW1 4RY
0798 6578086
fiekemm@hotmail.com

Rajan Amin
Zoological Society of London
Regents Park
London NW1 4RY
0207 7223333

Michael Waters
Department of Pathology
Royal Veterinary College
Hawkshead Lane
Hatfield AL9 7TA
0170 7666326

Scientific Paper submitted in part fulfilment of the requirements for the degree of Master of Science in Wild Animal Health

University of London, 2005

Abstract

Iron storage disease (haemochromatosis) is thought to be the cause of many diseases unique to captive black rhinoceroses (*Diceros bicornis*). Although it has been a common post mortem finding, assessing the onset and progression of iron storage disease during the life of a rhinoceros has thus far been difficult, due to the lack of reliable reference ranges for iron parameters and biochemistry. This study has used serum samples from eastern black rhinoceroses (*Diceros bicornis michaeli*) from a translocation programme in Kenya (n=27) to establish reference ranges against which to compare samples from captive individuals. Comparing serum samples from a seemingly healthy captive population in the UK (n=17) with these reference ranges has revealed significant differences in several biochemical parameters. Most remarkable were the elevations of the transferrin saturation, serum iron and GGT. These are considered important biochemical markers for haemochromatosis. However, when the obtained results of this study were compared with previously published data, these significant differences were obscured.

This study has demonstrated the importance of standardisation in the calculation of reference ranges and has shown the value of using free-ranging rhinoceroses of a similar subspecies to obtain reliable reference ranges for iron parameters and biochemistry of captive individuals, not in the least by analysing all the samples at set laboratories.

Keywords

Biochemistry, *Diceros*, iron, haemochromatosis, haemosiderosis, rhinoceros

Introduction

Black rhinoceroses (*Diceros bicornis*) are Critically Endangered browsing rhinoceroses from Africa (Owen-Smith and Berger, 2001). On post mortem examination of captive black rhinoceroses, haemosiderosis, the deposition of excess body iron into insoluble iron clusters in soft tissues (Jones, Hurt and King, 1997), is commonly found (Smith, Chavey and Miller, 1995). Remarkably, this is not seen in

free-ranging individuals (Kock, Foggin, Kock and Kock, 1992), nor in captive or free-ranging grazing rhinoceros species, such as the white rhinoceros (*Ceratotherium simum*) (Smith et al., 1995; Paglia and Dennis, 1999). Iron deposition starts shortly after capture (Kock and Morkel, 1993) and the total quantity appears to be correlated with the length of time in captivity (Miller, 2003). Possible adaptation to low dietary iron bioavailability in the wild could be the cause of enhanced intestinal iron absorption (Paglia, Dierenfeld and Tsu, 2001a). Certain diseases of unknown aetiology unique to captive black rhinoceroses, such as haemolytic anaemia, mucocutaneous ulcerative disorder, congenital leucoencephalomalacia and exceptional susceptibility to infections, seem to be related to this excessive iron storage (Paglia, Miller and Renner, 1996; Paglia and Radcliffe, 2000; Paglia, Kenny, Dierenfeld and Tsu, 2001b). This excess iron can interfere with normal physiological functioning due to its mechanical presence and formation of free oxi-radicals (Rolfs and Hediger, 1999; Beutler, Hoffbrand and Cook, 2003). This is termed haemochromatosis. Clinical signs may appear worse in black rhinoceroses in particular, because of inherent sensitivity to oxidative stress (Paglia and Miller, 1993) and natural low levels of biological antioxidants (Dierenfeld, du Toit and Miller, 1988).

These diseases unique to captive black rhinoceroses may be managed by adding iron chelators to the diet to reduce its bioavailability, increasing dietary antioxidants and, since interference is only successful at an early stage (Paglia and Dennis, 1999), by monitoring serum iron parameters and biochemistry to anticipate the onset and progression of iron overload (Olsman and Sloet van Oldruitenborgh-Oosterbaan, 2004). Reliable reference ranges will be needed against which to compare the blood results. Because of similarities of the gastrointestinal tract (Jones, 1979), the domestic horse, *Equus domesticus* (Order Perissodactyla, Family Equidae), has frequently been used as a nutritional model for all rhinoceros species (Dierenfeld, 1999; Dierenfeld, Atkinson, Craig, Walker, Streich and Clauss, 2005). Biochemical normal ranges derived from the horse have also been used for rhinoceroses, but these appear to be inadequate for some blood parameters (Clauss, Jessup, Norkus, Chen, Holick, Streich and Dierenfeld, 2002). Therefore, it might be more sensible to use physiological data acquired from free-ranging black rhinoceroses as a baseline against which to compare blood parameters of captive rhinoceroses. Recently, reference ranges have been established for the mineral status of both free-ranging black and white rhinoceroses

and captive black, white, Indian (*Rhinoceros unicornis*) and Sumatran rhinoceroses (*Dicerorhinus sumatrensis*) in the United States (USA) (Dierenfeld et al., 2005). However, inter-laboratory variation may render these reference ranges less helpful for use in the United Kingdom (UK).

The Darwin Initiative project of the Zoological Society of London (ZSL) is presently assisting the Kenya Wildlife Service (KWS) in implementing a five-year national conservation strategy for eastern black rhinoceroses (*Diceros bicornis michaeli*). As part of their biological management, adult black rhinoceroses from reserves approaching or exceeding ecological carrying capacity have been immobilised and translocated to increase the number of black rhinoceroses in existing sanctuaries and to create new populations. Additionally, rhinoceroses from existing populations have been immobilised and ear-notched for individual recognition to facilitate local population management. During these immobilisations, blood and tissue samples have been collected. Using those blood samples, this study aimed to establish reliable reference ranges from free-ranging eastern black rhinoceroses for iron parameters and biochemistry at laboratories based in the UK, to determine whether or not there are inter-region differences regarding these parameters in free-ranging rhinoceroses and to compare these with stored serum samples of a captive population to obtain a preliminary idea about the status of captive black rhinoceroses in the UK.

Materials and Methods

Sixty-four (64) serum samples from eastern black rhinoceroses inhabiting Nairobi National Park (NNP), Lake Nakuru National Park (LNNP) and the private sanctuaries of the Laikipia plateau in central Kenya (Solio, Ol Jogi and Lewa) were randomly selected, representing 64 individuals. All rhinoceroses had been immobilised with an intramuscular injection of etorphine [5.5 mg] (M99®, Novartis, Litlington) and xylazine [100 mg] (Rompun®, Bayter, Newbury), using a long-range rifle-type dart projector (Palmer Cap-Chur Inc., Powder Springs, USA). Immediately after induction, nalorphine [5 mg] (Nalorphine HCl Solution, Sigma-Aldrich, Gillingham) was administered intramuscularly for partial reversal and, after surgical preparation of the sampling site, blood samples were taken from the *vv. auriculares* of either ear using an 18-G hypodermic needle and a 20 ml disposable syringe. This blood was immediately transferred to 1.8 ml storage vials. Approximately one hour later the

samples were spun down for collection of the serum, which was subsequently stored in a deep freezer at -85°C. From storage, the samples were packed on dry ice and shipped to the UK under CITES licensing.

Twenty-four (24) stored serum samples from eastern black rhinoceroses residing at Port Lympne Wild Animal Park (Kent, UK) were randomly selected, representing 14 individuals. All samples had been taken from the *v. radialis* of either foreleg using a 21-G butterfly hypodermic needle and Vacutainer® system, either conscious or under general anaesthetic. Collected blood was left overnight for clotting and subsequently spun to separate the serum. This was transferred to separate vials and stored in a deep freezer at -85°C. After selection, the serum samples were packed on dry ice and sent to the Royal Veterinary College (RVC, Hatfield, Hertfordshire).

At the RVC, all serum samples were thawed after arrival and run through an ILAB™ 600 Clinical Chemistry System (Instrumentation Laboratory, Lexington, USA) for total protein (TP), albumin, globulin, sodium (Na), potassium (K), chloride (Cl), calcium (Ca), inorganic phosphorus (P), urea, creatinine, total bilirubin, bile acids, alanine transferase (ALT), aspartate aminotransferase (AST), creatinine kinase (CK), gamma-glutamyltransferase (GGT), alkaline phosphatase (ALP), glutamate dehydrogenase (GLDH) and sorbitol dehydrogenase (SDH).

Remaining serum was subsequently sent to the Veterinary Laboratory Agency (VLA, Shrewsbury, Shropshire). All samples were run through an Olympus® AU400 chemistry analyser (Olympus UK, Southall, Middlesex) for total iron and unbound iron binding capacity (UIBC). The total iron binding capacity (TIBC) was obtained by adding these two values and the percentage transferrin saturation was obtained by dividing the unbound iron binding capacity by the derived total iron binding capacity, multiplied by 100.

All results derived from haemolysed samples were discarded. Using SPSS 11.0 and MATLAB statistical software packages, results from 27 serum samples representing 27 free-ranging rhinoceroses from Kenya were used to establish reference ranges and compared with 17 serum samples representing 12 captive rhinoceroses from Port Lympne Wild Animal Park. The free-ranging population was divided into two subpopulations (the Laikipia plateau in central Kenya [Solio, Ol Jogi and Lewa, n=11] and the south-central lowland region [NNP and LNNP, n=16]) to determine more accurately which parameters differed significantly between the studied captive and free-ranging populations.

The Lilliefors goodness-of-fit hypothesis test was performed to determine normal distribution of the data. The significance level (P) was set at 0.05. For the parameters that followed normal distribution, the 95% confidence interval (mean ± 1.96SD) was used to establish the reference ranges of the free-ranging rhinoceroses. For the parameters that were not normally distributed, an attempt to transform the data to follow normal distribution using Logs to the base 10 was unsuccessful. Furthermore, because of the small sample size, the use of 95% percentiles would have resulted in a reference range based on all the data points, enabling outliers to severely distort this estimate. The reference ranges were therefore estimated as the 95% confidence interval of the median (Seigel, 1988).

To determine whether significant differences existed between the captive UK population and the free-ranging rhinoceroses from Kenya, the Student's t-test and the Analysis of Variance (ANOVA) (Petrie and Watson, 1999) were performed on the parameters that followed normal distribution in both captive and free-ranging populations and non-parametric approaches were used where either or both distributions (free-ranging and/or captive) did not follow normal distribution. The non-parametric Wilcoxon rank sum test was used to compare the parameters of free-ranging versus captive rhinoceroses and to compare the two different regions in Kenya (the Laikipia plateau and the South-central lowland region). The non-parametric Kruskal-Wallis test was undertaken for comparison of the parameters of the captive UK population with those of the free-ranging rhinoceroses from the different regions within Kenya. Where significant differences were found between the captive population and the Kenyan subpopulations, a multicomparison test on the ANOVA or the Kruskal-Wallis output parameters was performed to determine which population was significantly different from the other two populations. The nul-hypotheses (H_0) for this study assumed no significant differences between the serum iron parameters and biochemistry of the free-ranging and captive black rhinoceroses, no significant differences between the parameters of the free-ranging rhinoceroses from the two different regions and no significant differences between the parameters of the captive rhinoceroses and the two Kenyan regions.

Results

Bile acids results have been excluded from this study due to unreliable values. All data used are listed in Table 1. The results from the Lilliefors goodness-of-fit hypothesis test for normal distribution of the free-ranging populations, the two Kenyan regions and the captive population are displayed in Table 2. Serum iron and GGT followed normal distribution in both the captive and free-ranging populations. Transferrin saturation, inorganic phosphorus and ALP followed normal distribution in the free-ranging population, but not in the captive population. Albumin, sodium, chloride, calcium, urea and creatinine followed normal distribution in the captive population, but not in the free-ranging population. Total protein, globulin, potassium, total bilirubin, ALT, AST, CK, GLDH and SDH did not follow normal distribution in either population. The reference ranges derived from the free-ranging rhinoceroses from Kenya are displayed in Table 3 and the data ranges from the captive UK population are displayed in Table 4.

Several significant differences between the parameters of the free-ranging and captive rhinoceroses have been found. The captive black rhinoceroses showed a significant elevation of percentage transferrin saturation ($P<0.001$) and serum iron ($P=0.004$), and a significant reduction of the UIBC ($P<0.001$) (Figure 1). Of the other biochemical parameters, potassium ($P<0.001$) and inorganic phosphorus ($P=0.01$) were significantly decreased and urea ($P=0.004$), CK ($P=0.008$), GGT ($P<0.001$), ALP ($P=0.013$), GLDH ($P=0.044$) and SDH ($P=0.042$) were significantly increased.

Comparing the parameters of the rhinoceroses from the two regions in Kenya, significant differences could be found for TIBC ($P=0.042$) and SDH ($P=0.023$).

The two Kenyan subpopulations were subsequently compared to the captive population. The multicomparison test output for the iron parameters is displayed in Figure 2. Significant difference between the parameters of the captive population and the parameters of both free-ranging populations could be found for transferrin saturation ($P=0.001$), serum iron ($P=0.007$), UIBC ($P=0.001$), inorganic phosphorus ($P=0.02$), urea ($P=0.013$) and GGT ($P<0.001$) (Figure 3).

Discussion

In this study significant differences between several iron and biochemical parameters of captive and free-ranging eastern black rhinoceroses have been found. The marked differences of most iron parameters between a seemingly healthy captive population and its wild counterparts appear of particular importance. There was no significant difference between the TIBC of the captive and free-ranging rhinoceroses. This can be explained by the fact that this is a parameter inherent to the species (Smith, 1997), which is only altered in cases of reduced transferrin production due to hepatic failure (Brittenham, Weiss, Brissot, Laine, Guillygomarc'h, Guyader, Moirand and Deugnier, 2000). Compared to the parameters of the free-ranging black rhinoceroses, the serum transferrin saturation and the serum iron levels of the captive population were significantly elevated and the unbound iron binding capacity was significantly reduced. For comparison of these results with previously published data, a valid direct assessment is only possible where the ranges are calculated using the same approach. All the previous studies have based their range estimates on the data mean and standard deviation, which are both sensitive to non-symmetrical distributions and outliers, severely affecting the estimates. In this discussion, comparisons are only made using the normally distributed parameters. The transferrin saturation derived from the free-ranging black rhinoceroses from Kenya appears slightly higher than the range derived from a study of black rhinoceroses from Zimbabwe (Table 5a) (Paglia and Dennis, 1999). This might be because of the small sample size of that study (n=6), the possible difference in subspecies, differences in mineral content of local soil and vegetation or differences in the performed assays. Because of non-normal distribution, the transferrin saturation of the captive black rhinoceros population in this study could not be compared with the results from the study of Paglia and Dennis (1999). Comparing the normally distributed serum iron results with the published reference ranges listed in Table 5a and 5b, a number of differences can be identified. Firstly, when compared to the reference range for domestic horses (Smith, 1997), assumed to be [mean ± 1.96SD], the data from both the wild and captive population appear to be within normal limits, obscuring the significant difference between these two found in this study (Table 5b). The reference range given by ISIS, the International Species Information System (Flesness, 2002), is also assumed to be

[mean ± 1.96SD]. When comparing the serum iron reference range derived from free-ranging rhinoceroses and the data range of captive rhinoceroses obtained in this study to this reference range, it is noticeable that the captive results are considered within normal range and the significant difference between free-ranging and captive rhinoceroses is again obscured. This demonstrates that, because the ISIS data are derived from samples from captive individuals, the use of this reference ranges will not provide similar information as what would be obtained using a reference range derived from free-ranging individuals. Further, comparing the derived results with previously published reference ranges for black rhinoceroses (Table 5a), there is a marked difference between the serum iron measurements in this study and in a study of 27 free-ranging black rhinoceroses from Zimbabwe (Dierenfeld et al., 2005). Reasons for this discrepancy could again be the possible difference in subspecies, differences in mineral content of local soil and vegetation or differences in the performed assays. Additional comparison of the serum iron of the captive population from this study with that of a captive population from the USA [n=24] (Paglia and Dennis, 1999) shows that the serum iron levels of the captive population from the USA are considerably higher. This might again be due to differences in subspecies or performed assays, but are most likely related to significant dietary differences. This could benefit from further research, with samples from different zoological institutions analysed at one laboratory for direct comparisons of iron parameters using standardised statistical analysis, along with analysis of the different captive diets.

In humans, iron storage disease (haemochromatosis) is a common hereditary genetic disorder (Parkkila, Waheed, Britton, Bacon, Zhou, Tomatsu, Fleming and Sly, 1997; Brissot, Guyader, Loreal, Laine, Guillygomarc'h, Moirand and Deugnier, 2000), but can also be seen as an acquired disease (Hennigar, Greene, Walker and de Saussure, 1979; Gordeuk, 1992). Assessment of this disease in humans is based on screening individuals through measurement of the serum transferrin saturation (Brittenham et al., 2000; Hash, 2001). Transferrin saturations under 40% are considered normal and an elevated transferrin saturation of more than 60% on at least two occasions is considered indicative of haemochromatosis (Beutler et al., 2003). Although by these standards the results derived in this study from the free-ranging black rhinoceros population can be considered relatively high, most of the data acquired from the captive population could then be considered indicative of haemochromatosis. However, elevated transferrin saturation can also be seen with reduced transferrin

production due to hepatic failure of different origin, which should be ruled out first (Brittenham et al., 2000). For assessing haemochromatosis, the serum ferritin level is usually measured in conjunction with the assessment of the transferrin saturation. Being an acute phase reactant, the serum ferritin level should not be interpreted on its own, since elevations can be seen during several inflammatory processes (Limdi and Hyde, 2003). When seen with increased transferrin saturation, elevated serum ferritin can give an indication of the presence of liver cirrhosis, an irreversible consequence of haemochromatosis (Hash, 2001; Beutler et al., 2003). Equine serum ferritin assays (Smith, Moore, Cipriano and Morris, 1984) modified for black rhinoceroses are available (Cellular and Molecular Pathology Laboratory, College of Veterinary Medicine, Kansas State University) and have been used in several previous studies (Smith et al., 1995; Paglia and Dennis, 1999; Paglia et al., 2001b). Due to financial constraints, these assays could not be performed during this study.

After initial indication of haemochromatosis by elevated transferrin saturation and serum ferritin levels, liver enzymes are analysed to establish the extent of liver damage (Hash, 2001). In horses with haemochromatosis, elevations of bile acids, GGT, ALP and AST are usually seen and both bile acids and GGT are thought to be the most informative parameters for hepatic function (Olsman and Sloet van Oldruitenborgh-Oosterbaan, 2004). Although unfortunately the bile acids results could not be included, the captive rhinoceroses in this study did have significantly elevated inorganic phosphorus, urea and GGT levels when compared with free-ranging rhinoceroses. A case by case comparison would be required to draw conclusions about individual haemochromatosis or hepatic functioning of rhinoceroses from the captive population. Because this study focuses on the population as a whole, these comparisons have not been made here. Also, when an asymptomatic individual has abnormal blood parameters, it is advisable to take a new sample after overnight fasting and resubmit it for analysis prior to subsequent diagnostic steps (Limdi and Hyde, 2003). Thus, serial samples from one rhinoceros compared to these reference ranges would provide more information and could be considered for future study. For comparison of the differing parameters, values for inorganic phosphorus, urea and GGT are presently available from ISIS (Flesness, 2002). Assuming the reference range to be [mean ± 1.96SD], the ISIS reference range for GGT is much wider than the one obtained from the free-ranging rhinoceroses in this study and the significant difference between the captive and free-ranging

population is obscured (Table 6). The differences between these reference ranges could be explained by the fact that the ISIS range has been obtained using a much larger sample of solely captive black rhinoceroses, some of which could have been suffering from subclinical haemochromatosis, thus distorting the estimated ranges. Additionally, because the obtained data originate from different institutions, different laboratory techniques might have been used. Because the results for inorganic phosphorus and urea of both free-ranging and captive rhinoceroses were not normally distributed, these could not be compared with the ISIS data. Comparing the GGT results from this study with the reference range used for the domestic horse at the RVC (Table 6), the significant difference between the free-ranging and captive rhinoceroses is partly obscured in the wider normal range. This is remarkable, since the horse, belonging to the Order Perissodactyla, has been used in the past as a model for rhinoceroses. More importantly, the rhinoceros samples were analysed by the same laboratory (RVC, Hatfield), ruling out inter-laboratory variance. The non-normal distribution of the other data from the biochemistry analysis run at the RVC makes the comparison with the equine reference ranges, based on the assumption of normally distributed data (Table 7), very difficult. Because of haemolysis in many of the serum samples, the reference ranges in this study were derived from a relatively small sample size. More samples are presently being collected from black rhinoceroses in Kenya during the on-going translocation and ear-notching processes. The data derived from the analysis of those samples will be combined with our current data to obtain reference ranges that will be more representative. Thereafter, the comparison between equine and rhinoceros reference ranges will again be made to draw more definite conclusions.

Apart from humans, horses and black rhinoceroses, haemochromatosis has also been recorded in gum-eating marmosets, folivorous gorillas, hyrax and house shrews, and less commonly in forest-dwelling bongos, duikers, dik-diks and old orang-utans in captivity (Lowenstine and Munson, 1999). The main problem in both humans and animals is that clinical signs only appear at a later stage of the disease (Spelman, Osborn and Anderson, 1989; Lowenstine and Munson, 1999; Hash, 2001; Olsman and Sloet van Oldruitenborgh-Oosterbaan, 2004). The storage of excess iron will in time cause fibrosis of the affected organs. In the liver this is termed cirrhosis. This will disrupt the normal hepatic blood flow, causing passive congestion of the spleen and digestive organs. It is progressive, non-reversible and fatal (Jones et al., 1997).

Clinical signs of haemochromatosis are usually related to hepatic failure and are non-specific, such as lethargy and chronic wasting (Spelman et al., 1989; Lowenstine and Munson, 1999; Hash, 2001; Olsman and Sloet van Oldruitenborgh-Oosterbaan, 2004). Treatment of haemochromatosis is based on reducing the total iron content of the body to within its normal limits (Beutler et al., 2003). However, improvement of quality of life can only be achieved at an early stage of the disease, prior to irreversible damage to internal organs (Paglia and Dennis, 1999; Brissot et al., 2000; Brittenham et al., 2000). Several studies have shown that the amount of iron stored in black rhinoceroses increases with time kept in captivity (Smith et al., 1995; Paglia and Dennis, 1999; Paglia et al., 2001b). From as little as three weeks in boma confinement during translocations, a significant increase in hemosiderin deposition has been found (Kock and Morkel, 1993). Since the captive black rhinoceros population in this study had significantly higher serum iron levels and transferrin saturation than the free-ranging population, it may be sensible to assume that all captive black rhinoceroses are at risk of gradually developing haemochromatosis. Ideally, serial blood samples ought to be taken at regular intervals from a very early age onwards in order to determine the onset of excessive iron storage, to instigate further diagnostic testing, initiate possible dietary adjustments and therapeutic schedules without delay and to assess their effects. Just as in humans (Brittenham et al., 2000), monitoring of the serum transferrin saturation could be used as a screening tool in black rhinoceroses. Unfortunately, blood sampling a not-always-affable black rhinoceros, especially a calf in the presence of its dam, is not necessarily straightforward. Sophisticated diagnostic tools to determine tissue iron in human medicine, such as computed tomography (CT), magnetic resonance imaging (MRI) or magnetic susceptometry with a superconducting quantum interference device (SQUID) (Brittenham et al., 2000; Beutler et al., 2003) are unavailable for general zoo practice, due to expense and impracticality because of required cooperation and anatomical shape and size. Taking a liver biopsy under ultrasonographic guidance, such as performed in horses (Olsman and Sloet van Oldruitenborgh-Oosterbaan, 2004) may be more useful for black rhinoceroses to detect cirrhosis. However, it can be an inaccurate tool to determine hepatic iron content, because of uneven iron distribution (Brittenham et al., 2000; Beutler et al., 2003).

Serial phlebotomy appears to be the treatment of choice once haemochromatosis has been diagnosed (Paglia and Dennis, 1999). Again some cooperation of the rhinoceros

is essential for this treatment to succeed. Chelation therapy with deferroxamine has been proven successful in one case, but compliance and cost can pose serious problems (Paglia and Dennis, 1999). Because of the difficulties presented by the monitoring and treatment of haemochromatosis in black rhinoceroses, the focus of present research is on preventing the disease by improving the diet. Being a selective browser of twigs, bark, leaves and shrubs of a limited variety of plant species, the natural diet of free-ranging black rhinoceroses contains high levels of natural antioxidants and chelators like tannins (polyphenols), reducing the bioavailability of dietary iron (Clauss et al., 2002). Because in their captive diet both antioxidant levels and natural iron-chelators are low, black rhinoceroses are most likely suffering from the increased bioavailability of iron. Recent studies have focused on the addition of tannins to the diet, in the hope to reduce bioavailability and thus absorption of dietary iron (Clauss, Froeschle, Lechner-Doll, Hatt, Ganslosser and Dierenfeld, 2001). This research is presently on-going, but so far solutions have not been found. For this study, soil and browse samples from the different regions in Kenya have been collected. These samples have only recently arrived at the Institute of Zoology in London and will be forwarded to Direct Laboratories (Wolverhampton, UK) for analysis of the mineral composition and tannin (polyphenolics) content. Because of intestinal interaction of dietary minerals, not just the iron levels will be of interest, but also those of copper, cobalt, magnesium, manganese, molybdenum, selenium and zinc (Dierenfeld et al., 2005). These will then be compared with the dietary analysis of the black rhinoceroses from Port Lympne Wild Animal Park. We will expect a difference in mineral composition and chelator content to become evident and hopefully provide additional information to clarify the significant differences in biochemical parameters between this apparently healthy captive population and its free-ranging counterparts. Due to time constraints, these results could not be included here.

This study has demonstrated the importance of the standardisation of procedures, in particular of the calculation of reference ranges, to allow realistic comparisons between different studies. More importantly, it has shown that significant abnormalities of especially iron parameters can be obscured when comparing the analysis results with reference ranges derived from either captive rhinoceroses or the domestic horse and has demonstrated the value of using free-ranging rhinoceroses of a similar subspecies as a reference range for iron parameters and biochemistry of captive individuals, not in the least by analysing all the samples at set laboratories.

Acknowledgements

We would like to thank the Kenya Wildlife Service and Port Lympne Wild Animal Park for providing the serum samples, with special thanks to Benson Ouma and Richard McCort. The cooperation of Wayne Boardman of London Zoo and Edmund Flach of Whipsnade Wild Animal Park was greatly appreciated and we would like to thank Tony Sainsbury of the Institute of Zoology for his advice and support during the project set-up and coordination.

Last but not least, this study would not have been possible without the financial support of the Zebra Foundation of the British Veterinary Zoological Society and the Department of Pathology of the Royal Veterinary College, for which our sincerest gratitude.

Literature Cited

BEUTLER, E., A. V. HOFFBRAND AND J. D. COOK. 2003. Iron deficiency and overload. Hematology (The American Society of Hematology Education Program) 2003: 40-61

BRISSOT, P., D. GUYADER, O. LOREAL, F. LAINE, A. GUILLYGOMARC'H, R. MOIRAND AND Y. DEUGNIER. 2000. Clinical aspects of hemochromatosis. Transfusion Science 23: 193-200

BRITTENHAM, G. M., G. WEISS, P. BRISSOT, F. LAINE, A. GUILLYGOMARC'H, D. GUYADER, R. MOIRAND AND Y. DEUGNIER. 2000. Clinical consequences of new insights in the pathophysiology of disorders of iron and heme metabolism. Hematology (The American Society of Hematology Education Program) 2000: 39-50

CLAUSS, M., R. FROESCHLE, M. LECHNER-DOLL, J. M. HATT, U. GANSLOSSER AND E. S. DIERENFELD. 2001. Feeding tannins to captive black rhinoceros (*Diceros bicornis*): Results of a pilot study. *In* Proceedings of the International elephant and rhino research symposium: a research update on elephants and rhinos, Vienna, pp. 310

_____, D. A. JESSUP, E. B. NORKUS, T. C. CHEN, M. F. HOLICK, W. J. STREICH AND E. S. DIERENFELD. 2002. Fat soluble vitamins in blood and

tissues of free-ranging and captive rhinoceros. Journal of Wildlife Diseases 38: 402-413

DIERENFELD, E. S., R. DU TOIT AND R. E. MILLER. 1988. Vitamin E in captive and wild black rhinoceros (*Diceros bicornis*). Journal of Wildlife Diseases 24: 547-550

_____. 1999. Rhinoceros feeding and nutrition. *In* Zoo and Wild Animal Medicine, M. E. Fowler (ed.). Fourth Edition, WB Saunders, Philadelphia, pp. 568-571

_____, S. ATKINSON, A. M. CRAIG, K. C. WALKER, W. J. STREICH AND M. CLAUSS. 2005. Mineral concentrations in serum/plasma and liver tissue of captive and free-ranging rhinoceros species. Zoo Biology 24: 51-72

FLESNESS, N. 2002. Normal hematological values. International Species Inventory System (ISIS), Apple Valley, Minnesota.

GORDEUK, V. R. 1992. Hereditary and nutritional iron overload. Baillieres Clinical Haematology 5: 169-186

HASH, R. B. 2001. Hereditary hemochromatosis. Journal of the American Board of Family Practice 14: 266-273

HENNIGAR, G. R., W. B. GREENE, E. M. WALKER AND C. DE SAUSSURE. 1979. Hemochromatosis caused by excessive vitamin iron intake. American Journal of Pathology 96: 611-623

JONES, D. M. 1979. The husbandry and veterinary care of captive rhinoceroses. International Zoo Yearbook 19: 239-252

JONES, T. C., R. D. HURT AND N. W. KING. 1997. Veterinary Pathology. Sixth Edition, Williams & Wilkins, Baltimore, pp. 68-70, 804-805, 1027, 1098-1101

KOCK, M. D. AND P. MORKEL. 1993. Capture and translocation of the free-ranging black rhinoceros: medical and management problems. *In* Zoo and Wild Animal Medicine, M. E. Fowler (ed.). Third Edition, WB Saunders, Philadelphia, pp. 466-475

KOCK, N., C. FOGGIN, M. D. KOCK AND R. KOCK. 1992. Hemosiderosis in the black rhinoceros (*Diceros bicornis*): a comparison of free-ranging and recently captured with translocated and captive animals. Journal of Zoo and Wildlife Medicine 23: 230-234

LIMDI, J. K. AND G. M. HYDE. 2003. Evaluation of abnormal liver function tests. Postgraduate Medical Journal 79: 307-312

LOWENSTINE, L. J. AND L. MUNSON. 1999. Iron overload in the animal kingdom. *In* Zoo and Wild Animal Medicine, M. E. Fowler (ed.). Third Edition, WB Saunders, Philadelphia, pp. 260-268

MILLER, R. E. 2003. Rhinoceridae (Rhinoceroses). *In* Zoo and Wild Animal Medicine, M. E. Fowler and R. E. Miller (ed.). Fifth Edition, WB Saunders, Philadelphia, pp. 558-569

OLSMAN, A. F. AND M. M. SLOET VAN OLDRUITENBORGH-OOSTERBAAN. 2004. [Primary liver disease in the horse]. Tijdschrift voor Diergeneeskunde 129: 510-522

OWEN-SMITH, N. AND J. BERGER. 2001. Rhinoceroses. *In* The New Encyclopedia of Mammals, D. MacDonald (ed.). Oxford University Press, Oxford, pp. 476-481

PAGLIA, D. E. AND R. E. MILLER. 1993. Erythrocytes of the black rhinoceros (*Diceros bicornis*): susceptibility to oxidant-induced hemolysis. International Zoo Yearbook 32: 20-27

_____, _____ AND S. W. RENNER. 1996. Is impairment of oxidant neutralization the common denominator among diverse diseases of black rhinoceroses? *In* Proceedings of the American Association of Zoo Veterinarians, pp. 37-41

_____ AND P. DENNIS. 1999. Role of chronic iron overload in multiple disorders of captive black rhinoceroses (*Diceros bicornis*). *In* Proceedings of the American Association of Zoo Veterinarians, pp. 163-171

_____ AND R. W. RADCLIFFE. 2000. Anthracycline cardiotoxicity in a black rhinoceros (*Diceros bicornis*): evidence for impaired antioxidant capacity compounded by iron overload. Veterinary Pathology 37: 86-88

_____, E. S. DIERENFELD AND I. H. TSU. 2001a. Pathological iron overloads acquired in captivity by browsing (but not by naturally grazing) rhinoceroses. *In* Proceedings of the International elephant and rhino research symposium: a research update on elephants and rhinos, Vienna, pp. 217

_____, D. E. KENNY, E. S. DIERENFELD AND I. H. TSU. 2001b. Role of excessive maternal iron in the pathogenesis of congenital leukoencephalomalacia in captive black rhinoceroses (*Diceros bicornis*). American Journal of Veterinary Research 62: 343-349

PARKKILA, S., A. WAHEED, R. S. BRITTON, B. R. BACON, X. Y. ZHOU, S. TOMATSU, R. E. FLEMING AND W. S. SLY. 1997. Association of the

transferrin receptor in human placenta with *HFE*, the protein defective in hereditary hemochromatosis. Proceedings of the National Academy of Sciences of the United States of America 94: 13198-13202

PETRIE, A. AND P. WATSON. 1999. Statistics for Veterinary and Animal Science. Blackwell Science, Oxford.

ROLFS, A. AND M. A. HEDIGER. 1999. Metal ion transporters in mammals: structure, function and pathological implications. Journal of Physiology 518: 1-12

SEIGEL, A. F. 1988. Statistics and data analysis - an introduction. Wiley, New York.

SMITH, J. E., K. MOORE, J. E. CIPRIANO AND P. G. MORRIS. 1984. Serum ferritin as a measure of stored iron in horses. Journal of Nutrition 114: 677-681

_____, P. S. CHAVEY AND R. E. MILLER. 1995. Iron metabolism in captive black (*Diceros bicornis*) and white (*Ceratotherium simum*) rhinoceroses. Journal of Zoo and Wildlife Medicine 26: 525-531

_____. 1997. Iron metabolism and its disorders. *In* Clinical Biochemistry of Domestic Animals, J. J. Kaneko, J. W. Harvey and M. L. Bruss (ed.). Fifth Edition, Academic Press, San Diego, pp. 223-239

SPELMAN, L. H., K. G. OSBORN AND M. P. ANDERSON. 1989. Pathogenesis of hemosiderosis in lemurs: role of dietary iron, tannin and ascorbic acid. Zoo Biology 8: 239-251

Tables

Table 1a. Iron parameters and biochemistry of free-ranging Eastern black rhinoceroses (Kenya)

nr.	origin sample	%TS	Iron	TIBC	UIBC	TP	Alb	Glob	Na	K	Cl	Ca	P	Urea	Creat	Tbil	ALT	AST	CK	GGT	ALP	GLDH	SDH
1	Nakuru	52.4	32.1	61.3	29.2	75.6	28.1	47.5	136	9.7	87	2.65	1.68	5	110	0.8	4	109	95	14	27	3	14.5
2	Nakuru	39.8	21.4	53.8	32.4	81.6	25.6	56	142	5.3	90	2.82	1.66	1.9	191	0.9	2	26	41	9	46	3	15.3
3	Nakuru	28.4	11.2	39.5	28.3	91.6	22.2	69.4	141	6.9	103	2.32	1.48	8.8	115	0.1	6	410	1303	17	8	312	13.1
4	Nakuru	47.6	28	58.8	30.8	73.2	25.7	47.5	138	5.9	98	2.59	0.56	3.7	94	0.6	6	80	126	15	50	3	13
5	Nakuru	35.2	22.8	64.7	41.9	84.4	24.7	59.7	141	5.9	96	3.05	1.37	3.9	96	0.2	6	86	114	12	24	3	15.1
6	Nakuru	35.5	21.4	60.2	38.8	68.6	23.3	45.3	137	5.6	94	2.98	1.59	3.7	94	0.8	5	64	203	13	50	3	12.5
7	Nakuru	28.1	22.4	79.8	57.4	71.4	25.9	45.5	141	5.8	99	2.88	1.4	5.5	104	1.4	5	59	173	14	45	4	13.8
8	Nakuru	50.3	28.7	57.1	28.4	76.3	25.1	51.2	138	5.7	93	2.75	1.1	2.5	101	2.3	5	47	154	19	35	3	15.2
9	Nakuru	45.8	22.6	49.3	26.7	86.5	22.2	64.3	136	5.7	87	2.76	1.33	2.8	110	0.7	5	65	180	13	28	3	16.1
10	Nakuru	60.6	36.8	60.7	23.9	75.3	20.7	54.6	137	6.7	93	2.95	1.46	3.2	108	0.4	5	68	124	11	68	3	13.7
11	Nakuru	35.4	15.1	42.6	27.5	66.5	19.7	46.8	142	5.8	97	2.68	1.41	2.2	92	0	5	56	320	11	35	3	15.6
12	Nairobi NP	49.1	24.7	50.3	25.6	79.3	18.2	61.1	138.3	5.4	94.2	2.64	0.92	6.5	110	0.3	5	65	6	13	8	12	11.3
13	Nairobi NP	33.3	30	90	60	78	23.5	54.5	127.5	7.7	87.9	2.46	0.99	3.7	95	0	2	19	7	13	14	4	9.7
14	Nairobi NP	33.5	19.1	57.1	38	76.4	23.9	52.5	136.5	6	95.4	2.92	0.83	4.5	117	0	1	16	25	9	14	2	6.2
15	Nairobi NP	41.1	29.9	72.8	42.9	86.6	26.2	60.4	141	5.3	94	2.88	1.19	4.6	94	0.4	4	61	157	12	53	3	13.2
16	Nairobi NP	61.9	39	63	24	78.7	26.2	52.5	141	5.4	93	3.15	1.37	3.7	114	1.2	7	95	188	15	45	4	14.2
17	Ol Jogi	25.4	23.1	90.8	67.7	72.8	24.2	48.6	133	5.7	91	2.83	1.55	4.8	102	0.6	9	94	237	11	73	5	14.1
18	Ol Jogi	58.9	37.8	64.2	26.4	86.1	27.1	59	145	4.9	101	3	1.23	3.8	109	0.8	8	79	142	13	56	3	16.6
19	Ol Jogi	26.6	13.2	49.7	36.5	79.6	24.4	55.2	141	4.8	99	2.86	0.66	3.9	123	1.9	8	67	598	10	35	3	12.4
20	Ol Jogi	57.5	39.3	68.3	29	78.4	26.8	51.6	142	4.8	100	2.95	1.53	3.1	116	0.9	10	91	176	10	39	3	14.6
21	Solio	36.7	19	51.8	32.8	82	25.4	56.6	135.4	5.8	93	3	0.94	5.1	103	0	2	25	16	10	18	2	0
22	Solio	8.2	11.2	136.5	125.3	39.6	13	26.6	149.2	67.2	107.8	1.38	0.99	3.4	80	0	0	0	0	0	0	0	1.3
23	Solio	55.9	41.9	74.9	33	77.8	24.1	53.7	132.2	5.9	93.9	2.66	1.14	3.2	122	0.2	6	49	2	6	14	32	8.2
24	Solio	56	41.1	73.4	32.3	81.7	24.4	57.3	135.7	6.6	96.9	2.89	1.14	3.3	120	1.1	2	24	0	8	20	2	4.2
25	Solio	48.6	29.6	60.9	31.3	74.6	23.1	51.5	26.6	1.5	25.4	2.81	1.23	1.9	91	0	1	10	0	16	15	1	8.2
26	Solio	10.6	13.1	124	110.9	33	12.4	20.6	149.1	55.1	112.4	1.44	0.9	2.5	67	0	0	1	0	0	0	0	1.1
27	Lewa	87.5	79.6	91	11.4	98	29.3	68.7	153.6	7.6	113.3	3.09	0.65	11.9	145	0	22	896	13	20	4	218	11.3

Table 1b. Iron parameters and biochemistry of captive Eastern black rhinoceroses (United Kingdom)

nr.	origin sample	%TS	Iron	TIBC	UIBC	TP	Alb	Glob	Na	K	Cl	Ca	P	Urea	Creat	Tbil	ALT	AST	CK	GGT	ALP	GLDH	SDH
1	Port Lympne	78.4	42.8	54.6	11.8	81.1	25.1	56	133	4.9	92	2.26	0.62	3.6	85	0.6	10	48	381	20	3	3	14.5
2	Port Lympne	69.5	46	66.2	20.2	79.1	27.5	51.6	139	4.7	97	3.32	0.98	4.9	109	0.4	12	93	505	34	46	13	20.1
3	Port Lympne	66.6	36.5	54.8	18.3	96.9	21.2	75.7	134	4.9	96	2.97	0.68	5.4	104	0	8	22	72	36	38	30	16.8
4	Port Lympne	83.3	40.7	48.8	8.1	77.7	22	55.7	137	4.3	98	2.67	0.61	4	110	9.1	2	16	75	21	55	3	11.8
5	Port Lympne	78	39.8	51	11.2	78.7	22	56.7	139	4.3	101	2.79	0.56	4.2	120	11.1	8	19	71	23	58	3	23.3
6	Port Lympne	68.7	48.3	70.3	22	80	26.4	53.6	138	5.1	100	2.7	0.65	9.4	94	0	4	45	66	22	37	15	14
7	Port Lympne	78.5	37.3	47.5	10.2	62.5	16.1	46.4	140	5.5	99	2.48	0.24	5.5	77	0	12	206	8170	44	49	5	5.8
8	Port Lympne	55.9	35.5	63.5	28	80.3	28.7	51.6	142	4.4	101	3.18	0.46	6.3	137	0.8	3	39	411	32	51	7	14.9
9	Port Lympne	52.8	26.8	50.8	24	78.3	18.5	59.8	127	4.9	92	2.56	0.33	3.8	85	0.4	12	92	2603	18	18	5	9.7
10	Port Lympne	75	44.8	59.7	14.9	83	24.1	58.9	134	4.9	99	2.81	0.84	7.4	118	1.3	5	33	201	16	42	5	14.9
11	Port Lympne	70.5	38.2	54.2	16	80.3	24.5	55.8	141	4.7	101	2.88	1.63	5.6	135	0.7	4	69	287	24	36	5	15.7
12	Port Lympne	72.4	54.2	74.9	20.7	85.9	29	56.9	145	5.7	103	2.63	0.77	6.9	120	0	2	21	153	40	130	4	14.3
13	Port Lympne	77.4	50.6	65.4	14.8	83.5	28.5	55	143	5.4	101	2.21	0.73	6.5	119	0	2	18	150	39	118	2	15
14	Port Lympne	77.5	44	56.8	12.8	77.3	26.8	50.5	139	4.8	100	3.49	0.83	5.8	104	0.8	8	84	324	30	47	11	16.4
15	Port Lympne	43.5	49.7	114.2	64.5	57.9	28.1	29.8	146	4.7	97	2.85	2.32	1.5	90	0	5	6	62	7	304	2	14.4
16	Port Lympne	10.4	10.6	101.5	90.9	96	20.2	75.8	131	12.7	89	2.27	2.87	7.7	165	0	17	662	58517	10	42	11	5.1
17	Port Lympne	51.4	31.7	61.7	30	80	26.4	53.6	143	4.6	102	3.01	1.34	5.3	130	0	3	16	358	20	40	3	14.3

Legend: percentage transferrin saturation (%TS), iron (Iron) total iron binding capacity (TIBC), unbound iron binding capacity (UIBC), total protein (TP), albumin (Alb), globulin (Glob), sodium (Na), potassium (K), chloride (Cl), calcium (Ca), inorganic phosphorus (P), urea (Urea), creatinine (Creat), total bilirubin (TBil), alanine transferase (ALT), aspartate aminotransferase (AST), creatinine kinase (CK), gamma-glutamyltransferase (GGT), alkaline phosphatase (ALP), glutamate dehydrogenase (GLDH), sorbitol dehydrogenase (SDH). Bile acids have not been included, because of unreliable results.

Table 2. Results from Lilliefors goodness-of-fit test for normality on all the data [a]

Parameter	Free-ranging both regions	Lowland (NNP and LNNP)	Laikipia plateau	Captive
Transferrin saturation	0	0	0	1
Serum iron	0	0	0	0
Total iron binding capacity	1	0	0	1
Unbound iron binding capacity	1	0	1	1
Total protein	1	0	1	1
Albumin	1	0	1	0
Globulin	1	0	1	1
Sodium	1	0	1	0
Potassium	1	1	1	1
Chloride	1	0	1	0
Calcium	1	0	1	0
Inorganic phosphorus	0	0	0	1
Urea	1	0	1	0
Creatinine	1	1	0	0
Total bilirubin	1	0	0	1
ALT	1	1	0	1
AST	1	1	1	1
CK	1	1	1	1
GGT	0	0	0	0
ALP	1	0	0	1
GLDH	1	1	1	1
SDH	1	0	0	1

[a] H_0 assumes that the data follow normal distribution. The significance level is 0.05. The result of this hypothesis test is a Boolean value: 0 indicates the null hypothesis for normal distribution is not rejected, 1 indicates the nul hypothesis for normal distribution is rejected.

Table 3. Reference ranges for iron parameters and biochemistry of free-ranging eastern black rhinoceroses [a]

	FREE - RANGING (n = 27)			
Parameter	Median	Lower	Upper	Units
Transferrin saturation	42.6[b]	9.6[c]	75.6[c]	%
Iron	27.9[b]	0.7[c]	55.1[c]	µmol/l
Total iron binding capacity (TIBC)	61.3	57.1	73.4	µmol/l
Unbound iron binding capacity (UIBC)	32.3	28.3	38.8	µmol/l
Total protein	78.0	74.6	81.7	g/l
Albumin	24.4	23.1	25.7	g/l
Globulin	53.7	49.0	57.0	g/l
Sodium	138	136	141	mmol/l
Potassium	5.8	5.4	6.6	mmol/l
Chloride	94	93	99	mmol/l
Calcium	2.83	2.66	2.95	mmol/l
Inorganic Phosphorus	1.20[b]	0.57[c]	1.82[c]	mmol/l
Urea	3.7	3.2	4.6	mmol/l
Creatinine	108	95	115	µmol/l
Total Bilirubin	0.4	0.0	0.8	µmol/l
ALT	5	2	6	IU/l
AST	64	26	80	IU/l
CK	124	13	176	IU/l
GGT	12[b]	3[c]	21[c]	IU/l
ALP	28	14	45	IU/l
GLDH	3	3	4	IU/l
SDH	13.1	9.7	14.5	IU/l

[a] Of the data that did not follow normal distribution, all reference ranges have been calculated as the 95% confidence interval of the median.
[b] The mean is displayed for parameters following normal distribution.
[c] [mean ± 1.96SD] of the data for parameters following normal distribution

Table 4. Ranges of the data from the captive population in this study [a]

	CAPTIVE (n = 17)			
Parameter	Median	Lower	Upper	Units
Transferrin saturation [d e]	70.5	55.9	77.5	%
Iron [d e]	39.9 [b]	19.7 [c]	60.1 [c]	µmol/l
Total iron binding capacity (TIBC)	59.7	54.2	66.2	µmol/l
Unbound iron binding capacity (UIBC) [d,e]	18.3	12.8	24.0	µmol/l
Total protein	80.0	78.3	83.0	g/l
Albumin	24 [b]	17 [c]	32 [c]	g/l
Globulin	55.7	52.0	57.0	g/l
Sodium	138 [b]	128 [c]	148 [c]	mmol/l
Potassium [d]	5.4 [b]	1.6 [c]	9.2 [c]	mmol/l
Chloride [d]	99	97	101	mmol/l
Calcium	2.77 [b]	2.06 [c]	3.48 [c]	mmol/l
Inorganic Phosphorus [d,e]	0.73	0.61	0.98	mmol/l
Urea [d e]	5.5 [b]	1.9 [c]	9.1 [c]	mmol/l
Creatinine	112 [b]	68 [c]	156 [c]	µmol/l
Total Bilirubin	0.4	0.0	0.8	µmol/l
ALT	5	3	10	IU/l
AST	39	19	84	IU/l
CK [d]	287	75	411	IU/l
GGT [d,e]	26 [b]	5 [c]	46 [c]	IU/l
ALP [d]	46	38	55	IU/l
GLDH	5	3	11	IU/l
SDH [d]	14.5	14.0	15.7	IU/l

[a] Of the data that did not follow normal distribution, the have been calculated as the 95% confidence interval of the median.
[b] The mean is displayed for parameters following normal distribution.
[c] [mean ± 1.96SD] of the data for parameters following normal distribution
[d] Parameters significantly different from the entire free-ranging population in this study
[e] Parameters significantly different from both Kenyan regions (Laikipia plateau and Lowland)

Table 5a. Iron and transferrin results listed with previously published reference ranges for free-ranging and captive black rhinoceroses [a]

	Iron (µmol/l)		**Transferrin saturation (%)**	
	Lower	Upper	Lower	Upper
This study:				
Black rhino free-ranging Kenya (n=27)	14	41.8	25.8	59.4
Black rhino captive UK (n=17)	29.6	50.2	did not follow normal distribution	did not follow normal distribution
Dierenfeld et al., 2005 (mean ± SD):				
Black rhino free-ranging Zimbabwe (n=26-27)	59.2	106.2	**	**
Black rhino captive USA (n=12-34)	61.5	146.5	**	**
Paglia and Dennis, 1999 (mean ± SD):				
Black rhino free-ranging Zimbabwe (n=6)	31.5	46.2	22.0	34.0
Black rhino captive USA (n=24)	58.8	131.2	43.0	87.0

[a] Data displayed as [mean ± SD]

Table 5b. Iron results compared to the ISIS reference range for black rhinoceroses and a reference range for the domestic horse (*Equus domesticus*) [a]

	Iron	**(µmol/l)**
	Lower	Upper
This study:		
Black rhino free-ranging Kenya (n=27)	0.7	55.1
Black rhino captive UK (n=17)	19.7	60.1
Smith, 1997:		
Domestic horse [b]	19.2	76.2
ISIS, 2002:		
Black rhino captive [b] (n=237)	17.1	92.0

[a] Data displayed as [mean ± 1.96SD]
[b] Reference range based on SD, assumed to be [mean ± 1.96SD]

Table 6. Results for GGT compared to the ISIS reference range for black rhinoceroses and the RVC reference range for the domestic horse [a]

	GGT	**(IU/l)**
	Lower	Upper
This study:		
Black rhino free-ranging Kenya (n=27)	3	21
Black rhino captive UK (n=17)	5	46
ISIS, 2002:		
Black rhino captive [b] (n=573)	0	72
RVC, 2005:		
Domestic horse (n=40)	5	34

[a] Data displayed as [mean ± 1.96SD]
[b] Reference range based on SD, assumed to be [mean ± 1.96SD]

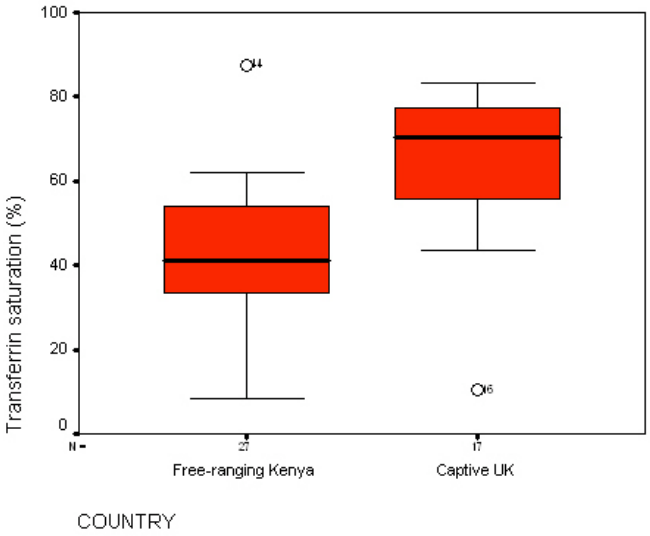
Table 7. Reference ranges for the domestic horse, *Equus domesticus*, used at the Royal Veterinary College (RVC, Hatfield) [a]

RVC reference ranges:	**Domestic horse**		
	Lower	Upper	Units
Total protein	50	64	g/l
Albumin	31	38	g/l
Globulin	16	30	g/l
Sodium	141	147	mmol/l
Potassium	2.4	4.9	mmol/l
Chloride	100	105	mmol/l
Calcium	2.71	3.14	mmol/l
Inorganic phosphorus	0.63	1.84	mmol/l
Urea	2.8	5.8	mmol/l
Creatinine	121	194	µmol/l
Total Bilirubin	8.5	49.3	µmol/l
AST	198	476	IU/l
CK	133	738	IU/l
GGT	5	34	IU/l
ALP	81	343	IU/l
SDH	1.8	6.7	IU/l

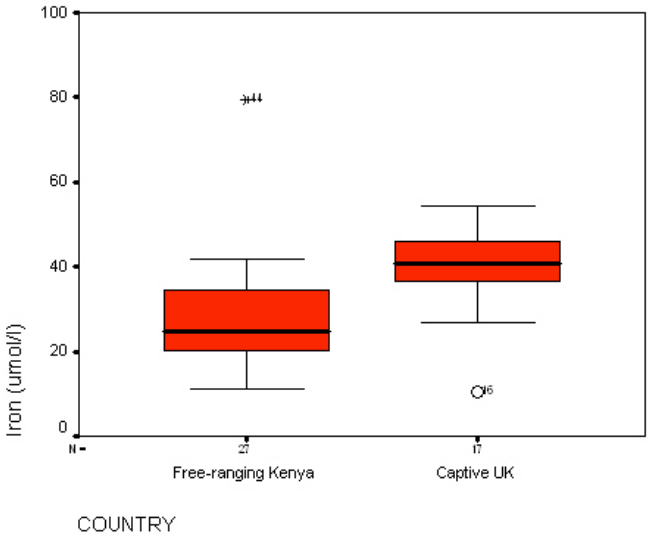
[a] The reference ranges from the RVC are established internally on an ILAB™ 600 Clinical Chemistry System (Instrumentation Laboratory, Lexington, USA) using approximately 40 samples, calculated as mean ± 1.96SD.

Figures

Figure 1. Box plots a-d. Comparison of serum iron parameters (transferrin saturation, serum iron, total iron binding capacity [TIBC] and unbound iron binding capacity [UIBC]) of captive black rhinoceroses with those of free-ranging black rhinoceroses

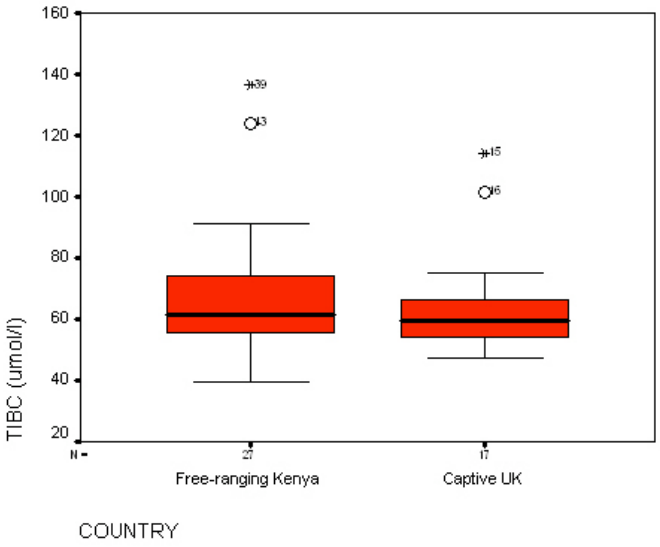


Box plot a. Transferrin saturation
In this study, there is a significant difference (P<0.001) between the transferrin saturation of the captive (n=17) and the free-ranging (n=27) black rhinoceroses.

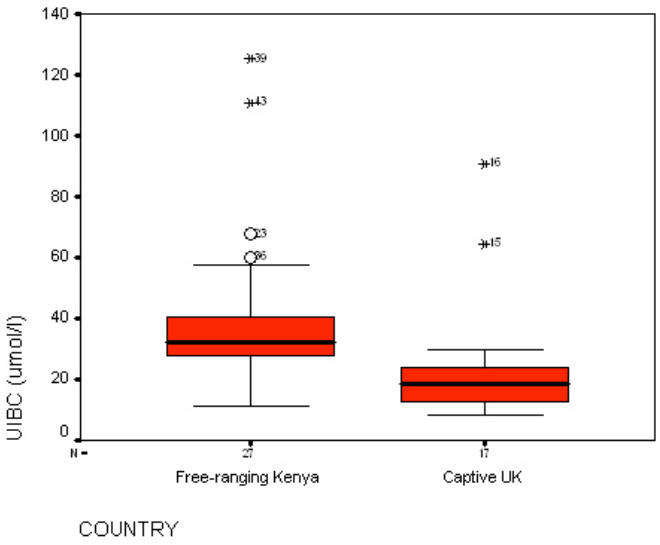


Box plot b. Serum iron
In this study, there is a significant difference (P=0.004) between the serum iron levels of the captive (n=17) and the free-ranging (n=27) black rhinoceroses.

Figure 1 continued.

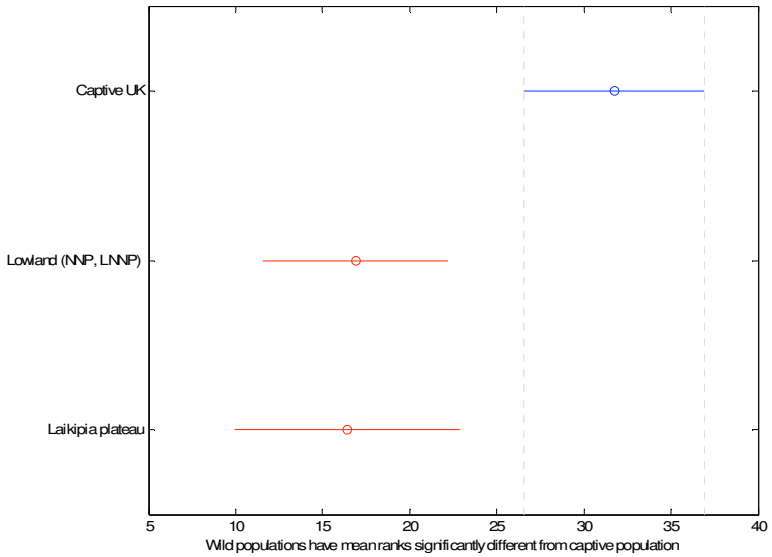


Box plot c. Total iron binding capacity (TIBC)
Since the total iron binding capacity is inherent to the species, the captive black rhinoceroses in this study (n=17) have comparable levels to the free-ranging (n=27) individuals.

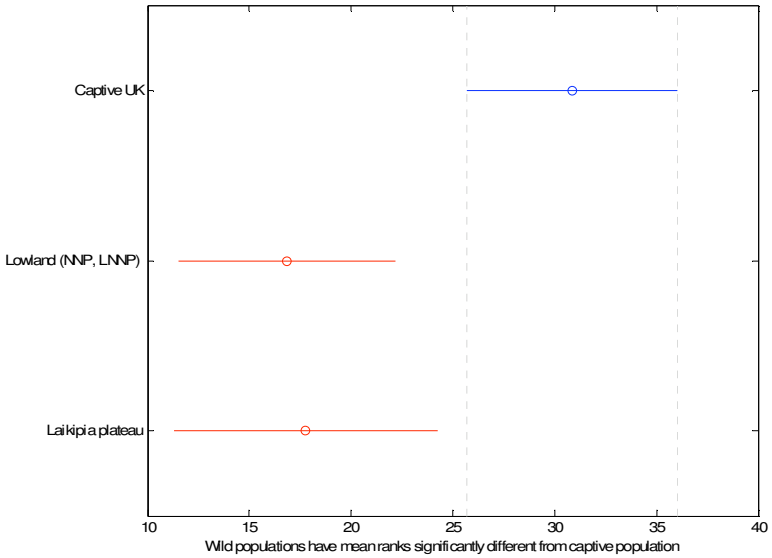


Box plot d. Unbound iron binding capacity (UIBC)
In this study, there is a significant difference (P<0.001) between the serum iron levels of the captive (n=17) and the free-ranging (n=27) black rhinoceroses.

Figure 2. Graph a-c. Output of the multicomparison test for transferrin saturation, serum iron and unbound iron binding capacity (UIBC)

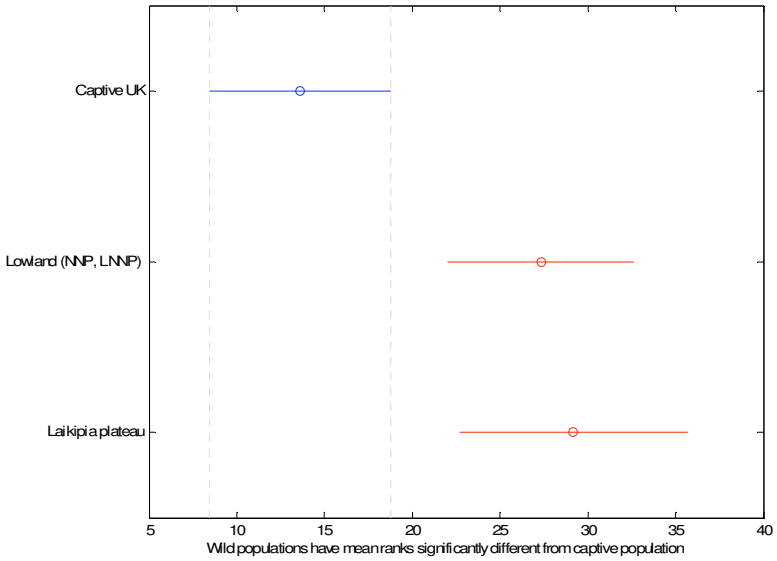


Graph a. Transferrin saturation
Multicomparison test output for the transferrin saturation results, displaying the significant difference (P=0.001) between the captive (n=17) and both free-ranging (wild, n=16/11) populations.



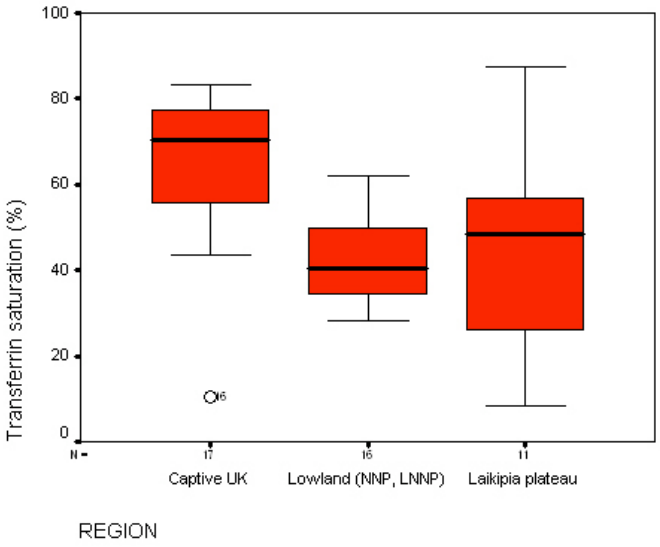
Graph b. Serum iron
Multicomparison test output for the serum iron results, displaying the significant difference (P=0.002) between the captive (n=17) and both free-ranging (wild, n=16/11) populations.

Figure 2 continued.

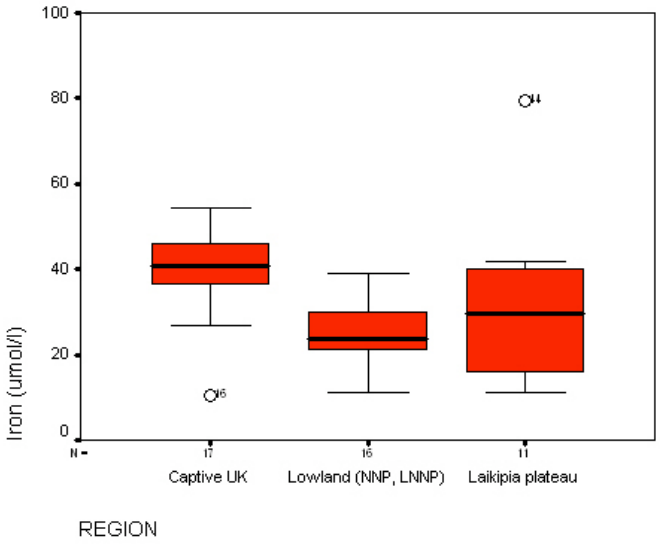


Graph c. Unbound iron binding capacity (UIBC)
Multicomparison test output for the UIBC results, displaying the significant difference (P=0.001) between the captive (n=17) and both free-ranging (wild, n=16/11) populations.

Figure 3. Box plots e-j. Comparison of all the parameters that display significant differences between the captive and both free-ranging populations using box plots

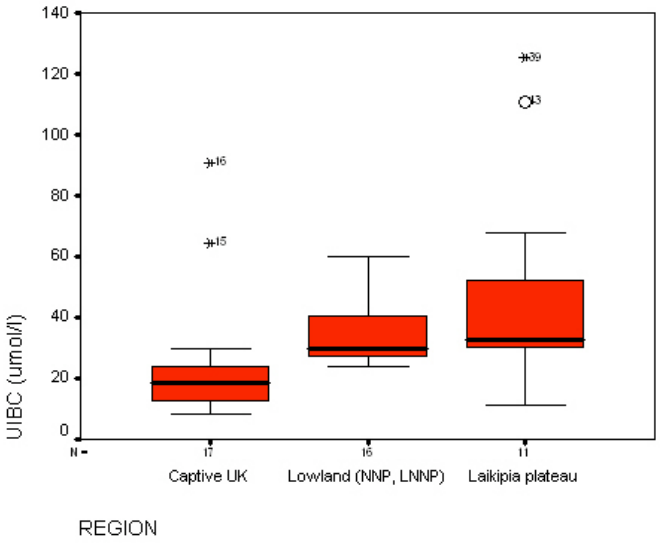


Box plot e. Transferrin saturation
In this study, there is a significant difference (P=0.001) between the transferrin saturation of the captive population (n=17) and both free-ranging populations (n=16/n=11) of black rhinoceroses.

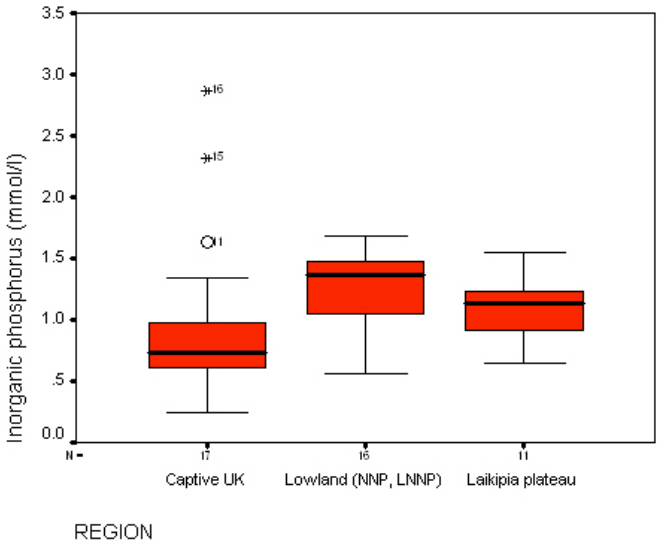


Box plot f. Serum iron
In this study, there is a significant difference (P=0.007) between the serum iron of the captive population (n=17) and both free-ranging populations (n=16/n=11) of black rhinoceroses.

Figure 3 continued.

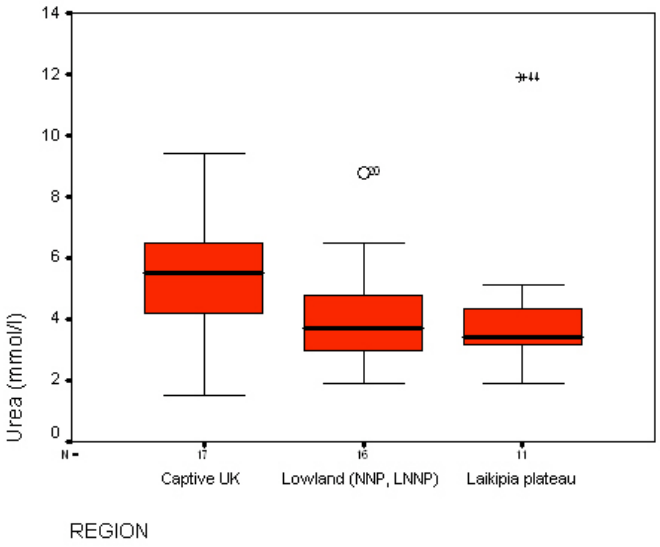


Box plot g. Unbound iron binding capacity (UIBC)
In this study, there is a significant difference (P=0.001) between the UIBC of the captive population (n=17) and both free-ranging populations (n=16/n=11) of black rhinoceroses.

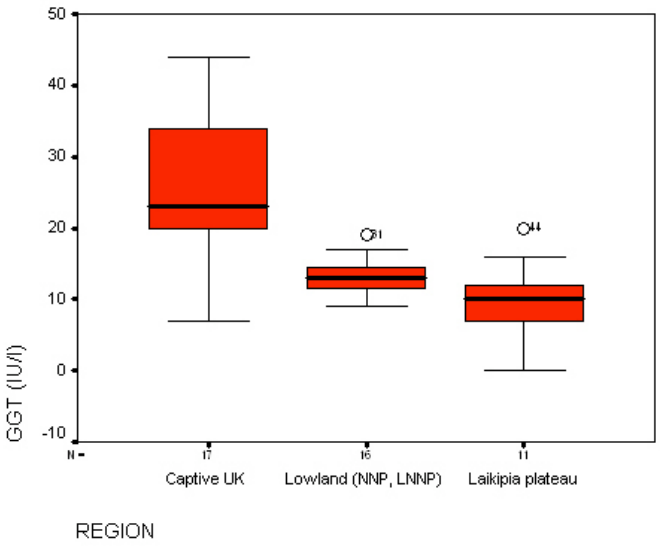


Box plot h. Inorganic phosphorus
In this study, there is a significant difference (P=0.02) between the inorganic phosphorus of the captive population (n=17) and both free-ranging populations (n=16/n=11) of black rhinoceroses.

Figure 3 continued.



Box plot i. Urea
In this study, there is a significant difference (P=0.013) between the urea of the captive population (n=17) and both free-ranging populations (n=16/n=11) of black rhinoceroses.



Box plot j. GGT
In this study, there is a significant difference (P<0.001) between the GGT of the captive population (n=17) and both free-ranging populations (n=16/n=11) of black rhinoceroses.

APPENDICES

Appendix I: *IUCN categories*

Appendix II: *CITES*

Appendix III: *Statistics output*

Appendix IV: *Task division*

APPENDIX I: IUCN categories

(Emslie and Brooks, 1999)

Extinct (EX)
A taxon is Extinct when there is no reasonable doubt that the last individual has died.

Extinct in the wild (EW)
A taxon is Extinct in the wild when it is known only to survive in cultivation, in captivity
or as a naturalised population (or populations) well outside the past range.

Critically endangered (CR)
A taxon is Critically Endangered when it is facing an extremely high risk of extinction in the wild in the immediate future, as defined by any of the following criteria (A to E):

A) Population reduction in the form of either of the following:
   1) An observed, estimated, inferred or suspected reduction of at least 80% over the last 10 years or three generations.
   2) A reduction of at least 80%, projected or suspected to be met within the next 10 years or three generations.

B) Extent of occurrence estimated to be less than 100km2 or area of occupancy estimated to be less than 10km2, and estimates indicating any two of the following:
   1) Severely fragmented or known to exist at only a single location.
   2) Continuing decline in habitat, subpopulations, mature individuals
   3) Extreme fluctuations in area, subpopulations, mature individuals

C) Population estimated to number less than 250 mature individuals and either:
   1) An estimated continuing decline of at least 25% within three years or one generation, whichever is longer or
   2) A continuing decline, observed, projected, or inferred, in numbers of mature individuals and population structure in the form of either:
      a) severely fragmented (i.e. no subpopulation estimated to contain more than 50 mature individuals)
      b) all individuals are in a single subpopulation.

D) Population estimated to number less than 50 mature individuals.

E) Quantitative analysis showing the probability of extinction in the wild is at least 50% within 10 years or three generations, whichever is the longer.

Endangered (EN)
A taxon is Endangered when it is not Critically Endangered but is facing a very high risk of extinction in the wild in the near future, as defined by any of the criteria (A to E):

A) Population reduction in the form of either of the following:
   1) An observed, estimated, inferred or suspected reduction of at least 50% over the last 10 years or three generations
   2) A reduction of at least 50%, projected or suspected to be met within the next 10 years or three generations.

B) Extent of occurrence estimated to be less than 5000km2 or area of occupancy estimated to be less than 500km2.

C) Population estimated to number less than 2500 mature individuals and either:
   1) An estimated continuing decline of at least 20% within five years or two generations, whichever is longer, or
   2) A continuing decline, observed, projected, or inferred, in numbers of mature individuals and population structure.

D) Population estimated to number less than 250 mature individuals.

E) Quantitative analysis showing the probability of extinction in the wild is at least 20% within 20 years or five generations, whichever is the longer.

Vulnerable (VU)

A taxon is Vulnerable when it is not Critically Endangered or Endangered but is facing a high risk of extinction in the wild in the medium-term future.

Lower risk (LR)

A taxon is Lower Risk when it has been evaluated, does not satisfy the criteria for any of the categories Critically Endangered, Endangered or Vulnerable. Taxa included in the Lower Risk category can be separated into three subcategories:

Conservation Dependent (cd)	Taxa which are the focus of a continuing taxon-specific or habitat-specific conservation programme targeted towards the taxon in question, the cessation of which would result in the taxon qualifying for one of the threatened categories above within a period of five years.
Near Threatened (nt)	Taxa which do not qualify for Conservation Dependent, but which are close to qualifying for Vulnerable.
Least Concern (lc)	Taxa which do not qualify for Conservation Dependent or Near Threatened.

Data deficient (DD)

A taxon is Data Deficient when there is inadequate information to make a direct, or indirect, assessment of its risk of extinction based on its distribution and/or population status. A taxon in this category may be well studied, and its biology well known, but appropriate data on abundance and/ or distribution is lacking. Data Deficient is therefore not a category of threat or Lower Risk.

Not evaluated (NE)

A taxon is Not Evaluated when it is has not yet been assessed against the criteria.

Reference:

EMSLIE, R. AND M. BROOKS. 1999. Status survey and conservation action plan: African rhino. IUCN/SSC African Rhino Specialist Group, Cambridge, pp. 83-85

APPENDIX II: CITES

CITES (Convention on International Trade in Endangered Species) works by subjecting international trade in specimens of selected species to certain controls. All import, export, re-export and introduction from the sea of species covered by the Convention has to be authorized through a licensing system.

Appendix I
Appendix I includes species threatened with extinction. Trade in specimens of these species is permitted only in exceptional circumstances. An import permit issued by the Management Authority of the State of import is required. This may be issued only if the specimen is not to be used for primarily commercial purposes and if the import will be for purposes that are not detrimental to the survival of the species. In the case of a live animal or plant, the Scientific Authority must be satisfied that the proposed recipient is suitably equipped to house and care for it. An export permit or re-export certificate issued by the Management Authority of the State of export or re-export is also required.
An export permit may be issued only if the specimen was legally obtained; the trade will not be detrimental to the survival of the species; and an import permit has already been issued.

Appendix II
This Appendix contains species that are protected in at least one country, which has asked other CITES Parties for assistance in controlling the trade. Changes to Appendix III follow a distinct procedure from changes to Appendices I and II, as each Party's is entitled to make unilateral amendments to it.
Appendix II includes species not necessarily threatened with extinction, but in which trade must be controlled in order to avoid utilization incompatible with their survival.
An export permit or re-export certificate issued by the Management Authority of the State of export or re-export is required for Appendix II specimens.

Appendix III
This Appendix contains species that are protected in at least one country, which has asked other CITES Parties for assistance in controlling the trade. Changes to Appendix III follow a distinct procedure from changes to Appendices I and II, as each Party's is entitled to make unilateral amendments to it.

http://www.cites.org/eng/disc/how.shtml

APPENDIX III: Statistics output

STUDENT'S T-TEST
for serum iron and GGT
captive vs. free-ranging

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	Df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
									Lower	Upper
iron	**Equal variances assumed**	0.73	0.398	-3.042	42	0.004	-11.923	3.919	-19.8322	-4.0144
	Equal variances not assumed			-3.253	40.693	0.002	-11.923	3.6658	-19.3282	-4.5185
GGT	**Equal variances assumed**	18.68	0	-6.034	42	0	-14.02	2.323	-18.705	-9.33
	Equal variances not assumed			-5.138	19.851	0	-14.02	2.728	-19.711	-8.324

WILCOXON RANK SUM TEST and MANN-WHITNEY U TEST
captive vs. free-ranging

	% transferrin saturation	TIBC	UIBC	total protein	ALBUMIN	GLOBULIN	SODIUM
Mann-Whitney U	72.5	203.5	78.5	179.5	194	196.5	224.5
Wilcoxon W	450.5	356.5	231.5	557.5	572	574.5	377.5
Z	-3.784	-0.627	-3.64	-1.205	-0.856	-0.796	-0.121
Asymp. Sig. (2-tailed)	0	0.531	0	0.228	0.392	0.426	0.904

Grouping Variable: COUNTRY

	potassium	CHLORIDE	CALCIUM	inorganic phosphorus	UREA	creatinine	total bilirubin
Mann-Whitney U	77.5	154.5	214.5	122.5	108.5	200.5	204.5
Wilcoxon W	230.5	532.5	367.5	275.5	486.5	578.5	357.5
Z	-3.67	-1.811	-0.362	-2.58	-2.918	-0.7	-0.618
Asymp. Sig. (2-tailed)	0	0.07	0.718	0.01	0.004	0.484	0.536

Grouping Variable: COUNTRY

	AST	creatinine kinase	ALP	GLDH	SDH
Mann-Whitney U	193.5	120	127	148.5	145
Wilcoxon W	346.5	498	505	526.5	523
Z	-0.868	-2.64	-2.472	-2.015	-2.037
Asymp. Sig. (2-tailed)	0.385	0.008	0.013	0.044	0.042

Grouping Variable: COUNTRY

STUDENT'S T-TEST
for normally distributed parameters

Laikipia plateau vs. South-central region

		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference
Transferrin saturation (%)	**Equal variances assumed**	9.386	0.005	-0.078	25	0.938	-0.525	6.7231
	not assumed			-0.069	12.729	0.946	-0.525	7.662
Iron (umol/l)	**Equal variances assumed**	7.072	0.013	-1.184	25	0.248	-6.393	5.4001
	not assumed			-1.022	11.937	0.327	-6.393	6.2572
TIBC (umol/l)	**Equal variances assumed**	7.076	0.013	-2.561	25	0.017	-20.438	7.9789
	not assumed			-2.255	12.904	0.042	-20.438	9.0618
Inorganic phosphorus (mmol/l)	**Equal variances assumed**	0.079	0.781	1.518	25	0.142	0.184	0.12118
	not assumed			1.534	22.412	0.139	0.184	0.11994
total bilirubin	**Equal variances assumed**	0.115	0.737	0.54	25	0.594	0.131	0.2431
	not assumed			0.538	21.445	0.596	0.131	0.2438
GGT (IU/l)	**Equal variances assumed**	4.741	0.039	2.169	25	0.04	3.67	1.692
	not assumed			1.901	12.649	0.08	3.67	1.931
ALP	**Equal variances assumed**	0.923	0.346	1.191	25	0.245	9.47	7.95
	not assumed			1.13	17.587	0.274	9.47	8.38
SDH	**Equal variances assumed**	12.623	0.002	2.962	25	0.007	4.918	1.6605
	not assumed			2.585	12.476	0.023	4.918	1.9021

WILCOXON RANK SUM TEST and MANN-WHITNEY U TEST
for non-normally distributed parameters
Laikipia plateau vs. South-central lowland region

	UIBC (umol/l)	Total protein	ALBUMIN	GLOBULIN	SODIUM	Potassium	CHLORIDE	CALCIUM
Mann-Whitney U	70	88	83	83	81.5	82	55	78.5
Wilcoxon W	206	154	219	149	217.5	148	191	214.5
Z	-0.888	0	-0.247	-0.247	-0.323	-0.297	-1.632	-0.469
Asymp. Sig. (2-tailed)	0.374	1	0.805	0.805	0.747	0.767	0.103	0.639
Exact Sig. [2*(1-tailed Sig.)]	.394(a)	1.000(a)	.827(a)	.827(a)	.753(a)	.790(a)	.110(a)	.645(a)

a not corrected for ties

b Grouping variable REGION

	Urea (mmol/l)	creatinine	ALT	AST	creatinine kinase	GLDH
Mann-Whitney U	81	79	78	71	52	63
Wilcoxon W	147	215	214	137	118	129
Z	-0.346	-0.445	-0.5	-0.839	-1.779	-1.311
Asymp. Sig. (2-tailed)	0.729	0.657	0.617	0.401	0.075	0.19
Exact Sig. [2*(1-tailed Sig.)]	.753(a)	.680(a)	.645(a)	.422(a)	.080(a)	.231(a)

a not corrected for ties

b Grouping variable REGION

KRUSKAL-WALLIS TEST
all regions

(UK, Laikipia plateau, South-central region)

	Transferrin saturation (%)	TIBC (umol/l)	UIBC (umol/l)	Total protein	ALBUMIN	GLOBULIN	SODIUM	Potassium
Chi-Square	14.53	5.404	13.548	1.46	0.807	0.693	0.253	13.963
df	2	2	2	2	2	2	2	2
Asymp. Sig.	0.001	0.067	0.001	0.482	0.668	0.707	0.881	0.001

a Kruskal Wallis test
b Grouping variable REGION

	CHLORIDE	CALCIUM	Inorganic phosphorus (mmol/l)	Urea (mmol/l)	creatinine	total bilirubin	ALT	AST
Chi-Square	6.991	0.231	7.867	8.644	0.643	1.004	1.465	1.657
df	2	2	2	2	2	2	2	2
Asymp. Sig.	0.03	0.891	0.02	0.013	0.725	0.605	0.481	0.437

a Kruskal Wallis test
b Grouping variable REGION

	creatinine kinase	ALP	GLDH	SDH
Chi-Square	9.38	7.333	4.865	8.029
df	2	2	2	2
Asymp. Sig.	0.009	0.026	0.088	0.018

a Kruskal Wallis test
b Grouping variable REGION

ONE-WAY ANOVA

for serum iron and GGT
all regions

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
iron	**Between Groups**	1749.473	2	874.737	5.549	0.007
	Within Groups	6462.729	41	157.628		
	Total	8212.202	43			
GGT	**Between Groups**	2137.549	2	1068.775	19.25	0
	Within Groups	2276.36	41	55.521		
	Total	4413.909	43			

APPENDIX IV: Task division

Fieke Molenaar has been responsible for

- organising the CITES import certificate
- applying for additional funding from the Zebra Foundation
- assisting Richard McCort in obtaining fresh samples from rhinoceroses at Port Lympne Wild Animal Park
- selecting stored serum samples from
    - Port Lympne Wild Animal Park
    - Whipsnade Wild Animal Park (not analysed)
    - London Zoo (not analysed)
- supervising repackaging of serum samples from Kenya
- negotiating analysis costs with the Veterinary Laboratory Agency (VLA)
- writing the literature review
- organising all obtained data
- the basic statistical analysis of the data
- writing the scientific paper
- organising the Department for Environment, Food and Rural Affairs (DEFRA) soil import licence (soil analysis has fallen outside the scope of this project due to time constraints)

Rajan Amin has been instrumental in

- the cooperation with the Kenya Wildlife Service (KWS)
- assisting with CITES and DEFRA applications
- the statistical analysis of the data
- corrections to the literature review and scientific paper

Michael Waters has been responsible for

- biochemical analysis at the Royal Veterinary College (RVC)
- funding of biochemical analysis
- corrections to the literature review and scientific paper