COPPER DEFICIENCY IN BLESBOK (*DAMALISCUS PYGARGUS PHILLIPSI*) FROM THE KAROO NATURE RESERVE

by

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Abstract

The copper status of blesbok was evaluated by measuring the hepatic and plasma copper concentrations, and erythrocyte superoxide dismutase activity. The copper status of blesbok from the Karoo Nature Reserve, Eastern Cape was shown to be significantly lower than blesbok from the Willem Pretorius and Gariep Dam Game Reserves, Free State.

Analysis of soil and water in the Karoo Nature Reserve suggests that alkaline soil, associated with organic matter, may be responsible for decreased availability of copper to plants.

It was shown that leaching of copper could take place when liver samples are stored in 10% buffered formalin. Storage of samples in liquid nitrogen was found to be the only reliable method of maintaining erythrocyte superoxide dismutase activity for extended periods of time.

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List of Abbreviations Used

ANOVA	Analysis of variance
df	Degrees of freedom
EC	Electrical conductivity
EC-SOD	Extracellular superoxide dismutase
EDTA	Ethylenediamine tetra-acetic acid
ESP	Exchangeable sodium percentage
GLM	General linear model
HGB	Haemoglobin
per. comm.	Personal communication
ppm.	Parts per million
rpm	Revolutions per minute
S	Siemens
SAR	Sodium adsorption ratio
SOD	Superoxide dismutase
TCA	Trichloroacetic
UK	United Kingdom
USA	United States of America

CHAPTER 1

Introduction

During 1980, nine male and thirteen female blesbok were introduced into the Karoo Nature Reserve, Graaff-Reinet, South Africa. The blesbok had been obtained from a farm in the Steynsburg district. They are believed to be of pure historical Cape stock, which have survived without the introduction of animals from other gene pools. In 1992, it was noticed that the hair-coats of many blesbok had paled considerably. Ataxia soon developed and some animals died. 'Swayback' was later diagnosed histopathologically in one affected blesbok and on the basis of copper levels in the blood, faeces and liver of the affected animal (Penrith, Tustin, Thornton & Burdett, 1996).

By 1993, the population of blesbok had reached 160 animals but the population had declined to 88 by 1994 and high mortalities among young animals were reported (see Figure 1.1). None of the lambs born in 1994 survived (Penrith et al., 1996). Since 1995, there has been a slow, but constant increase in the blesbok population. At present, there are 99 animals on the reserve.

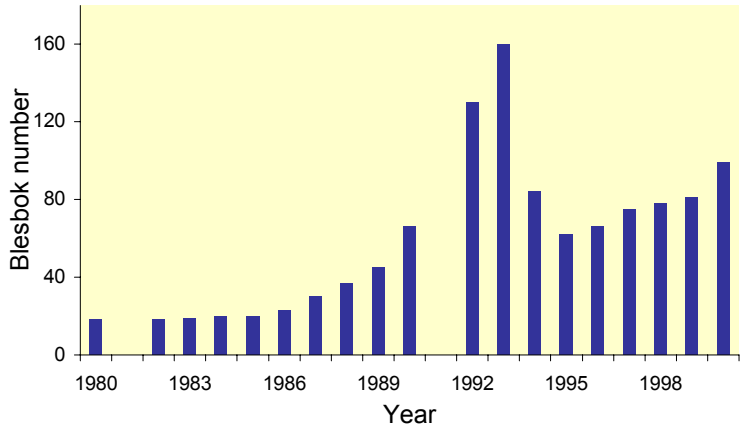

Figure 1.1: Total number of blesbok in the Karoo Nature Reserve, Graaff-Reinet, 1980 - 2000 (Department of Economic Affairs, Environment and Tourism, Eastern Cape Province, 2000).

This study was planned to evaluate the copper status of a representative proportion of the blesbok population in the Karoo Nature Reserve, by measuring copper concentrations in liver and plasma, and superoxide dismutase activity in erythrocytes of these animals. These various indicators were assessed to see if there was any correlation between them and to suggest an indicator that best represented the clinical picture.

Before a study of the blesbok in the Karoo Nature Reserve could be undertaken, it was necessary to do some preliminary work. The effect of formalin storage on the concentration of copper in livers of blesbok was studied. It was hypothesised that there would be a significantly lower level of copper in formalin fixed liver when compared to frozen liver.

As it was not possible to analyse blood samples immediately after collection in the field, the effect of storage temperature on the activity of superoxide dismutase (SOD) was determined. In addition, an evaluation of equipment used in the measurement of haemoglobin concentration was also undertaken.

As there are no published data on normal copper or superoxide dismutase values for blesbok, blesbok from Willem Pretorius Game Reserve, Free State and Gariep Dam Game Reserve, Free State, were sampled and used as controls. Blesbok from these reserves were used as controls as the reserves fall within the natural distribution of the blesbok (Skinner & Smithers, 1990), the animals there appear healthy and the populations thrive. Appropiate null hypotheses were set:

- Hypothesis 1 - The copper concentration measured in the plasma and liver, and erythrocyte superoxide dismutase activity of blesbok in the Karoo Nature Reserve is not lower from that found in the control populations.
- Hypothesis 2 - There is no correlation between copper values in plasma, liver and erythrocyte superoxide dismutase.

Earlier studies had not established the cause of the swayback in blesbok from the Karoo Nature Reserve. It was suspected that the dam in the reserve was causing minerals to be leached from the soil and that low copper levels in the soil and herbage were the result (Penrith et al., 1996). The assumption that the Dam Area (the area covered when the dam is full) was the source of the copper deficiency problem seemed reasonable, as the blesbok spend the majority of their time there. Copper deficiency associated with periodically flooded soils has previously been reported (Ramírez, Mattioli, Tittarelli, Giuliodori & Yano, 1998).

The next step of this study was to determine the cause of the copper deficiency. An attempt was made to classify the soil of the Karoo Nature Reserve into saline, saline-alkali or nonsaline-alkali soils (Tan, 1993). It appeared likely that the soil in the reserve was saline/sodic, as the area occurs in a semi-arid region, the surrounding area is highly eroded (personal observation) and a dam in the reserve holds back silt and has the effect of raising the water table.

To test the hypothesis that the Dam Area contained low levels of copper, soil samples were collected from the Dam Area and compared to control samples taken from the area surrounding the dam that had remained unflooded. In addition to the analysis of copper in the soil, factors affecting copper availability in the soil, viz. pH, Zn, CO_3^{2-} and organic matter were included in the soil analysis (McBride, 1981; Moraghan & Mascagni, 1991; Shuman, 1991). Trace elements that interact with copper in the mammalian system, viz. molybdenum, sulphur and zinc (Underwood & Suttle, 1999) were also included in the soil analysis. Data was correlated to determine if any relationships exist between any of the measured parameters. The appropiate null hypotheses were:

- Hypothesis 3 – Measured soil parameters in samples from the Dam Area of the Karoo Nature Reserve do not differ significantly from the control areas.

- Hypothesis 4 - There is no correlation between any of the measured soil parameters.

CHAPTER 2

Literature Review

2.1. The importance of copper in living systems

Copper, an element named after Cyprus, which was the richest known source of copper in ancient times, occupies a space on the periodic table with other elements known as the first transition series elements, elements 21-30. The first transition series includes scandium, titanium, vanadium, chromium, manganese, iron, cobalt, nickel, copper and zinc. These elements share common physical and chemical properties and like copper, most of them can be classified as biological trace elements. Copper plays an essential role in the biochemistry of living matter. Although the amounts needed are minuscule, copper is an essential component of many enzymes and biological catalysts.

In the distant past, an evolutionary crisis would have emerged when oxygen levels in the atmosphere increased. Although oxygen is essential for life, highly damaging intermediate products, such as superoxide free radicals (O_2^-) are formed during metabolism (McCord, Keele & Fridovich, 1971) from the univalent reduction of molecular oxygen. In the body, free radicals are converted to harmless substances by the catalytic properties of copper and iron. Copper, as part of superoxide dismutase, converts O_2^- into H_2O_2 according to the equation:

$O_2^- + O_2^- + 2H^+ \rightarrow O_2 + H_2O_2$ (McCord & Fridovich, 1968; McCord & Fridovich, 1969)

Fe (as part of catalase) is able to convert the H_2O_2 into H_2O and O_2. Thus, the potential of O_2 to maximise energy production from organic compounds was released.

Three chemical properties of copper are important for carrying out metabolic processes in the mammalian system:

- Copper ions react with amino acids or proteins more strongly than other metallic ions to form very stable chelates.
- Copper is a very effective catalytic agent and is probably the most versatile catalyst of all the elements.
- Copper is able to exist in various ionisation states. It exists as the free, neutral atom (Cu), as the cuprous ion (Cu^{+}) and the cupric ion (Cu^{2+}). As a result, copper is able to easily donate or accept electrons (Frieden, 1968).

2.2. Copper metabolism

Copper metabolism has been reviewed by Evans (1973), Underwood (1977) and Camakaris (1987).

2.2.1. Absorption of copper

In most species, dietary copper is poorly absorbed. Copper absorption takes place in the stomach and small intestine of most mammals (Evans, 1973). Considerable net absorption takes place in the large intestine of the sheep (Grace, 1975). The extent of this absorption is influenced by the amount and chemical form of the copper ingested; by the dietary level of several other metal ions and organic substances; and by the age of the animal (Underwood, 1977).

The chemical form of copper greatly affects the availability of copper to animals. The intestinal uptake of ^{64}Cu from several inorganic compounds by beef cattle has been tested (Chapman & Bell, 1963). The relative amount of ^{64}Cu appearing in the blood was in the following order: $CuCO_3$ > $Cu(NO_3)_2$ > $CuSO_4$ > $CuCl_2$ > Cu_2O > CuO (powder) > CuO (needles) > Cu (wire).

Changes in the chemical forms of copper in plants appear to affect its availability. Fresh green herbage is less effective in adding to body copper stores than hay or dried herbage with an equivalent total copper content (Hartmans & Bosman, 1970). Apparently, changes occur in the chemical forms of copper during the curing or drying process.

Several metals are able to depress copper absorption. The most important are molybdenum, sulphur, zinc and iron (Underwood, 1977). The interaction between these metals and copper is reviewed in *2.3. Copper Interactions.*

Crampton, Matthews & Poisner (1965), found that copper is not absorbed into the serosal fluid by simple diffusion, that the mechanism of absorption was energy dependant and of limited capacity in hamsters. It has been shown that L-amino acids facilitate the intestinal absorption of copper (Kirchgessner & Grassman, 1970). Since it is known that trans-membrane amino acid transport is energy dependent, it is likely that the energy dependant mechanisms described by Crampton et al. (1965), may represent copper transported as a copper-amino acid complex. On the other hand, Marceau, Aspin & Sass-Kortsak (1970), observed the same absorption rates of ^{64}Cu whether or not the metal was bound to histidine.

Proteins also play an important role in copper absorption. Metallothionein, a low molecular weight sulphydryl-rich protein binds a number of heavy metals such as copper and zinc. It is an ubiquitous protein which has been highly conserved throughout evolution (Camakaris, 1987). Metallothionein has no apparent enzymatic function but probably acts as an intracellular binding ligand. Intestinal metallothionein may play a passive role in providing binding sites to copper and thus facilitates its absorption. It may also play a role in preventing the absorption of toxic amounts of copper as well as other trace elements (Evans, 1973). Various physiological stimuli, such as food restriction, physical stress, bacterial infection and copper administration may all induce the synthesis of metallothionein. In addition, metals such as cadmium and zinc also induce metallothionein (DiSilvestro & Cousins, 1983; Camakaris, 1987).

2.2.2. Transport to the liver

Once absorption has taken place, copper binds to albumin (Bearn & Kunkel, 1954), which has a high affinity, specific binding site for the metal (Camakaris, 1987). A proportion of this Cu-albumin complex may form a ternary complex in association with L-histidine (Lau & Sarkar, 1971). There is evidence that another protein, 'transcuprein', which is larger than albumin at 270 000 daltons, may be involved in transport of copper to the liver (Linder, Weiss & Wirth, 1985). Transcuprein has a greater affinity for copper than albumin and is saturated with copper at a lower concentration. As a result, it is able to compete with albumin for copper, despite its lower concentration in the plasma and the high affinity of the copper ion for albumin.

It has been suggested that hepatic copper transport is a passive process (Saltman, Alex & McCornack, 1959). Others have reported evidence of facilitated transport of copper as a free ion (Schmitt, Darwish, Cheney & Ettinger, 1983). Albumin may slow the rate of transport of copper into hepatocytes by binding and therefore reducing the concentration of free Cu^{2+} in solution (Darwish, Cheney, Schmitt & Ettinger, 1984). As a result, the dissociation of copper from albumin may be the rate-limiting step for copper entry into hepatocytes (Camakaris, 1987). It has been suggested that copper uptake by the liver involves a two stage process, first binding to glutathione and then metallothionein (Bremner, 1993).

2.2.3. Hepatic copper

The main storage organ of copper, the liver, plays a role in copper metabolism. In the liver, copper is incorporated into enzymes and proteins, stored, or excreted. Copper is divided between various fractions within the hepatic cell (Evans, 1973):

- 50% of hepatic copper occurs in the cytosol. A large proportion occurs as metallothionein, which may play a role in the detoxification and storage of trace metals. A small proportion of copper in the cytosol occurs in copper-dependant enzymes.
- 20% of hepatic copper occurs in lysosomes and mitochondria. Lysosomal copper may be a temporary storage pool or a pool destined for biliary excretion. Mitochondria contain the copper-dependant enzyme cytochrome-*c* oxidase.
- 20% of hepatic copper occurs in the nucleus, where it is stored and where it may be involved in metabolic processes.
- 10% of hepatic copper occurs in microsomes where it probably exists as newly synthesised copper-proteins.

2.2.4. Copper distribution in the body

Copper is distributed throughout the body and found in varying concentrations in different organs. High levels are found in the liver, brain, heart and kidney, with intermediate levels in the lung, intestine and spleen. Hepatic copper accounts for about 10% of the total amount of copper in the body. Muscle and bone have the lowest concentrations of copper, but contain approximately 50% of the total body copper because of their large mass (Evans, 1973).

Copper is divided among various fractions in whole blood. In plasma, the major proportion of copper is carried in ceruloplasmin, other proteins and amino acids carry the rest. In humans, 60 - 65% of total plasma copper is associated with ceruloplasmin. Albumin and transcuprein carry another 15% and 10% is associated with lower molecular weight components such as amino acids (Linder et al., 1985). The majority of copper present in the red cell is associated with superoxide dismutase.

2.2.5. Functional forms of copper

2.2.5.1. *Ceruloplasmin (EC 1.16.3.1)*

Ceruloplasmin was first isolated and characterised by Holmberg & Laurell (1948). As the derivation of the name from Latin (*caeruleus* – sky blue) suggests, it is a dark blue protein from plasma. It is synthesised in the liver, secreted into the blood and makes up the majority of the copper content in plasma, varying between 56 - 99%, depending on species (Gubler, Lahey, Cartwright & Wintrobe, 1953; Evans & Wiederanders, 1967).

Ceruloplasmin is classified as a glycoprotein with a molecular weight of 132 000 daltons, each molecule containing six atoms of copper (Camakaris, 1987). It is a true oxidase involved in iron utilisation and in promoting the rate of iron saturation of transferrin (Osaki, Johnson & Frieden, 1966). Transferrin, which binds ferric iron, is the only known protein that supplies iron to the marrow cells for haematopoiesis. Iron entering the blood from storage cells is in the ferrous state and is oxidised by ceruloplasmin. In addition, ceruloplasmin also oxidises adrenalin, noradrenalin, serotonin and melatonin. It is also an acute-phase protein and levels increase in the bloodstream during periods of inflammation or infection (Evans, 1973).

There has been considerable debate as to whether ceruloplasmin acts as a copper transport protein. The *in vitro* exchangeability of copper ions in ceruloplasmin has never been shown, although several studies have provided evidence that copper from ceruloplasmin can act as a source of copper for other enzymes (Hsieh & Frieden, 1975; Harris & DiSilvestro, 1981).

The activity of ceruloplasmin in plasma correlates highly with plasma copper concentration. The measurement of ceruloplasmin concentration in plasma may be a better indicator of copper insufficiency than measuring copper itself. Ceruloplasmin analysis is unaffected by exogenous contamination of samples by trace elements, and a smaller sample is required for analysis (Todd, 1970). It has been further shown that ceruloplasmin is more indicative of copper status in an animal than plasma copper concentration when high dietary concentrations of dietary molybdenum (> 8mg Mo/kg diet) are present (Underwood & Suttle, 1999).

2.2.5.2. *Superoxide dismutase (EC 1.15.1.1)*

The first description of superoxide dismutase (SOD) was of a copper-containing protein isolated from erythrocytes and liver and called hemocuprein and hepatocuprein, respectively (Mann & Keilin, 1938). Later, cerebrocuprein was isolated from human brain tissue (Porter & Folch, 1950). Human hepatocuprein, cerebrocuprein and erythrocuprein (hemocuprein) were shown to be identical to each other (Carrico & Deutsch, 1969). The enzymatic function for erythrocuprein, viz. the dismutation of superoxide radicals, was discovered in 1969 (McCord & Fridovich, 1969).

Although oxygen is essential for life, highly damaging intermediate products, such as superoxide free radicals (O_2^-) are produced from the univalent reduction of molecular oxygen. In aerobic organisms, O_2^- may, for example, be generated from reactions involving non-haem iron proteins or flavoproteins, or the autoxidation of adrenalin. The O_2^- radical may cause lipid peroxidation (Lawrence & Jenkinson, 1987), react with thiol groups and trypophan residues, kill bacteria and render viruses non-infective, as well as inactivate ribonuclease (Malmström, Andréasson & Reinhammar, 1975). The catalytic property of copper provides the means of converting superoxide free radicals to harmless substances (McCord et al., 1971).

Superoxide dismutase occurs in most mammalian tissue (Marklund, 1984; Paynter, 1987), with high activities in the liver and red cells (Paynter & Allen, 1982). Two forms of the enzyme are found intracellularly. One form has a molecular weight of 32,000 and can be divided into two subunits. Each subunit contains an atom of copper and zinc (Carrico & Deutsch, 1970). Zinc plays a role in the stability of the enzyme (Forman & Fridovich, 1973). This form is cyanide-sensitive and appears to be predominantly cytoplasmic. High activities of the enzyme are found in metabolically active organs, such as liver and kidney (Paynter & Allen, 1982; Marklund, 1984). The second form contains manganese instead of copper or zinc, is cyanide-insensitive and appears to be mitochondrial in origin (Weisiger & Fridovich, 1973). High

activities of this enzyme are found in organs with a high metabolic rate and thus a high rate of respiration e.g. liver, kidney and myocardium (Marklund, 1984).

A superoxide dismutase isoenzyme (EC-SOD) that is composed of four equal non-covalently bound subunits, can be found extracellularly. It is cyanide-sensitive, contains four copper atoms and probably also four zinc atoms (Marklund, 1982). EC-SOD is by far the dominant SOD isoenzyme in plasma (Marklund, 1984).

Superoxide dismutase appears to be of primary importance in the utilisation of cellular copper. A ten-fold reduction in hepatic copper in rats only resulted in a two-fold reduction in SOD activity and the amount of apoenzyme remained at normal levels (Chung, Romero, Tinker, Keen & Amemiya, 1987). In addition, the rate of depletion of copper superoxide dismutase activity in copper depleted rats is very dependent on the tissue type. The greatest changes are observed in the liver and red cells of most species, with least changes in the brain and skeletal muscle (Paynter, Moir & Underwood, 1979; Prohaska, 1990).

In red cells, the enzyme appears to be synthesised at the time of erythropoiesis, and its half-life in blood correlates with the half-life of the red cells (McMurray, 1980). Differences are observed in the rate of decrease of plasma and red cell copper concentrations in response to decreasing copper availability, with depletion in plasma occurring at a far greater rate than that in the red cells. Due to the relatively long half-life of SOD, the rate of decrease of this enzyme will be slower than other measurements of copper status, such as plasma/liver copper or ceruloplasmin. Whereas ceruloplasmin activity decreases exponentially in the acute stages of copper insufficiency, decreases in red cell superoxide dismutase activities are more linear in nature. Low superoxide dismutase values therefore have diagnostic significance, indicating a chronic or intense deficiency of available copper (Suttle & McMurray, 1983).

Despite the more rapid turnover of leukocytes, leukocyte superoxide dismutase appears to be less labile than its erythrocyte equivalent in sheep and cattle. The mean leukocyte superoxide dismutase depletion in induced copper deficiency was only about a half of that found in erythrocytes. Leukocyte copper/superoxide dismutase activity may, however, still be a sensitive indicator of copper deficiency. Manganese-dependent superoxide dismutase in mitochondria was not thought to play a significant role (Jones & Suttle, 1981). In copper deficient-induced steers, whole blood SOD was reduced at a stage when animals were clinically normal, mean liver copper concentration was marginal and plasma copper concentration was normal (Xin, Waterman, Hemken & Harmon, 1991).

Only a few references could be found that deal with the stability of superoxide dismutase. Beckman & Pakarinen, in Beckman, Lundgren & Tärnvik (1973), reported that human placental extracts kept at -20°C for eight years showed no decrease in staining activity of superoxide dismutase. No significant changes in serum SOD activity took place in samples that had been kept for at least two months at -20°C (Kawaguchi, Sakurabayashi, Yamanaka & Kawai, 1983). Heparinised human blood samples could be stored at 4°C for up to ten days (Winterbourn, Hawkins, Brian & Carrell, 1975) or three days (DiSilvestro & Marten, 1990) without a measurable loss of activity. Loss of activity of samples stored at room temperature was variable. After three days, SOD activity was reduced by 0 - 30%. Activity loss at room temperature was accelerated in samples collected in EDTA. Serum SOD activity was the same as that of plasma (Kawaguchi, Sakurabayashi, Yamanaka & Kawai, 1983).

Values for red cell copper superoxide dismutase activities associated with chronic copper insufficiency have been reported (Table 2.1).

No reports that deal with superoxide dismutase measurements in non-domestic animals could be found.

Table 2.1: Red cell copper superoxide dismutase activities associated with chronic copper insufficiency.

Species	Red cell copper SOD activity (U/g HGB)* Marginal	Severe
Ovine	200-400	<200
Bovine	250-450	<250
Cervine		
(Fallow)	100-200	<100
(Red)	100-300	<100

* *One activity unit is equivalent to the activity of 1μg of purified bovine copper superoxide dismutase. HGB = haemoglobin*
From Paynter, 1987

2.2.5.3. *Cytochrome-c oxidase (EC 1.9.3.1)*

Cytochrome-*c* oxidase is the terminal enzyme in oxidative phosphorylation and is essential in cellular metabolism. It catalyses the oxidation of reduced cytochrome-*c* by molecular oxygen, and in the process oxygen is reduced to water. Each monomer of cytochrome-*c* oxidase contains one molecule of haeme and one atom each of iron and copper (Evans, 1973).

2.2.5.4. *Amine oxidases*

Lysyl oxidase (EC 1.4.3.13), an enzyme containing four atoms of copper per molecule, is required to catalyse the oxidative deamination of lysine residues in the peptide chain of elastin, the major protein of vascular tissue (Hill, Starcher & Kim, 1967). The product formed is desmosine, which forms the cross-links in elastin. A reduction in cross-linkage by desmosine results in lack of elasticity. The stability of collagen, as part of connective tissue and bone, also depends on the oxidative deamination of lysine residues (Chou, Savage & O'Dell, 1969). Deficiency in copper therefore results in fragility of the vascular and skeletal tissue.

2.2.5.5. *Tyrosinase (EC 1.14.18.1)*

A copper enzyme, tyrosinase is essential in the pigmentation process of hair or fur, since this enzyme catalyses the first two steps in the synthesis of melanin pigments from tyrosine (Lerner & Fitzpatrick, 1950).

2.2.5.6. *Other copper-containing enzymes*

The above enzymes only represent a few of the copper containing enzymes in the mammalian system. Other enzymes include: laccase, ascorbic acid oxidase, uricase, δ-aminolevulinic acid dehydrase, dopamine-β-hydroxylase and galactose oxidase (Evans, 1973).

2.2.6. Excretion of copper

The liver provides the major pathway of copper excretion via the bile. In the bile, copper becomes associated with macromolecules that prevent reabsorption of the metal. Hormones of the adrenal cortex affect copper excretion as a result of their influence on choleresis. Urinary copper excretion is negligible under normal conditions, as most of the copper in circulating blood is bound to plasma proteins or in red blood cells (Evans, 1973).

2.3. Copper interactions

An extensive review of copper interactions is provided by Gawthorne (1987).

The majority of elements that interact with copper are first transition series elements and thus share common characteristics. The interaction between these metals is considered to be competition for transport systems and storage proteins (Gawthorne, 1987).

2.3.1. Copper, molybdenum and sulphur

The ingestion of molybdenum as molybdate decreases the copper availability to animals. If sulphide is present, this antagonism takes place at low

concentrations of molybdenum (< 10 ppm) commonly found in foodstuffs. This interaction is only significant in the ruminant, as micro-organisms in the rumen are able to oxidise the organic and inorganic sulphur in the diet to sulphide. Since protein carries sulphur with it, in the form of sulphur containing amino acids, by-pass protein (protein that escapes bacterial breakdown in the rumen) can significantly decrease copper absorption in the intestine (Gawthorne, 1987).

It has been hypothesised that thiomolybdates are formed in the rumen following reactions between hydrogen sulphide and molybdate. These thiomolybdates react with, or are bound to proteins, and it is this complex that chelates copper strongly, making the copper unavailable for absorption. In the circulation, thiomolybdates are reversibly bound to albumin and modify the way in which copper binds to it, slowing the rate of delivery of the metal to the liver. The Cu-Mo-S complex is insoluble in trichloroacetic acid (TCA) and it is very likely that a decrease in the availability of copper for absorption is related to the amount of TCA-insoluble copper measured (Gawthorne, 1987).

As the concentration of molybdenum in the diet increases in the presence of small amounts of sulphur, a progressive decrease in the concentration of copper takes place in the liver and the kidneys, followed by a decline in copper-containing enzymes. If the imbalance in the molybdenum:copper intake ratio becomes more pronounced, molybdenum-containing compounds can be absorbed into the systemic circulation, causing an accumulation of TCA-insoluble copper in blood and tissue (Gawthorne, 1987). It was initially thought the TCA-insoluble copper was unavailable for utilisation by an animal. However, Suttle & Small (1993), showed that an increase in TCA-insoluble copper caused an increase in erythrocyte superoxide dismutase. The Cu-Mo-albumin complexes are not excretable, but are slowly hydrolysed, thereby providing a slow, sustainable release of copper from the TCA-insoluble copper pool (Mason, 1986).

2.3.2. Copper and zinc

Very high dietary concentrations of zinc will lead to a lower tissue concentration of copper. Conversely, low dietary concentrations of zinc can lead to signs of copper toxicity. The mechanism whereby high concentrations of dietary zinc induces copper deficiency has been ascribed to decreased copper absorption. Zinc is known as a potent inducer of metallothionein and copper may be trapped by metallothionein in intestinal mucosal cells (Hall, Young & Bremner, 1979; DiSilvestro & Cousins, 1983).

2.3.3. Copper and iron

A high dietary intake of iron will decrease copper levels in animals. A high intake of iron is likely in animals fed silage or consuming soil as a contamination of pasture. The generation of sulphide in the rumen is probably a necessary part of the inhibitory effect of iron on copper. Iron sulphide formed in the rumen may act as a carrier of sulphide to the abomasum, where acidity releases sulphide and promotes the formation of insoluble copper sulphide (Suttle & Peter, 1985; Gawthorne, 1987).

2.3.4. Copper and cadmium

The excessive intake of cadmium induces signs of copper deficiency. Cadmium is a potent inducer of metallothionien synthesis, which suggests that copper is entrapped by metallothionein in intestinal mucosal cells, in a manner similar to that proposed for high zinc intakes (Gawthorne, 1987).

2.3.5. Copper and selenium

Selenium supplementation given to ewes on low copper pastures raised blood and liver copper concentrations in ewes and their lambs (Thomson & Lawson, 1970). In adult sheep, selenium supplements have not had a significant effect on copper status in the animal (Lee & Jones, 1976).

2.3.6. Copper and other metals

Metals, such as lead and nickel may also interact with copper. Calcium, through an interaction with zinc, indirectly affects copper metabolism (Gawthorne, 1987).

2.4. The effect of different sample storage methods on the copper concentrations measured in the liver

Only a limited number of references could be found that deal with the effect of different sample storage methods on mineral concentrations in the liver.

Lead toxicity was induced in calves by dosing them with Pb acetate (Bratton, Zmudzki & Richardson, 1985). Liver samples collected from dead or euthanased calves after seven days were either frozen or fixed in formalin before being analysed six months later. Their results supported the use of fixed liver as a means of estimating the Pb concentration in fresh tissue at the time of death in calves exposed to Pb. Conversely, copper levels in these formalin fixed samples were lower than those of the frozen samples. The correlation between the copper content of fixed and frozen samples was not significant.

The effect of formalin fixation on the concentration of selenium (Se) in porcine liver was examined by Sullivan, Pando, Everson & Robinson (1992). Selenium, like copper, forms part of mammalian enzymes. No statistically significant difference between values for Se in fresh and frozen liver was found, but a highly significant difference was found when values for Se in formalin-fixed liver was compared to values for Se in fresh or frozen liver. The mean concentration of Se in formalin-fixed livers was 86% of that in either fresh or frozen livers.

2.5. The pathogenesis and clinical signs of copper deficiency

This subject is extensively reviewed by Fell (1987) and Underwood & Suttle (1999).

The manifestations of copper deficiency vary with the species, age, sex of animal, severity and duration of the deficiency. The process of pigmentation and keratinization of wool are the first to be affected by a lowered copper status in sheep. At certain levels of copper intake these defects can develop without any other signs of copper deficiency being present. Neonatal ataxia occurs readily in lambs from copper deficient ewes in some areas but only rarely occurs in calves from the same area. This probably reflects differences in the development of the foetal central nervous system. In sheep brain myelination is rapid in mid-pregnancy, while myelination is slower in calves (Suttle, 1987). Copper deficient pigs and chicks develop cardiovascular lesions that have not been observed in copper deficient cattle (Underwood, 1977).

The development of a copper deficiency may be divided into four stages: depletion, deficiency, dysfunction and disease (see Figure 2.1). During depletion, there is loss of the trace element from storage sites in the body, such as the liver, although plasma concentrations may remain constant. When liver supplies are exhausted, circulating levels of copper decline and the result is a deficiency state. The next stage, dysfunction, is reached when copper containing enzymes in the tissues begin to decline. As these enzymes decline, the result is a disease state with clinical signs such as achromotrichia and ataxia (Suttle, 1986).

2.5.1. Blood

Anaemia is common in all species where a deficiency of copper is prolonged or severe. In copper-deficient pigs, rabbit and rats, the anaemia is microcytic and hypochromic, similar to that found in iron deficiency (Fell, 1987). In ewes and cattle, the anaemia is macrocytic and hypochromic (Bennetts, Beck, Harley & Evans, 1941). Factors that may be involved in the mechanism of anaemia in copper deficiency are as follows (Fell, 1987):

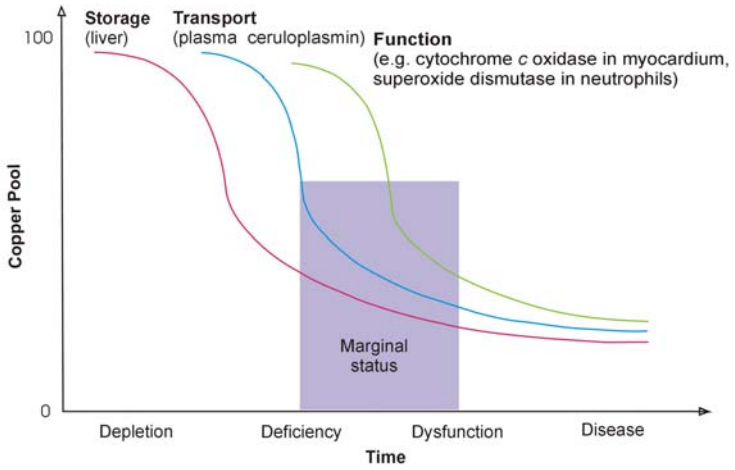

Figure 2.1: The sequence of biochemical changes following copper deprivation in animals (Underwood & Suttle, 1999).

- Depression of cytochrome oxidase activity may result in the defective synthesis of phospholipids, and so delay the maturation of red blood cells (Van Wyk et al., 1953, in Fell, 1987).
- High cytochrome oxidase activity is needed for haematopoiesis, as the uptake of iron by mitochondria of the developing erythrocytes is energy-dependant and requires the production of ATP by oxidative phosphorylation (Cooper, Webster & Harris, 1963).
- Ceruloplasmin plays an essential role in iron saturation of transferrin. Thus, low levels of ceruloplasmin may decrease the rate of haemoglobin synthesis.
- As a result of decreased levels of superoxide dismutase found during chronic copper deficiency, red blood cells are more susceptible to peroxidation damage from superoxide radicals. There is evidence that the lifespan of erythrocytes is decreased in copper deficient pigs (Bush, Jensen, Athens, Ashenbrucker, Cartwright & Wintrobe, 1956).

2.5.2. The circulatory system

Cytochrome oxidase activity in the myocardium is decreased in copper deficient animals (Gubler, Cartwright & Wintrobe, 1957). Another copper-dependent enzyme, dopamine-β-monooxygenase (EC.1.14.17.1), that catalyses the formation of noradrenaline from dopamine, has been shown to become rate-limiting in the hearts of copper-deficient mice (Gross & Prohaska, 1990). Myocardial hypertrophy has been noted in cattle (Mills, Dalgarno & Wenham, 1976). The cardiac involvement may result in sudden death ("falling disease") if the deficiency is prolonged or severe. The lesion appears to be cardiac arrest associated with patchy, fibrous replacement of the myocardium (Bennetts, Harley & Evans, 1942). Heart failure, attributable to pathological changes of this type has not been reported in copper-deficient sheep (Fell, 1987).

A decrease in lysyl oxidase in copper deficient animals results in the formation of elastic tissue with reduced elastin content, more lycine, less desmosine and isodesmisine. This weakness in arterial walls can lead to dissecting aneurysms and sudden death from rupture of major arteries. Aneurysm formation has been noted in copper-deficient chickens, pigs, rabbits and guinea pigs (Fell, 1987). Rupture of the aorta is not a feature of copper-deficiency in cattle nor sheep, although histological changes in the elastic tissue of the aorta and ligamentum nuchae of sheep (Cleary & Fanning, 1975), and cattle (Mills et al., 1976), have been noted.

2.5.3. The reproductive system

With the exception of sheep, most neonatal animals have higher concentrations of copper in the liver than do adults (Underwood, 1977). Newly born mammals need an adequate hepatic store of copper and a subsequent intake of copper through the colostrum in milk to survive. The foetus demand for copper is a priority and may place a severe drain on a copper deficient

mother. If this demand is not met, the offspring may be born with deficient stores of copper or the pregnancy may be terminated (Fell, 1987).

It is not clear whether a relationship exists between fertility and copper status. In cattle, conflicting reports have been published (Fell, 1987). In sheep, attempts to breed ewes depleted of copper under experimental conditions were unsuccessful; the results were either failure to conceive, or foetal death and abortion (Howell, 1968).

2.5.4. The nervous system

The most common clinical manifestation of copper deficiency involving the nervous system has been termed “swayback”, synonymous with “lamkruis” or enzootic ataxia. It is mainly a disease of newborn or unweaned animals and is characterised by uncoordinated movements of the hindlimbs, a staggering gait and swaying of the hindquarters. Two forms are recognised:

2.5.4.1. *Congenital (cortico-spinal) swayback*

The essential lesion here is absence or destruction of the white matter of the cerebral hemisphere. Copper appears to be essential for myelin formation, but it is not clear whether the mechanism involves a specific effect on a particular synthetic process or a more general impairment of energy metabolism (Fell, 1987). A possible explanation is that copper deficiency causes a depression in cytochrome oxidase activity, which leads to an inhibition of aerobic metabolism and phospholipid synthesis. This leads to inhibition of myelin synthesis, since myelin is composed largely of phospholipids (Underwood, 1977). The condition is non-inflammatory and appears very rapidly in late gestation (Fell, 1987). Another copper-dependent enzyme, peptidylglycine α-amidating monooxygenase was shown to be reduced in the brain of newborn rats from copper-depleted dams (Prohaska & Bailey, 1995).

Venous stasis, perivenous cerebral oedema and a resultant cerebral hypoxia are early lesions in congenital swayback. Combined with the occlusive pressure on the cerebral blood vessels and reduced cytochrome oxidase activity, this may be enough to interfere with myelin synthesis. Destruction of white matter in the motor tracts of the spinal chord also occurs, resembling Wallerian degeneration with dying back of axons (Fell, 1987).

Animals with congenital swayback may be born blind, paralysed or moribund.

2.5.4.2. *Delayed (spinal) swayback*

Delayed swayback is a milder form of congenital swayback, where clinical signs of copper deficiency only appear after birth. In general, the longer the period of onset of clinical signs after birth, the milder the disease (Innes & Saunders, 1962). In delayed swayback, demyelination does not occur in the cerebral hemisphere, but is confined largely to the brain stem and motor tracts in the spinal chord (Fell, 1987).

2.5.5. The alimentary system

Diarrhoea may occur in copper deficient animals. This may be the result of malabsorption (Fell, Dinsdale & Mills, 1975), or disturbances in the gastrointestinal motility caused by a decline in the concentration of noradrenalin in the intestinal musculature, as noradrenalin is believed to have an inhibitory role in the regulation of intestinal motility (Lawrence, Davies & Mills, 1982). Noradrenalin synthesis is mediated by dopamine-β-hydroxylase, a copper-containing enzyme.

2.5.6. The renal system

Degenerative lesions in the kidneys have been noted in copper deficient animals. As cytochrome oxidase activity is quite well conserved in the kidney, a direct effect of copper deficiency on cell respiration or energy supply is

unlikely. Rather, circulatory failure insufficient to cause necrosis but sufficient to impair function of the kidney is more likely (Fell, 1987).

2.5.7. The skeletal system

Reports of brittleness of bone, spontaneous fractures and loss of compact bone from the shafts of long bones in copper deficient animals are common. The primary biochemical lesion in such cases is a reduction in the activity of the lysyl oxidase, leading to diminished stability and strength of bone collagen, in a manner analogous to the effect on aortic elastin (Underwood, 1977).

Osteoblast function is also defective in copper deficient animals, as evidenced by a failure of bone deposition, while osteoclast activity appears to proceed as normal. The function of osteoblasts is to produce the collagen and glycoprotein-rich ground substance of organic bone matrix (Fell, 1987).

2.5.8. The respiratory system

As in bone, connective tissue defects in copper-deficient lungs have been attributed to a decline in lysyl oxidase activity. Emphysema has been reported in several species (Fell, 1987).

2.5.9. The integument

Achromotrichia is the earliest clinical sign of copper deprivation in all species and is a very sensitive index of copper deficiency (Underwood & Suttle, 1999). The copper dependent enzyme tyrosinase is the enzyme involved in the production of melanin from tyrosine.

In copper deficient sheep, crimp in the wool becomes less distinct, until the fibres emerge as almost straight, hair-like growths, to which the descriptive terms “stringy” or “steely” wool have been given. The tensile strength of steely wool is reduced, the elastic properties are abnormal and it tends to set

permanently when stretched. Crimp is dependent on the presence of disulphide groups that provide the cross-linking of the keratin and on the alignment of the long chain keratin fibrillae in the fibre. Both are adversely affected in copper deficiency (Underwood, 1977).

2.6. Copper deficiency in blesbok and other ungulates

There are only a limited number of references dealing with copper deficiency in blesbok.

Hepatic copper levels were determined, using neutron activation analysis, in apparently healthy blesbok in the Mountain Zebra National Park at Cradock (32°15'S / 25°41'E) and blesbok in the Golden Gate Highlands National Park at Bethlehem (28°31'S / 29°37'E) (Turkstra, de Vos, Biddlecombe & Dow, 1978). It was not specified from which liver lobe the samples were taken. Samples were stored in formalin until analysis. The mean copper concentration in liver samples of the two blesbok populations were: $\bar{x}$ = 101.3 ppm[i], *s* = 16.4, *n* = 8 and $\bar{x}$ = 126.0 ppm, *s* = 65.9, *n* = 8. All samples were measured on a dry mass basis.

Jones (1980), investigated the deaths of blesbok at the Whipsnade Park, England, where 18 blesbok from a herd died over a four year period. Varying degrees of damage to the liver and gall bladder were found, and it was postulated that the lesions might have been caused by the ingestion of fungal toxins from decaying matted grass at the base of the growing stems. This in turn was suggested to have led to a secondary copper deficiency. He also reported that the North of England (Chester) Zoological Society had experienced a similar problem, although they believed that the weight loss in the blesbok was due to a primary copper deficiency.

Copper deficiency was reported by Dierenfeld, Dolensek, McNamara & Doherty (1988), in a herd of captive blesbok in New York Zoological Park.

[i] ppm X 15.74 = μmol/l

Copper deficiency was diagnosed on clinical signs, low serum copper levels and pathology. Feed and water samples were also analysed. They concluded that signs of copper deficiency were observed with blood serum Cu levels of less than 0.8 μg/ml. The cause of the deficiency appears to have been multi-factorial and may have been exacerbated by pregnancy. They also suggested that the copper requirements and/or metabolism in blesbok might differ from those of domestic bovidae as copper levels in feed and water were in the normal range for domestic bovids.

An isolated population of blesbok in the Heidelberg district (34°18'S / 20°51'E) of the Western Cape, a known mineral deficient area, was studied by Bigalke & Van Hensbergen (1991). The animals were small, showed achromotrichia and were found to have decreased skeletal strength, as measured with an Instron strength testing machine. The mean ($\bar{x}$) liver copper levels (in ppm dry weight) of culled animals from this population were as follows: female (*n* = 43), $\bar{x}$ = 48; male (*n* = 34), $\bar{x}$ = 55; lamb (*n* = 8), $\bar{x}$ = 72; subadult (*n* = 17), $\bar{x}$ = 17; adult (*n* = 52), $\bar{x}$ = 47.

Swayback in a blesbok and a black wildebeest from the Karoo Nature Reserve was discussed in a case report (Penrith et al., 1996). The diagnosis of swayback was based on the clinical finding of ataxia and microscopic lesions of myelopathy typically found with swayback in other species, associated with low copper concentrations in the liver. The copper concentration in the liver of the affected blesbok was 3 ppm on a wet mass basis[ii]. The mean ($\bar{x}$) copper concentration in the livers of five animals that were shot elsewhere was 21.6 ppm on a wet mass basis, *s* = 18.1. In addition to copper, concentrations of Zn, Mn, Fe and Co in the liver were also analysed.

The bontebok is closely related to the blesbok, the species being divided taxonomically on a sub-species level (Essop, Harley, Lloyd & Van Hensbergen, 1991). Van der Walt & Ortlepp, 1960, discussed the movement

[ii] ppm (wet basis) X 3 = ppm (dry basis)

of bontebok (*Damaliscus dorcas dorcas*) from Bredasdorp to Swellendam in South Africa. The animals were moved, because in 1957, they noted that 15 young animals had succumbed to swayback and 49 other animals had died of parasitic infestation. The area is known to be deficient in copper and cobalt and was a “flat, unhospitable, limestone area, with sparse and innutritious vegetation”.

Twenty seven hair samples and 13 liver samples of bontebok in the Cape of Good Hope Nature Reserve, South Africa, were analysed for copper (Zumpt & Heine, 1978). Copper concentration, as ppm dry mass in hair of bontebok had a mean of 7.5 with a range of 2-12 ppm. In the liver samples, the mean was 83.5 with a range of 18-208 ppm. These values were compared with liver samples from sheep in the western Cape, where the mean was 163 ppm, range 25-600 ppm dry mass. They concluded that the reserve was in a copper-deficient belt and the bontebok showed signs of copper deficiency, such as pale coats and bone fractures. There was no indication that fertility was affected by the copper deficiency in these animals.

Hepatic copper levels were determined, using neutron activation analysis, in bontebok (*Damaliscus dorcas dorcas*) from the Bontebok National Park in Swellendam (34°02’S / 20°25’E) (Turkstra et al., 1978). The copper concentration was $\bar{x}$ = 460.4 ppm, *s* = 138.3, *n* = 8. These values were compared to blesbok and the higher hepatic copper concentrations in the bontebok were ascribed to geographical or genetic differences.

Besides blesbok and bontebok, reports of a copper deficiency syndrome in other African ungulates include black wildebeest (*Connachaetes gnou*) (Penrith et al., 1996) and gemsbok (*Oryx gazella*) (Gillespie, D'Andrea & Lockaby, 1995). Reports of copper deficiency in other ungulates include bactrian camels (*Camelus bactrianus*) (Zong-Ping, Zhuo & You-Jia, 1994), moose (*Alces alces*) (Flynn, Franzman, Arneson & Oldemeyer, 1977; Rehbinder & Peterson, 1994) and the red deer (*Cervus elephus*) (Tölgyesi & Bencze, 1970; Peet & Hepworth, 1993; Audigé, Wilson, Morris & Davidson,

1995). The deficiency in red deer, characterised as enzootic ataxia, differs from the condition in sheep, in that it is not found in neonates, but rather in young adult or even mature deer (Wilson, 1984, in Peet & Hepworth, 1993).

2.7. The pathogenesis and clinical signs of copper toxicity

Small amounts of copper in the diet are necessary for survival, but conversely, high concentrations may be toxic. The sudden ingestion of large quantities of copper may lead to an acute poisoning. More common, however, is ingestion of copper in levels slightly higher than that required for homeostatic control. Most of the copper is stored in the liver and to a lesser extent in the kidney. In such circumstances, the animal is known to be 'copper loaded' (Howell & Gooneratne, 1987). The capacity for hepatic copper storage varies greatly among species and differences among species in tolerance to high copper intake is also great. Sheep are particularly susceptible to high copper intake, whereas cattle are more tolerant (Underwood, 1977).

2.7.1. Acute copper poisoning

This syndrome is relatively uncommon and may arise in two ways:

2.7.1.1. *Oral ingestion*

This condition is uncommon, but presents as an acute gastroenteritis with abdominal pain, diarrhoea and sometimes death. The wall of the gut is congested and haemorrhages may occur (Howell & Gooneratne, 1987).

2.7.1.2. *Parenteral administration*

Overdosage of an injectable copper preparation may cause animals to become weak and lethargic with icteric mucous membranes, or the animal may be found dead. Haemolysis and haemoglobinuria may occur. There may

be marked elevation of the activity of liver-specific enzymes such as sorbitol dehydrogenase in the blood (Howell & Gooneratne, 1987).

2.7.2. Cumulative (chronic) copper poisoning

This is the most common form of poisoning and occurs readily in sheep as they appear to be the most susceptible animals to this condition. The induction of copper toxicity may be due to either a high copper/low molybdenum ratio in the diet, for example, in animals fed subterranean clover (*Trifolium subterraneum L.)*, or the ingestion of hepatotoxic plants such as *Senecio spp.* The damage produced by the alkaloids may make the liver incapable of metabolizing and excreting the normal quantities of copper absorbed from the gastrointestinal tract (Howell & Gooneratne, 1987).

There are three distinct phases in the manifestation of chronic copper toxicity, viz. the pre-haemolytic, the haemolytic and the post-haemolytic phases. During the pre-haemolytic or copper-loading phase, copper accumulates in tissue, particularly in the liver, over a period of time ranging from a few weeks to months. During this time, the animal is clinically normal. The haemolytic phase is often precipitated by stress when the homeostatic mechanisms of the body break down. It is the clinical phase of the disease characterised by the sudden development of haemolysis and is often referred to as the “haemolytic crisis”. The animal becomes dull, lethargic, anorexic and may display polydipsia. Mucous membranes are a dirty brown colour, due to a combination of jaundice and high concentrations of methaemoglobin in the blood. The urine is dark and may have the colour of port wine. Many animals die during or shortly after a haemolytic crisis, but animals with ‘mild’ haemolysis may survive into a post-haemolytic phase, when the clinical appearance of the animal returns to normal (Howell & Gooneratne, 1987).

2.8. The role of soil in determining copper availability in animals

Soil is a mixture of inorganic and organic particles with variable amounts of water and air. The most important factors in the formation of soil are the nature of the parent rock and the climatic conditions of the region (Watkeys, 1999). A description of the geology and climate of the Karoo Nature Reserve is covered in Chapter 3.

2.8.1. Soil of arid regions

When the average precipitation is less than 500 mm per annum, more water is lost from soil from evapotranspiration than is gained from precipitation (Tan, 1993). This results in the accumulation of dissolved inorganic solutes in soil. In this process of salinization, salts accumulate in inverse proportion to their solubility. In non-saline or slightly saline soils, the soil solution is dominated by the Ca^{2+}, Mg^{2+} and HCO_3^- ions. As salinization increases, the more soluble salts become dominant. Conversely, with desalinization, the soluble salts are leached out first (Szabolcs, 1989). Nutrients in the soils can also concentrate at the wetting front which may result in their patchy distribution as the water evaporates (Hunter, Romney & Wallace, 1982). In general, alkali soils occur in drier regions, whereas acidity is associated with leached soils of wetter areas (Watkeys, 1999).

Soil salinity is evaluated by the electrical conductivity of the saturation extract (EC). Soil sodicity, which refers to the presence of excessive amounts of sodium in soil, is evaluated by the exchangeable sodium percentage (ESP) or the sodium adsorption ratio of the saturation extract (SAR). The SAR is more conveniently and accurately determined (Rhoades & Miyamoto, 1990). Both are nearly the same in value over the range of 0 - 30 (US Salinity Laboratory Staff, 1954).

Salt affected soils can be divided into three broad groups: saline soils (EC > 0.4 Sm^{-1} at 25°C, ESP < 15%), saline-alkali soils (EC > 0.4 Sm^{-1} at 25°C, ESP > 15%) and nonsaline-alkali soils (EC < 0.4 Sm^{-1} at 25°C, ESP > 15%) (Tan, 1993).

Excess salinity adversely affects plant growth, as salt increases the energy that must be expended by the plant to absorb water from the soil. This energy would normally be used for growth. In contrast to saline soils, sodic soils have reduced permeabilities and poor tilth (Rhoades & Miyamoto, 1990).

Secondary salinization can occur with the use of poor quality water for irrigation or as a result of the rise in the groundwater table that occurs from irrigation. Raising the groundwater table can cause salinization by transporting salts from the deeper soil layers to the surface layers, or by hindering the natural drainage and leaching of salts. Soluble salts are able to travel by capillary action from the groundwater table into the soil profile (Szabolcs, 1989).

2.8.2. Copper

The average copper concentration in the earth's crust is about 70 ppm (Goldschmidt, 1954, in Hodgson, 1963). The parent material of soil plays an important role in determining the copper concentration of that soil, especially where the majority of the soil is relatively young. Soils derived from coarse-grained material, like sands and sandstones, or from acid igneous rocks, contain lower concentrations of copper than those derived from fine-grained sedimentary rocks, like clays and shales, or from basic igneous rocks (Jarvis, 1981). Mispah-type soil has been associated with low hepatic copper levels in cattle (Grant, Biggs & Meissner, 1996). Copper is translocated with clay and tends to be most abundant when clay content is high (Fagbami, Ajayi & Ali, 1985).

Copper is one of the least mobile of the trace elements in soil profiles (Hodgson, 1963), with the result that there is little variation of total copper concentration with depth and very little copper will be leached into groundwater. Movement of copper from highly leached surface horizons into lower horizons has, however, been observed in podzols (McBride, 1981). Copper is not subject to oxidation/reduction reactions under changing drainage conditions. Its mobility and supply to plants may be indirectly affected by the reduction of hydrous oxides of Fe and Mn, and the breakdown of organic matter in poorly drained soils (Kubota, Lemon & Allway, 1963; Beckwith, Tiller & Suwadji, 1975; Jarvis, 1981). Flooding of soil has been reported to reduce water-soluble copper levels, presumably due to intense reduction and the formation of insoluble sulphides (Ponnamperuma, 1985).

Chalcopyrite is the most common copper mineral in sedimentary rocks,. Other copper sulphides also occur, as well as secondary minerals such as copper carbonates and silicates (chrysocolle $CuSiO_3.2H_20$) formed by weathering of sulphides and ferromagnesium silicate minerals (Delhaize, Loneragan & Webb, 1987). Of the sedimentary rocks, sandstones are composed of minerals that weather with difficulty and in general, only contain small amounts of trace elements (Thornton, 1983). In addition, copper can be occluded within secondary minerals (e.g. $CaCO_3$ and $MgCO_3$). Occluded copper is non-diffusible and makes up the majority of soil copper.

Copper is found largely in unavailable forms. Apart from the presence of copper in the crystal lattices of primary and secondary minerals, copper can be divided into other pools in soil. These are soil solution and exchangeable copper, adsorbed, organic-bound and a hydrous oxide segment (Pickering, 1981). The following equilibria has been proposed between these pools, with the equilibrium shifting to the left as pH drops (McClaren & Crawford, 1973):

$$Exchangeable-and\ soluble-Cu \Leftrightarrow Specifically\ adsorbed-Cu \Leftrightarrow Organically\ bound-Cu$$

Clays, organic matter and hydrous oxides, particularly those of Mn, all provide strong sorption sites for copper (Jarvis, 1981). Oxides may play an important role in limiting the mobility of copper and hence its availability to plants (Jarvis, 1981).

The soil solution pool is the central focus of soil chemistry, as it is from this pool that plants derive nutrients and absorb trace elements. In soil solution, 90 - 99% of copper is complexed in organic form (McBride, 1981). Copper is bound more strongly to organic matter than any other cation and copper organic complexes play an important role in regulating copper mobility and availability in soil (Mengel & Kirby, 1987). High molecular weight organic compounds, such as lignins, are essentially immobile and serve to immobilise elements associated with them. Short-chain organic acids and bases serve to promote solubility and movement. Both humic and fulvic acids have been found to bind to Cu^{2+} (Stevenson & Fitch, 1981). Both soluble and insoluble complexes can be formed, depending upon the pH, presence of salt (ionic strength effect) and degree of saturation (Stevenson, 1991).

When copper is present in low amounts in highly organic soils, it may be so tightly complexed with humic acids that it is unavailable to plants. The fulvic acids complexes are generally more soluble and, although they only constitute a small proportion of total soil copper, they may play an important role in making copper available to plants. Thus, as copper concentration in the soil increases, the stronger binding sites of humic acid become saturated and more copper becomes solubilized through the action of fulvic acids (Delhaize et al., 1987).

Simple biochemical substances produced by micro-organisms (e.g. simple aliphatic acids, amino acids, phenols) may also chelate Cu^{2+}. This means that high concentrations of copper may be attained in localised zones that favour the growth of micro-organisms, such as near decomposing residues and in the rhizosphere, the soil zone directly influenced by plant roots (Stevenson & Fitch, 1981).

Inorganic copper is not readily available to plants, as copper is held very tightly on inorganic exchange sites. However, cation exchange for Cu^{2+} and $CuOH^{+}$ can take place and it appears to be the most effective ion in this respect (Mengel & Kirby, 1987). Copper will hydrolyse in aqueous solution according to the equation:

$$Cu^{2+} + H_2O \leftrightarrow CuOH^{+} + H^{+} \ (pK_a = 7.5)$$

(Smith & Martell, 1989, in Harter, 1991)

At low pH, it is unlikely that copper is hydrated. As the pH reaches neutrality, copper is rapidly hydrolysed to $Cu(OH)_2^0$ (Table 2.2).

Table 2.2: The effect of pH on copper ion composition in soil solution. Values expressed as percent in solution (Harter, 1983).

pH	**4**	**5**	**6**	**7**	**8**
Cu^{2+}	100	100	96	33	1
$CuOH^{+}$			2	7	1
$Cu(OH)_2^0$			2	56	92

As the pH of soil approaches about 8, the overall solubility of Cu^{2+} decreases to a minimum, then increases above pH 8 as carbonate and anionic complexes become important. It has been suggested that the major inorganic form of complexed Cu^{2+} in neutral and alkaline soil solution is $CuCO_3^{-}$ (McBride, 1981; Shuman, 1991).

An increase in soil pH will decrease the bioavailablility of copper by redistributing copper in the exchangeable fraction to other fractions such as the precipitated and occluded fractions (McClaren & Crawford, 1973; Elsokkary & Lag, 1978; Sims & Patrick, 1978). Strong adsorption of copper onto mineral colloid surfaces occurs at high soil pH (James & Barrow, 1981; Jarvis, 1981; Mengel & Kirby, 1987).

A normal range of 2 - 60 mg/kg copper in soil has been suggested (Thornton, 1983). Values for total extractable copper of 0.5 – 3 ppm (Aubert & Pinta, 1977), 4 - 6 ppm on mineral soils and 20 - 30 ppm on organic soil (Delhaize et al., 1987), or less than 0.75 ppm (Haque, Aduayi & Sibanda, 1993), have been suggested as being deficient for the growth of plants. Because of the relative immobility of copper, the major proportion of the copper supplied to plants is through root interception, as compared to diffusion and mass-flow (Oliver & Barber, 1966). The uptake of copper by plants is thought to involve the initial immobilization of copper on the root surface, followed by the formation of soluble copper-amino acid complexes within the free space of the root (Pickering, 1981). Copper uptake seems to be a metabolically mediated process and there is evidence that copper strongly inhibits the uptake of Zn and *vice versa*. It is still not clear whether copper is taken up as Cu^{2+} or as a Cu chelate (Mengel & Kirby, 1987). However, given the variety of forms in which copper occurs in the soils, no simple relationship has yet been found between total soil copper and copper concentrations in plants (Delhaize et al., 1987). There has also been no close correlation between 'available' copper in soil and the copper content in plants (Mortvedt, 1977), due to other soil properties, such as pH or organic matter.

Application of nitrogen, phosphorous and zinc has been reported to accentuate copper deficiency in plants (Moraghan & Mascagni, 1991)

2.8.3. Molybdenum

A range of < 1.00 – 5.00 mg/kg (Thornton, 1983), an average of 2.00 ppm total molybdenum content and an average of 0.20 ppm (Cheng & Oullette, 1973) or 0.01 – 12.00 ppm (Aubert & Pinta, 1977) available content in soil has been reported. Molybdenum availability is determined more by pedogenic processes than by the parent rock (Aubert & Pinta, 1977). Molybdenum is released fairly easily from primary minerals by weathering and remains mobile as potentially soluble molybdates (Mo^{6+}) (Gupta & Lipsett, 1981). Many peats, alkali soils and poorly drained soils with high water tables tend to produce

forages high in molybdenum (Kubota et al., 1963; Allaway, 1977). Conversely, well-drained or leached soils tend to be low in molybdenum content.

Molybdenum in soil can occur either in soil solution, adsorbed by positively charged surfaces, such as hydrous oxides of Fe and Al, present in organic matter or held in the crystal lattice of minerals (Gupta & Lipsett, 1981). In soil solution, molybdenum normally occurs in anionic form (MoO_4^{2-}) (Stevenson, 1991) and it is soluble under a wide range of conditions. It is probably the most mobile of all metallic elements (Chesworth, 1991). Water-soluble MoO_4 and organically complexed molybdenum are considered to be relatively available to plants. Root interception and mass flow are considered to be the important mechanisms controlling the movement of molybdenum from soil to plants (Gupta & Lipsett, 1981).

Molybdate is adsorbed by sequioxides and clay minerals (Mengel & Kirby, 1987). Adsorption of molybdenum is pH dependent, with maximum adsorption occurring at pH 4. As pH increases, molybdenum adsorption decreases (Harter, 1991) and availability for plants thus increases. The MoO_4^{2-} concentration increases 100-fold for each unit increase in pH.

Soluble phosphorous will enhance the uptake of molybdenum by plants, while soluble sulphur will decrease molybdenum uptake. The inhibitory antagonistic effect of SO_4^{2-} on molybdenum content has been suggested to occur primarily during the absorption process (Gupta & Lipsett, 1981).

2.8.4. Zinc

Parent material has a greater effect on soil Zn content than do pedogenic factors. Basic eruptive rocks usually contain more Zn than acid eruptive rocks, metamorphic rocks or sedimentary rocks such as limestone or sandstone (Aubert & Pinta, 1977). A normal range of 25 – 200 mg zinc/kg soil has been reported (Thornton, 1983). A range of plant available (EDTA) Zn in soils varied from 1.9 – 13 ppm (Aubert & Pinta, 1977).

Most of the total zinc in soil exists in unavailable forms (Shuman, 1991). This trace element may be found on exchange sites of clay minerals, organic matter or adsorbed on soil surfaces. Both soluble and insoluble complexes are formed with organic matter (Mengel & Kirby, 1987). Total and extractable Zn levels tend to increase with increasing soil acidity, finer texture and increasing organic content (Pickering, 1981).

Zinc moves to the plant root primarily by diffusion. Anything that affects the mobility of zinc will, therefore, affect the availability of Zn to plants. (Moraghan & Mascagni, 1991). Increasing pH decreased Zn in the water-soluble and the exchangeable fractions, due to increased adsorption (Shuman, 1991). A mutually competitive interaction exists between Cu^{2+} and Zn^{2+} as both ions are absorbed through common carrier sites in the roots of plants (Bowen, 1987).

2.8.5. Sulphates

The valency of sulphur ranges from –2 to +6, with sulphides and sulphates representing the two extremes. Sulphur can thus occur in many forms, from the free state to inorganic and organic compounds.

Clays, shales and slates are usually rich in sulphur. Most of the sulphur in rocks is in the sulphide form e.g. iron, nickel and copper sulphides. During the weathering process, sulphur oxidises to sulphates, the form that plants take up. Sulphates are adsorbed by organic matter, clay colloids and hydrous oxides. Soluble and adsorbed sulphates make up only a small proportion of the total sulphur in soil. Over 90% of the total soil sulphur occur in the organic matter and is derived from plant and animal residues returned to the soil. Micro-organisms convert some of the sulphur to the sulphate form (Murphy, 1980).

Sulphates can be removed from the superficial horizons by leaching. A high pH can accelerate this process as very little sulphate is adsorbed at pH 6.5 or

above. In calcareous soils, insoluble sulphate associated with calcium carbonate may form an important fraction of the total sulphur (Williams & Steinbergs, 1962; Murphy, 1980).

Atmospheric sulphur can play an important part in sulphur content in plants. It has been estimated that 30% of the sulphur in plants can be supplied from atmospheric sources, even when the supply from the soil is adequate (Murphy, 1980).

2.8.6. Carbonates

Inorganic carbonates occur in soils primarily as calcite ($CaCO_3$) and dolomite ($CaCO_3 \cdot MgCO_3$). Calcite is one of the most common inorganic mineral salts found in soil. It can be found in soils from every climatic zone, but is characteristic of steppe, semiarid and arid soils. Due to the very low solubility of calcite, mobility is also low (Szabolcs, 1989). Among the soil-forming rocks, the occurrence of calcium carbonate is chiefly associated with sedimentary rocks (Nelson, 1982). Sodium carbonate, magnesium carbonate and hydroxycarbonate are common in evaporates or in regions of high-salt deposition in soil.

Dissolved carbonates are in equilibrium with solid-phase carbonates and CO_2 and will thus increase in systems with high partial pressures of CO_2 such as in flooded soils and microenvironments of high microbial activity, or in sodic soils, because of the high solubility of Na_2CO_3 (Loeppert & Suarez, 1996).

If the calcic horizon is close to the surface, plants may exhibit P and Zn deficiency and Fe chlorosis. A calcic horizon below the surface may impede water movement (Nelson, 1982).

CHAPTER 3

Materials and Methods

3.1. The study areas

3.1.1. Karoo Nature Reserve, Eastern Cape

The Karoo Nature Reserve was established in 1979. It is approximately 14 500 ha in size and almost surrounds the town of Graaff-Reinet (32°15'S / 24°33'E) in the Eastern Cape Province, South Africa. The greater portion of the reserve is situated between 740 and 1480 metres above sea level in the foothills of the Sneeuberg mountain range.

Before the establishment of the reserve by the South African Nature Foundation, the area was formerly town commonage administered by the Graaff-Reinet municipality. For the past two hundred years, a variety of domestic stock (cattle, sheep, goats, horses) had been allowed to graze in the area, leading to extensive veld degradation (Thornton, D., pers. comm.).

3.1.1.1. *Geology*

The major geological formations in the reserve are derived from the Adelaide Subgroup of the Beaufort series of the Karoo system, and post-Karoo dolerite intrusions (Visser, 1986). The geological system consists of very thick layers of near horizontal strata of sedimentary rocks. It is characterised by strong sandstone layers separated from one another by thick shale and mudstone, red, red-purple to grey and green-grey in colour. This sandstone is usually rich in feldspar, which erodes easily under the prevailing climatic conditions. The shales and mudstones are also unstable and will easily erode. Plateau and gully erosion is evident on the talus slopes and pediments. The material originating from this weathering and erosion process is usually clay-like and

rich in alkaline salts. Intersecting the sedimentary strata are vertical dolerite dykes and horizontal or near horizontal uneven dolerite plates, the latter being more common (Van Riet & Minnaar, 1977).

The majority of the plains (usually flood-plains) are covered with alluvium – shingle, sand, mud and wash stones of recent origins, with characteristic superficial calcrete. The thickness of the alluvium varies between 7-13 m, although in some places it can reach a thickness of 23 m. The shingles play an important role in the characteristics of the underground water in the area (Van Riet & Minnaar, 1977).

3.1.1.2. *Soil*

Ellis & Lambrechts (1986), mapped units of uniform terrain that form soil patterns known as pedosystems in the Karoo. The pedosystems of the Karoo Nature Reserve area has been broadly classified as shallow soil of pedologically young landscapes. The soil is weakly structured and developed in pedisediments overlying hard rock at shallow depth. The genesis of the soil is one of deposition.

At a finer level, the soils of the reserve range from the shallow (< 120 cm), Mispah-rock complex, to the deep (> 120 cm), red-brown calcareous duplex soils of the Shigalo-Limpopo Association (Figure 3.1). The Mispah-rock complex is associated with the dolerite sills and dykes, which intrude the sedimentary beds of the Beaufort Group. Dolerite plays an important role in the formation of soil in the area – the presence of dolerite boulders overlying the basic soils of the pediments enhances soil quality by reducing alkalinity and improving the water-holding capacity (Van Riet & Minnaar, 1977).

The pediment soils are red, apedal, weakly structured, freely drained soils with a high base status. The soils are generally calcareous duplex forms of a secondary nature, having been deposited as alluvium on the impermeable sandstone (Palmer, 1989a). In general, the A horizon of this type of soil is rich

in most plant nutrients, but very high values for zinc and boron have been recorded in soil of depression areas (Ellis & Lambrechts, 1986).

3.1.1.3. *Rainfall*

The region falls in the 300.0 – 400.0 mm isohyet of mean annual rainfall, with a mean annual rainfall of 341.1 mm per year for the past 43 years (Figure 3.3) (Van Riet & Minnaar, 1977). Between 60 - 70% of rainfall occurs during the summer (Figure 3.4) (Venter, Mocke & De Jager, 1986). Rainfall is the primary driving variable in determining landscape-scale vegetation (Woodward, 1987; Desmet & Cowling, 1999).

3.1.1.4. *Temperature*

The average annual temperature for the Graaff-Reinet area is a minimum of 11.0°C and a maximum of 24.3°C. The hottest month is January with an average maximum of 31°C and the coldest month June with an average minimum of 6.3°C (Van Riet & Minnaar, 1977).

3.1.1.5. *The Vanryneveldspas dam and other water supply*

The Vanryneveldspas dam was built in 1924, lies within the reserve and covers 1094 ha when full. The area of catchment covers 3681 km^2 with a mean annual runoff of 7.70 mm (36 X 10^6 m^3). The main source of the dam is the Sundays River, with other contributions from the Pienaars and Broederstroom Rivers. Total dissolved solids are in the region of 200 - 1000 mgl^{-1} (Görgens & Hughes, 1986). A 50 year sediment volume for the Vanryneveldspas dam has been estimated at 28.00 X 10^6 m^3 (Rooseboom, 1978, in Görgens & Hughes, 1986). In effect, the dam is 50% silted, rendering the sluice gates non-functional and having the effect of raising the water table (Van Riet & Minnaar, 1977). The water content of the dam since 1988 is shown in Figure 3.5.

There are two functional boreholes in the Game Viewing Area of the Karoo Nature Reserve. Groundwater occurs in cracks in Karoo Sandstone and along dolerite intrusions. The rock type in which the groundwater occurs is one of the main determining factors controlling the quality of the water (Hodgson, 1986).

3.1.1.6. *Vegetation*

The area is arid, sub-desert and the vegetation is variable, being influenced by rainfall (Woodward, 1987). The vegetation types in the Reserve have been classified as False Central Lower Karoo, False Karroid Broken Veld, Succulent Mountain Veld and Karroid *Merxmuellera* Mountain Veld by Acocks (1988), or as Eastern Mixed Nama Karoo, Central Lower Nama Karoo and Valley Thicket (Low & Rebelo, 1998). The blesbok occur almost exclusively in the False Central Karoo or the Eastern Mixed Nama Karoo area, the area known as the Game Viewing Area.

A more detailed analysis of the vegetation of the reserve has been undertaken (Palmer, 1989a; Palmer, 1989b) (Figure 3.2). A total of 327 indigenous plant species and three distinct physiognomic classes of vegetation occur on the reserve. These are Shrubland, Succulent Thicket and Dwarf Shrubland. In the Game Viewing Area, a Degraded Dwarf Shrubland and Grassy Dwarf Shrubland occur. The flood plain area of the dam was not classified, but is dominated by *Cynodon dactylon* and weedy species, such as *Salsola kalki* (personal observation). At the time of his study, Palmer (1989a), could not confirm the large-scale anthropogenic transformation of the vegetation that was supposed to have occurred. He suggested that the Succulent Dwarf Shrubland represents the prevailing climax, rather than an extremely slow recovery rate from previous mismanagement.

At present, the vegetation of the reserve appears to be highly overgrazed (Figures 3.6-3.9).

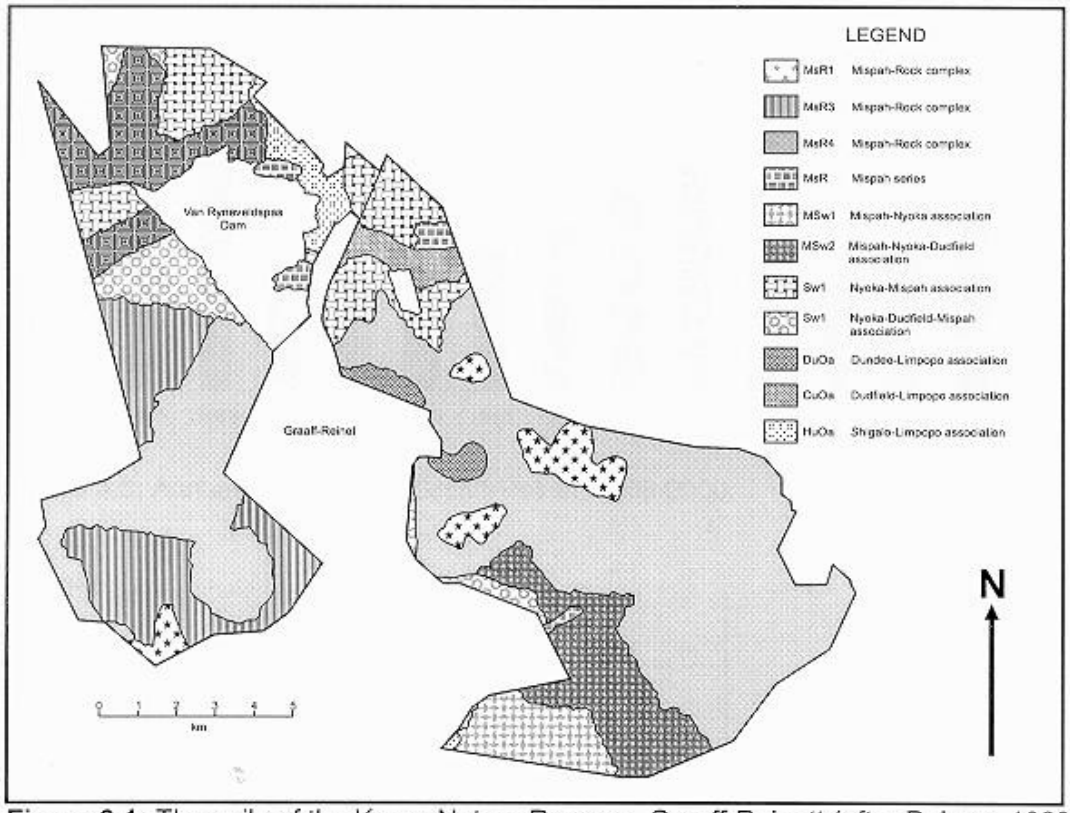

Figure 3.1: The soils of the Karoo Nature Reserve, Graaff-Reinett (after Palmer, 1989)

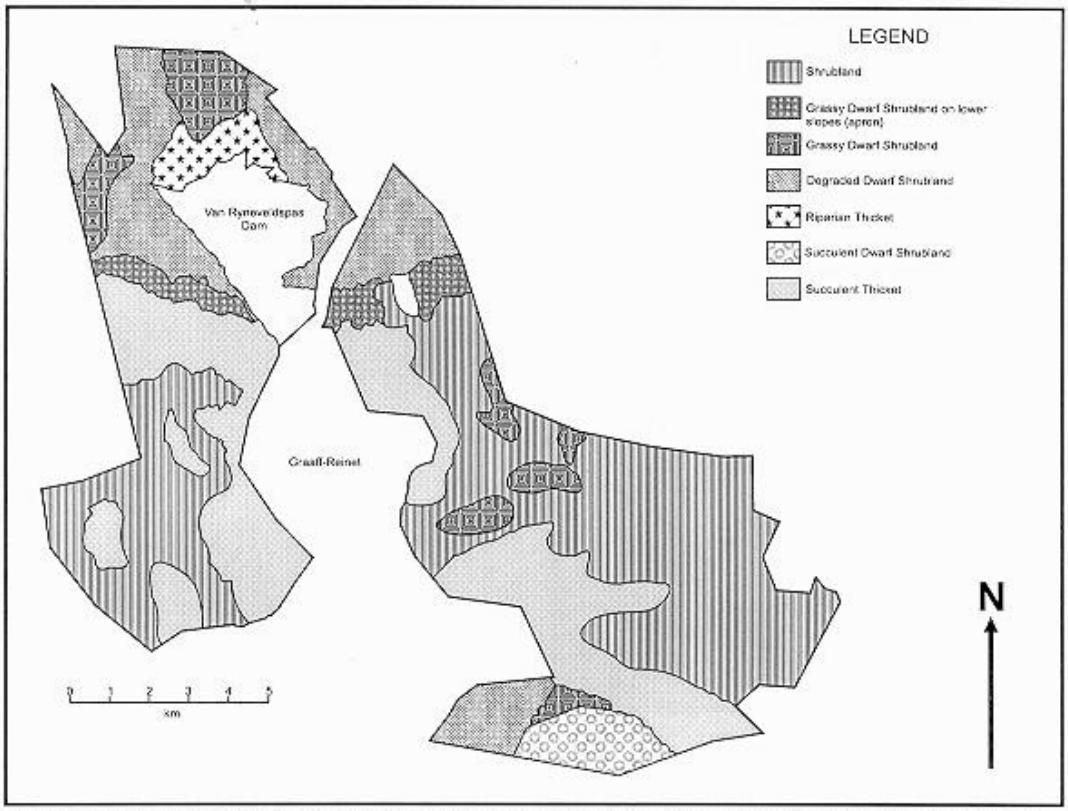

Figure 3.2: The vegetation of the Karoo Nature Reserve, Graaff-Reinet (after Palmer, 1989)

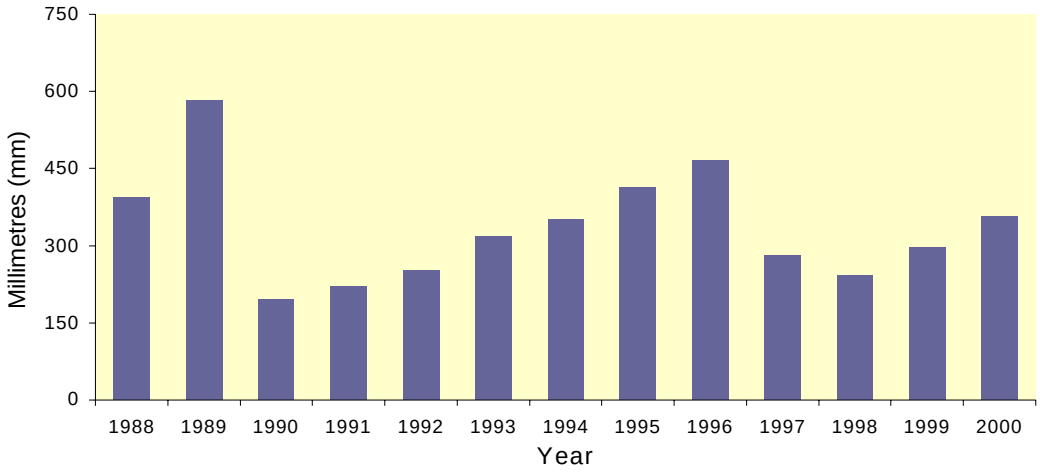

Figure 3.3: Annual precipitation, Graaff-Reinet, 1988-2000.

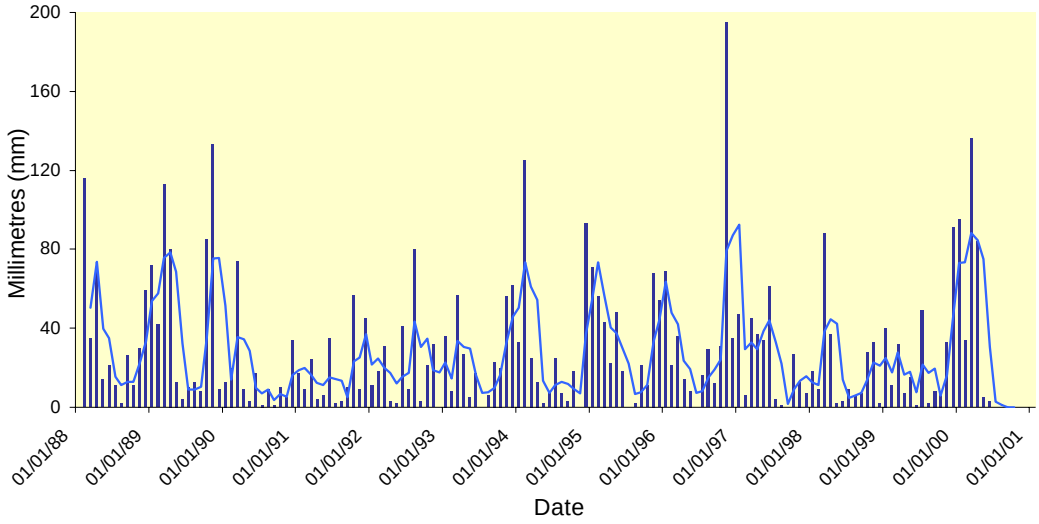

Figure 3.4: Monthly precipitation, Graaff-Reinet, 1988-2000. (Trendline = moving average, three per period).

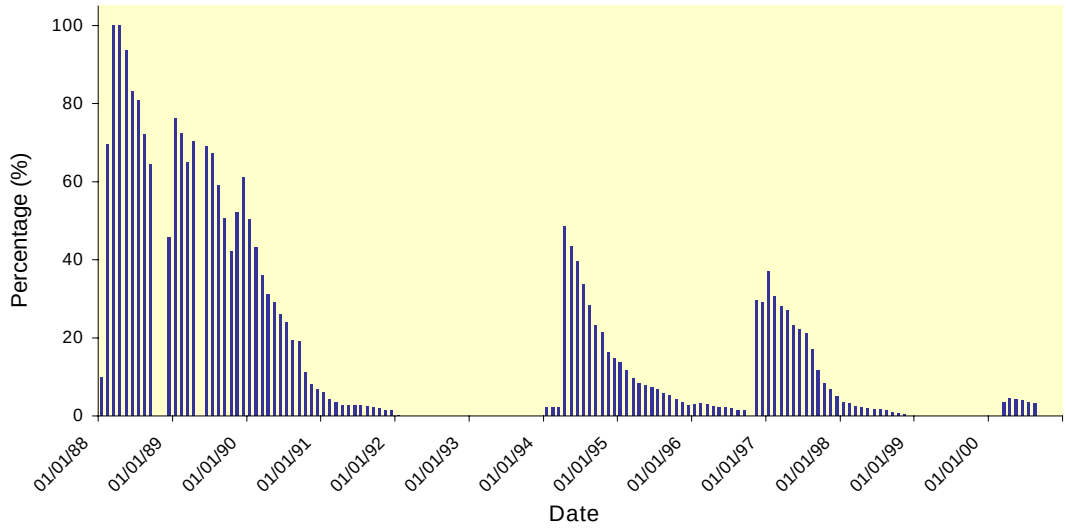

Figure 3.5: Vanryneveldspas Dam water content, Graaff-Reinet, 1988-2000.

igure 3.6: View of the Karoo Nature Reserve, August 2000. An area of the Reserve dominated by *Atriplex lindleyi*, 32°11'15 S, 24°30'59 E, looking NE.

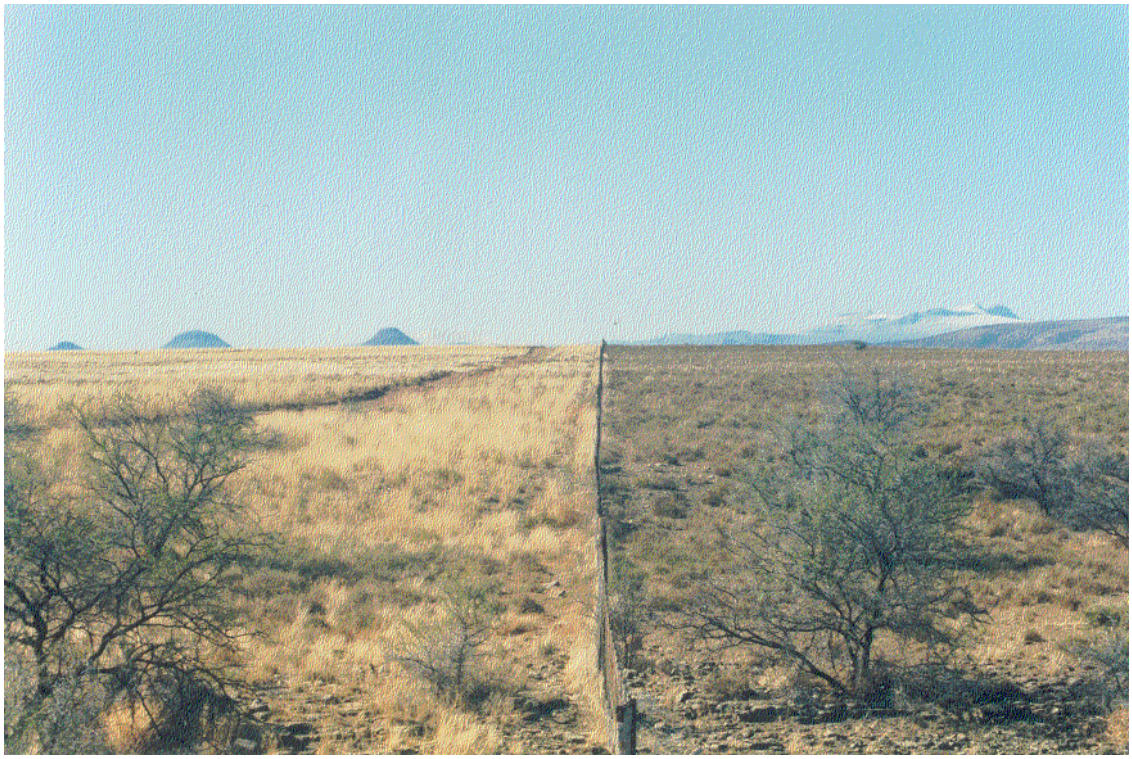

Figure 3.7: View of the Karoo Nature Reserve, August 2000. Fence line with the Reserve on the right hand side, 32°10'16 S, 24°29'31 E looking E.

Figure 3.8: View of the Karoo Nature Reserve, August 2000. Fence line with the Reserve on the left, 32°10'18 S, 24°30'40 E, looking W.

Figure 3.9: View of the Karoo Nature Reserve, August 2000. A fenced camp within the Reserve, 32°10'31 S, 24°30'20 E, looking E.

3.1.1.7. *Animals*

Besides blesbok, other herbivores occurring on the reserve include African buffalo (*Syncercerus caffer*), gemsbok (*Oryx gazella*), red hartebeest (*Alecephalus buselaphus*), greater kudu (*Tragelaphus strepsiceros*), springbok (*Antidorcas marsupialis*) and black wildebeest (*Connachaetes gnou*).

3.1.2. Willem Pretorius Game Reserve, Free State

The vegetation types in the Reserve are classified as Transitional *Cymbopogon-Themeda* Veld and Dry *Cymbopogon-Themeda* Veld (Acocks, 1988) or as Moist Cool Highveld Grassland (Low & Rebelo, 1998).

3.1.3. Gariep Dam Game Reserve, Free State

The vegetation types in the Reserve are classified as False Upper Karoo (Acocks, 1988) or as Eastern Mixed Nama Karoo (Low & Rebelo, 1998).

3.2. Blesbok

3.2.1. Blesbok observations

A number of observations were made between the 15th August 2000 and 5th September 2000 in the Game Viewing Area of the Karoo Nature Reserve. Observations were made from a vehicle, throughout the day, between 07h15 and 18h30. It was not possible to observe activities during the night. The Game Viewing Area was divided into blocks (Figure 3.10), based on the layout of the roads. All the blesbok were counted in each block during one observation period. In total, 21 observation periods were logged (Table 4.1).

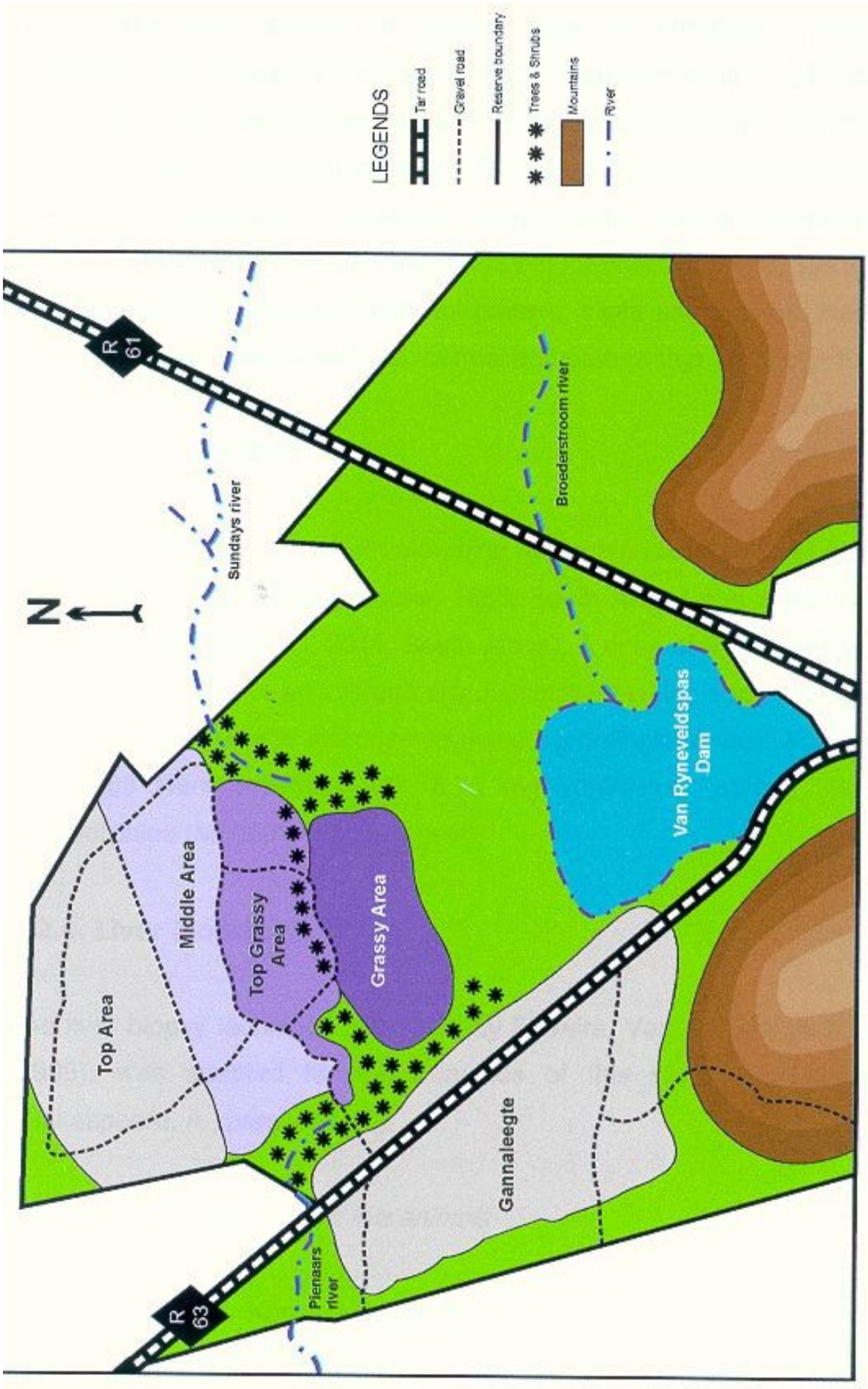

Figure 3.10: The observation areas in the Game Viewing Area, Karoo Nature Reserve, Graaff-Reinett

3.2.2. Animal groups

The blesbok were divided into groups: male and females; juvenile (< 12 months of age), subadult (12-24 months of age) and adult (> 24 months of age). Age was determined according to incisor eruption (Ludbrook & Ludbrook, 1981; Olivier & Greyling, 1991; Watson, Skinner, Erasmus & Dott, 1991) and general size of the animal. Body condition was graded from zero to five, using the body condition scoring system for sheep (Russel, 1991). Ataxia was subjectively categorised into not present, slight and marked. Hair colour was subjectively categorised into, normal and pale (with a scale of + to 3+).

3.2.3. Immobilisation

Blesbok were immobilised with etorphine hydrochloride (M99® - Logos Agvet, Private Bag X115, Halfway House, 1685, South Africa) and xylazine (Kyron, PO Box 27329, Benrose, 2011, South Africa). A dose of 3 mg M99 and 5 mg xylazine was used for an average sized animal, weighing approximately 70 kg (Burroughs, 1993), and the dose adjusted accordingly to size. The effects of the drugs were reversed with 8.75 mg yohimbine (Kyron) and 6 mg diprenorphine (M5050® - Logos Agvet).

3.2.4. Liver biopsies

The liver biopsy technique described by Ferreira, Van der Merwe & Slippers (1996), was adapted for the purposes of this study (Schultheiss, W., Shakespeare, A., pers. comm.).

3.2.4.1. *Liver biopsies from live animals*

Blesbok from the Karoo Nature Reserve were chemically immobilised to obtain the liver biopsies. Hair was shaved at the biopsy site on the right side of the body, over the 11th intercostal space and a hand's breadth below the

processus spinosus. The area was cleaned and disinfected with 70% ethyl alcohol. A sterile, surgical drape was placed over the animal. The skin was stabbed with a scalpel blade and a five-gauge biopsy needle inserted perpendicularly through the skin incision and the intercostal muscles. The needle was then directed towards the olecranon of the left forelimb. After the liver was penetrated, the stylet of the biopsy needle was removed and the needle was rotated while being advanced a further three centimetres. Suction was applied to the needle before removing it. A 20 ml plastic syringe attached to the needle was used for this purpose. The liver tissue sample was then ejected from the biopsy needle onto a sterile drape. Any non-hepatic tissue, if present, was separated from clearly identified liver tissue. The liver sample was placed in a test tube containing approximately one millilitre of heparin saline to remove any excess blood. The contents of the test tube were poured onto the sterile drape to absorb the heparin saline. The liver tissue was placed in a test tube kept in a container cooled with ice and placed in a freezer at -20°C as soon as possible.

After the biopsy had been obtained, the wound was sprayed with Supona Aerosol (Fort Dodge, PO Box 1785, Kempton Park, 1620, South Africa). An antibiotic (Duplocillin – Intervet SA, PO Box 4278, Edenvale, 1610, South Africa) was injected intramuscularly at a dosage of 1 ml/25 kg.

The biopsy needle was sterilised with pure alcohol and washed with distilled water before each procedure.

3.2.4.2. *Liver biopsies from culled animals*

Whole livers were obtained from blesbok in Willem Pretorius Game Reserve and Gariep Dam Game Reserve, culled as part of population control measures.

Liver samples were taken with a five-gauge biopsy needle without a stylet, in a manner simulating the procedure in a live animal. The dorsal right lobe of

the liver was penetrated and the biopsy needle directed in a cranio-ventro-medial direction, with the liver in an anatomically correct position. Samples were frozen at -20°C as soon as possible after collection. The approximate dry mass of the sample was 0.15 g.

The biopsy procedure was repeated on twenty blesbok livers selected at random. The second biopsy was performed next to the original liver biopsy site. The original sample was stored at -20°C, the second biopsy was stored in a serum test tube filled with 10 mls 10% buffered formalin. Samples were stored for approximately four and a half months before analysis.

3.2.5. Analysis of hepatic copper levels

The samples were placed onto clean watch glasses and dried in an oven at 90°C overnight to remove all moisture. The samples were then weighed on an analytical balance (Mettler AE100) and transferred to a clean, acid washed digestion flask containing three glass digestion beads. Ten millilitres of Acid Mix (1:5 70% HNO_3:65% $HClO_4$) (Merck) was added to each flask for biopsies weighing < 0.40 g (15 mls for biopsies weighing > 0.40 g). The flasks were allowed to stand for approximately 15 minutes and then gently heated to a maximum temperature of 250°C until digestion was complete. The liver biopsies with a mass < 0.40 g were diluted to 10 ml with deionised water or 20 mls where the mass of the samples was greater than 0.40 g. The copper levels were measured by flame atomic absorption spectrophotometer (GBC SDS-270 Avanta, GBC Scientific Equipment Pty Ltd, Victoria, Australia).

3.2.6. Blood sample collection

Blesbok from the Karoo Nature Reserve were chemically immobilised before collecting the blood samples. Jugular blood samples were collected from Willem Pretorius Game Reserve and Gariep Dam Game Reserve blesbok, as soon as possible after the animal was shot. Samples were collected in sodium

heparin tubes (Becton Dickinson, USA) and placed immediately into a cooler box.

Five additional blood samples were obtained from chemically immobilised blesbok, on the premises of MSD Pty Ltd., Gauteng, for the purposes of haemoglobin concentration determination.

For determination of the effect of temperature on the stability of superoxide dismutase, blood samples were collected from three Jersey cows housed at the Onderstepoort Faculty of Veterinary Science, University of Pretoria. The experiment was repeated using five blood samples collected from culled blesbok in the Gariep Dam Game Reserve.

3.2.7. Blood sample preparation

For determination of plasma copper levels, blood samples were centrifuged at 3000 rpm for 10 mins, within five hours after collection. Plasma was removed and placed in plastic vials. The plasma samples were frozen until analysis.

For the erythrocyte superoxide dismutase assay, a 0.5 ml sample whole blood was taken and centrifuged for 10 minutes at 3 000 rpm. The plasma was aspirated off and the erythrocytes washed three times with a 0.9% NaCl solution, centrifuging for 10 minutes at 3 000 rpm after each wash. Washed bovine erythrocytes were stored at different temperatures, viz. room temperature (22°C), fridge (4°C), freezer (-20°C) and liquid nitrogen (-200°C) and the activity of superoxide dismutase measured over time. Blesbok samples were stored in a fridge (4°C), freezer (-20°C) and liquid nitrogen (-200°C).

Washed erythrocyte samples from the Karoo Nature Reserve and Willem Pretorius Game Reserve were placed immediately after washing into liquid nitrogen until further analysis.

3.2.8. Haemoglobin determination

After gentle mixing, the haemoglobin concentration of all the blood samples was determined within six hours using a visual, filter photometer - AO Spencer Hb-meter (American Optical Company, USA). Whole blood was used and the red blood cells lysed using saponin sticks.

Where possible, blood samples were re-analysed for haemoglobin content within 48 hours, using a coulter counter (Coulter T-890, Beckman, USA). The apparatus employs a lytic reagent to lyse the erythrocytes and converts the haemoglobin to a stable cyanide-containing pigment. A transmission wavelength of 525 nm was used.

3.2.9. Analysis of plasma copper levels

Five mls of 7% trichloroacetic acid (Sigma-Aldrich) was added to one millilitre of plasma. The mixture was allowed to stand for 10 mins and then centrifuged for 20 mins at 4000 rpm. The supernatant was decanted and then re-centrifuged for 10 mins at 3000 rpm. One, two, four and eight ppm copper standards were prepared from 1000 ppm stock (Merck). The samples were analysed with a flame atomic absorption spectrophotometer (GBC SDS-270 Avanta, GBC Scientific Equipment Pty Ltd, Victoria, Australia). The analysis was controlled with Assayed Human Sera Level 1 (Cat. #: HN 1530, Randox Laboratories, Crumlin, United Kingdom).

A range for plasma copper concentration in blesbok was determined using liver samples collected from Willem Pretorius and Gariep Dam Game Reserves. The range was defined as mean $\pm$ 2 $\times$ standard deviations.

3.2.10. Superoxide dismutase assay

A commercial assay kit (RANSOD Cat. No. SD 125 - Randox Laboratories Ltd., Crumlin, United Kingdom) was used to measure superoxide dismutase

activity. The method employs xanthine and xanthine oxidase to generate superoxide radicals which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) to form a red formazan dye. The superoxide dismutase activity is measured by the degree of inhibition of this reaction.

In the laboratory, the samples, if frozen, were defrosted at room temperature. Samples were made up to 2.0 ml with redistilled water, mixed and left to stand at +4°C for 15 minutes. The lysate was further diluted with 0.01 mmol/l phosphate buffer (pH 7.0) to a final dilution of 1:100 for blesbok samples and 1:200 for bovine samples. The dilution was adjusted so that the percentage inhibition would fall between 30 and 60%. The sample was mixed with the mixed substrate. Absorbance was read using a spectrophotometer (Beckman DU 650, USA). The wavelength was set at 505 nm and the temperature at 37°C. The initial absorbance was read 30 seconds after addition of xanthine oxidase. The final absorbance was read three minutes after the first reading. A change in absorbance per minute was calculated and the percentage inhibition determined by comparing the reaction to the uninhibited reaction (xanthine oxidase added to the mixed substrate). A standard curve was used to determine the superoxide dismutase activity (U/ml) from the percentage inhibition.

Each sample was analysed three times and the results averaged. Measurements not within a 30 - 60% inhibition range were discarded and a different dilution made. Precision was controlled by using a RANSOD control (Cat. #: SD 126 – Randox Laboratories, Crumlin, United Kingdom).

A range of erythrocyte superoxide dismutase activity in blesbok was determined. The range was defined as mean $\pm$ 2 $\times$ standard deviations.

3.3. Soil and water

3.3.1. Collection of soil samples

Twelve soil samples were taken in the Dam Area and eight soil samples in the area surrounding the Dam Area, the latter to serve as control samples. The Dam Area was defined as the area below 785 m (the level of the dam when full), as the riparian thicket occurs between 785 and 790m. Control samples were taken between 790 and 795 m. Sample sites were selected using contour boundaries but were randomly selected within contour boundaries. Five sample sites were randomly selected below 780 m, seven sites between 780-785 m and eight sites between 790-795 m (see Figure 3.11). Positions were determined using a hand-held Global Positioning System (GPS) and 1:10 000 orthophoto maps (Chief Directorate: Surveys and Mappings, Rhodes Avenue, MOWBRAY, Private Bag X10). As different reference systems were used (WGS 84 and modified Clarke 1880 respectively), a standard correction, as determined from the centre of Graaff-Reinet, was used viz. GPS – 43.49m = L_0(Y); GPS – 293.39m = L_0(X) (Van der Walt, L., pers. comm.).

Soil samples were collected by taking a 30 cm deep, core sample and then stored in plastic containers (Peterson & Calvin, 1996). The samples were air-dried and stored for approximately two months before analysis.

3.3.2. Collection of water samples

Samples of water were taken from the two functional boreholes and three samples from the dam (Figure 3.11). In all cases, two, clean, one litre glass bottles were filled from source (boreholes). To one of the bottles, one millilitre concentrated HNO_3/100 ml water was added for the metal analyses.

3.3.3. Measurement of pH

Analysis was performed by the soil laboratory of the Institute for Soil, Climate and Water (ISCW), Agricultural Research Council (ARC).

The pH of the soil was determined with a 1:2.5 soil/water ratio suspension on a mass basis using a Radiometer (PHM 82) with a combination electrode (GK2401C). Twenty five cm^3 de-ionised water was added to 10 g dried soil in a glass beaker. The contents were stirred immediately for five seconds with a

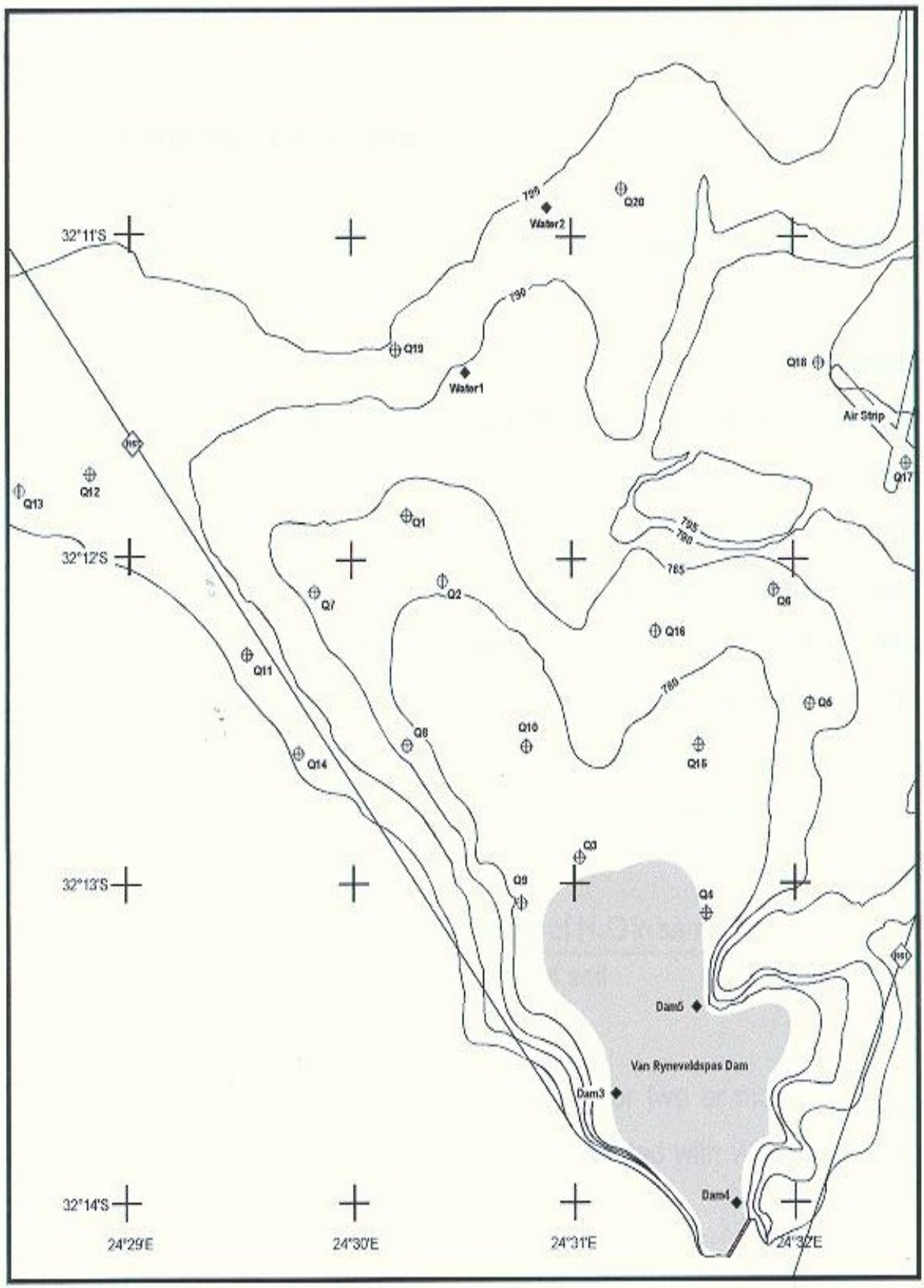

Figure 3.11: Contour map of the Game Viewing Area of the Karoo Nature Reserve showing the soil (⊕) and water (◆) sample sites, August 2000.

glass rod and again after 50 min. The mixture was then allowed to stand for 10 min before the reading was taken.

For water samples, no preparation took place and the samples were analysed as is.

3.3.4. Determination of salinity

Determination of salinity was performed by the soil laboratory, ISCW, ARC.

A saturation water paste extraction was used in the determination of salinity (Rhoades, 1982, in Janzen, 1993)). Two hundred and fifty grams of soil with known moisture content was weighed into a container with a lid. Sufficient deionized water was added to saturate the soil sample. The saturated sample would glisten, flow slightly when the container was tipped and slide cleanly from a spatula. The sample was allowed to stand for at least one hour, after which the container was rechecked for saturation and then weighed. The increase in weight (from the added deionized water) was recorded and the saturation percentage (SP) calculated according to the following equation:

$$SP = \frac{(\text{weight of } H_2O \text{ added} + \text{weight of } H_2O \text{ in sample})}{\text{oven–dry weight of soil}} \times 100$$

After allowing the saturated soil paste to stand for two or more hours, the sample was transferred to a Buchner filter funnel fitted with Whatman No. 42 filter paper and a vacuum applied.

Standard 0.010 and 0.100 *N* KCl solutions were prepared. A Radiometer CDM 83 with a through flow cell was rinsed and then calibrated with the standard KCl solution. The conductivity flow cell was used to determine the electrical conductivity of the samples, corrected to 25°C (Rhoades & Miyamoto, 1990).

3.3.5. Determination of sodicity

The sodicity, using the sodium adsorption ratio (SAR), was determined by the soil laboratory, ISCW, ARC using the following method (Rhoades & Miyamoto, 1990):

A saturation water paste extraction (see 3.3.4) was performed. An atomic absorption spectrophotometer (Varian AA40), fitted with an oxidising air-acetylene flame, was used to measure the concentrations of Ca, Mg and Na. The SAR was calculated as follows:

$$SAR = Na/\sqrt{(Ca + Mg)/2}$$

Where the total cation concentrations in the saturation extract are in mmol/l.

3.3.6. Determination of Cu, Mo, Zn and Fe concentrations

Analysis was performed by the soil laboratory of the ISCW, ARC. Plant available (di-ammonium EDTA) trace element analysis was used for Cu, Mo and Zn analysis (Trierweiler & Lindsay, 1969, as modified by Beyers & Coetzer, 1971).

The air-dried soil was crushed with a roller mill and refined to a fineness of ≤ 1 mm in a porcelain mortar. Five grams of air-dry soil was placed in an extraction bottle. Fifteen cm^3 0.02mol.dm^{-3} $(NH_4)_2$ EDTA solution was then added to the soil and the container sealed with a stopper. The container was shaken horizontally for 60 mins at 180 oscillations/minute in a reciprocating shaker at a constant temperature of $20 \pm 2°C$. The sample was centrifuged in the same container for five minutes at 2000 rpm and then filtered through Whatman no. 40 paper into suitable containers, using silicone stoppers. The metals Cu, Mo and Zn were analysed with an inductively coupled plasma mass spectrometer (ICP-MS), model VG PlasmaQuad PQ2 Turbo Plus (VG

Elemental UK). Iron was measured with an atomic absorption spectrophotometer (Varian AA40), fitted with an oxidising air-acetylene flame.

For water samples, no preparation took place and the samples were analysed as is.

3.3.7. Determination of sulphate concentration

A saturation water paste extraction was performed, as in 3.3.4, by the soil laboratory, ISCW, ARC, to analyse SO_4^{2-} concentration in soil.

Samples were analysed with a Dionex ion chromatograph (DX 120) fitted with an ASA4 anion exchange column, an AG4A guard column and an ASRS-11-anion suppresser.

For water samples, no preparation took place and the samples were analysed as is.

3.3.8. Determination of carbonate concentration

Carbonate concentration in soil was performed by the author, in the pharmacology laboratory, Department of Pharmacology, Faculty of Veterinary Science, University of Pretoria.

A gravimetric method for loss of carbon dioxide, modified from US Salinity Laboratory Staff (1954) and Allison & Moodie (1965), was used to determine the carbonate concentration in soil.

A 75 ml Erlenmeyer flask containing 10 ml of 3 *M* HCl and a magnetic stirrer was covered with plastic film and 3 holes using an 18G needle was made in the film. The flask was weighed to the nearest 0.1 mg. Ten grams of soil (ground in a mortar and pestle and then passed through a 2mm mesh to remove stones) was added to the flask and reweighed. The flask was placed

on a stirrer and weighed every 15 mins. The final weight was determined when the weight of the flask did not change by more than 1 to 2 mg.

The weight of CO_2 = difference between initial and final weights (flask + acid + soil)

$$CO_3\text{-}C,\% = \left(\frac{g\,CO_2\ lost}{g\ soil}\right)\left(\frac{g\,C\,mol^{-1}}{g\,CO_2 mol^{-1}}\right)(100)$$
$$= \left(\frac{g\,CO_2\ lost}{g\ soil}\right)(0.2727)(100)$$

3.3.9. Determination of organic carbon

The determination of organic carbon was undertaken by the soil laboratory, ISCW, ARC, using the Walkley-Black method (Walkley, 1935).

A 0.5 mol.dm^{-3} iron (II) ammonium sulphate solution was standardised against 10 dm^3 0.167 mol.dm^{-3} $K_2Cr_2O_7$ (see later). The soil sample was ground with a porcelain mortar and pestle to pass through a 0.35 mm sieve. One gram (0.5 g or less if high in organic matter) air-dried soil was added to a 500 cm^3 Erlenmeyer flask, containing ten cm^3 $K_2Cr_2O_7$ solution. The flask was swirled to disperse the soil in the solution. Twenty cm^3 concentrated (AR grade) sulphuric acid was rapidly added to the flask and the flask swirled for one minute. The flask was cooled on a sheet of asbestos for 30 min and 150 cm^3 de-ionised water and 10 cm^3 concentrated ortho-phosphoric acid then added.

One cm^3 indicator (0.4% barium diphenylamine sulphonate) was added and the excess dichromate titrated with the iron (II) ammonium sulphate solution. The determination was repeated with less soil if more than 75% of the dichromate was reduced.

The carbon content was calculated according to the following formula, using a recovery factor of **f** = 1.3:

$$\text{Concentration of } Fe(NH_4)_2(SO_4)_2 \text{ mol.dm}^{-3} = \frac{10\ cm^3\ K_2Cr_2O_7 \times 0.167 \times 6}{cm^3\ Fe(NH_4)_2(SO_4)_2}$$

$$\text{Organic C \%} = \frac{[cm^3\ Fe(NH_4)_2(SO_4)_2\ \text{blank} - cm^3\ Fe(NH_4)_2(SO_4)_2\ \text{sample}] \times \mathbf{M} \times 0.3 \times \mathbf{f}}{\text{soil mass (g)}}$$

where **M** = concentration of the $Fe(NH_4)_2(SO_4)_2$ in $mol.dm^{-3}$.

3.4. Statistical analysis

Results were analysed with either SigmaStat® ver 2.03 (SPSS Inc.) or SAS®. The SAS® system is an integrated system of software providing complete control over data management, analysis and presentation (SAS Institute South Africa Pty. Ltd., 93 Central Street, HOUGHTON, 2041, Republic of South Africa).

CHAPTER 4

Results

4.1. Blesbok

4.1.1. Grazing utilisation by blesbok in the Karoo Nature Reserve

Table 4.1: The average number or blesbok noted per observation period ($\bar{x}$) in the Game Viewing Area, Karoo Nature Reserve, 15th August 2000 – 5th September 2000.

Observation period	Grassy area	Top grassy area	Middla area	Top area	Gannaleegte
1	21	1			
2	13				
3	15				
4	13	1			
5	1				
6	13				
7	17	3			
8	16				
9	14	1	3		
10	4				
11	11				
12	10				
13	20	1			
14	20				
15	21				
16	16				
17	7				
18	17	1	1		
19	10				
20	3				
21	8				
$\bar{x}$	12.8	0.4	0.2		

From Table 4.1, some blesbok were seen 100% of the time in the grassy area, 29% of the time in the top grassy area and 10% of the time in the middle area. The rest of the blesbok were situated south of the dam area, closer to the Vanryneveldspas dam and were too far away from any roads to be counted accurately. Therefore, during the entire observation period, less than 4% of the blesbok population were observed outside the area surrounding the Vanryneveldspas dam, 10% of the time.

4.1.2. Storage of liver biopsies

The mean ($\bar{x}$ = 2094.6 μmol/kg, *s* = 927.4, *n* = 19) liver copper concentration in formalin was significantly lower than the mean ($\bar{x}$ = 2274.4 μmol/kg, *s* =

935.0, *n* = 19) copper concentration measured in frozen livers (paired t-test: t = -4.13, df = 18, P < 0.001).

4.1.3. Comparison of haemoglobin measuring instruments

Linear regression of haemoglobin results from the photometer and coulter counter yielded a regression equation of $y = -1.368 + 1.148x$, where y = coulter counter result and x = photometer result (see Figure 4.1). The regression was tested for curvilinearity and the quadratic term was found to be not significant (P > 0.05).

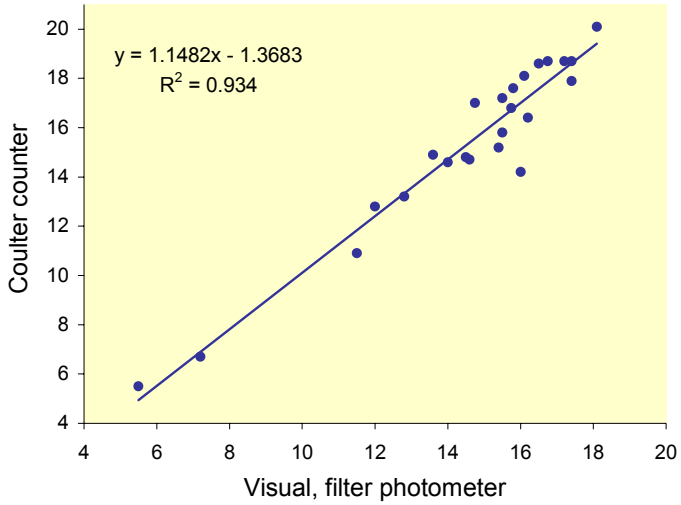

Figure 4.1: Regression of results obtained (in g/dl) from measuring blesbok blood samples (*n* = 24) with a visual, filter photometer (AO Spencer Hb-meter) and a coulter counter (Coulter T890, Beckman, USA).

4.1.4. The effect of time and temperature on the activity of SOD

The effect of time and temperature on the activity of SOD is represented graphically in Figure 4.2 and 4.3. The data obtained from blesbok SOD samples stored at 4°C and -20°C was fitted to a general linear model (GLM) and a repeated measures ANOVA performed. The contrast variable (time) was compared to the initial time (day 0). Results are shown in Table 4.2.

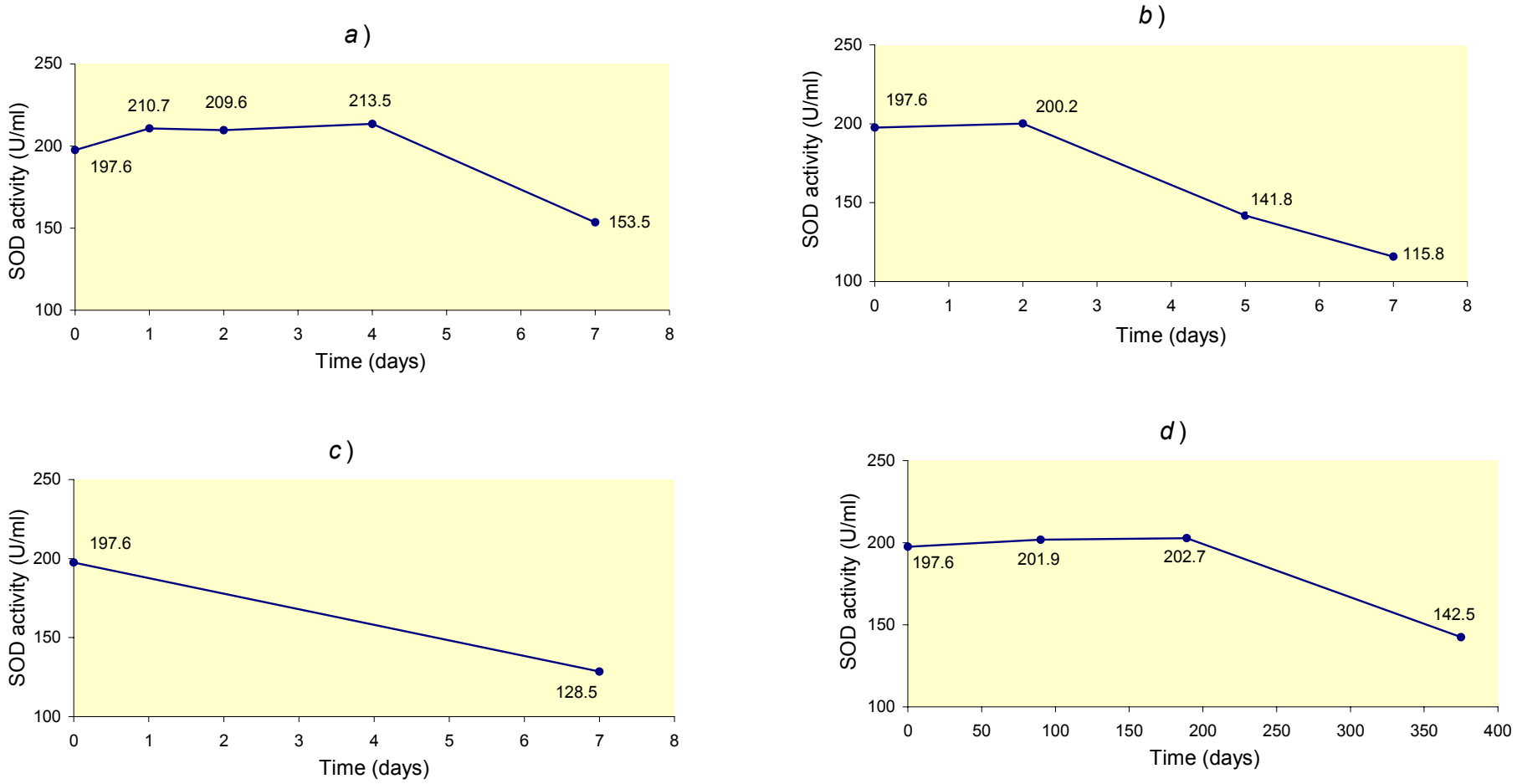

Figure 4.2: The effect of temperature on the stability of bovine (Jersey cows) erythrocyte superoxide dismutase over time: (*a*) room temperature, 22°C; (*b*) fridge, 4°C; (*c*) freezer, -20°C; (*d*) liquid nitrogen, -200°C. Values represent the mean activity from three animals.

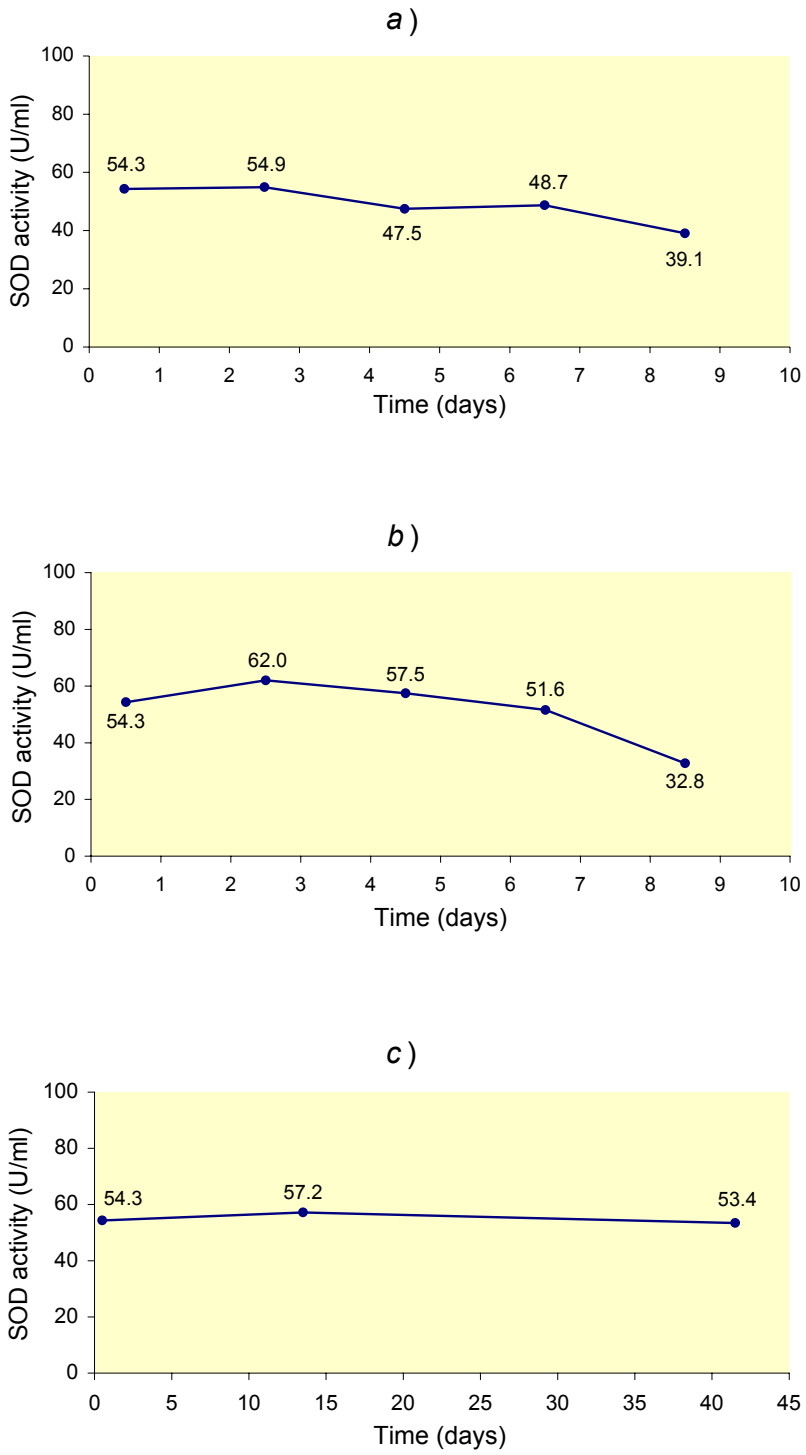

Figure 4.3: The effect of temperature on the stability of blesbok erythrocyte superoxide dismutase over time: (*a*) fridge, 4°C; (*b*) freezer, -20°C; (*c*) liquid nitrogen, -200°C. Values represent the mean activity from five animals.

The data obtained from blesbok samples stored at -200°C was analysed separately as the data did not fit into the GLM. A repeated measures ANOVA showed no significant differences (P > 0.05) between the different days at a temperature of -200°C.

Table 4.2: The effect of temperature (4°C and -20°C) on the activity of blesbok superoxide dismutase over time. Results of a repeated measures ANOVA of the contrast variable (time) compared to the initial time (day 0).

Contrast variable	Parameter	df	F	P
Day 2	Time	1	4.50	0.1013
	Temperature	1	3.29	0.1440
Day 4	Time	1	0.11	0.7527
	Temperature	1	0.81	0.4190
Day 6	Time	1	7.65	0.0506
	Temperature	1	0.89	0.3984
Day 8	Time	1	126.48	0.0004
	Temperature	1	3.69	0.1271

4.1.5. The copper status of blesbok

Results from the analyses of blesbok samples obtained are summarised in Table 4.3, Figures 4.4 - 4.6. The SOD analysis of samples obtained from Willem Pretorius Game Reserve was performed 34 ± 2 days after collection; those from Graaff-Reinet was performed 13 ± 2 days after collection.

Table 4.3: The copper status of blesbok in the Karoo Nature Reserve, Willem Pretorius and Gariep Dam Game Reserves, 2000/20001. * Units per gram haemoglobin.

Indicator of copper status	Mean ($\bar{x}$)	Standard deviation (*s*)	Sample size (*n*)
Liver copper concentration (μmol/kg)			
Karoo Nature Reserve	251.43	228.10	14
Willem Pretorius Game Reserve	2785.38	1906.18	37
Gariep Dam Game Reserve	1509.61	1024.20	11
Plasma copper concentration (μmol/l)			
Karoo Nature Reserve	0.95	0.35	14
Willem Pretorius Game Reserve	25.77	2.62	23
Gariep Dam Game Reserve	21.99	3.22	5
Erythrocyte superoxide dismutase (U/ml)			
Karoo Nature Reserve	19.09	11.48	14

Willem Pretorius Game Reserve	95.33	19.09	20
Gariep Dam Game Reserve	53.46	9.06	6
Erythrocyte superoxide dismutase (U/g Hb*)			
Karoo Nature Reserve	136.99	62.19	14
Willem Pretorius Game Reserve	602.20	123.55	20
Gariep Dam Game Reserve	379.90	77.15	6

A summary of reported hepatic copper concentration in clinically healthy blesbok is presented in Table 4.4., with no regard to sampling, storage or analytical methods.

Table 4.4: Hepatic copper levels (μmol/kg dry mass) in South African blesbok. All unpublished data is taken from blesbok in the Free State. The mean value in bold type is a weighted mean (the sample size was taken into account).

Reference	Mean ($\bar{x}$)	Standard deviation (*s*)	Sample size (*n*)
Turkstra, de Vos, Biddlecombe, & Dow, 1978	1789	757	16
Penrith, Tustin, Thornton, & Burdett, 1996	1020	853	5
Willem Pretorius Game Reserve (this study)	2785	1906	37
Gariep Dam Game Reserve (this study)	1510	1024	11
P. Nel (unpublished data)	1278	384	21
S. Stead (unpublished data)	1592	1100	7
All references	**1973**	**1465**	**97**

The copper concentration in the plasma of clinically healthy blesbok is 20.41 – 29.95 μmol/l and the erythrocyte superoxide dismutase activity is 35.98 – 135.35 U/ml; 254.83 – 846.97 U/g haemoglobin.

The blesbok data was fitted to a GLM and analysed using F tests and multiple comparison procedures. Results are shown in Table 4.5.

Table 4.5: Results of multiple comparison procedures to differentiate treatment groups. * Units per gram haemoglobin.

	P
Liver copper concentration (μmol/kg)	
Willem Pretorius vs. Karoo	< 0.0001
Gariep Dam vs. Karoo	0.0487
Plasma copper concentration (μmol/l)	
Willem Pretorius vs. Karoo	< 0.0001
Gariep Dam vs. Karoo	< 0.0001
Erythrocyte superoxide dismutase (U/ml)	
Willem Pretorius vs. Karoo	< 0.0001

Gariep Dam vs. Karoo	< 0.0001
Erythrocyte superoxide dismutase (U/g Hb*)	
Willem Pretorius vs. Karoo	< 0.0001
Gariep Dam vs. Karoo	< 0.0001

4.1.6. Correlation of blesbok data

The relationship between liver copper, plasma copper and erythrocyte SOD activity was examined. The blesbok data was fitted to a GLM and a correlation performed on the residuals after removing the effect of location. A significant relationship ($r = 0.40509$, $n = 35$, $P = 0.0158$) between liver and plasma copper levels was observed.

No significant correlation ($P > 0.05$) was obtained between the copper status of blesbok and degree of ataxia, achromotrichia and condition score.

4.1.7. Gender and reproduction

The effect of gender on copper status in blesbok was examined. The blesbok data was fitted to a GLM and no significant differences ($P > 0.05$) between males and females was observed, after the effect of location was removed.

Out of a total population of 99 blesbok in the Karoo Nature Reserve (Figure 1.1), only five lambs (< 1 year old) were observed. The recruitment rate was therefore 5%. In addition, one dead juvenile was found in the dam area. Of seven adult female blesbok caught about 4 months after the peak of the rut at Karoo Nature Reserve, only one was pregnant. An estimated pregnancy rate was therefore 14%. The only female blesbok at Willem Pretorius Game Reserve (May, 2000) that was noted to be pregnant was found to have the highest liver copper level (4092 μmol/kg).

4.2. Soil and water

Results are summarised in Table 4.6 and 4.7. The individual ion analyses for the calculation of SAR are presented in Appendix 2.

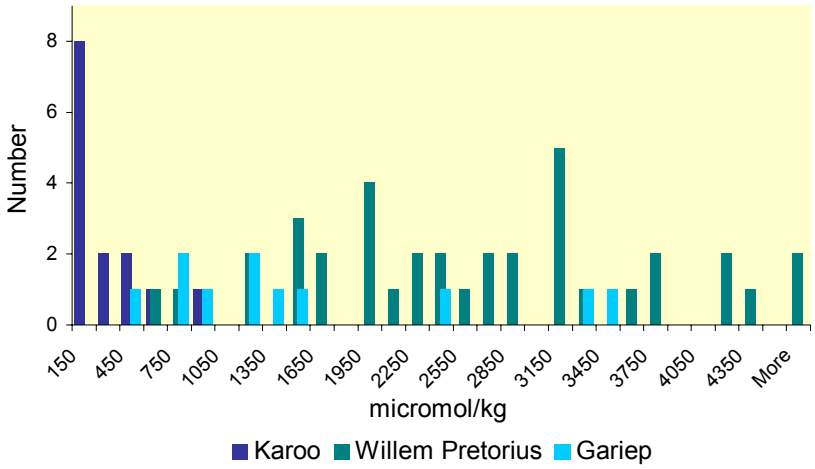

Figure 4.4: Histogram of liver copper concentrations in blesbok from Karoo Nature Reserve (*n* = 14), Willem Pretorius (*n* = 37) and Gariep Dam Game Reserves (*n* = 11), 2000/2001.

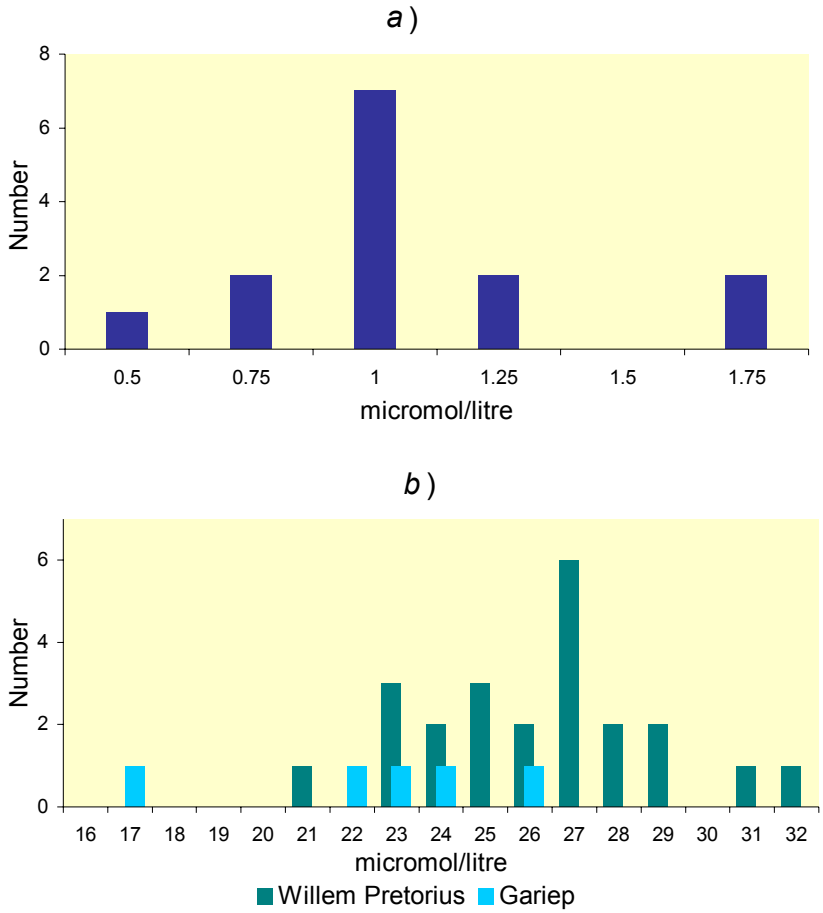

Figure 4.5: Histogram of plasma copper concentrations in blesbok from *a*) the Karoo Nature Reserve (*n* = 14); *b*) Willem Pretorius (*n* = 23) and Gariep Game Reserves (*n* = 5), 2000/2001.

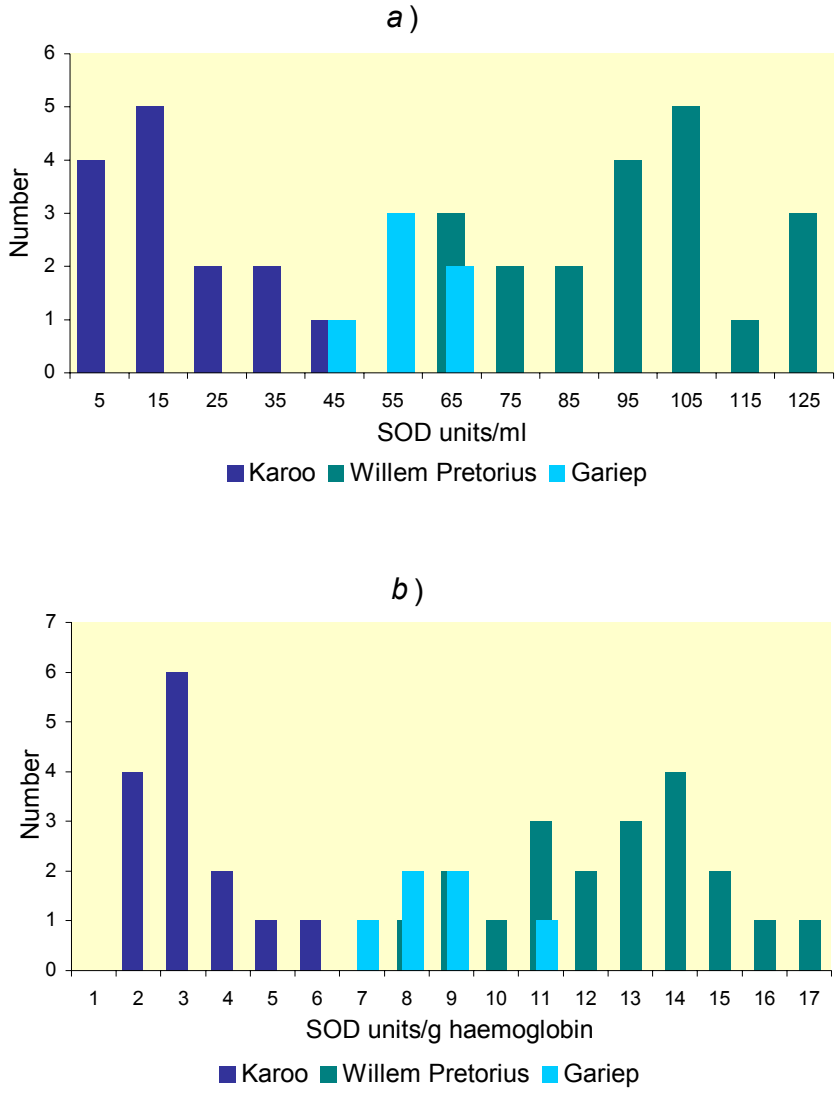

Figure 4.6: Histogram of erythrocyte superoxide dismutase activity in *a*) units/ml and *b*) units/g haemoglobin, in blesbok from the Karoo Nature Reserve (*n* = 14), Willem Pretorius (*n* = 20) and Gariep Game Reserves (*n* = 6), 2000/2001.

Table 4.6: Analysis of soil samples from the Karoo Nature Reserve, Graaff-Reinet, September 2000. Trace element concentrations were determined with di-ammonium EDTA extraction. $\bar{x}$ = mean, *s* = standard deviation, SAR = sodium adsorption ratio, EC = electrical conductivity.

ID	Elevation	pH	EC	SAR	Cu	Mo	Zn	SO_4^{2-}	CO_3^{2-}	C
	M		S/m		Mg/kg	mg/kg	mg/kg	mg/kg	%	%
Q 3	780	8.34	0.108	1.28	3.670	<0.0001	0.989	296.0	0.093	1.52
Q 4	780	7.76	1.930	8.63	8.285	<0.0001	1.733	11591.4	0.129	1.71
Q 9	780	8.10	0.310	2.28	4.777	0.0500	1.798	1216.0	0.290	2.66
Q 10	780	8.30	0.136	1.65	6.298	0.0020	1.882	445.1	0.109	1.40
Q 15	780	8.37	0.091	1.48	2.359	0.0235	1.126	83.0	0.172	2.05
Q 1	785	8.14	0.085	0.58	0.050	<0.0001	0.034	34.8	0.042	1.71
Q 2	785	8.46	0.082	0.50	6.075	0.0220	2.713	119.4	0.115	0.68
Q 5	785	10.18	0.216	19.19	6.144	0.0070	2.637	345.8	0.083	0.74
Q 6	785	9.21	0.068	6.22	5.246	0.0440	2.062	23.9	0.091	0.61
Q 7	785	9.14	0.056	0.80	4.464	0.0025	1.018	35.8	0.276	0.38
Q 8	785	8.87	0.044	2.41	2.521	<0.0001	0.728	52.1	0.028	0.38
Q 16	785	8.94	0.094	6.24	9.435	<0.0001	2.748	4.7	0.133	1.00
$\bar{x}$		*8.99*	*0.092*	*5.13*	*4.848*	*0.019*	*1.706*	*88.1*	*0.130*	*0.79*
s		*0.65*	*0.057*	*6.69*	*2.971*	*0.019*	*1.104*	*119.3*	*0.081*	*0.46*
Q 11	795	8.42	0.102	2.45	6.365	<0.0001	1.407	43.1	0.029	0.36
Q 12	795	7.30	0.567	0.68	4.194	0.0307	0.999	267.8	0.029	0.53
Q 13	795	8.37	0.022	0.29	4.002	0.0532	1.635	17.3	0.043	0.34
Q 14	795	8.56	0.035	0.38	3.362	0.0046	0.898	11.4	0.031	0.39
Q 17	795	8.95	3.010	36.13	5.780	0.0320	0.958	564.4	0.320	0.39
Q 18	795	7.96	0.099	2.34	2.009	0.0256	0.867	176.9	0.019	0.47
Q 19	795	8.98	0.030	0.75	3.378	0.0172	1.297	5.4	0.023	0.24
Q 20	795	8.80	0.029	0.34	3.268	0.0238	1.250	8.5	0.127	0.33
$\bar{x}$		*8.42*	*0.487*	*5.42*	*4.045*	*0.027*	*1.164*	*136.9*	*0.078*	*0.38*
s		*0.56*	*1.036*	*12.44*	*1.418*	*0.015*	*0.276*	*197.9*	*0.104*	*0.09*

Table 4.7: Analysis of trace element concentrations in water samples taken from the Karoo Nature Reserve, Graaff-Reinet, September 2000. $\bar{x}$ = mean, *s* = standard deviation.

ID	pH	Cu	Mo	Zn	SO_4^{2-}	Fe
		ppb	ppb	ppb	ppm	ppm
Water1	7.88	8.594	0.96	253.50	51.22	0.05
Water2	7.71	21.342	0.69	404.49	80.99	0.10
Dam3	8.14	3.189	0.00	180.09	96.93	0.53
Dam4		5.014	7.84	15.22	150.00	1.24
Dam5		6.962	8.21	15.57	135.31	0.64
$\bar{x}$		*9.020*	*3.54*	*173.78*	*102.89*	*0.51*
s		*7.182*	*4.11*	*165.67*	*40.17*	*0.48*

4.2.1. Dam and control areas

Soil parameters in the Dam and control areas of the Karoo Nature Reserve were compared with either a Student's t-test or a Mann-Whitney Rank Sum test, depending on whether data was normally distributed. A significant difference ($T = 36.00$, $P < 0.001$) in the soil organic matter percentage of the Dam and control area was detected. No significant differences ($P > 0.05$) in the pH (H^+ concentration), EC, SAR, copper, molybdenum, zinc, sulphate, carbonate, calcium, magnesium and sodium concentration were detected.

4.2.2. Correlation of soil parameters

Table 4.8: Correlation of various soil parameters (*n* = 20) of the Karoo Nature Reserve, September 2000. Figures in *italics* are the correlation coefficients. Below them are the P-values in normal print. P-values < 0.05 are printed in bold. EC = electrical conductivity, SAR = sodium adsorption ratio.

EC S/m	**SAR**	**Cu mg/kg**	**Mo mg/kg**	**Zn mg/kg**	**SO_4^{2-} mg/kg**	**CO_3^{2-} %**	**C %**	**Ca mg/kg**	**Mg mg/kg**	**Na mg/kg**	
0.390	*-0.219*	*-0.162*	*-0.011*	*-0.238*	*0.442*	*-0.116*	*0.447*	*0.629*	*0.601*	*0.056*	**H+**
0.087	0.347	0.488	0.962	0.308	**0.050**	0.621	**0.048**	**0.003**	**0.005**	0.807	
	0.642	*0.483*	*-0.066*	*0.114*	*0.838*	*0.308*	*0.586*	*0.788*	*0.872*	*0.838*	**EC**
	0.002	**0.031**	0.777	0.626	**0.000**	0.182	**0.007**	**0.000**	**0.000**	**0.000**	
		0.531	*-0.189*	*0.236*	*0.457*	*0.219*	*0.286*	*0.194*	*0.343*	*0.904*	**SAR**
		0.016	0.418	0.311	**0.042**	0.347	0.218	0.407	0.136	**0.000**	
			-0.157	*0.765*	*0.289*	*0.387*	*0.154*	*0.230*	*0.281*	*0.538*	**Cu**
			0.504	**0.000**	0.213	0.091	0.508	0.324	0.225	**0.014**	
				0.120	*0.055*	*0.134*	*-0.164*	*-0.088*	*-0.070*	*-0.017*	**Mo**
				0.608	0.816	0.568	0.484	0.705	0.762	0.942	
					0.027	*0.354*	*0.197*	*-0.092*	*-0.057*	*0.296*	**Zn**
					0.906	0.122	0.399	0.695	0.807	0.201	
						0.297	*0.547*	*0.699*	*0.856*	*0.651*	**SO_4^{2-}**
						0.198	**0.013**	**0.000**	**0.000**	**0.002**	
							0.425	*0.428*	*0.390*	*0.387*	**CO_3^{2-}**
							0.060	0.058	0.088	0.089	
								0.575	*0.659*	*0.567*	**C**
								0.008	**0.002**	**0.009**	
									0.931	*0.475*	**Ca**
									0.000	**0.034**	
										0.600	**Mg**
										0.005	

CHAPTER 5

Discussion

5.1. Plants and copper

Truter & Louw (1959), analysed natural pastures in South Africa and Namibia to determine if pastures were deficient in copper. They concluded that pastures in the Graaff-Reinet district had sufficient copper content.

Analysis of the copper content in plants from the Karoo Nature Reserve was not performed, as the data that would have been obtained was regarded as unreliable for the following reasons:

- It was not possible to determine the diet of the blesbok as the flight distance of the blesbok was in excess of 60 m. Other studies on the grazing habits of blesbok (Du Plessis, 1968; Killian & Fairall, 1983) could not be used, as none of them took place in the Karoo or in an area of similar vegetation type.
- Sample selection of plant material for trace element analysis often does not correlate with what an animal ingests. Selective grazing and ingestion of soil as a result of grazing close to the ground are two important factors responsible for these differences (Judson & McFarlane, 1998).
- Under grazing systems with artificially planted pastures, samples plucked to simulate grazing may provide an approximate estimate of mineral content of the plants selected by sheep and cattle (Langlands & Holmes, 1978). Under extensive conditions, however, such sampling methods may not be valid due to the marked variability in plants selected. For instance, in semi-arid rangeland areas, it has been shown that 56% of the diet of sheep was composed of plant species that made up only 1% of the forage on offer (Leigh & Mulham, 1966), and 72% of the diet selected by cattle was from species making up 3% of forage on offer (Rosiere, Beck & Wallace, 1975).

- The quantity of soil ingested will increase under certain conditions, such as high stocking rates, in areas of poor drainage or where forage is sparse (Judson & McFarlane, 1998). Under farming conditions in Britain, soil has been shown to account for 1 to 18% of the dry-matter intake in cattle, and 10 to 14% of sheep, rising to perhaps as much as 40% in winter months (Thornton & Abrahams, 1981). In one study, soil ingestion provided 3-36% of the total zinc, and 6-16% of the total copper intake in cows (Thornton & Kinniburgh, 1978). Soil ingestion is an important source of trace elements where levels of the trace elements are appreciably higher in soil than in plants. Iron, a macromineral, would fall into this category. Soil of the Karoo Nature Reserve appeared to contain a high concentration of iron, as the metal was visible to the naked eye when a magnet was placed in the soil. When concentrated HCl was added to a soil sample, the soil discoloured the acid to a bright yellow colour (Huyser, D., pers. comm.). Iron is known to inhibit copper (Gawthorne, 1987) and to have an idea of iron intake in the blesbok, the degree of pasture contamination and soil intake would have to be quantified. This is possible by performing titanium assays of pasture and faeces, as titanium is present in relatively high concentrations in soils and in very small amounts in herbage (Thornton, 1983). Titanium is not absorbed by animals (Judson & McFarlane, 1998).
- The measurement of copper concentration in soil or the soil parent material does not relate directly with copper levels in plants. The levels in plants are modified by a range of factors, such as the different forms of copper in soil, the presence of other soil components and by the age, developmental stage, species and even cultivar of the plant. In young soils, however, the likely development of copper deficiency in plants tends to follow the concentration of copper in soils and geochemistry of the parent material (Delhaize, Loneragan & Webb, 1987). Sims (1986), found a positive relationship between exchangeable, organic and Fe-oxide bound copper and plant uptake, yet was unable to predict copper uptake in plants.

5.2. Blesbok

5.2.1. Grazing utilisation by blesbok in the Karoo Nature Reserve

It has been shown that blesbok graze almost exclusively in the Dam area of the Karoo Nature Reserve. During earlier years, the blesbok ranged more extensively than present day (Burdett, P., pers. comm.). A possible factor for the contraction of the range of the blesbok could be the degradation of the habitat and conversion of the landscape from grassland to dwarf shrubland (see Figure 3.6 – 3.9).

5.2.2. Storage of liver biopsies

The leaching of copper in liver samples stored in formalin has been shown. This may lead to significantly lower results for formalin fixed liver tissue when compared to frozen samples. Therefore, when the storage method of liver samples differs, the results of hepatic copper concentration obtained cannot be compared directly with each other. As the degree of leaching cannot be controlled, this study suggests that frozen liver should be used for the estimation of liver copper concentration.

5.2.3. Comparison of haemoglobin measuring instruments

The visual, filter photometer was found to be a satisfactory measurement of haemoglobin concentration in the blood. The photometer has the advantages of being portable, inexpensive, simple, quick and easy to use.

5.2.4. The effect of time and temperature on the activity of superoxide dismutase

Temperature and time was found to have an important effect of the activity of erythrocyte SOD. In the initial study involving Jersey cows, the activity of SOD activity appears to have been maintained for approximately four days at 22°C;

at least two days at 4°C, less than a week at -20° and for at least 6 months in liquid nitrogen.

The experiment was repeated, using five blesbok samples. It was shown that the activity of SOD was maintained for up to four days and less than six days (P = 0.0506 was taken as significant) when samples were kept at 4°C and -20°C. The activity of SOD was maintained for the duration of the study period (41 days), when samples were placed in liquid nitrogen.

There was a slight increase in SOD activity after the initial measurement for both bovine and blesbok samples. This might suggest that slight dehydration of the samples had taken place. The degree of dehydration was evaluated by monitoring the haemoglobin concentration of each sample throughout the experimental period. No increase in haemoglobin concentration was noted and it was concluded that no dehydration of the samples had taken place.

From this data, it is recommended that bovine and blesbok blood samples should be kept cool and analysed as soon as possible within 4 days for an accurate measurement of SOD activity. Storage of samples in liquid nitrogen is the only method of maintaining the activity of SOD for more than four days.

5.2.5. The copper status of blesbok

5.2.5.1 Hepatic copper concentration

The principal reason for measuring hepatic copper concentration in an animal is to provide an indication of the level of copper reserves. Additional reports in the literature were used to determine a mean hepatic copper level in clinically normal blesbok. As the standard deviation of these results was very large, a normal range for hepatic copper concentration in blesbok could not be determined. This variability occurs even when animals are fed the same diet and increases considerably in animals grazing at pasture (Paynter, 1987). A mean of 840 μmol/kg (*n* = 77) was determined in a study of copper-deficient

blesbok (Bigalke & Van Hensbergen, 1991). The lowest mean obtained from 'normal' blesbok was 1020 μmol/kg (Penrith et al., 1996). A hepatic copper value of < 950 μmol/kg is therefore suggested as being indicative of a copper deficient state in blesbok.

There are disadvantages in using hepatic copper concentration as an indicator of copper status in blesbok:

- A number of factors, such as species, age, diet and disease may affect hepatic copper concentration. Age of the animal affects copper concentration in the liver, in that greater hepatic copper concentrations are found in the foetus and the newborn when compared to the adult (Underwood, 1977). In bovines and cervines, foetal and neonatal hepatic copper concentrations may be five times higher that that of the dam, even when the dam is deficient in copper (Gooneratne & Christensen, 1985; Paynter, 1987). No preferential transfer of copper to the foetus appears to occur in sheep and goats (Owen, Proudfoot, Robertson, Barlow, Butler & Smith, 1965; Suttle & Field, 1968). In these species, hepatic copper concentration rises steadily from birth.
- Hepatic copper concentration may not always accurately reflect copper availability to the animal. For example, when molybdenum and sulphur interfere with copper metabolism, the measurement of hepatic copper concentration has the potential to overestimate copper availability to the animal, as a proportion of the copper in the liver may be unavailable to the animal.
- The distribution of copper in the liver itself is uneven (O Cuill, Hamilton & Egan, 1970), the caudate lobe having higher concentrations than other lobes (Bingley & Dufty, 1972).
- Due to the large variation in hepatic copper concentration, a normal range cannot be determined in this species. Care needs to be taken in the interpretation of low liver copper concentration, as values in healthy animals can also fall below the accepted level of normality (Suttle, 1986).

- Hepatic biopsies are invasive, time-consuming, sterile procedures have to be practised and there is the potential for complications.

For the above reasons, hepatic biopsies cannot be recommended as a routine procedure to evaluate copper status in blesbok.

5.2.5.2. Plasma copper concentration

In normal adult animals, copper concentration in the blood show an approximately equal distribution between the red cells and plasma, with platelets and leukocytes contributing little. In both cattle and sheep, serum copper concentrations are consistently lower than those in plasma. The difference is attributed to a rapid sequestration of ceruloplasmin into the clot during clot formation (Paynter, 1982).

When the dietary molybdenum concentration is > 8mg/kg, measurement of total plasma copper concentrations in the sheep may underestimate the degree of insufficiency, as nearly 40% of the total plasma copper may be unavailable to the animal. As a result, a non-ceruloplasmin copper fraction, which is insoluble in dilute TCA acid at ambient temperatures, appears in the plasma (Smith & Wright, 1975).

Plasma sampling has advantages over liver sampling in determining the copper status of blesbok. The procedure is minimally invasive and samples are quick and easy to obtain, with very little equipment required. This study shows that the variation in plasma copper concentration is less than for hepatic copper concentration. The TCA acid-soluble copper concentration in plasma is therefore recommended as the indicator of choice to evaluate copper status in blesbok.

5.2.5.3. Erythrocyte superoxide dismutase

Erythrocyte SOD activity is a valuable indicator of chronic copper deficiency as it is a measurement of the functional deficiency of copper present in an animal. In adult animals, relatively few factors, apart from dietary copper availability have been reported to affect copper superoxide dismutase activity. Changes do not occur with stress, infection, parasitism (McMurray, 1980; Arthington, Corah & Blecha, 1996), inflammation (DiSilvestro & Marten, 1990), or dietary iron intake (Reffett, Spears & Prabowo, 1986). Severe zinc deficiency also does not affect SOD activity (Bettger, Fish & O'Dell, 1978)

Advantages to the use of red cell superoxide dismutase become more limited when the copper insufficiency is acute. In these situations, the half-life of the enzyme is likely to be longer than the half-lives of copper enzymes in other tissues (Paynter, 1987). Another disadvantage is that the assay can be difficult to perform. It is important that the erythrocytes are thoroughly washed, as plasma inhibitors may affect the results. In addition, the lysates must be diluted to minimise interference from red blood cell constituents, which probably include haemoglobin (Underwood & Suttle, 1999). As the normal range of erythrocyte superoxide dismutase activity in blesbok was lower than that reported in domestic animals, such as bovines, it was required that the samples be less diluted. This increased the possibility of interference by plasma inhibitors or haemoglobin. The detection limits of SOD activity was approached with the assay kit in the analysis of the blesbok samples from the Karoo Nature Reserve. The assay is not performed at most diagnostic laboratories in South Africa and therefore, the measurement of SOD activity to evaluate copper status in blesbok cannot be recommended.

Gärtner & Weser, 1983, reported that 95% of erythrocyte copper was found in superoxide dismutase. Measurement of erythrocyte copper should, therefore, correlate well with superoxide dismutase and would be less problematical and as useful as measurement of SOD activity (Underwood & Suttle, 1999). However, copper associated with superoxide dismutase has been reported to only make up as little as 60% of the total red blood cell copper (Underwood, 1977; Evans, 1973). The second fraction of erythrocyte copper is in a labile pool. Neumann & Silverberg (1967), suggested that this compartment

contains copper complexed with amino acids and may function as a source of copper for superoxide dismutase. Masters, Smith & Casey (1985), obtained equivocal results when examining the relationship between the activity of erythrocyte superoxide dismutase and erythrocyte copper concentrations. Their results indicated that in some circumstances, TCA acid-soluble copper could exist in bovine erythrocytes without contributing to SOD activity.

5.2.6. Correlation of copper data

A significant correlation between hepatic and plasma copper concentration was shown. This provides an additional advantage for the use of plasma copper concentration as the indicator of choice to evaluate copper status in blesbok.

It was not possible to predict copper status from the degree of ataxia, coat colour and condition score. The degree of ataxia represents the copper status at the time of myelination of the central nervous system and not necessarily a reflection of current copper status. Coat colour is not a specific sign of copper deficiency and achromotrichia may, for example, be caused by vitamin or cobalt deficiencies (Underwood & Suttle, 1999). Condition score is affected by numerous factors such as nutrition and verminosis and is not specific for copper status.

5.2.7. Copper deficiency and fertility

Even though there has been an increase in blesbok numbers in the Karoo Nature Reserve since 1995, the fertility of the blesbok from the Karoo Nature Reserve appears to be affected. The 2000 recruitment rate of 5% for blesbok in the reserve compares poorly with recruitment rates of 57% in a good year at Rietvlei (Du Plessis, 1972), a mean of 33% for 21 blesbok herds in Kwazulu-Natal (Marchant, 1987) and 34% for blesbok from Brakkekuil (Bigalke & Van Hensbergen, 1991). The pregnancy rate of 14% for the

blesbok in the Karoo Nature Reserve compares poorly with 84% (*n* = 473) (Du Plessis, 1972) and 64% (Bigalke & Van Hensbergen, 1991).

The above records are mainly observational and the sample size is too small to draw any definitive conclusions. Although it has been suggested that copper deficiency affects reproductive performance (Howell, 1968; Flynn et al., 1977; Fell, 1987), poor reproductive performance can be influenced by a wide variety of factors, such as malnutrition or stress, for example.

An attempt was made to compare the copper status of lactating and non-lactating females. The sample size of animals was, however, too small to draw any definitive conclusions.

5.2.8. Other factors that affect the copper status of blesbok

The Game Viewing Area of the Karoo Nature Reserve appears to be heavily overgrazed (Figure 3.6 – 3.9), due to the high stocking density of animals and ostriches. Census figures for 2000 showed the following numbers in the Game Viewing Area: black wildebeest (*Connochaetes gnou*) – 141; buffalo (*Syncerus caffer*) – 102; gemsbok (*Oryx gazella*) – 148; kudu (*Tragelaphus strepsiceros*) – 194; ostrich (*Struthio camelus*) – 138; red hartebeest (*Alcelaphus buselaphus*) – 105; springbok (*Antidorcas marsupialis*) – 151; steenbok (*Raphicerus campestris*) – 48. All the animals concentrate around the Dam area, which covers about 1000 ha. The total area of the Game Viewing area is estimated at 3 500 ha. The high stocking density will impact on the vegetation, with the result that animals are forced to graze more unpalatable and innutritious vegetation. Trace element deficiencies may be the result.

Seasonal trends in the copper status of animals have been shown. A decline in copper status in winter with a rise in spring has been reported (Flynn et al., 1977; Audigé et al., 1995). In contrast, a rise in hepatic copper concentration during winter has also been reported (Grant, Biggs & Meissner, 1996). Samples from Willem Pretorius Game Reserve were collected in early May,

2000, while samples from Karoo Nature Reserve were collected at the end of August, 2000. Although the two sets of samples were collected in different months, it still remained within the same season i.e. the beginning and end of winter. It was assumed that a seasonal trend in the copper status of blesbok did not play an important role in this study.

A genetic predisposition of blesbok to the development of copper deficiency is possible. The majority of reports of copper deficiency in non-domestic ungulates involve blesbok or their close relatives – bontebok (*Damaliscus dorcas dorcas*). The normal range of copper values in blesbok is a lot lower than in domestic animals, such as cattle and sheep.

5.3. Soil and water

5.3.1. Extraction method

A di-ammonium EDTA extraction was performed for various trace elements to provide an estimate of the concentration of trace elements available to plants and thus the concentration of trace elements in the plants themselves. EDTA forms soluble metal-chelate complexes with the free-metal ions in solution. Replenishment of free-ions in the soil solution from solid phases occurs in response to this complexation. The solution is buffered near neutrality (pH 7.3) to avoid dissolution of carbonate minerals that could release unavailable micronutrients. The quantity of micronutrients extracted by a chelate reflects both the initial concentration in the soil solution (intensity factor) and the ability of the soil to maintain this concentration (capacity factor) (Viets & Lindsay, 1973). The process of nutrient removal by plant roots and the subsequent replacement of ions from labile solid-phases in soil is thus mimicked by chelating agents. Although neither the total contents of copper in plants, nor concentration in shoots is strongly related to extractable Cu, absorption per unit of root can be predicted from the measurement of available copper in soil (Jarvis & Whitehead, 1981).

5.3.2. Salinity and sodicity

According to the US Salinity Laboratory Staff (1954), soil of the Karoo Nature Reserve is not saline nor sodic. In the arid regions of South Africa, ESP is two to three times higher than SAR, as a result of the high calcium and magnesium content in the soil (Nel, P., pers. comm.). Even taking this into account, it is unlikely that the soil is sodic. The lack of salinity or sodicity may possibly be explained by the vertical movement of salts to subsurface horizons, which were not sampled.

5.3.3. Dam and control areas

Copper uptake by plants has been reported to be predicted by inclusion of soil organic matter content with the level of extractable copper (Martens, 1968). A significant difference was found in the organic matter content between the Dam and control areas. This can possibly be explained by the different vegetation types - the Dam Area is dominated by monocotyledons (*Cynodon spp.*) with adventitious root systems and the surrounding area by dicotyledonous bushes with tap root systems. When soil is sampled, more root tissue will be taken with the sample if the area is dominated by monocotyledonous plants rather than with dicotyledonous plants. The difference in vegetation types may be explained by differing availability to water and the process of soil enrichment (Palmer, Novellie & Lloyd, 1999). The process of soil enrichment is associated with the replacement of tall, tufted grass by prostrate creeping species, such as *Cynodon spp.* These replacement species are adapted to conditions of high nutrient availability under conditions of intense grazing ('greedy feeders') (Roux, 1969). Such conditions occur with blesbok as they focus their grazing on specific patches and territorial males tend to defecate in middens (Du Plessis, 1972). These middens are usually surrounded by lawns of creeping grass. A positive feed back system probably occurs as the herbivores favour these patches due to the increased nutritional value of these grasses. The process appears to be

driven primarily by nutritional availability, rather than selective grazing (Roux, 1969).

For the other measured parameters, the null hypothesis that there is no statistical difference in soil parameters from the Dam and control areas of the Karoo Nature Reserve cannot be rejected. A few interesting observations can, however, be made.

None of the measured parameters in soil were unusual for the general area, except for the higher pH (Loock, A., Nel, P., pers. comm.). The highest pH of soil occurs between the 780 to 785m contour levels. The dam was last 100% full in 1988. Since then, this area has been exposed and it is this area where the blesbok spend the majority of their time.

It is suggested that the high pH of soil, associated with organic matter, is responsible for a decreased availability of copper to plants.

The interpretation of the results was made difficult by the lack of published reports on copper concentrations in southern African soils and the factors affecting it. It would have been beneficial to analyse soil samples at Willem Pretorius and Gariep Dam Game Reserves, but this was not possible due to financial constraints.

5.3.4. Correlation of soil data

Correlation of various soil parameters in the Karoo Nature Reserve produced no significant negative correlations although a number of significant positive correlations were found. Only significant correlations with copper will be discussed.

A highly significant correlation between copper and zinc was obtained. Copper and zinc are similar in nature and processes that affect the availability of copper will affect the availability of zinc in a similar manner.

Copper was positively correlated with sodium and therefore, also with SAR and EC. Copper availability decreases with increasing pH (McClaren & Crawford, 1973; Elsokkary & Lag, 1978; Sims & Patrick, 1978) and as sodium influences pH through the formation of NaOH, it was expected that there would be a negative correlation with sodium/SAR. Why a positive correlation was obtained is difficult to explain. It may be that the pH range of soil in the Karoo Nature Reserve was fairly narrow (7.30 – 10.18) and the correlation of sodium and copper is therefore unreliable. Alternatively, it may be possible that through the formation of saline or sodic soils by dehydration, copper may also concentrate in the soil. Copper is, however, one of the most immobile elements in soil (Hodgson, 1963).

5.3.5. Water

The importance of water quality in the development of copper deficiency in blesbok in the Karoo Nature Reserve is difficult to determine. Two boreholes and the dam in the reserve are potential water sources. During the whole observation period, no blesbok were observed to drink from these water sources, although a blesbok was once noted near the top borehole (32°10'50S; 24°30'48E). Free standing pools and puddles in the Sundays and Pienaars riverbeds are numerous and it is assumed that the blesbok meet their water needs at these water points. These water points could not be sampled due to the thick vegetation and the threat from buffalo.

The water quality in the boreholes and dam appears to play an insignificant role in the copper status of the blesbok in the Karoo Nature Reserve.

CHAPTER 6

Summary and Conclusions

During 1980, nine male and thirteen female blesbok were introduced into the Karoo Nature Reserve, Graaff-Reinet, South Africa. In 1992, achromotrichia and ataxia developed in some blesbok and some animals died. 'Swayback' was diagnosed histopathologically in one affected blesbok and on the basis of copper levels in the blood, faeces and liver of the affected animal (Penrith, Tustin, Thornton & Burdett, 1996).

By 1993, the population of blesbok had reached 160 animals but the population had declined to 88 by 1994 and high mortalities among young animals were reported. None of the lambs born in 1994 survived (Penrith et al., 1996). Since 1995, there has been a slow, but constant increase in the blesbok population.

This study was planned to evaluate the copper status of a representative proportion of the blesbok population in the Karoo Nature Reserve, by measuring copper levels in liver and plasma, and superoxide dismutase activity (SOD) in erythrocytes of these animals. The results were compared to normal values determined from blesbok in Willem Pretorius Game Reserve and Gariep Dam Game Reserve, Free State. In addition, any correlation between the various indicators was determined and an indicator that best represented the clinical picture suggested.

The next part of this study was to determine the cause of the copper deficiency. It was suspected that the dam in the reserve was causing minerals to be leached from the soil and that low copper levels in the soil and herbage were the result (Penrith et al., 1996).

Soil samples were collected from the Dam Area (the area covered when the dam is full) and compared to control samples taken from the area surrounding the dam that has remained unflooded. In addition to the analysis of copper in the soil, factors affecting copper availability in the soil, viz. pH, zinc, carbonate and organic matter were included in the soil analysis (McBride, 1981; Moraghan & Mascagni, 1991; Shuman, 1991). Trace elements that interact with copper in the mammalian system, viz. molybdenum, sulphate and zinc (Underwood & Suttle, 1999) were also included in the soil analysis. Data was correlated to determine if any relationships exist between any of the measured parameters.

The following conclusions were made:

- Storage of liver samples in formalin can cause leaching of copper into the formalin.
- A visual, filter photometer was found to compare well with a coulter counter for the measurement of haemoglobin.
- To obtain accurate measurements of SOD activity, samples should be kept cool and analysed as soon as possible within four days.
- Storage of blood samples in liquid nitrogen is the only effective method of maintaining SOD activity for longer than four days.
- Trichloracetic acid-soluble copper concentration in plasma is recommended as the indicator of choice to evaluate copper status in blesbok.
- A hepatic copper value of < 950 μmol/kg is suggested as being indicative of a copper deficient state in blesbok. The normal range of copper concentration in blesbok plasma is 20.41 – 29.95 μmol/l. The normal range of erythrocyte superoxide dismutase activity in blesbok is 35.98 – 135.35 U/ml; 254.83 – 846.97 U/g haemoglobin.
- The copper status of blesbok from the Karoo Nature Reserve is significantly lower than the expected normal range for blesbok.
- The surface horizons of soil from the Karoo Nature Reserve is not saline nor sodic.

- The null hypothesis: Measured soil parameters in samples from the Dam Area of the Karoo Nature Reserve do not differ significantly from the control areas could not be rejected.
- Leaching of the surface horizons of soil does not appear to play a role in the development of copper deficiency in blesbok from the Karoo Nature Reserve.
- It is suggested that alkaline soil, associated with organic matter, is responsible for a decreased availability of copper to plants.
- The water quality of the Van Ryneveldpas dam and boreholes is thought to play an insignificant role in the development of copper deficiency in blesbok from the Karoo Nature Reserve.

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Appendix 1 – Blesbok data

Date	Identification	Sex	Age	Liver Cu (formalin)★	Liver Cu (frozen)★	Plasma Cu✧	HGB (Hb-meter)❖	HGB (coulter counter)❖	SOD (Units/ml)	SOD (Units/g HGB)	Condition score	Ataxia score	Achromotrichia	Comments
Willem Pretorius Game Reserve														
05/05/00	3	F	3P	1700	1841	26.91	16.10	18.10	106.6	662.0				Lact.
05/05/00	4	F	1P	551	614	24.23	15.50	17.20	66.0	426.1				
05/05/00	5	M	1P	2423	2376	24.23	17.40	17.90	90.1	518.1				
05/05/00	14	M	1P	2408	2675	28.01	16.75	18.70	86.1	514.0				
05/05/00	17	M	2P	2927	3116	26.44	14.75	17.00	93.9	636.4				
05/05/00	20	M	3P	3761	4233	27.54	14.50	14.80	102.1	703.8				
05/05/00	18	M	1P		1070	26.44	17.20	18.70	127.0	738.6				
06/05/00	11	F	3P		2785	26.59	15.75	16.80	84.7	537.9				Lact.
06/05/00	60	M	3D		1888	27.38	18.10	20.10	66.3	366.3				
06/05/00	63				11661	23.29	15.80	17.60	72.0	456.0				
05/05/00	13				3163	26.75	15.90		100.0	629.2				
05/05/00	7	F	3D		2770	25.49	15.60		127.0	813.8				
06/05/00	8	F	3P		2093	25.97	17.40		106.8	613.8				Lact.
06/05/00	9	M	3P		4107	30.06	16.10		110.3	684.9				
06/05/00	10	M	1P		1888	22.50	16.25		97.0	596.7				
06/05/00	62	M	1P		3604	24.86	13.80		77.4	561.2				
05/05/00	1	M	1P	1180	1385	28.96	15.60		124.1	795.5				
05/05/00	2	M	1P	1825	2187	22.35	15.60		103.9	665.9				
05/05/00	15	M	1P	2518	2298	22.50	14.00		96.0	685.4				
05/05/00	21	F	3D	504	551	20.77	15.80		69.3	438.6				
05/05/00	6	M	1P	2486	2565	23.29	17.40	18.70						
05/05/00	16	M	3P	3368	3541	31.32	13.60	14.90						
06/05/00	61	M	3P	991	1479	26.91	16.50	18.60						
05/05/00	22	F	3D	2880	3069									
05/05/00	51	F	3P	1511	1637									
05/05/00	52	F	3P	2282	2486									
05/05/00	A3	M	1P	3210	3132									
05/05/00	Anon1			1621	1888									
05/05/00	Anon2			1652	2140									
06/05/00	24	F	3P		4092									Preg.
06/05/00	25	F	1P		1180									
06/05/00	26	F	3P		3147									
06/05/00	64	M	3P		3667									
05/05/00	A4	F	3D		1369									
06/05/00	A5	M	3P		6672									
06/05/00	A6	F	3D		3069									
06/05/00	A7	F	3P		1621									

Date	Identification	Sex	Age	Liver Cu (formalin)★	Liver Cu (frozen)★	Plasma Cu✧	HGB (Hb-meter)❖	HGB (coulter counter)❖	SOD (Units/ml)	SOD (Units/g HGB)	Condition score	Ataxia score	Achromotrichia	Comments
Gariep Dam Game Reserve														
05/02/01	X1					25.28	16.40	16.20	63.1	389.7				
05/02/01	X2					21.98	13.20	12.80	39.1	305.7				
05/02/01	X3					22.45	14.60	14.00	52.8	377.4				
05/02/01	X4					23.55	15.80	15.50	53.6	346.0				
05/02/01	X5					16.69	12.80	12.00	63.0	525.0				
05/02/01	X6						14.70	14.60	49.0	335.7				
05/02/01	X7				708									
05/02/01	X8				850									
05/02/01	X9				2267									
05/02/01	X10				1118									
05/02/01	X11				1354									
05/02/01	X12				693									
05/02/01	X13				425									
05/02/01	X14				1338									
05/02/01	X15				3305									
05/02/01	X16				1181									
05/02/01	X17				3368									
Graaff-Reinet Nature Reserve														
11/07/00	GR1	M	Adult		377	0.63	12.40		8.0	64.2	3.0	NP	+	
19/07/00	GR2	M	Adult		426	1.10	14.70		39.0	265.0	3.0	NP	+	
17/08/00	GR3	M	Adult		138	1.10	7.75		6.3	80.9	3.0	NP	++	
31/08/00	GR4	M	Adult		116	0.79	14.00		16.2	115.6	3.0	+++	+	
31/08/00	GR5	F	Adult		79	0.79	8.00		5.0	62.9	2.5	++	+++	✞
31/08/00	GR6	F	Adult		82	0.47	12.80		21.3	166.4	3.0	NP	-	
31/08/00	GR7	M	Adult		132	0.94	13.70		15.8	115.2	3.0	+++	+	CM
31/08/00	GR8	M	Adult		215	1.57	14.75		21.9	148.5	2.0	-	++	
31/08/00	GR9	M	Adult		195	0.63	14.20		19.3	135.6	3.0	+++	-	
01/09/00	GR10	F	Adult		574	0.94	16.60		32.5	196.0	2.5	++	+	Preg. ✞
01/09/00	GR11	F	Adult		93	0.94	10.50		8.8	84.2	2.5	+++	+	
01/09/00	GR12	F	Adult		105	0.79	13.50		15.8	117.3	3.0	-	+	
01/09/00	GR13	F	Adult		138	0.94	13.60		16.1	118.5	3.0	++	+	CM
01/09/00	GR14	F	Adult		850	1.73	16.70		41.3	247.5	3.0	+	-	
MSD Pty Ltd.														
26/11/00	Z1	M	Adult			ND	7.20	6.70	14.2	196.5				

Date	Identification	Sex	Age	Liver Cu (formalin)★	Liver Cu (frozen)★	Plasma Cu✧	HGB (Hb-meter)❖	HGB (coulter counter)❖	SOD (Units/ml)	SOD (Units/g HGB)	Condition score	Ataxia score	Achromotrichia	Comments
26/11/00	Z2	F	Adult			ND	5.50	5.50	8.6	156.1				
26/11/00	Z3	F	Adult			ND	11.50	10.90	11.8	102.8				
26/11/00	Z4	F	Adult			2.51	15.40	15.20	60.3	391.8				
26/11/00	Z5	F	Adult			2.83	16.00	14.20	58.3	364.6				

Legends

★	Values in μmol/kg
✧	Values in μmol/l
❖	Values in g/dl
✞	Found dead the day after collection of samples. Post-mortem revealed the cause of death to be capture myopathy
CM	Chew marks on dorsal neck, an indication of mineral deficiency (Meltzer, DGA, pers. comm.)
D	Deciduous incisors
F	Female
HGB	Haemoglobin
Lact.	Lactating
M	Male
ND	No copper detected
NP	Not present
P	Permanent incisors
Preg.	Pregnant
SOD	Superoxide dismutase

Appendix 2 – Soil data

ID	Calcium				Magnesium				Sodium				Saturation
	mg/l	$mmol^+/l$	mg/kg	$cmol^+/kg$	mg/l	$mmol^+/l$	mg/kg	$cmol^+/kg$	mg/l	$mmol^+/l$	mg/kg	$cmol^+/kg$	%
Q 1	109.42	5.36	64.69	0.32	28.98	2.38	17.13	0.14	26.22	1.14	15.50	0.07	59.1
Q 2	78.84	3.86	41.21	0.21	35.86	2.95	18.74	0.15	21.28	0.93	11.12	0.05	52.3
Q 3	111.70	5.48	72.94	0.36	35.96	2.96	23.48	0.19	60.59	2.64	39.57	0.17	65.3
Q 4	1359.45	66.64	815.67	4.07	1030.20	84.76	618.12	5.09	1725.33	75.05	1035.20	4.50	60.0
Q 5	21.60	1.06	9.48	0.05	10.82	0.89	4.75	0.04	435.45	18.94	191.09	0.83	43.9
Q 6	17.82	0.87	9.01	0.04	5.71	0.47	2.89	0.02	117.17	5.10	59.21	0.26	50.5
Q 7	49.10	2.41	22.95	0.11	16.17	1.33	7.56	0.06	24.99	1.09	11.68	0.05	46.7
Q 8	19.26	0.94	8.15	0.04	7.89	0.65	3.34	0.03	49.44	2.15	20.93	0.09	42.3
Q 9	345.07	16.92	246.77	1.23	98.12	8.07	70.17	0.58	185.20	8.06	132.44	0.58	71.5
Q 10	141.18	6.92	91.13	0.45	41.71	3.43	26.92	0.22	86.14	3.75	55.61	0.24	64.6
Q 11	67.49	3.31	31.63	0.16	20.10	1.65	9.42	0.08	88.88	3.87	41.66	0.18	46.9
Q 12	683.51	33.51	345.66	1.72	273.16	22.47	138.14	1.14	82.10	3.57	41.52	0.18	50.6
Q 13	21.45	1.05	7.43	0.04	6.89	0.57	2.39	0.02	6.02	0.26	2.08	0.01	34.6
Q 14	26.21	1.28	10.79	0.05	7.08	0.58	2.91	0.02	8.46	0.37	3.48	0.02	41.2
Q 15	83.00	4.07	56.85	0.28	29.72	2.45	20.36	0.17	61.20	2.66	41.92	0.18	68.5
Q 16	33.09	1.62	19.09	0.10	7.70	0.63	4.44	0.04	152.24	6.62	87.85	0.38	57.7
Q 17	950.99	46.62	430.04	2.15	348.26	28.65	157.48	1.30	5095.63	221.65	2304.24	10.02	45.2
Q 18	53.25	2.61	25.91	0.13	31.09	2.56	15.13	0.12	86.40	3.76	42.05	0.18	48.7
Q 19	26.81	1.31	10.43	0.05	5.99	0.49	2.33	0.02	16.32	0.71	6.35	0.03	38.9
Q 20	33.19	1.63	12.91	0.06	4.76	0.39	1.85	0.02	7.94	0.35	3.09	0.01	38.9