High serum concentrations of iron, transferrin saturation and gamma glutamyl transferase in captive black rhinoceroses (*Diceros bicornis*)

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Iron storage disease (haemochromatosis) is thought to be the cause of many disorders unique to captive black rhinoceroses (*Diceros bicornis*). To establish reliable reference ranges for iron parameters, serum samples from 27 eastern black rhinoceroses (*Diceros bicornis michaeli*) from a translocation programme in Kenya were analysed and compared with the samples from 17 captive individuals. The transferrin saturation, serum iron concentration and gamma glutamyl transferase were significantly higher in the captive rhinoceroses, but these elevations were not evident when the results were compared with previously published data.

BLACK rhinoceroses (*Diceros bicornis*) are critically endangered browsing rhinoceroses from Africa (Owen-Smith and Berger 2001). Postmortem examinations of captive black rhinoceroses commonly reveal haemosiderosis, the deposition of excess iron in insoluble clusters in soft tissues (Jones and others 1997). In a study comparing captive black rhinoceroses with captive white rhinoceroses (*Ceratotherium simum*), the quantity of hepatic non-haem iron was significantly greater in the black rhinoceroses and appeared to be increasing with the time kept in captivity (Smith and others 1995). In contrast, haemosiderosis is not observed in free-ranging black rhinoceroses (Kock and others 1992), although iron deposits begin to develop shortly after they have been captured (Kock and Morkel 1993). It has been suggested that the animals' adaptation to the low bioavailability of iron in the wild could be the cause of enhanced intestinal iron absorption in captivity (Paglia and others 2001a).

Certain disorders 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 and others 1996, 2001b, Paglia and Radcliffe 2000). Excess iron can interfere with normal physiological functions owing to its mechanical presence and the formation of tissue-damaging free oxy-radicals (Rolfs and Hediger 1999, Beutler and others 2003), a process termed haemochromatosis. Clinical signs may appear worse in black rhinoceroses in particular because of their inherent sensitivity to oxidative stress (Paglia and Miller 1993) and naturally low levels of biological antioxidants (Dierenfeld and others 1988). These disorders unique to captive black rhinoceroses can be treated by adding iron chelators to the diet to reduce its bioavailability, increasing dietary antioxidants and, because treatment is successful only if the disease is detected 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 are needed against which to compare the biochemical results. 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 (*Dicerorhinus sumatrensis*) rhinoceroses in the USA (Dierenfeld and others 2005). However, inter-laboratory variations may render these reference ranges less useful in the UK.

In this study, blood samples collected from free-ranging eastern black rhinoceroses (*Diceros bicornis michaeli*) during translocation procedures in Kenya have been used to establish reliable reference ranges for iron and other biochemical variables at laboratories in the UK. The results have been used to demonstrate whether there were differences in these parameters between rhinoceroses from different regions, and to compare them with the results from stored serum samples from a population of captive black rhinoceroses in the UK.

MATERIALS AND METHODS

Adult eastern black rhinoceroses inhabiting Nairobi National Park (NNP), Lake Nakuru National Park (LNNP) and the private sanctuaries of the Laikipia district in central Kenya (Solio Ranch, Ol Jogi Game Reserve and Lewa Wildlife Conservancy) were immobilised with an intramuscular injection of 5·5 mg etorphine (M99; Novartis) and 100 mg xylazine (Rompun; Bayer) using a long-range rifle-type dart projector (Palmer Cap-Chur). Immediately after they had been immobilised, 5 mg nalorphine (Nalorphine HCl Solution; Sigma-Aldrich) was administered intramuscularly for partial reversal and, after surgical preparation of the sampling site, blood samples were taken from an ear vein with an 18 G hypodermic needle and a 20 ml disposable syringe and transferred to 1·8 ml blood collection tubes. Approximately one hour later, the samples were centrifuged and the serum was separated and stored at –85°C. From storage, the samples were packed in dry ice and shipped to the UK under CITES licensing.

Blood samples from 12 eastern black rhinoceroses at Port Lympne Wild Animal Park in Kent (UK), were taken from the medial radial vein of either foreleg with a 21 G butterfly hypodermic needle and Vacutainer system (Fig 1), either with the animals conscious or under general anaesthesia. The blood was left overnight to clot and then centrifuged to separate the serum, which was transferred to blood tubes and stored at –20°C. These serum samples were packed in dry ice and sent to the Royal Veterinary College (RVC) – Hatfield.

At the RVC, all the serum samples were thawed and the samples that showed no visual signs of haemolysis were run through an ILAB 600 Clinical Chemistry System (Instrumentation Laboratory) for standard biochemical analysis, consisting of total protein, albumin, globulin, sodium, potassium, chloride, calcium, inorganic phosphorus, urea, creatinine, total bilirubin, bile acids, alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatine kinase (CK), gamma glutamyl transferase (GGT), alkaline phosphatase

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FIG 1: Taking a blood sample from the medial radial vein of the right foreleg of a trained captive black rhinoceros (*Diceros bicornis*), using a 21G butterfly hypodermic needle and Vacutainer

(ALP), glutamate dehydrogenase (GLDH) and sorbitol dehydrogenase (SDH).

Residual serum samples were sent to Veterinary Laboratories Agency (VLA) – Shrewsbury, where they were run through an AU400 chemistry analyser (Olympus) 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 calculated by dividing the serum iron concentration by the TIBC.

Serum samples were obtained from 27 free-ranging rhinoceroses (14 males and 13 females). Their precise ages were unknown, but 17 of them were considered to be adults over seven years of age and 10 were considered between three-and-a-half and seven years of age. Serum samples were obtained from 17 captive animals. Nine of them had been captive-bred in European zoos, six were originally wild-caught in Africa but had lived at Port Lympne Wild Animal Park for over 25 years, and two were of unknown origin. The male:female ratio was 3:5 and the age range was four to 34 years, with an average age of 24 years.

Statistical analyses

The results from the free-ranging population and the captive population were compared using SPSS 11.0 and MATLAB (MathWorks 2004). The free-ranging population was divided into two subpopulations (the 11 rhinoceroses from the Laikipia district in central Kenya and the 16 from the south-central region in the NNP and LNNP) to determine more accurately which parameters differed significantly between the captive and free-ranging populations. The significance level was set at $P<0{\cdot}05$.

The Lilliefors goodness-of-fit hypothesis test was applied to determine whether the data were normally distributed (Conover 1980). For the parameters that were normally distributed, the 95 per cent confidence interval (mean ± 1·96 sd), was used to establish the reference ranges of the free-ranging rhinoceroses. For the parameters that were not normally distributed, due to the small sample sizes, the use of 95 per cent percentiles would have resulted in a reference range based on all the data points, so that outliers would severely distort the estimates. The reference ranges were therefore estimated as the 95 per cent confidence interval of the median (Seigel 1988).

To determine whether there were significant differences between the values from the rhinoceroses in the Laikipia district and the south-central region, the non-parametric Wilcoxon rank sum test was used. To compare the captive UK population with the free-ranging rhinoceroses from the different regions within Kenya, an analysis of variance (Petrie and Watson 1999) was used to compare the parameters that were normally distributed in all three populations and the non-parametric Kruskal-Wallis test was used for the parameters that were not normally distributed. Where significant differences were found, a multi-comparison test of the outputs of the analysis of variance or the Kruskal-Wallis test was used to determine which population was significantly different from the other two populations.

RESULTS

The reference ranges derived from the data from the free-ranging rhinoceroses from Kenya are shown in Table 1 and the ranges from the captive rhinoceroses are shown in Table 2. A mechanical fault resulted in unphysiologically low bile acid measurements, requiring these results to be excluded. Serum iron concentrations and GGT activities were normally distributed in both the captive and free-ranging populations. Transferrin saturation, inorganic phosphorus and ALP activities were normally distributed in the free-ranging population, but not in the captive population. The concentrations of albumin, sodium, chloride, calcium, urea and creatinine were normally distributed in the captive population, but not in the free-ranging population. The concentrations of total protein, globulin, potassium and total bilirubin, and the activities of ALT, AST, CK, GLDH and SDH, were not normally distributed in either population.

There were significant differences between the rhinoceroses from the two regions of Kenya in the TIBC ($P=0{\cdot}042$) and SDH ($P=0{\cdot}023$) concentration.

In comparison with the two Kenyan populations subpopulations, the captive population of rhinoceroses had significantly higher values of transferrin saturation ($P=0{\cdot}001$), serum iron ($P=0{\cdot}007$), urea ($P=0{\cdot}013$) and GGT ($P<0{\cdot}001$), and significantly lower values of UIBC ($P=0{\cdot}001$) and inorganic phosphorus ($P=0{\cdot}02$) (Figs 2 to 7).

DISCUSSION

There were significant differences between several of the biochemical parameters in the captive and free-ranging eastern black rhinoceroses. In comparison with the free-ranging rhinoceroses, the serum transferrin saturation and the serum iron levels of the captive population were significantly higher and the UIBC was significantly lower. A valid direct comparison of these results with previously published data is possible only if the ranges have been calculated using the same statistical methods. All the previous studies have based their range estimates on the means and standard deviations of the data, which are sensitive to both non-symmetrical distributions and outliers. In this discussion, comparisons have been made only between the parameters that were normally distributed.

The transferrin saturation range derived from the free-ranging black rhinoceroses from Kenya was slightly higher than the range derived from a study of black rhinoceroses from Zimbabwe (Table 3) (Paglia and Dennis 1999), possibly

TABLE 1: Reference ranges for serum biochemistry derived from 27 free-ranging eastern black rhinoceroses (*Diceros bicornis michaeli*) calculated as the median (95 per cent confidence interval [CI] of the median) except where otherwise indicated

Parameter	Median	95% CI
Transferrin saturation (%)	42·6*	9·6†-75·6
Iron (µmol/l)	27·9*	0·7†-55·1
TIBC (µmol/l)	61·3	57·1-73·4
UIBC (µmol/l)	32·3	28·3-38·8
Total protein (g/l)	78·0	74·6-81·7
Albumin (g/l)	24·4	23·1-25·7
Globulin (g/l)	53·7	49·0-57·0
Sodium (mmol/l)	138	136-141
Potassium (mmol/l)	5·8	5·4-6·6
Chloride (mmol/l)	94	93-99
Calcium (mmol/l)	2·83	2·66-2·95
Inorganic phosphorus (mmol/l)	1·20*	0·57†-1·82
Urea (mmol/l)	3·7	3·2-4·6
Creatinine (µmol/l)	108	95-115
Total bilirubin (µmol/l)	0·4	0·0-0·8
ALT (iu/l)	5	2-6
AST (iu/l)	64	26-80
CK (iu/l)	124	13-176
GGT (iu/l)	12*	3†-21
ALP (iu/l)	28	14-45
GLDH (iu/l)	3	3-4
SDH (iu/l)	13·1	9·7-14·5

* Mean value,
† Mean ± 1·96 sd
TIBC Total iron binding capacity, UIBC Unbound iron binding capacity, ALT Alanine aminotransferase, AST Aspartate aminotransferase, CK Creatine kinase, GGT Gamma glutamyl transferase, ALP Alkaline phosphatase, GLDH Glutamate dehydrogenase, SDH Sorbitol dehydrogenase

TABLE 2: Reference ranges for serum biochemistry derived from 17 captive eastern black rhinoceroses (*Diceros bicornis michaeli*), calculated as the median (95 per cent confidence interval [CI] of the median) except where otherwise indicated

Measurement	Median	95% CI
Transferrin saturation (%)‡§	70·5	55·9-77·5
Iron (µmol/l)‡§	39·9*	19·7†-60·1
TIBC (µmol/l)	59·7	54·2-66·2
UIBC (µmol/l)‡§	18·3	12·8-24·0
Total protein (g/l)	80·0	78·3-83·0
Albumin (g/l)	24*	17†-32
Globulin (g/l)	55·7	52·0-57·0
Sodium (mmol/l)	138*	128†-148
Potassium (mmol/l)‡	5·4*	1·6†-9·2
Chloride (mmol/l)‡	99	97-101
Calcium (mmol/l)	2·77*	2·06†-3·48
Inorganic phosphorus (mmol/l)‡§	0·73	0·61-0·98
Urea (mmol/l)‡§	5·5*	1·9†-9·1
Creatinine (µmol/l)	112*	68†-156
Total bilirubin (µmol/l)	0·4	0·0-0·8
ALT (iu/l)	5	3-10
AST (iu/l)	39	19-84
CK (iu/l)‡	287	75-411
GGT (iu/l)‡§	26*	5†-46
ALP (iu/l)‡	46	38-55
GLDH (iu/l)	5	3-11
SDH (iu/l)‡	14·5	14·0-15·7

* Mean value,
† Mean ± 1·96 sd
‡ Parameters significantly different from the free-ranging rhinoceroses as a whole
§ Parameters significantly different from both the rhinoceroses in the Laikipia plateau and from those in the Lowland regions of Kenya
TIBC Total iron binding capacity, UIBC Unbound iron binding capacity, ALT Alanine aminotransferase, AST Aspartate aminotransferase, CK Creatine kinase, GGT Gamma glutamyl transferase, ALP Alkaline phosphatase, GLDH Glutamate dehydrogenase, SDH Sorbitol dehydrogenase

because that studied included only six animals, or because of the possible difference between subspecies, differences in the mineral content of local soil and vegetation or differences in the assays used. Because of their non-normal distribution, the transferrin saturation values of the captive black rhinoceros population in this study could not be compared with the results of Paglia and Dennis (1999).

There were no significant differences between the reference ranges for serum iron given by either the LYNX (ZSL 1991) or ISIS (Flesness 2002) reference ranges for captive black rhinoceroses, both assumed to be (mean ± 1·96 sd), and the range observed in the captive black rhinoceroses in this study (Table 4). The serum iron concentrations observed by Dierenfeld and others (2005) in 27 free-ranging black rhinoceroses in Zimbabwe were much higher than those in the free-ranging rhinoceroses in Kenya (Table 3). The differences may be due to the possible difference in subspecies, differences in the mineral content of local soil and vegetation, or differences in the assays used. The serum iron concentration of the captive population in this study was considerably lower than that in 24 captive rhinoceroses in the USA (Paglia and Dennis 1999). Again, the differences may be due to differences in subspecies or the assays used, but are more likely to be related to significant dietary differences.

In human beings, iron storage disease (haemochromatosis) is a common hereditary genetic disorder (Parkkila

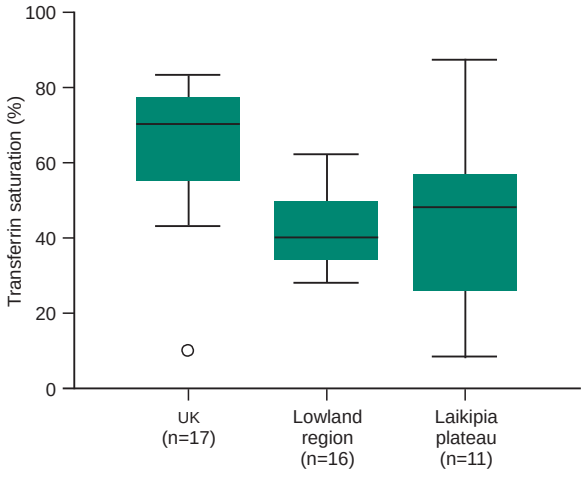

FIG 2: Box and whisker plots of the serum transferrin saturation of 17 captive black rhinoceroses (*Diceros bicornis*) and 27 free-ranging animals in two regions of Kenya

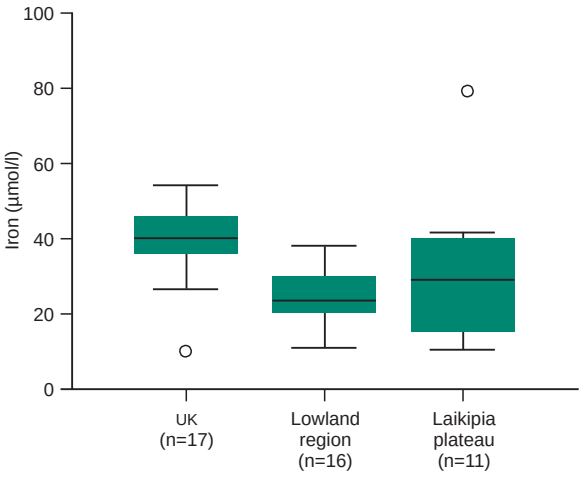

FIG 3: Box and whisker plots of the serum iron concentrations in 17 captive black rhinoceroses (*Diceros bicornis*) and 27 free-ranging animals in two regions of Kenya

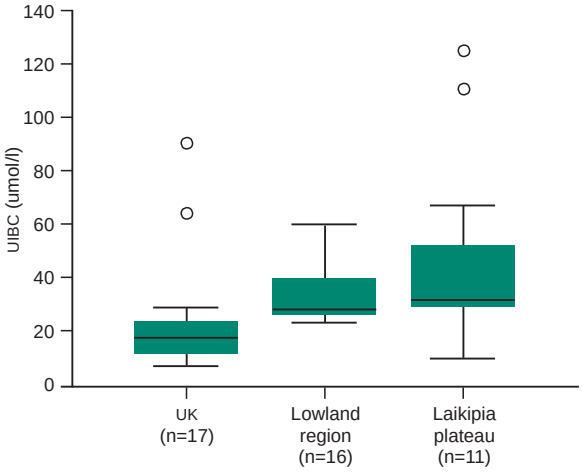

FIG 4: Box and whisker plots of the serum unbound iron binding capacity (UIBC) of the 17 captive black rhinoceroses (*Diceros bicornis*) and 27 free-ranging animals in two regions of Kenya

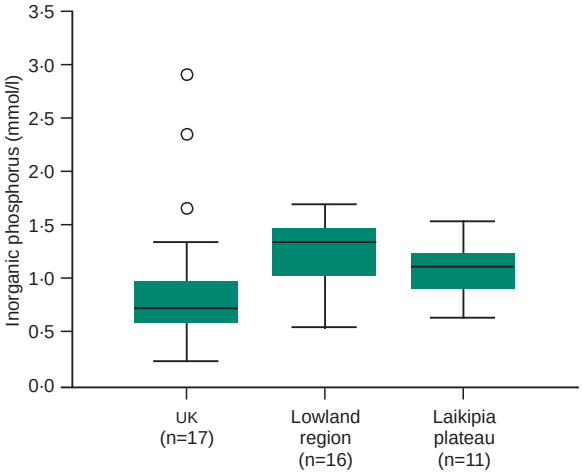

FIG 5: Box and whisker plots of the serum inorganic phosphorus concentrations in 17 captive black rhinoceroses (*Diceros bicornis*) and 27 free-ranging animals in the two regions of Kenya

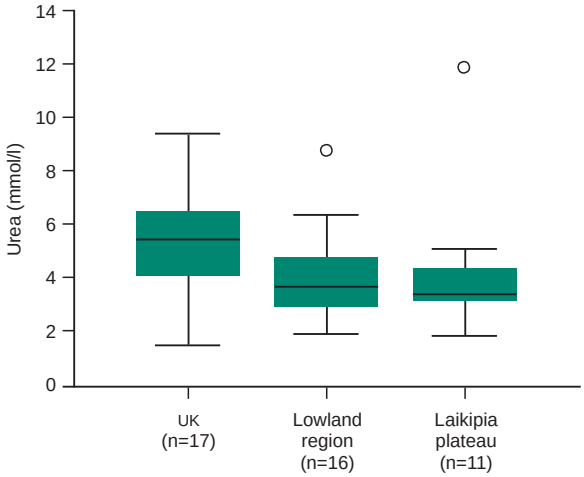

FIG 6: Box and whisker plots of the serum urea concentrations in 17 captive black rhinoceroses (*Diceros bicornis*) and 27 free-ranging animals in two regions of Kenya

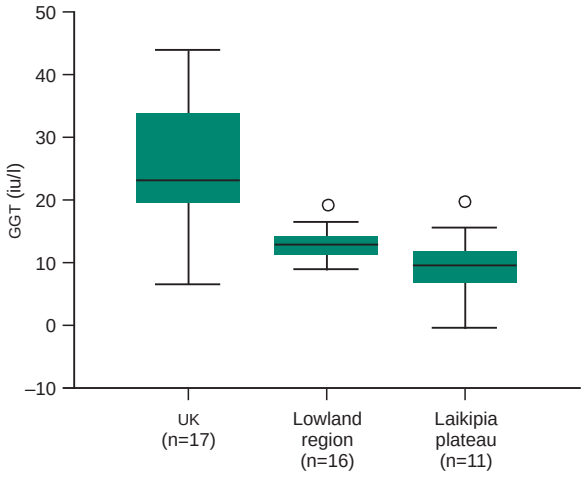

FIG 7: Box and whisker plots of the serum gamma glutamyl transferase (GGT) activities in 17 captive black rhinoceroses (*Diceros bicornis*) and 27 free-ranging animals in two regions of Kenya

and others 1997, Brissot and others 2000), but it can also be an acquired disease (Hennigar and others 1979, Gordeuk 1992). The disease is assessed by measuring the patient's serum transferrin saturation (Brittenham and others 2000, Hash 2001). Transferrin saturations under 40 per cent are considered normal, but a transferrin saturation of more than 60 per cent on at least two occasions is considered to indicate haemochromatosis (Beutler and others 2003). By these standards, most of the captive black rhinoceroses in this study could be considered to have haemochromatosis, and the results derived from the free-ranging population can also be considered to be relatively high. However, a high transferrin saturation can be due to a reduction in transferrin production owing to hepatic failure of different origin, and this needs to be ruled out (Brittenham and others 2000) before the diagnosis can be confirmed.

After an initial indication of haemochromatosis by elevated transferrin saturation, liver enzymes are analysed to establish the extent of liver damage (Hash 2001). In horses with haemochromatosis, increases in bile acids, GGT, ALP and AST are usually observed, and bile acids and GGT are thought to be the best indicators of hepatic function (Olsman and Sloet van Oldruitenborgh-Oosterbaan 2004). The captive rhinoceroses in this study did have significantly higher activities of GGT than the free-ranging rhinoceroses. A reference range for GGT is available from ISIS (Flesness 2002); assuming it is based on (mean ± 1·96 sd), the range is much wider than the one derived from the free-ranging rhinoceroses in this study, and compared with it, the range derived from the captive rhinoceroses is not significantly elevated (Table 4). The differences between the reference ranges could be explained by the fact that the ISIS range was derived from a much larger sample of captive black rhinoceroses, some of which could have been suffering from subclinical haemochromatosis. Furthermore, because the data originate from different institutions, different laboratory techniques may have been used. Different blood sampling techniques can also affect biochemical measurements. Studies of the effects of delayed separation on biochemical analytes showed that if whole blood was left standing for 24 hours at 21°C, there would not be an increase in the GGT level of more than 2 per cent, and maximum increases of 1·8 per cent in ALT and 3·0 per cent in AST (Clark and others 2003). More importantly, serum analytes appeared to be stable in whole blood kept at 20°C during the first eight to 12 hours after collection (Stahl and Brandslung 2005). In the present study, several of the samples from the captive black rhinoceroses were left overnight at approximately 15°C before the serum was separated. This could have increased the GGT levels slightly, but not significantly. On the other hand, the activity of AST decreases if the separated serum is not frozen but stored at room temperature (Heins and others 1995), especially if left for more than three days (Saeed and others 1995). In the present study the samples were frozen directly after separation. These variations demonstrate the importance of standardising the procedures for blood sampling, sample handling and sample storage for reliable

TABLE 3: Comparisons of the ranges of serum iron and transferrin saturation, calculated as the means (sd), of the black rhinoceroses in this study and previously published ranges

	Iron (µmol/l)	Transferrin saturation (%)
This study		
27 free-ranging, Kenya	16·5-35·4	26·3-55·4
17 captive, UK	29·6-50·2	Not normally distributed
Dierenfeld and others (2005)		
26-27 free-ranging, Zimbabwe	59·2-106·2	Not available
12-34 captive, USA	61·5-146·5	Not available
Paglia and Dennis (1999)		
6 free-ranging, Zimbabwe	31·5-46·2	22·0-34·0
24 captive, USA	58·8-131·2	43·0-87·0

TABLE 4: Comparisons of the ranges of serum iron and gamma glutamyl transferase (GGT), calculated as the means (±1·96 sd), of the black rhinoceroses in this study with the ranges given by LYNX and ISIS, both assumed to be means (±1·96 sd)

	Iron (µmol/)	GGT (iu/l)
This study		
27 free-ranging, Kenya	0·7-55·1	3-21
17 captive, UK	19·7-60·1	5-46
LYNX (ZSL 1991)		
18 captive	20·6-68·9	Not available
ISIS (Flesness 2002)		
237 captive	17·1-92·0	0-72

comparisons between biochemical parameters of different populations and/or individuals.

Of the eight captive rhinoceroses with transferrin saturation and/or GGT levels exceeding the reference ranges, five had ulcerations on their back, legs and/or feet when they were sampled. Detailed information about the extent, duration and response to treatment of these skin lesions was not available, and there were too few animals to draw any conclusions about a possible relationship between skin problems and abnormal biochemical parameters.

In time, the storage of excess iron causes fibrosis of the affected organs. In the liver this is termed cirrhosis, a condition that disrupts the normal hepatic blood flow and causes passive congestion of the spleen and digestive organs. It is progressive, irreversible and fatal (Jones and others 1997). The clinical signs of haemochromatosis are usually related to hepatic failure and are non-specific, for example, lethargy and chronic wasting (Spelman and others 1989, Lowenstine and Munson 1999, Hash 2001, Olsman and Sloet van Oldruitenborgh-Oosterbaan 2004). The treatment of haemochromatosis aims to reduce the total iron content of the body to within normal limits (Beutler and others 2003). However, clinical improvement can be achieved only when the disease is at an early stage, before the internal organs have suffered irreversible damage (Paglia and Dennis 1999, Brissot and others 2000, Brittenham and others 2000). It has been shown that the amount of iron stored by black rhinoceroses increases with the time they are kept in captivity (Smith and others 1995, Paglia and Dennis 1999, Paglia and others 2001b). A significant increase in haemosiderin deposition has been observed in rhinoceroses kept in a boma for only three weeks during translocation (Kock and Morkel 1993). Because the captive black rhinoceroses in this study had significantly higher serum iron levels, transferrin saturation and GGT than the free-ranging animals, it may be sensible to assume that all captive black rhinoceroses are at risk of developing haemochromatosis. Ideally, serial blood samples should be taken at regular intervals to determine when excessive iron storage begins, so that further diagnostic testing, possible dietary adjustments and therapeutic schedules can be introduced without delay and their effects can be assessed. As in human beings (Brittenham and others 2000), measurements of the serum transferrin saturation could be used to monitor the condition of black rhinoceroses.

The results of this study demonstrate the importance of standardising procedures, particularly the statistical methods used to establish reference ranges, so that reliable comparisons can be made between studies. More importantly, the study has demonstrated the value of using reference ranges derived from free-ranging rhinoceroses to compare with reference ranges derived from captive animals of the same species with all the samples being analysed at the same laboratories.

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