

Alpha-tocopherol and fatty acids of major browse plant species of black rhinoceros in the Great Fish River Reserve

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Received 23 September 2003. Accepted 16 March 2004

The black rhinoceros (*Diceros bicornis*) is an endangered species, and a number of these animals have been re-introduced into the Great Fish River Reserve where the vegetation is mostly succulent thorny shrub characterized by high species richness. Health problems have been observed in captive wild animals that may be linked to nutritional factors. In particular, vitamin E deficiency may result in diseases in the rhinoceros such as haemolytic anaemia and neurodegenerative disorders. Plant samples collected from the reserve were analysed for vitamin E and fatty acid profiles, as certain fatty acids may play a role in the absorption of vitamin E. Vitamin E content of the plant species studied was variable, with the highest amount of 729 µg/g dry weight found in *Euclea undulata*. The other plant species investigated had vitamin E levels between 100 and 700 µg/g dry weight. The fatty acid composition varied with plant species and season with the predominant fatty acids being C₁₄, C₁₆ and C_{18:3}. Spring growth had higher levels of C₁₄ fatty acid compared to the winter growth, and in winter, the levels of unsaturated fatty acids were higher than in spring. Generally, plants low in vitamin E had low levels of unsaturated fatty acids but no systematic relationship was found between vitamin E and total fatty acids.

Key words: black rhinoceros, alpha-tocopherol, vitamin E, browse fatty acids, HPLC, GC-MS.

INTRODUCTION

Extinction pressure on the black rhinoceros (*Diceros bicornis*) has resulted in considerable research into their health and nutrition. Ecological studies have shown that the animals are primarily browsers, compared with the white rhinoceros, which is a grazer (Dierenfeld *et al.* 1995; Grant 1999). In captivity, the white rhinoceros has survived better than the black rhinoceros, a phenomenon that has been attributed to their different feeding strategies and hence different nutritional needs (Dierenfeld *et al.* 1988; Miller 1993).

This study was carried out on the Great Fish River Reserve (GFRR) in the Eastern Cape Province of South Africa (35 km from Grahamstown). The vegetation type is mostly succulent thorny shrub (valley Bushveld) about 2–3 m high and species richness is relatively high. Forty-six black rhinos of both sexes and of varying ages were introduced into the GFRR between 1986 and 1997 from KwaZulu-Natal. The animals graze freely over about 37 000 ha and are protected from the surrounding area by electric fencing.

Free-ranging animals exert a choice in the plant

species and plant parts that they consume, and as such, their choice affects their nutritional uptake (Mayes & Dove 2000). Several nutrients, including vitamins, are essential in animal nutrition and a vitamin E deficiency, recorded in several animal species, has been associated with pathology (Dierenfeld & Traber 1992). Vitamin E is fat-soluble and its biological activity is exhibited in the tocopherols, which are an important class of compounds with antioxidant activity. α-Tocopherol is the predominant form and exhibits the greatest biological activity (Goodman *et al.* 1991; Hofius & Sonnewald 2003).

Fatty acid composition is an important aspect of herbivore nutrition, due to the requirement of specific fatty acids to fulfil various metabolic functions. Vitamin E is absorbed from the gut in association with fatty acids and low levels of dietary fatty acids may inhibit availability due to a compromised uptake. Specifically, the presence of polyunsaturated fatty acids (PUFA) has been shown to sequester vitamin E in amounts commensurate with their own concentration and degree of saturation (Ghebremeskel *et al.* 1991; Dierenfeld 1999).

Previous studies have reported on plant vitamin E in black rhino browse collected from various geographical regions (Ghebremeskel *et al.* 1991;

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Dierenfeld *et al.* 1995). Our study is the first report on plant vitamin E and fatty acids in the main plant species browsed in the selected area and aimed at providing a possible insight into habitat assessments in areas before the introduction of animals.

MATERIALS AND METHODS

Materials

All the solvents used were HPLC grade. Methanol, 95% ethanol, hexane, TLC plates, sodium dodecyl sulphate (SDS), α -tocopherol and α -tocopherol acetate were all obtained from Merck (South Africa). Standard saturated fatty acid methyl esters (FAMES) were obtained from Analabs (South Africa), while the polyunsaturated methyl esters were purchased from Sigma Aldrich (South Africa), as was the internal standard, heptadecanoic acid. Gases were purchased from Afrox (South Africa).

Plant collection

Plant species selection was based on a previous study that back-tracked the black rhinoceros on the GFRR, observing its feeding habits (Ausland & Sveipe 2000). In this study the list of the plant species most often browsed by the black rhinos on this game reserve was adopted with slight modifications and included *Euclea undulata*, *Jathropha capensis*, *Schotia afra*, *Plumbago auriculata*, *Ehretia rigida*, *Maytenus capitata*, *Euphorbia bothae*, *Azima tetraacantha* and *Grewia robusta*.

Sample preparation

Stock solutions of α -tocopherol and α -tocopherol acetate were prepared in acetonitrile. Standard fatty acid methyl esters (C_{12} - C_{20} saturated) and heptadecanoic acid internal standard were prepared in hexane.

Vitamin E extraction

Plant twigs were obtained from different branches of individually marked bushes, kept in plastic bags and transported to the laboratory for analysis within two hours of collection. Leaf material was removed, shredded and 1 g samples ground with 5 ml 1% ascorbic acid in water. Equivalent amounts were also weighed out for determination of dry weight. The mixture was transferred to 20 ml polystyrene tubes and 3 ml ethanol and 2 ml 1% SDS added, and then extracted into 4 ml hexane containing α -tocopherol acetate (internal standard). The mixture was extracted twice more into

hexane, the extracts pooled and evaporated to dryness under nitrogen. The dried extracts were reconstituted in 2 ml methanol and analysed by Reversed Phase High Performance Liquid Chromatography (RP-HPLC).

Detection and quantitation of vitamin E

Vitamin E analysis was carried out initially by thin layer chromatography (TLC), and then by RP-HPLC. Crude plant extracts and varying amounts of standard α -tocopherol were spotted on silica gel TLC plates and the plates developed in chloroform-hexane (10:3 v/v). TLC was used as a rapid screening technique prior to quantitative analysis.

The HPLC system was a Varian 5000 instrument with a Rheodyne injection valve equipped with a 20 μ l sample loop. A Hewlett Packard Agilent Zorbax SB-C18 column (4.6 mm \times 250 mm \times 5 μ m particle size) was utilized with a methanol and water elution gradient (Dierenfeld *et al.* 1995). An initial 95% methanol mobile phase was increased to 100% in 5 min, and maintained at 100% methanol for 25 min. The flow rate was 0.5 ml/min and detector settings were 292 nm and 0.2 AUFS using a Linear Instruments UVIS 200 HPLC detector. α -Tocopherol was used as an external standard, and quantitation was performed by calculating the peak area ratios of α -tocopherol and α -tocopherol acetate (Alvarez & De Mazancourt 2001).

Quantitation of vitamin E

Vitamin E quantitation by RP-HPLC used an α -tocopherol external standard with a standard curve obtained for each batch of samples analysed. Standard curves were obtained with 5 different concentrations on a single day and linearity between 1 μ g and 5 μ g α -tocopherol on column was satisfactory. The ratio of the α -tocopherol to α -tocopherol acetate peak areas was plotted (y-axis), against the amount of α -tocopherol (μ g) (x-axis). α -Tocopherol acetate was used as internal standard and a recovery study performed indicated 98.2% recovery of the internal standard. Stability studies were carried out for vitamin E in plant leaves and their extracts and vitamin E was found to be more stable in plant extracts than when stored as fresh leaves.

Fatty acid analysis

Lipid extraction and hydrolysis. Total lipids were extracted and hydrolysed using a method described by Christie (1989). The plant leaves

were dried by microwave heating until the dry weight was constant. The dried leaves were crushed to a fine powder and shaken in a solution of chloroform:methanol (2:1, v/v) at a ratio of 1:10 (w/v) for one hour at room temperature.

The plant tissue was then filtered, and the residue washed with 2 ml methanol. The filtrate was collected, mixed with 2 ml 1% saline and the lower layer containing the lipids collected and evaporated under nitrogen.

The isolated lipid extract was hydrolysed with a 1M solution of NaOH in ethanol for 1 h at 80°C. Heptadecanoic acid standard (2 ml of 1 mg/ml in ethanol) was then added to the mixture. Saline (2 ml of 10% solution) was added and the mixture extracted into hexane to remove all the neutral organic substances. The aqueous phase was acidified to pH less than 2, using a 1M solution of hydrochloric acid and then extracted again three times into 2 ml hexane. The hexane extracts containing the free fatty acids were pooled and dried under nitrogen.

The fatty acids were methylated for GC and GC-MS analysis by addition of sulphuric acid (2 ml of 1 % in methanol) to the fatty acid mixture and kept at room temperature overnight. The mixture was made alkaline (pH > 8) using a 1M solution of NaOH and extracted into hexane. The extract was dried with 0.5 g NaHCO₃, evaporated to dryness under nitrogen and reconstituted with 1 ml hexane and analysed by GC and GC-MS. Total fatty acids were quantified and seasonal fatty acid profiles obtained.

GC analysis of fatty acid methyl esters (FAMES). A Hewlett Packard model 5890A capillary gas chromatograph fitted with a flame ionization detector and a split/splitless injector (split ratio 45:1) was used. A HP-INNOWax fused silica capillary column (30 m × 0.25 mm i.d. and 0.25 µm film thickness) was temperature-programmed from 150–220°C at 2°C/min and held at 220°C for 15 min. Helium was used as carrier gas at a flow rate of 25 cm/sec. Injector and detector temperatures were maintained at 220°C and 240°C, respectively, and 0.5 µl sample containing 1 µg heptadecanoic acid methyl ester was injected. Peaks were identified from their retention times compared with standard FAMES.

GC-MS analysis of fatty acid methyl esters. The gas chromatograph was a HP 6890 with a HP 5973 mass selective detector. A HP-5 capillary column (30 m × 0.25 mm i.d. and 0.2 µm film thickness) was temperature-programmed from

150°C to 240°C at 4°C/min and then maintained at 240°C for 15 min giving a total run time of 37.5 min. The inlet temperature was 260°C and 0.1 µl sample was injected using splitless injection mode (0.2 min). Helium was used as carrier gas at a flow rate of 0.7 ml/min. The Wiley 275 mass spectral library was used in identification as well as by comparison with the mass spectra of available authentic standards.

RESULTS AND DISCUSSION

Studies carried out in Kenya (Ghebremeskel *et al.* 1991) and Zimbabwe (Dierenfeld *et al.* 1995) have shown black rhino to utilize a wide variety of plant species, while concentrating on a few preferred species. Studies over longer periods refer to a higher number of browsed plant species compared to studies over brief periods (Gilchrist & Mackie 1983). The reason for the wide selection is unclear, but it is speculated that the various plants may contain certain unique characteristics essential to the black rhino. Black rhinos tend to shift their feeding preferences according to the circumstances and abundance of plants in their environment. As a result it has proven difficult to compare studies on the species that they prefer in different vegetation areas. Furthermore, it is speculated that the choice of plants would differ seasonally (Ausland & Sveipe 2000).

The amount of vitamin E as exhibited in the activity of only α-tocopherol was used in quantification. Quantitative analysis of vitamin E, determined by RP-HPLC, indicated that vitamin E levels varied according to plant species, with *E. undulata* containing the highest amounts (729 µg/g dry weight) and *E. rigida* the lowest (Fig. 1). The other plant species selected had vitamin E levels between 100 and 700 µg/g dry weight. These values were considerably higher than values reported from previous studies on plants eaten by the black rhino, 57.5–341 µg/g dry weight (Dierenfeld *et al.* 1995) and 0.4–153 µg/g dry weight (Ghebremeskel *et al.* 1991) on plant species collected in Zimbabwe and Kenya, respectively. However, the considerably lower vitamin E values may have been due to the fact that both studies used twig samples and worked out a leaf/twig ratio, which is more representative browsing rather than just leaves as analysed in this study.

While no distinct relationship was observed between plant vitamin E levels and total unsaturated fatty acid methyl esters, plant species low in vitamin E generally had low levels of unsaturated

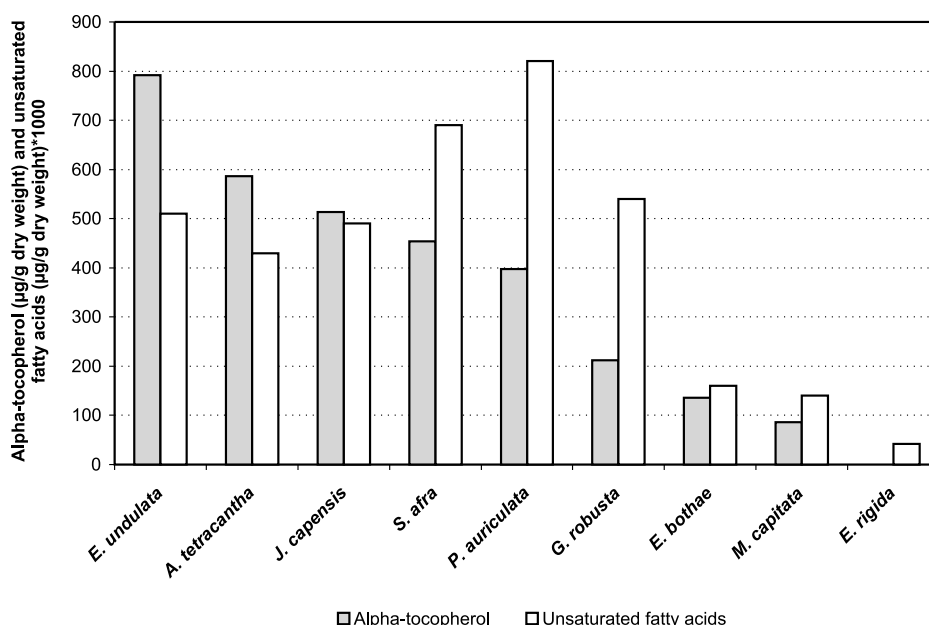


Fig. 1. Vitamin E and total unsaturated fatty acid methyl ester levels in various plant species.

fatty acids (Fig. 1).

Black rhinos on the GFRR were shown to browse most often on *E. bothae*, which was found to contain relatively low levels of vitamin E. The high preference index may have been due to the abundance of this plant species on the GFRR. One may conclude that in identifying the principal food plant for an animal species, it is important to take into account the preference index as well as the amount of material ingested from the plants (Ausland & Sveipe 2000).

From this study it cannot be concluded that the animal's feeding habit was influenced by nutrient composition, and specifically, vitamin E content. Vitamin E analysis of plants that are rejected by the black rhino, such as *Acacia* species, would be recommended. Furthermore, since vitamin E is not the only important nutrient in black rhino nutrition, a wider range of nutrients would have to be analysed in order to make a sound conclusion.

The vitamin E levels reported in this study were also higher than previously reported values on forages fed in zoos. Generally, browse samples have been shown to provide higher amounts of vitamin E than standard zoo diets, a factor which suggests that captive black rhinoceros risk a vitamin E deficiency unless supplemented (Dierenfeld *et al.* 1990). The values obtained were also higher than the United States' National Research Council (NRC) recommended values

(50–80 µg/g dry weight) for vitamin E in the diet of equids, a group to which the black rhino belongs (Dierenfeld *et al.* 1990).

Although the frequency of browsing on each plant species has been determined (Ausland & Sveipe 2000), the actual amount of plant material ingested by black rhino was not determined. As a result, no study on free-ranging animals can report with absolute certainty how much of a nutrient the animals get from their wild diet. Because of this, the data obtained from plant analysis need to be considered along with the physical condition of the animals. Since this population is thriving, it can be concluded that the animals are getting adequate nutrition.

FAME peaks on the GC and GC/MS were identified using retention times and mass spectra of standard mixtures and quantitation used heptadecanoic acid as an internal standard. Total plant fatty acids were quantified by summing the areas of the different fatty acid methyl ester peaks and the results are shown in Fig. 2.

Relative fatty acid proportions were determined for all plant species in winter and in spring. Figures 3, 4 and 5 show seasonal fatty acid profiles of selected species, *S. afra*, *M. capitata* and *E. undulata*.

The fatty acid composition varied with plant species and season. This was in agreement with previous studies on fatty acid composition of

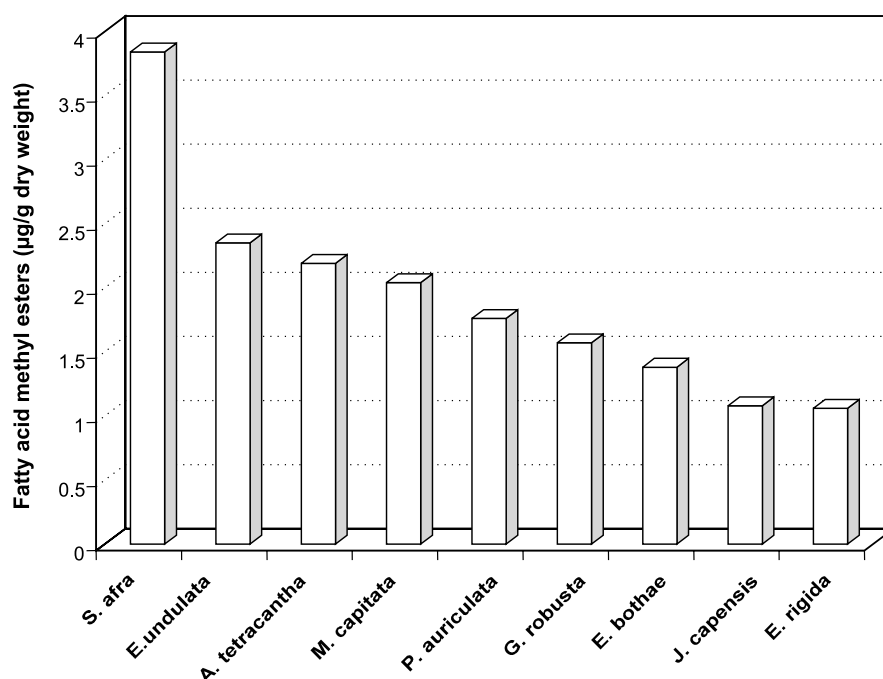


Fig. 2. Total fatty acid methyl esters of various plant species.

selected plant species in South Africa (Davidson *et al.* 1990; Davidson 1998). The predominant fatty acids were C_{14} , C_{16} and $C_{18:3}$ as shown in Figs 3, 4 & 5 which are selected examples used to illustrate the seasonal variation of plant fatty acid patterns. Spring growth had high levels of C_{14} fatty acid compared to the winter growth. In the winter, the levels of unsaturated fatty acids were generally much higher than in the spring. Generally, C_{16} fatty acid was found in higher amounts than C_{18} fatty acid which in turn was found in comparable amounts to $C_{18:1}$ fatty acid. C_{20} fatty acids were

found in marginal amounts in winter growth and only in a few species in spring growth. No unsaturated C_{16} fatty acids were found. Ghebremeskel *et al.* (1991) reported detectable levels of C_{15} , $C_{16:1}$, C_{17} and C_{22} fatty acids, which were not found in this study.

The overall profile of all species was an increased amount of unsaturated C_{18} fatty acids with no C_{12} fatty acid in the winter. A transition from winter growth into spring growth resulted in a shift to lower carbon chain saturated C_{12} and C_{14} fatty acids. Davidson (1998) reported an overall

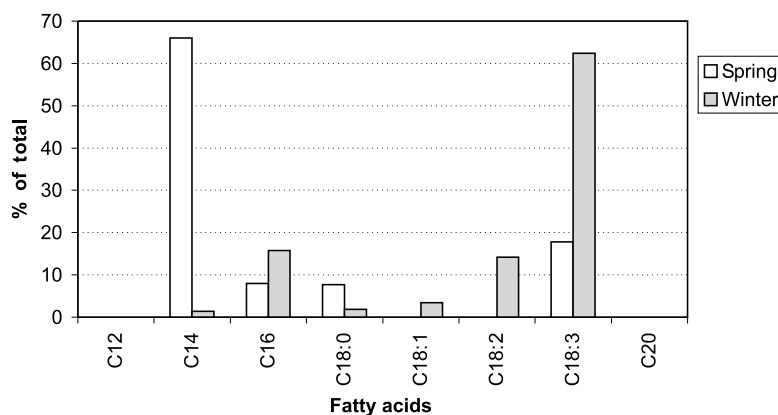


Fig. 3. *Schotia afra* fatty acid methyl ester profiles.

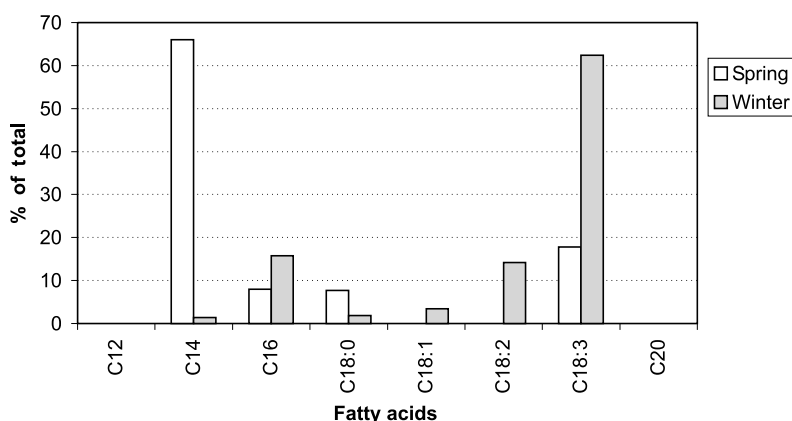


Fig. 4. *Maytenus capitata* fatty acid methyl ester profiles.

maximum amount of C_{18:3} essential fatty acids but no parallel increase in C_{18:2}. Our findings were contrary to this, as there was an overall increase in both the polyunsaturated fatty acids in winter growth.

No relationship was found between vitamin E and total fatty acids. *S. afra* was reported to have the highest fatty acid content and yet it ranked fourth in vitamin E levels. On the other hand, *E. rigida*, whose vitamin E levels were below the detection limit, also had the lowest amount of fatty acids. Because no pattern was found in vitamin E and total fatty acid content, vitamin E content was compared with that of mono-unsaturated and poly-unsaturated fatty acids (Fig. 1). Generally,

plants low in vitamin E had low levels of unsaturated fatty acids, with the exception of *E. rigida* with undetectable levels of vitamin E. *M. capitata* and *E. bothae*, which were low in vitamin E (86 and 136 µg/g, respectively) were also low in unsaturated fatty acids (140 and 160 µg/g, respectively). The tocopherols are well-known antioxidants whose presence in plants is often correlated with a relative abundance in unsaturated fatty acids (Bruni *et al.* 2002). This is due to an increase in unsaturated fatty acids warranting an increase in the requirement for vitamin E to avoid oxidation of these fatty acids. Howitt *et al.* (1972) reported that the nature of associated fats was critical for the absorption of α-tocopherol since it interacts with

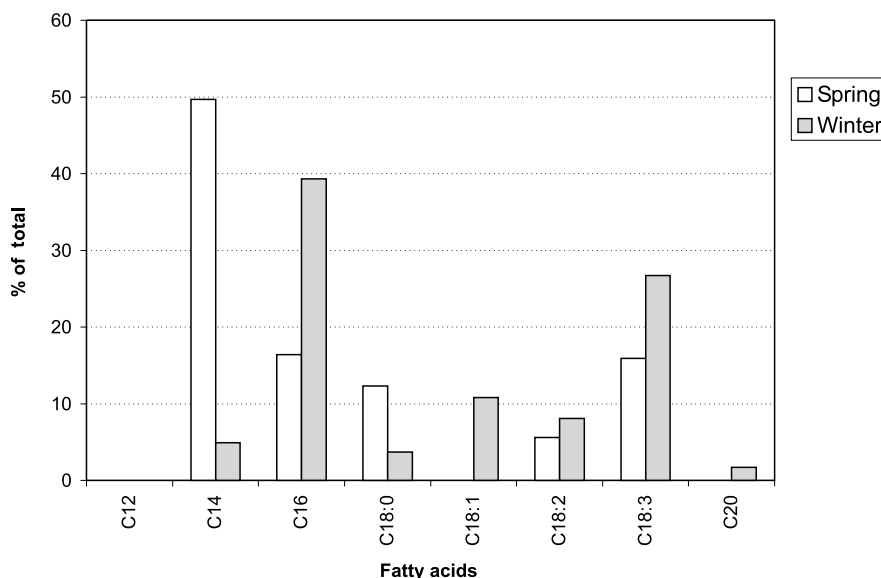


Fig. 5. *Euclea undulata* fatty acid methyl ester profiles.

polyunsaturates within the intestinal lumen. In the nutrition of the black rhinos this could be important, as the feed given to captive rhinos, would be high in concentrates and low in fibre. This would result in an increased rate of passage through the gastrointestinal tract, thereby potentially reducing the absorption of α -tocopherol and other nutrients (Gilchrist & Mackie 1983; Grant 1999). The essential fatty acids are important in the absorption of vitamin E and their deficiency may result in disease conditions characterized by inadequate fatty acids, such as scaly skin disorders, as well as those typical of low α -tocopherol levels (Grant 1999).

ACKNOWLEDGEMENTS

We are grateful to the Management of the GFRR, in particular Brad Fike, for cooperation in plant species identification and collection; the National Research Foundation, Pretoria, and the Mellon Foundation, New York, for financial support.

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