PHOSPHORUS AND CALCIUM EXTRACTION FROM BONE DIGESTION IN THE RUMEN OF SHEEP (*OVIS ARIES*)

by

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SUMMARY

Phosphorus and Calcium extraction from bone digestion in the rumen of sheep (*Ovis aries*).

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Sheep were used to model the digestion of bone in the rumen. The model of ruminal bone digestion was used to identify a possible source of phosphorus and calcium for ruminants, with particular focus on giraffes. The daily requirements for phosphorus and calcium by giraffes to sustain

skeletal growth and maintenance are large. The source of sufficient calcium is browse. The source of necessary phosphorus is obscure, but it could be via osteophagia, a frequently observed behaviour in giraffes. The possibility of ingested bone being digested in the rumen was assessed. Bone samples from cancellous and dense bones were immersed in distilled water, artificial saliva, and in the rumens of five sheep, for a period of up to 30 days. Distilled water had no effect on bones. Dense (metacarpal shaft) bone samples were softened by exposure to saliva and rumen fluid, but neither calcium nor phosphorus concentration was affected. Cancellous (cervical vertebrae) bone samples also softened and the mass and volume of the samples decreased over the period, especially as a result of exposure to saliva, but they also lost little calcium and phosphorus. In conclusion the use of sheep to model the possible rumen digestion of bone established that although saliva and rumen fluid can soften ingested bones, it is unlikely that ingested bone provides any significant source of minerals while in the rumen, for giraffes and ruminants in general.

Keywords: osteophagia, phosphorus, calcium, giraffe (*Giraffa camelopardalis*).

Chapter I

INTRODUCTION

The thought of an ungulate seeking out bones to feed on is one that most scientists and travellers would consider bizarre. The fact that such a bizarre behaviour is a frequent occurrence in giraffes (*Giraffa camelopardalis*) (Linnaeus, 1758) was the driving force behind this research project.

The giraffe is a truly fascinating animal and is unique in both appearance and demeanour. Limited research that has been done on this species focused on evolution, habits, feeding ecology and some physiological attributes (cardiovascular, respiratory and thermoregulation), and most recently skeletal physiology and biomechanics.

Two main factors lead to questions regarding the phosphorus (P) and calcium (Ca) intake of giraffes:

- Phosphorus and Ca daily requirements to sustain the growth and maintenance of their skeletons are large.
- The source of sufficient Ca has been identified as browse however the source of necessary P is obscure.

Due to the nature of this project it was impossible to conduct the research using giraffes as a model to test whether bones are digested in the rumen. Sheep were therefore used as test subjects to model the possible extraction of P and also Ca during possible digestion of bone in the rumen. The aim was to establish if bone fragments entering the rumen undergo digestion.

1.1 LITERATURE REVIEW & BACKGROUND

Osteophagia, the depraved appetite

A depraved appetite or 'pica' is a well documented phenomenon in African ungulates and occurs in ruminants both domestic and wild (Theiler *et al*. 1924; Green 1925; Sutcliffe 1973; Anderson 1974; Sekulic & Estes 1977; Hampton 2002). Pica is associated with all forms of abnormal appetites, which can be referred to as allotriophagia. The practise of osteophagia, the appetite for bone, is the particular form of pica focused on in this study.

The peculiar behaviour of osteophagia was recorded as early as 1796 in the southern African region, where cattle (*Bos taurus / indicus*) were observed seeking out bones lying in the veld (Denton 1982). It was Theiler *et al.* (1924) who were the pioneers of the study of P deficiency in cattle and they established the relationship between osteophagia and botulism.

They identified that P was a crucial factor for the growth rate and maintenance of live weight of cattle under ordinary veld grazing conditions, and by supplementary feeding with P they were able to eliminate both osteophagia and botulism.

Theiler *et al.* (1924) also established that P deficiency was manifest all year round however it declined or temporarily disappeared in some animals in the spring when youngest new growth of grass becomes available. They also established that the supplementation of compounds such as chalk, iron sulphate, sulphur and so on had no influence on the occurrence of osteophagia.

Osteophagia occurring in natural conditions has a distinct geographical distribution that depends largely on the P content of the parent rock on which food plants are growing (Sutcliffe 1973). Underwood and Suttle (1999) elaborated further by indicating that the widespread areas of P deficiency for grazing livestock occur throughout the world and arise mostly from a combination of soil and climatic effects on herbage P concentrations. Factors such as excessive Ca, aluminium or iron, can also reduce the availability of P to plants (Sutcliffe 1973). It is well known that veld pastures of southern Africa are generally deficient in P (Theiler *et al*. 1924; Sutcliffe 1973; Denton 1982; Underwood & Suttle 1999) and Theiler

et al. (1924) identified osteophagia as the most obvious clinical symptom of P deficiency in cattle.

The appetite for bone

The appetite for bones observed in P deficient cattle is innate, specific and cued mainly by the smell of bones (Denton *et al*. 1986; Blair-West *et al.* 1989). Studies conducted by Denton *et al*. (1986) and Blair-West *et al*. (1989) show that by exteriorising the parotid salivary duct, which prevents the recycling of P, and feeding a low P diet to cattle will induce an appetite for bones. A complete block of the appetite for bones can be achieved within one hour by intravenous infusion of sodium phosphate sufficient to raise the inorganic phosphate fraction of blood plasma to normal levels thus indicating that the phosphate concentration in blood appears to regulate bone appetite.

Osteophagia can thus be associated with a decline in the inorganic phosphate fraction of blood plasma and also with the withdrawal of Ca and P from the reserves in bones (Denton 1982; Denton *et al*. 1986; Blair-West *et al*. 1989; Underwood & Suttle 1999). Phosphorus deficiency in cattle is obtained when the concentration of inorganic P in blood plasma drops below 1.0 mmol/l, above which no interest in bones is shown (Denton *et al*. 1986). Phosphorus deficient cattle presented with various

trays containing crushed bone or Ca or P salts (calcium sulphate, calcium phosphate, sodium phosphate and calcium carbonate) illustrate an active exploratory behaviour and seek out the crushed bone, which is then chewed vigorously (Denton *et al*. 1986).

The risks of eating bone

A diet deficient in P and Ca or the severe lack of vitamin D over a prolonged period results in abnormalities, subnormal growth, impaired fertility and the development of pica (Blair-West *et al.* 1989; Underwood & Suttle 1999). The subsequent development of osteophagia can result in a number of problems. It can be linked to injuries or even death from lodged bones (Anderson (1974); it reduces grazing time and causes teeth wear (Barrette 1985); and it increases the risk of contracting botulism from ingestion of the Type D toxin produced by the anaerobic bacterium (*Clostridium botulinum*) (Theiler *et al.* 1924; Denton 1982; Denton *et al*. 1986; Blair-West *et al*. 1989; Underwood & Suttle 1999).

Ruminal digestion

Ruminants have evolved a four chambered stomach that allows for pre-gastric digestion, which is made possible through the presences of microorganisms. Rumination is the maximum specialization in

fermentation digestion (Merchen 1988). The first two compartments of the stomach, the rumen and reticulum (reticulo-rumen), allow for the retention of fibrous food, which is an essential mechanism for the maximum extraction of energy (Van Soest *et al*. 1988). The maximum extraction of energy from ingesta is made possible by the complex structure of the reticulo-rumen and the omasum, which collectively sort the slower digesting fibre from the more easily digestible portions of the diet (Van Soest *et al*. 1988). The abomasum and small intestine are essential for the digestion and absorption of lipids, protein, vitamins, many minerals and any non-structural carbohydrates that have escaped ruminal fermentation (Merchen 1988).

Food is diluted with copious amounts of saliva, first during initial ingestion and again during rumination (Van Soest *et al*. 1988; Yano *et al*. 1991; McDonald *et al*. 2002). Rumen contents can be separated into two layers, a lower liquid layer in which the finer food particles are suspended, and a drier upper layer (raft) of coarse fibrous material (Church 1976; McDonald *et al*. 2002). The breakdown of food is accomplished partly by physical and partly by chemical means (Church 1976). The contents of the rumen are continually mixed by rhythmic contractions and during rumination material at the anterior end is drawn back into the oesophagus and returned to the mouth where coarser material undergoes mastication

before being returned to the rumen (Van Soest *et al*. 1988; McDonald *et al*. 2002).

The reticulo-rumen provides a continuous culture system for anaerobic bacteria, protozoa and fungi. Food and water enter the rumen and the food is partially fermented to yield principally volatile fatty acids (VFA), microbial cells, and the gases methane and carbon dioxide (Owens & Goetsch 1988). The gases are lost through eructation and the VFA are mainly absorbed through the rumen wall (Merchen 1988). The microbial cells, together with undegraded food components, pass to the abomasum and small intestine where they are digested by enzymes and the products of digestion are absorbed (Merchen 1988; Van Soest *et al*. 1988). An important note to add is that although giraffes are frequently observed chewing bones I was unable to find any reports, which have investigated the presence of bones in the rumen or intestine.

All ingesta passing from the oesophagus into the stomach must pass through the reticulo-omasal orifice before proceeding to the omasum and then to the abomasum, where gastric digestion can occur (Reid *et al.* 1991). To do so the size of the particles must be small. The reticulo-rumen is effective at achieving small particle size: in sheep digestive organs beyond the reticulo-omasal orifice contain few particles greater than 1 mm in length (Poppi *et al.* 1980).

Ruminant saliva

Ruminants secrete large amounts of saliva from the salivary glands, which are swallowed and enter the rumen; sheep are estimated to secrete 10 liters/day (Van Soest *et al*. 1988; Yano *et al.* 1991; McDonald *et al*. 2002). Ruminant saliva helps to maintain a desirable physico-chemical environment for microbial fermentation, especially counteracting the tendency for rumen pH to fall, by its bicarbonate concentration (Church 1988; Jacques et al. 1989).

Calcium concentrations in saliva are similar to plasma Ca concentrations however P concentrations are considerably higher than that of plasma (Yano *et al*. 1991). The salivary glands of ruminants therefore have an important part to play in the regulation and homeostasis of P (Clark *et al*. 1973; Tomas & Somers 1974). Phosphorus supply from saliva is also important for rumen microbial nutrition (Milton & Ternouth 1984; Ternouth *et al*. 1985).

Availability of calcium and phosphorus to ruminants

Calcium and P are the two most abundant mineral elements in the animal body. The availability of Ca and P in different feeds may vary considerably

according to their chemical combination or physical association with other compounds (McDowell 1992; Underwood & Suttle 1999). They are frequently found in insufficient quantities in common feedstuffs to meet requirements of livestock, with a P deficiency being predominately a condition of grazing ruminants whereas a Ca deficiency is more a problem of animals fed mostly on concentrates (McDowell 1992; Perry *et al.* 1999; Underwood & Suttle 1999).

Phosphorus enters the rumen as mono-, di- and tri-substituted inorganic phosphates, and also as organic compounds namely phytates (or phytic acid), phospholipids, and phosphoproteins. (Georgievskii 1982). Hydrolysis of phytates occurs in the reticulo-rumen by action of bacterial phytases, gastric digestion dissolves the soluble and also some of the insoluble phosphates, additionally the splitting of the phosphoric acid from the organic compounds takes place predominately in the small intestine under the effect of phosphatases (Georgievskii 1982). The bulk of Ca in various chemical forms within different feeds is predominantly converted to calcium chloride via gastric digestion, which is almost completely dissociated into ions, and Ca in its ionic form is the main Ca form absorbed in the small intestine (Georgievskii 1982).

Absorption of calcium and phosphorus in ruminants

Absorption of ingested Ca and P in the GIT of ruminants is well known to occur in the small intestine (Phillipson & Storry 1965; Church 1979; Grace 1981; Georgievskii 1982; Scharrer 1985; Yano *et al.* 1991; Underwood & Suttle 1999), via both active and passive absorption (diffusion) (Wasserman 1981; Braithwaite 1984). However it has also been established that P and Ca absorption takes place from the reticulo-rumen (Höller *et al.* 1988; Beardsworth *et al.* 1989; Care *et al.* 1989). In support of these findings Wadhwa and Care (2002) have recently identified the reticulo-rumen as being recognized to be an important site of net absorption of phosphate ions from ruminal fluid containing phosphate concentrations at normal levels. The intra-ruminal phosphate concentration has also been found to play a role in the absorption of Ca and magnesium (Mg) from the forestomach of sheep (Dua & Care 1999).

According to Care (1994) under normal dietary conditions in which the P intake of a ruminant is adequate, the absorption of P ions takes place from both the reticulo-rumen and the small intestine. The rate of absorption in the small intestine is however three times greater than in the reticulo-rumen. When absorption of P ions in the reticulo-rumen is severely reduced as in dietary P deficiency, the absorption of both Ca and Mg ions from the reticulo-rumen becomes impaired.

The ability of an animal to absorb and utilize P and Ca from the GIT depends on vitamin D (McDowell 1992; Underwood & Suttle 1999). Field herbage is not normally rich in vitamin D, occurring only in sun-dried roughages and dead leaves of growing plants (Underwood & Suttle 1999; McDonald *et al.* 2002). The extent of vitamin D formation from dietary precursors, such as ergosterol and 7-dehydrocholesterol, by ultraviolet (UV) irradiation of the ruminant's skin is therefore important (Underwood & Suttle 1999).

According to McDonald *et al.* (2002) provitamins such as the precursor's ergosterol and 7-dehydrocholesterol of vitamin D_2 and D_3 respectively have no vitamin value and must be converted into calciferols before they are of any value. For this conversion it is necessary to impart a definite quantity of energy to the sterol molecule and UV light present in sunlight facilitates this conversion. The chemical transformation occurs in the skin and also in the skin secretion, which are known to contain the precursors. In southern Africa ruminants are exposed to sufficient irradiation from sunlight to ensure adequate vitamin D formation from dietary precursor synthesis (Underwood & Suttle 1999).

Dietary vitamin D is absorbed from the small intestine and is transported in the blood to the liver, where conversion to 25-hydroxycholecalciferol takes

place, which is then transported to the kidney, where it is converted into 1,25-$(OH)_2D$ (McDonald *et al*. 2002). 1,25 dihydroxycholecalciferol is then transported via the blood system to various target tissues such as bone and the intestine, where it is responsible for the absorption of Ca and P from the intestinal lumen (McDowell 1992; McDonald *et al*. 2002).

Resorption of calcium and phosphorus from bone

Phosphorus and Ca requirements are highly dependent on the physiological state of an animal, with factors such as growth rate, lactation and pregnancy resulting in an increased demand for Ca and P (Denton 1982; McDowell 1992).

McDonald *et al*. (2002) emphasize that the skeleton is not a stable unit in the chemical sense because large amounts of the Ca and P in bone can be liberated by reabsorption. Bone continually undergoes a process of resorption with mobilization, and formation with restorage, of Ca and P between the bone, the blood supply, and other parts of the body.

The three principal components in bone tissue include: the organic base (95% collagen), the inorganic fraction and water, and all three have a close structural interconnection (Georgievskii 1982). The organic matrix of bone in which the mineral salts are deposited consists of a mixture of

proteins, predominately ossein (Maynard *et al*. 1979). This soft fibro-organic matrix is composed mostly of collagen fibres and to a lesser extent mucopolysaccharide gel, and the protein matrix in bone can only become calcified when the proper levels of Ca, P, magnesium (Mg) and other minerals are present (McDowell 1992).

The inorganic portion or mineral component of mature bone is approximately 32%, which is comprised mostly in the form of calcium phosphate [Ca_3 (PO_4) $_2$] and hydroxyapatite [Ca_{10} $(PO4)$ $_6(OH)$ $_2$] (Georgievskii 1982; McDowell 1992). Of this mineral component of mature bone Ca and P generally constitute 36% and 17% respectively (Underwood & Suttle 1999).

Resorption and formation of bone are dependent on two different types of bone cells, osteoclasts and osteoblasts, the activity of which is regulated by PTH, 1,25 $(OH)_2D$ and calcitonin. Osteoblasts can form new bone on surfaces of bone previously resorbed by osteoclasts (McDonald *et al*. 2002). Osteoblasts are also actively involved in the synthesis of matrix components of bone and potentially facilitate the movement of mineral ions between extracellular fluid and bone surfaces (McDonald *et al*. 2002).

Resorption of Ca is controlled by the action of the parathyroid gland (Yano *et al*. 1991). A low-Ca diet results in the ionic Ca concentrations in the extracellular fluids to fall, the parathyroid gland is stimulated and PTH and

1,25(OH) $_2$D are secreted causing resorption of bone, which liberates Ca to meet the requirements of the animal (Yano *et al*. 1991; McDonald *et al.* 2002). Since Ca is combined with P in bone, the P is also liberated and excreted by the animal (McDonald *et al*. 2002). Thus ruminants are able to regulate the concentration of Ca and also P in blood plasma (Yano *et al.* 1991).

A dietary Ca:P ratio between 1:1 and 2:1 is assumed to be ideal for growth and bone formation, however ruminants can tolerate a wide range of dietary Ca:P particularly when their vitamin D status is high (McDowell 1992; McDonald *et al.* 2002). One such cause for a wide range in Ca:P can be found in a diet consisting predominantly of legumes, which have a high Ca content relative to P (Ca:P ratio of 6 to 10:1) (McDowell 1992). The average Ca:P ratio of 2.1:1 in bone is near constant, however it can decrease or increase slightly in response to deficiencies of Ca or P (Underwood & Suttle 1999).

Metabolism of calcium and phosphorus in ruminants

The metabolism of Ca and P is closely interconnected and interactions in the gastrointestinal tract (GIT), in intercellular fluids, and in the bone-blood system are essentially regulated by similar biological and physicochemical mechanisms (Church 1979; Denton 1982; Georgievskii 1982; Denton *et*

al. 1986). Underwood and Suttle (1999) do however argue that there is no specific or effective hormonal regulation of P metabolism. Instead the hormonal regulation on Ca metabolism has a direct effect on P. These mechanisms ensure optimum absorption and endogenous excretion of Ca and P in the digestive tract; maintenance of their normal concentrations and proportions in blood and in the intercellular fluid; deposition of Ca and P as hydroxyapatite in bone tissue and their liberation during resorption; realization of the ion-exchange function of the skeleton; and regulation of Ca and P excretion by variations in their reabsorption or active secretion in the renal ducts (Georgievskii 1982; McDowell 1992; Underwood & Suttle 1999).

Although the regulation of Ca and P is similar to the maintenance of the concentration of these minerals in blood plasma varies. The priority of all mammals is to maintain Ca concentrations in blood plasma and extracellular fluids close to 2.5 mmol/l throughout fluctuations in demand and supply (Hurwitz 1996). The control of P metabolism differs in that, provided it is present in absorbable forms in the diet, it will be extensively absorbed, even when supplied in excess (Underwood & Suttle 1999). Thus normal levels of inorganic P can range above and below the renal threshold of 2-3 mmol/l in a healthy animal, which is due to the absence of a tight hormonal control (Underwood & Suttle 1999).

The system that controls and coordinates these mechanisms includes: the parathyroid hormone (PTH); calcitonin; and vitamin D and its derivatives. According to Hurwitz (1996), blood Ca concentration is maintained within very narrow limits by several hormones. These hormones are thyrocalcitonin (calcitonin) and PTH and they function in a delicate relationship with the active form of vitamin D 1,25 dihydroxycholecalciferol (1,25-$(OH)_2D$) to control blood Ca and P levels (McDowell 1992; Underwood & Suttle 1999). The production rate of 1,25-$(OH)_2D$ is under physiological as well as dietary control (McDowell 1992). Calcitonin, contrary to the other two, regulates high plasma Ca levels by depressing gut absorption, halting bone demineralisation, and reducing reabsorption by the kidneys (McDowell 1992).

Calcium absorption is adjusted in response to requirements. When there is an increase in demand for Ca the result is the fall in plasma Ca concentration, which increases the release of PTH, which in turn stimulates the increased production of calcium-binding protein (CaBP) and so accelerates Ca absorption (McDowell 1992; McDonald *et al.* 2002). In a reverse manner, an increase in plasma Ca concentration causes suppression of PTH release, a reduction in 1,25-$(OH)_2D$ production, and reduced Ca absorption (McDowell 1992; McDonald *et al.* 2002).

The interrelationship between Ca and P ions in the GIT has a marked influence on gastrointestinal absorption on one another (Braithwaite 1984; Walker & Ali-Ali 1987). Thus similarities in metabolism of Ca and P may indicate that osteophagia is more related to maintaining a proper ratio (Ca:P) rather than simply increasing the intake of one of the two (Barrette 1985).

Osteophagia in giraffe and other wild ruminants

In wild African ruminants the craving for bones, a probable result of a deficiency of P in the diet, has been identified in a number of species (Anderson 1974; Sutcliffe 1973; Field 1975; Sekulic & Estes 1977) however it is especially frequently observed behaviour in giraffes (*Giraffa camelopardalis*) (Pattern 1940; Nesbit-Evans 1970; Western 1971; Wyatt 1971; Leuthold & Leuthold 1972; Hall-Martin 1975; Langman 1978; Kok & Opperman 1980; Hampton 2002), and this frequency increases in the winter months when the nutrient quality of browse declines (Langman 1978).

It has been assumed that this behaviour reflects a need for large amounts of Ca or most likely P. Giraffes exhibit extreme selectivity in the wild where herbaceous forage is selected not according to relative availability in a given habitat but according to quality, that is nutritional content,

digestibility and succulence (Pellew 1984). A giraffe's diet of browse on average has a Ca:P ratio of 7.7:1 in comparison to grass, in which the ratio is approximately 2:1 (Pellew 1984; Mitchell & Skinner 2003). The high Ca: P in browse indicates that giraffes can obtain four to five times more Ca from browse than grass (van Schalkwyk *et al.* 2004). A diet fed to cattle with a Ca:P ratio at this level (7.7:1) could result in clinical signs of P deficiency, essentially resulting in osteophagia (McDowell 1992; Underwood & Suttle 1999). The conclusion is that it is the inability to balance Ca and P intake that is possibly the most important influence resulting in osteophagia in giraffe.

Potential dietary imbalances are compounded by the fact that the giraffe skeleton is unique amongst mammals. It constitutes a greater proportion of body mass, it elongates faster than any other, and it must support a large body mass (Hall-Martin 1975). In addition the skeleton of a female giraffe is also subjected to periodic demineralisation because of a 15-month gestation period, a full-term foetus weighing 102kg, a 15-month lactation period during which they have to provide an estimated seven grams of Ca daily for milk (Aschaffenburg *et al*. 1962; Mitchell & Skinner 2003), and a calving interval of 550 – 650 days (Mitchell & Skinner 2003). Thus giraffe cows will have larger requirements for Ca and P. Furthermore, giraffes are non-seasonal so it could be predicted that a female giving birth between April and October (winter) when the nutritional

value of herbage declines, would have a higher variation in bone mineral content than for males. This prediction is supported by observations of Hall-Martin (1975) that it is mostly females that show signs of osteophagia, and with Langman's (1978) observation that 90% of incidents of osteophagia occur between April and October.

Analysis of bone density and Ca and P concentration in giraffe bones by van Schalkwyk *et al.* (2004) and Mitchell *et al*. (2005) found that density and mineral concentration was similar to that of African buffaloes (*Syncerus caffer*). Density of cervical vertebrae for giraffes was 1.3±0.1 and 1.4±0.1 for buffaloes (P>0.05) (van Schalkwyk *et al.* 2004), and the Ca concentrations were 0.185±0.01 and 0.198±0.01 respectively and P concentrations were 0.090±0.005 and 0.093±0.006 respectively (Mitchell *et al.* 2005). However, the absolute amounts of P and Ca required by giraffes was two to three fold more than the amount required by buffaloes (Body mass of mature buffalo bulls 603 ± 62kg; mature giraffe bulls 1184 ± 70kg, Skinner & Smithers 1990), thus indicating that giraffes accumulate significantly more P and Ca during growth (Mitchell *et al*. 2005). This study also found that the amount of Ca in the diet of giraffes was probably adequate but the origin of sufficient P was unknown. Osteophagia is a possible source.

The nutritional value of bone ingested into the rumen is poorly understood. It is however well recognized that in the form of bone meal it provides a suitable source of P and Ca for ruminants (Ammerman *et al.* 1995; Perry *et al.* 1999). Bone meal is considered to have a relative Ca bioavailability value of 95% in comparison to calcium carbonate (Soares 1995a). In comparison to several highly available standard phosphate sources bone meal has a bioavailability of greater than 95% (Soares 1995b).

When included in the diet for livestock the benefits of bone meal are apparent, however the small particle size of bone in bone meal allows entry to the abomasum without a need for rumen digestion, and therefore ensures adequate absorption. Moreover the processed form of bone (bone meal) allows for larger quantities of bone to be consumed by livestock.

For osteophagia to be an effective adaptive behaviour to supply P (and/or Ca), and assuming that ingested bones or bone fragments enter the rumen, then the ingested bones must be small enough to pass through to the abomasum and small intestine or must be able to be digested in the rumen and P and Ca extracted in a soluble, absorbable form. As far as could be determined no study on rumen digestion of bones has been done, so this report discusses the results of a study designed to assess

whether bone digestion occurs in a rumen, to provide an adequate source of P (and/or Ca) to alleviate osteophagia.

1.2 OBJECTIVES OF THIS STUDY

1. To establish whether digestion of bone could occur in a rumen and result in the extraction of P and Ca from bone.

2. To compare P and Ca extraction from different types of bone, cervical vertebrae (porous bone) and metacarpus shaft (compact bone).

1.3 HYPOTHESES

1. Significant digestion of bone occurs in the rumen.

2. Phosphorus and Ca are extracted to the same magnitude from compact and cancellous bone in the rumen.

Chapter II

MATERIALS & METHODS

2.1 ORIGIN, TYPES AND PREPARATION OF BONE SAMPLES

Phosphorus and Ca extraction from cancellous (porous) and compact (dense) bone was determined. Bone samples used were obtained from giraffe bones because the composition of both types has been established in previous studies (van Schalkwyk *et al.* 2004; Mitchell *et al.* 2005). The large size of giraffe bones allowed for samples to be cut from fewer bones thus increasing standardization.

For the present study cancellous bone was obtained from the third, fourth and fifth cervical vertebrae of five mature giraffe bulls. A mature bull's metacarpus was used for the compact bone samples in the trial. All bones were from bulls older than six years (Hall-Martin 1976). The specific ages of the giraffes were unknown. All samples were collected from the Kruger National Park (and surrounding areas). For disease control purposes all samples were autoclaved at 121° C for 15 min at 6.8 kg pressure in an autoclave (Almor P-09A, Almor Ltd., UK) at the State Veterinary office, Skukuza. The sterilizing method of autoclaving has no significant effect on the P and Ca composition of bone (Moreno & Forriol 2002).

To insure uniformity of bone density the cervical vertebrae were sawn into portions of similar porosity, and the portions that could be further sawn into cubes with side lengths of approximately 1.7 cm were used. The fifty most uniform cubes were used as cancellous bone samples for the trial. Benzie *et al.* (1955) and Benzie *et al.* (1959) found that there was a significant difference in density of different parts of long bones of sheep (*Ovis aries*). For this reason only the mid-shaft of the metacarpal diaphyseal was used to insure uniformity in bone density. The shaft was sawn laterally into discs of approximately 1.7 cm in width and cubes were then cut from each disc. The fifty most uniform cubes were used as the compact bone samples in the trial. The surface area of each cube was thus about 17.5 cm^3 and their volumes about 5 cm^3 (Tables 6, 7, 8). All bones were sawn using an electric band saw in the Department of Anatomy and Physiology, Faculty of Veterinary Science, University of Pretoria.

2.2 EXPERIMENTAL ANIMALS

Five mature, rumen-fistulated Merino wethers (*Ovis aries*) were used for the trial. The Animal Use and Care Committee of the Faculty of Veterinary Science, University of Pretoria approved the trial work (approval number V068/04). Sheep were used as experimental animals because they are

easy to handle and because they have a history of osteophagia (Brothwell 1976; Bazely 1989). The sheep were housed in individual cement floor covered pens at the husbandry facility at the experimental farm, Faculty of Biological and Agricultural Science, University of Pretoria. They were fed a ration of good quality teff hay (*Eragrostis tef*) (Table 1) and water was available *ad libitum*. The hay was milled to 1cm lengths and mixed. To ensure standardization and avoid any gastrointestinal problems the sheep were fed the ration for 16 days prior to the trial. Feed consumption rate was estimated at approximately 2 kg dry matter (DM)/wether/day, which is above normal intake for rams on a maintenance diet (Perry *et al*. 1999). The sheep were fed twice daily, with equal quantities being fed at each feeding, and they were given *ad libitum* access to the feed. Their body masses ranged from 38.1 kg to 72.0 kg at the start of the trial and they maintained body mass for the duration of the trial.

Table 1: Teff hay (*Eragrostis tef*) fed to the sheep throughout the trial. All values are on a dry matter (DM) basis.

DM (g/100g)	GE (MJ/kg)*	Moist (g/100g)	Ash (g/100g)	CP (g/100g)**	CF (g/100g)	Ca (g/100g)	P (g/100g)
100	18.5	0	4.72	7.16	34.91	0.28	0.32

* According to van Ryssen (per. comm.)

** "Dumas" method was used in the crude protein analysis, AOAC official method 990.03 (Horwitz 2000).

- GE = Gross Energy; Moist. = Moisture; CP = Crude Protein; CF = Crude Fibre.

2.3 SHEEP MONITORING

The sheep's rumen fluid pH was monitored at the start of the trial and at the different time intervals (10, 20 and 30 days respectively) using the Model IQ 150 handheld pH/mV/ Temperature Meter or Model IQ 120 pH meter with silicon chip sensor (I.Q. Scientific Instruments, Inc., San Diego, U.S.A.). Distilled water and artificial saliva pH readings were also monitored at the start of the trial and at the respective time intervals.

Blood plasma samples from all five sheep were taken at the start of the trial and at the respective time intervals. Each sheep had 10 ml of blood drawn from the jugular vein at each time interval, using vacutainer needles

(1.2 mm X 38 mm) and 5 ml tubes. Heparinised tubes were used for the collection of blood plasma. To minimize the release of organic P from erythrocytes, sample tubes were slowly rolled to ensure adequate mixing (Little *et al*. 1971). Tubes were placed in a container with ice packs and were centrifuged within one hour. Preparation of blood plasma for inorganic P analysis was done following Little *et al*. (1971). After precipitation the solutions were filtered through glass microfibre paper (9.0 cm GF/A Whatman Ltd., England) into acid-cleaned 30 ml McCartney bottles. The remaining plasma in the centrifuged tubes was pipetted into individual, sealable tubes for analysis of inorganic Ca levels. All samples were refrigerated at 5°C.

Calcium and P concentrations in plasma were analysed using the same methods described above for bone sample analysis, except that to minimise interference by P on the spectrophotometer, the plasma samples for Ca analysis were diluted fifty times with lantanechloride ($LaCl_2$ 0.1%).

2.4 EXPERIMENTAL DESIGN

Five samples of each bone type selected at random were placed in individual 50 ml glass sealable containers for the duration of the trial. These samples were control samples. Five samples of each bone type of the remaining 90 samples (45 of each bone type) were randomly selected for the other nine treatments (Table 1).

Table 2: Ten treatments used in the trial. Samples of both bone types were used in each treatment.

Treatment	Solution	Duration (days)
1	Control*	30
2	Distilled Water	10
3	Distilled Water	20
4	Distilled Water	30
5	Artificial Saliva	10
6	Artificial Saliva	20
7	Artificial Saliva	30
8	Rumen Fluid	10
9	Rumen Fluid	20
10	Rumen Fluid	30

* Bone samples placed in sealed containers at day zero of trial.

All bone samples were placed in individual "*in situ*" nylon bags (pore size 53 µm; Nutrilab) and tied-off using thin nylon cord.

Fifteen of the 45 samples of each bone type were placed in distilled water (pH 6.4 ± 0.5), 15 in an artificial saliva solution, for mixture see Table 2, to which hydrochloric acid (4ml/L) was added to the solution to reduce the pH to rumen pH of 6.6 at the start of the trial. Six containers of distilled water and six of artificial saliva each containing five samples of the same bone type were placed in a water bath at a temperature of 39°C, i.e. rumen temperature. According to McDonald *et al.* (2002) the temperature of the liquid portion of rumen content remains close to that of the animal (38 - 42°C).

Table 3: A standard formula for artificial saliva (McDougall, 1948).

Chemical	**Concentration (g/L)**
$NaHCO_3$	9.80
KCl	0.57
NaCl	0.47
$MgSO_4.7H_20$	0.12
$CaCl_2$ (anhydrous)	0.04
Na_2HPO_4 without H_2O	3.17

Fifteen samples of each of the bone types were placed in the rumens of the five sheep. The average rumen pH was 6.5 ± 0.2. Three samples of each bone type were placed in each of the five rumens. The *in situ* nylon bags were attached to a 120g disc shaped weight using thin nylon cord

approximately 20cm. The weights suspended the bags below the fluid surface in the rumens of the sheep.

Samples were kept in distilled water, artificial saliva and rumen fluid for three time intervals (Table 2). At the appropriate time intervals bone samples were removed from the nylon bags and washed thoroughly for approximately two minutes under tap water and oven dried following Harris (1970), for analysis.

The use of the in situ technique

The intra-ruminal in sacculus, *in situ*, digestion technique is routinely used for studying effects of the ruminal environment (Uden & Van Soest 1984; Meyer & Mackie 1986; Nocek 1988; Uden 1992; Michalet-Doreau & Ould-Bah 1992; Stern *et al.* 1997), but it has been most commonly used to estimate microbial protein degradation in the rumen (Mehrez & Ørskov 1977; Stern *et al.* 1997). Caution must be taken in interpreting results on feed evaluation and rates of degradation when using the *in situ* technique because of the large degree of variation that can occur. One such variation is the microbial population inside the bag differs from that of the surrounding ruminal ingesta, however the use of a nylon bag with pore sizes of 53 µm allows for a maximum (62% – 82%) total culturable bacteria count and equal protozoal counts within the bag in comparison to

the surrounding ingesta when a high roughage diet is fed (Meyer & Mackie 1986).

In comparison to laboratory methods, particularly the *in vitro* digestion method (Tilley & Terry 1963), the *in situ* technique is valuable as the digestion occurs in the rumen of a living animal (Stern *et al.* 1997). Several factors (Table 4) that can affect estimates of nutrient digestion were controlled in order for the technique to be standardized.

Table 4: Recommendations for standardizing the *in situ* digestion procedure.

Variable	Recommendation	Reference
Bag Porosity	40 to 60 µm	Nocek 1988; Michalet-Doreau & Ould-Bah 1992
Particle Size	2 - 5 mm, because contents would not have undergone mastication and rumination.	Nocek 1988
Sample size to bag surface area	20 mg/cm2, because over filling the bag causes delayed bacterial attachment.	Nocek 1988; Michalet-Doreau & Ould-Bah 1993
Diet	Feed to meet nutrient requirements of test animal.	Nocek 1988; Michalet-Doreau & Ould-Bah 1994
Microbial movement into the nylon bags	Animals fed frequently increases bacteria entering bags.	Meyer & Mackie 1986
Nylon bag procedure	Insert all the bags at the same time and remove them at different time intervals	Michalet-Doreau & Ould-Bah 1994
Postruminal washing	Rinse in tap water until water is clear (about 90s/bag with moderate manipulation).	Nocek 1988
Microbial contamination	Reduce samples to ash to eliminate the organic component.	van Ryssen per. comm.

The recommended particle size (Table 4) was not used in the experiment because the objectives for the project involved the ingestion of larger bone fragments into the rumen. This would have had an effect on the sample size to bag surface area (Table 4) however due to the duration of the bone samples in the rumen there was more than adequate time for bacterial attachment (van Ryssen per comms.).

2.5 PREPARATION OF BONE SAMPLES FOR P AND CA ANALYSIS

Cancellous bone samples were ground to a powder using a custom-made iron pestle and mortar. The coarse powder of the compact bone samples, ground in the pestle and mortar, was further ground using a motor-driven mill (Mikro-Feinmühle-Culatti MFC, Janke IKA® - Labortechnik, 50/60Hz, 200W) to particles of approximately 1mm^3 in size.

Duplicate samples of powdered bone samples, weighing 0.5 ± 0.003g were oven dried to determine DM (Harris 1970), and were ashed in a muffle furnace at 550°C for 4.0 h. Samples were left to cool overnight and placed in a desiccator for 30 min prior to determining the ash mass. The ash residue was dissolved in an acid solution, filtered and diluted to a volume of 100 ml following the dry ashing technique, AOAC official method 999.11 (Horwitz 2000). Dissolved ash solutions for Ca analysis

were diluted 50 times with distilled water and a further 10 times with lantanechloride ($LaCl_2$ 0.5%). Solutions for P analysis were diluted 50 times with distilled water.

Phosphorus levels were measured using an Auto Analyser II (Techicon™, Bran & Lübbe, Germany) according to the AOAC official method 965.17 (Horwitz 2000). Calcium levels were measured with an Atomic Absorption Spectrophotometer (Perkin-Elmer, 5100PC, USA) using the AOAC official method 935.13 (Horwitz 2000).

2.6 MEASUREMENT OF BONE MASS, VOLUME AND DENSITY

Initial mass of the bone samples was recorded using a Mettler Toledo Bloc PB 153-S scale (Mettler, Microsep, RSA) to an accuracy of 0.1g. After initial mass was recorded, all samples were defatted using petroleum ether as described by the Association of Official Analytical Chemists (AOAC) official method 945.16 (Horwitz 2000) at Nutrilab, Department of Animal and Wildlife Science, Faculty of Natural and Agricultural Sciences, University of Pretoria. Lipid free samples were weighed (±0.001g) and then oven dried following Harris (1970), allowed to cool for 30 min in a desiccator and weighed to determine dry mass (± 0.001g).

Volumes for the bone samples were determined by the displacement of water in volumetric flasks, which measured changes in volume to 1.0 ml. A study by Khan *et al.* (1997) showed that the temperature at which the density of water is closest to 1 g/ml is at 4°C, which is when it reaches 0.9999720 g/ml. At 20°C the density of water is equal to 0.9982041. For sufficient accuracy in this study water at room temperature (approximately 20°C) was used, and the density of water was rounded off to 1 g/cm^3. Volumetric density was calculated by dividing dry mass by volume and recorded as g/cm^3 (Gaynor Evans 1973). Volume and mass measurements were repeated pre- and post-treatments to determine any significant changes. All samples were oven dried following Harris (1970) after volume measurements were taken, and before mass was determined.

2.7 PHOSPHORUS AND CALCIUM CONCENTRATIONS IN BONE

A difference of less than 10% between the values obtained from duplicate bone samples was regarded as being within experimental error. For larger differences analysis of those particular samples was repeated. Ash concentration was determined and multiplied by the mass of the bone sample post treatment to determine actual ash content (Total Ash). Calcium and P readings were converted to mg/L ([volume * dilution * reading] / sample mass), and percentages were determined. The mean

percentage multiplied by actual mass of the sample post treatment was used to calculate Total Ca (g) and Total P (g).

2.8 STATISTICAL ANALYSIS

A two-tailed Student's t-test was used to compare differences between samples. Difference in mass, volume and density could be compared using the bone samples that were used in the different treatments, however P and Ca readings required the destruction of the bone samples , sample were reduced to ash to eliminate the organic component, therefore comparisons for these measurements were made with the control samples. *P*-values < 0.05 were regarded as significant.

Chapter III

RESULTS

3.1 RUMEN, SALIVA, DISTILLED WATER, AND BLOOD CHEMISTRY

Rumen and distilled water pH remained constant over the 30 days of the experiment at 6.5 ± 0.3 and 6.4 ± 0.4 respectively (Table 5). The pH of saliva increased significantly over time presumably because of the excellent buffering capacity of the solution. The increase in saliva pH was not a result of the presence of bones, even though bones are buffers, because there was an increase in pH in the saliva solution without bone samples (Table 5).

Plasma Ca concentration remained constant at 2.4 ± 0.1 mmol/l (Table 6). Plasma P concentration was more variable ranging from 1.4 ± 0.3 to 1.7 ± 0.7 mmol/l (Table 6). These P and Ca values are within normal range (Church 1979; Hurwitz 1996; Underwood & Suttle 1999) and there were no significant differences between the values measured at each time interval.

Table 5: pH readings recorded for rumen fluid, artificial saliva and distilled water ($D.H_2O$) pre-trial and at the relevant time intervals during the trial.

Fluid Type	N (animals/ containers)	Before treatment	10 days	20 days	30 days
Rumen (mean±SD)	5	6.4±0.2	6.5±0.2	6.5±0.3	6.5±0.3
Saliva + no bones	1	6.6	7.5	7.7	-
Saliva+ Mc•.	3	6.9	7.2	7.3	7.4
Saliva+ Vert•.	3	6.8	7.0	7.4	7.3
Saliva (mean±SD)	7	6.8±0.2	7.2±0.2	7.4±0.2	7.3
$D.H_2O$ + no bones	1	6.6	6.6	7.2	-
$D.H_2O$ + Mc.	3	6.5	5.9	6.3	6.7
$D.H_2O$ + Vert.	3	6.5	5.8	6.0	6.1
$D.H_2O$ (mean±SD)	7	6.5 ± 0.1	6.1 ± 0.4	6.5 ± 0.7	6.4 ± 0.4

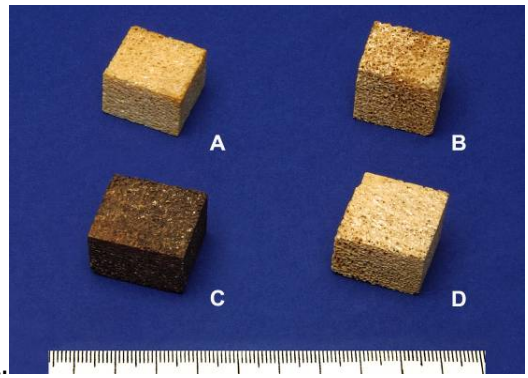
- Mc. = Metacarpus shaft samples.
- Vert. = Cervical vertebrae samples.

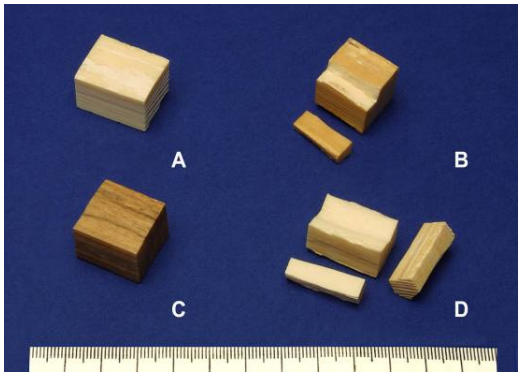
Table 6: Plasma Ca and P values for the five sheep throughout the trial.

Animal Number	Ca (mmol/l)				P (mmol/l)			
	0 days	10 days	20 days	30 days	0 days	10 days	20 days	30 days
1	2.5	2.3	2.3	2.4	1.1	1.3	1.8	1.2
2	2.3	2.4	2.3	2.6	2.0	1.9	2.2	1.9
3	2.7	2.4	2.3	2.2	1.4	1.2	2.5	2.1
4	2.1	2.4	2.5	2.5	1.5	1.3	0.9	1.2
5	2.6	2.3	2.5	2.9	1.2	1.3	0.9	1.1
Mean ± SD	2.4±0.2	2.4±0.1	2.4±0.1	2.5±0.3	1.4±0.4	1.4±0.3	1.7±0.7	1.5±0.5

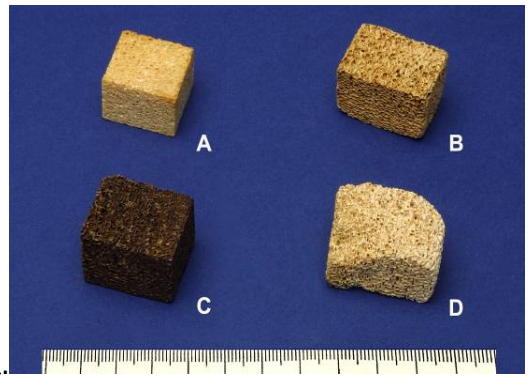
3.2 PHYSICAL APPEARANCE OF BONE SAMPLES

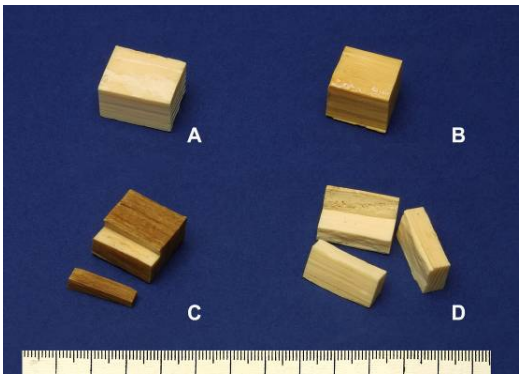
The physical appearance of the two types of bones after exposure to the various treatments is shown in Figures 1, 2, and 3. Distilled water had no obvious macroscopic effects. Artificial saliva produced visible erosion of the vertebrae but had no effect on samples derived from the metacarpus. Rumen fluid had no visible effect on the bones other than to discolour them. In addition both rumen fluid and artificial saliva caused softening of the cervical vertebrae samples, and cracking of the metacarpus shaft samples.

a. 

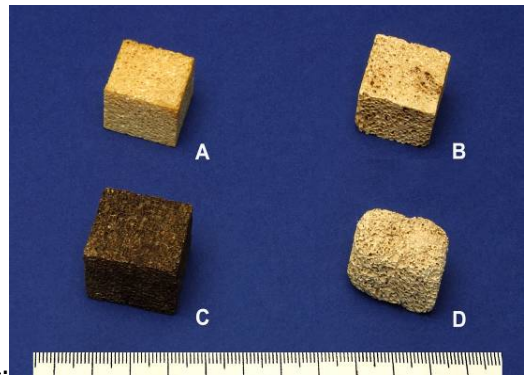
b. 

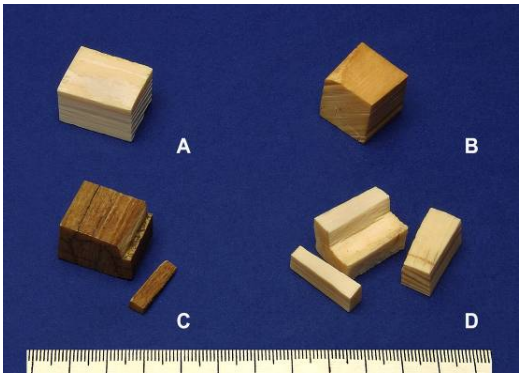
Figures 1a&b: Cervical vertebrae (a) and metacarpus shaft (b) samples after 10 days: A – control; B – distilled water; C – rumen fluid; D – artificial saliva.

a. 

b. 

Figures 2a&b: Cervical vertebrae (a) and metacarpus shaft (b) samples after 20 days: A – control; B – distilled water; C – rumen fluid; D – artificial saliva.

a. 

b. 

Figures 3a&b: Cervical vertebrae (a) and metacarpus shaft (b) samples after 30 days: A – control; B – distilled water; C – rumen fluid; D – artificial saliva.

3.3 PHYSICAL AND CHEMICAL COMPOSITON OF BONE SAMPLES

Analysis of mass, volume, density and mineral content of the two types of bone as a result of the treatments are shown in Tables 7, 8 and 9. To estimate the effects of immersion in the three fluids over the three time intervals mass, volume and density were determined prior to immersion on all five bone samples of each type used in each treatment. Ash, Ca and P content pre-treatment were determined from five bone samples selected randomly from all samples cut from each bone type, and were assumed therefore to be representative of all samples. This assumption is supported by data from samples where no measurable digestion occurred (Table 7). This procedure was necessary and differed from that used to measure mass, volume, and density, because analysis of ash, Ca and P content destroyed the samples and prevented their re-use.

3.3.1 EFFECTS OF DISTILLED WATER

Table 7: Effects of distilled water.

Bone Variable	N bone samples	Before treatment/ Control	10 days	20 days	30 days
Metacarpus Shaft					
Mass (g)*	15	9.3±1.0	9.5±0.8	8.7±0.7	9.5±1.3
Volume (ml)*	15	5.0± 0.6	4.9±0.2	4.8±0.3	5.0±0.6
Density*	15	1.9±0.1	1.9±0.1	1.8±0.1	1.9±0.1
Total Ash (g)	5	7.0±1.0	6.8±0.6	6.0±0.4	6.9±0.9
% Ca	5	25.6±0.9	25.1±1.4	24.4±1.1	25.4±0.5
% P	5	11.6±0.2	11.4±0.3	10.7±0.5	11.5±0.2
Total Ca (g)	5	2.5±0.4	2.4±0.3	2.1±0.1	2.5±0.3
Total P (g)	5	1.1±0.2	1.1±0.1	0.9±0.1	1.1±0.2
Total Ash – Ca+P		3.3±0.5	3.2±0.2	2.9±0.3	3.4±0.4
Cervical Vertebrae					
Mass (g)*	15	5.0±0.7	4.2±0.5	4.1±0.7	5.2±0.6
Volume (ml)*	15	4.7±0.8	4.0±0.0	4.2±0.4	5.4±0.9
Density*	15	1.1±0.1	1.1±0.1	1.0±0.1	1.0±0.1
Total Ash (g)	5	2.6±0.4	3.0±0.4	3.0±0.3	**3.4±0.5**
% Ca	5	21.1±0.7	23.6±1.4	23.6±1.6	22.0±0.8
% P	5	9.5±0.6	10.8±0.6	10.1±1.0	10.2±0.8
Total Ca (g)	5	1.0±0.1	1.1±0.1	1.1±0.1	1.2±0.2
Total P (g)	5	0.4±0.1	0.5±0.1	0.5±0.1	0.6±0.1
Total Ash – Ca+P		1.2±0.2	1.4±0.2	1.4±0.2	**1.6±0.2**

* N bone samples for before treatment means = 15; N bone samples for 10, 20 and 30 day intervals = 5.

- Bold = significant (P<0.05) using the t-test, from controls.

Table 7 shows that, apart from an anomalous increase in total ash and the total ash minus total Ca and P (i.e. non-Ca and P minerals) in cancellous bone samples after 30 days in distilled water, there was no effect on bone composition, as might have been predicted. The results shown in this table confirm previous findings on giraffe compact and cancellous bone, which determined that limb bones are far denser than vertebrae (van Schalkwyk *et al.* 2003; Mitchell *et al.* 2005). The mass, and density, and total ash and total Ca and P concentration of vertebrae are significantly less than those in the metacarpus, although the percentage of Ca and P is the same in both bone types.

3.3.2 EFFECTS OF ARTIFICIAL SALIVA

Table 8 shows that bone samples from the metacarpus are unaffected by the effects of saliva. However, the saliva solution had several significant effects on cancellous bone (Table 8).

Table 8: Effects of artificial saliva

Bone Variable	N bone samples	Before treatment/ Control	10 days	20 days	30 days
Metacarpus Shaft					
Mass (g)*	15	10.0±0.9	9.5±0.6	9.6±1.1	10.7±0.4
Volume (ml)*	15	5.3±0.4	5.1±0.3	5.2±0.5	5.5±0.4
Density*	15	1.9±0.1	1.9±0.1	1.9±0.1	1.9±0.1
Total Ash (g)	5	7.0±1.0	6.9±0.5	6.9±0.8	7.6±0.3
% Ca	5	25.6±0.9	25.4±0.7	25.2±1.3	24.7±1.0
% P	5	11.6±0.2	11.4±0.2	11.7±0.4	11.7±0.2
Total Ca (g)	5	2.5±0.4	2.4±0.2	2.4±0.3	2.7±0.1
Total P (g)	5	1.1±0.2	1.1±0.1	1.1±0.1	1.3±0.1
Total Ash – Ca+P		3.3±0.5	3.3±0.3	3.3±0.3	3.7±0.1
Cervical Vertebrae					
Mass (g)*	15	4.8±0.9	4.7±0.9	4.2±0.5	**3.5±0.5**
Volume (ml)*	15	4.5±0.9	4.6±1.3	**3.8±0.4**	**3.5±0.5**
Density*	15	1.1±0.2	1.0±0.2	1.1±0.2	1.0±0.1
Total Ash (g)	5	2.6±0.4	**3.6±0.7**	3.1±0.7	3.0±0.4
% Ca	5	21.1±0.7	**24.3±1.2**	**24.3±1.8**	**25.5±1.9**
% P	5	9.5±0.6	**10.9±1.1**	**11.3±0.8**	**11.6±1.1**
Total Ca (g)	5	1.0±0.1	1.3±0.3	1.1±0.2	1.1±0.2
Total P (g)	5	0.4±0.1	0.6±0.1	0.5±0.1	0.5±0.1
Total Ash – Ca+P		1.2±0.2	1.7±0.4	1.4±0.3	1.4±0.1

* N bone samples for before treatment means = 15; N bone samples for 10, 20 and 30 day intervals = 5.

- Bold = significant (P<0.05) using the t-test, from controls.

The mass and volume of the samples decreased significantly over the 30 day period, confirming the visible effects of saliva noted above. Conversely the percentage of Ca and P in the samples increased significantly over the period, presumably because the bone samples absorbed Ca and P from the saliva solution. This percentage increase in Ca and P did not however translate into increased absolute amounts of Ca and P because the mass of the bones decreased. Discounting this apparent absorption, Ca and P loss from the bones themselves can be calculated to be 0.5 g Ca and 0.1 g P over the 30 day period, which are trivial amounts, when compared to the amount of Ca and P required daily.

3.3.3 EFFECTS OF RUMEN FLUID

Table 9 shows that, as in the case of saliva, bone samples derived from metacarpal bones are unaffected by the effects of rumen fluid except in so far as bones soften after immersion suggesting that the collagen matrix is digested. Similarly cancellous bone softens. In neither case though is digestion of matrix accompanied by a decrease in bone mass or volume as there was for saliva, nor was there removal of Ca and P.

Table 9: Effects of the rumen fluid.

Bone Variable	N Bone samples	Before treatment/ Control	10 days	20 days	30 days
Metacarpus Shaft					
Mass (g)*	15	10.0±0.9	9.6±0.4	10.1±0.6	10.0±1.4
Volume (ml)*	15	5.2±0.4	5.1±0.2	5.3±0.4	5.3±0.5
Density*	15	1.9±0.1	1.9±0.0	1.9±0.1	1.9±0.1
Total Ash (g)	5	7.0±1.0	6.9±0.4	7.2±0.4	7.2±1.1
% Ca	5	25.6±0.9	25.7±0.9	25.3±0.6	25.6±1.4
% P	5	11.6±0.2	11.6±0.3	11.4±0.2	11.4±0.5
Total Ca (g)	5	2.5±0.4	2.5±0.2	2.6±0.1	2.6±0.5
Total P (g)	5	1.1±0.2	1.1±0.1	1.2±0.1	1.1±0.2
Total Ash – Ca+P		3.3±0.5	3.3±0.2	3.4±0.3	3.4±0.5
Cervical Vertebrae					
Mass (g)*	15	5.5±0.5	5.6±0.3	5.7±0.7	5.6±0.4
Volume (ml)*	15	5.5±0.6	5.5±0.9	5.2±0.3	5.7±0.4
Density*	15	1.0±0.1	1.0±0.1	1.1±0.1	1.0±0.1
Total Ash (g)	5	2.6±0.4	**3.6±0.5**	**3.3±0.4**	**3.2±0.2**
% Ca	5	21.1±0.7	21.9±1.1	21.3±0.4	21.8±0.8
% P	5	9.5±0.6	9.9±0.9	9.9±1.1	9.7±0.5
Total Ca (g)	5	1.0±0.1	1.3±0.1	1.1±0.1	1.2±0.1
Total P (g)	5	0.4±0.1	0.6±0.1	0.5±0.1	0.5±0.1
Total Ash – Ca+P		1.2±0.2	1.8±0.3	1.6±0.2	1.6±0.1

* N bone samples for before treatment means = 15; N bone samples for 10, 20 and 30 day intervals = 5.

- Bold = significant (P<0.05) using the t-test, from controls.

The total ash content of cancellous bones increased, probably because of absorption of minerals other than Ca and P from the rumen fluid because it is the non-Ca +P fraction of the ash that appears to increase (although not reaching the level of significance).

Chapter IV

DISCUSSION

The assumptions underlying this study are that wild ruminants, like domestic ruminants, have a requirement for dietary P, and that diets deficient in P result in the phenomenon of osteophagia. A further assumption is that osteophagia is a method of balancing their P and Ca intake. Calcium and P are two major constituent minerals of the ruminant body. The skeleton contains 99% of the total Ca and 78% of the total P, mainly as hydroxyapatite. Only a residual 1% of the total Ca exists in the soft tissues and the extracellular fluid. About 20% of the total P may have a role in constituting cell membrane, in maintaining the acid-base balance in the body fluid, and in supplying vital energy for metabolism.

The phenomenon of osteophagia is prevalent throughout a range of ruminants, both domestic and wild and is especially apparent in giraffe. The types of bones chewed or swallowed range from compact bones to bones that are often brittle and highly porous. Bones from carcasses in different stages of decomposition are also selected (Kok 1982; Hampton 2002). Bone is one of the few natural available sources of P, which is why there is a strong belief that a nutritional gain is the cause for the phenomenon of osteophagia. Phosphorus is a major mineral component of bone. According to McDonald *et al.* (2002) the dry matter of bone,

which is highly complex in structure, consists of approximately 460g mineral matter / kg, 360g protein / kg, and 180g fat / kg. The composition does vary according to the age and nutritional status of the animal but Ca and P remain the two most abundant mineral elements in bone (36% and 17% respectively). However the ability of a ruminant to utilize this source of P and possibly Ca is poorly understood.

If osteophagia has evolved as an adaptation for providing minerals it could be expected to be both directed and selective. Moreover ingested bones or bone fragments too large to enter the lower digestive tract directly could be digested in the reticulo-rumen. There is no evidence that animals select, for example, cancellous bone, which is easier to crush, over dense bone that even with sophisticated machinery is difficult to reduce to powder or small particles. The many observations made of osteophagia in giraffes show that bones selected range from dense nonporous bones to bones that are highly porous and often brittle, however smaller sized bones are preferred (Langman 1978). Furthermore giraffes and ruminants in general do not have mouthparts designed for crushing and grinding bone. Their molar teeth are predominantly used for grinding herbage, although Sutcliffe (1973) has provided evidence that deer chew bones in a “cigar like manner” which results in fork shaped bones with zigzag margins.

Nevertheless, because bone is a potentially large source of minerals the study reported here analysed the possibility that bones deposited in the rumen could be digested there, and release P and Ca. A second outcome is that bone digestion in the rumen reduces bones or bone fragments to a size that allows them to pass through the reticulo-omasal orifice to enter the abomasum and small intestine where further digestion is more certain. Either of these outcomes would make osteophagia a highly advantageous behaviour, especially if the rumen is itself adapted to digest bone.

4.1 THE USE OF SHEEP TO MODEL THE DIGESTION OF BONE

It is possible that the reason for the lack of clear digestion of bones in the rumen is because the ability of sheep rumen fluid to digest bone differs from that of other ruminants, especially browsers. This explanation is, however, unlikely. Hofmann and Stewart (1972) and Hofmann (1989) have identified the anatomical variations, which allow ruminants to be classified into three distinct feeding types according to their choice of forage and the morpho-physiological adaptations of their digestive system. However Gordon and Illius (1994) and Robbins *et al.* (1995) have suggested that there is little difference in digestive capabilities of African ruminants with different morphological adaptations of the gut. Moreover, the digestive capability of giraffe rumen fluid seems to be unremarkable. For example Jones *et al.* (2001) showed that their rumen fluid has no greater ability to

digest nitrogen in tanninferous feeds than did rumen fluid from sheep. They did show that the rumen fluid of browsers has a lower rumen fluid pH than that of grazers however my results show that pH has little effect on the digestion of ingested bones. This assessment suggests that, although no comparative investigation of bone digestion in rumens has been done, differences in ruminal digestion of feed between browsers and grazers are unlikely to be large.

4.2 BONE DIGESTION IN THE RETICULO-RUMEN

The results of this study show that there is little digestion of bone in the rumen. Thereby allowing the rejection of the first hypothesis, namely that significant digestion of bone does not occur in the rumen. Significant amounts of P and Ca were not removed from either dense or cancellous bones by immersion in either artificial saliva or rumen fluid for up to 30 days (refer to Tables 8 & 9). Thus the second hypothesis for this study was also rejected.

Both types of bone soften because of digestion of the collagen matrix but this digestion is not associated with significant loss of P or Ca. Compared with rumen immersion, immersion in artificial saliva did, however, result in some digestion of vertebrae (refer to Table 8; significant loss in mass in bone samples after 30 days). In saliva cancellous bone not only softens,

its mass and volume decrease, and P and Ca are removed in proportion to the decline in mass (bone density remains constant). The elution of minerals by saliva could simply be an effect of pH, however this is unlikely because at the start of the trials the pH of distilled water, saliva and rumen fluid was acidic and similar, but only saliva had the effects reported (Table 5). Moreover the pH of the saliva solution became more alkaline with time. Thus the mechanism of digestion in artificial saliva must lie in chemical reactions between the minerals in the saliva and those in bone. The mineral concentration and mineral make-up of the artificial saliva at the "onset" of the trial was not compared to that at the "end-of-trial".

4.3 SOFTENING OF BONE IN THE RETICULO-RUMEN

The softening of cancellous bone and dense bone, taken together with the observation that the samples became softer the longer they were in the fluids, suggests that immersion could facilitate mechanical digestion during rumination. The possibility exists therefore that, if giraffe saliva contains digestive enzymes and if it is of similar chemical composition to artificial saliva, then through the mechanical effect of chewing, and the chemical action of saliva, minerals may be released from bones, especially cancellous bones. A more important consequence of this effect is that re-chewed, softened, bones are likely to be of the size that could

pass through the rumen without further digestion to enter the more caudal regions of the digestive tract.

4.4 AN ALTERNATIVE TO RUMINAL BONE DIGESTION

An alternative to digestion of bone in the rumen is chewing bones for a long time, which may partially break up bone into smaller particles. These would pass through the rumen into the abomasum and beyond, where there is certainly digestion and the absorption of P and Ca, especially via the anterior duodenum.

However considering all these factors it should be noted that ruminants that eat bones do not in fact show a decline in osteophagia, confirming that bones that may be deposited in the rumen, or even bypass it, do not seem to provide adequate amounts of P and/or Ca. Moreover the blood data (Table 6) shows that plasma Ca and phosphate concentrations did not change and were therefore not affected by the presence of bones in the rumen. This indicates that either the concentration of P and Ca in blood is tightly regulated or that there is little absorption of these minerals when they are in the rumen.

4.5 CONCLUSION

Although osteophagia is a potentially large source of Ca and P, there appears to be little digestion of large bone fragments in the rumen that cannot pass through the reticulo-omasal orifice, at least in the model used. Furthermore the eating or chewing of bones does not lead to a decline in osteophagia suggesting that if absorbable forms of P and Ca were obtainable they would be in insufficient amounts for the animals requirements. The origin of sufficient dietary P for giraffes therefore remains an enigma.

Another reason altogether, for example, could be the appetite for bone is a simple curiosity and that the taste is appealing. Although this seems unlikely, the evidence available for domestic ruminants provides a strong case for osteophagia providing a potential mineral source in their diets.

It is known that bone in a processed form, included in a diet as a feed additive, can provide domestic ruminants with the necessary Ca and P requirements. This understanding along with the evidence of osteophagia provides sufficient evidence to indicate that osteophagia is a behaviour based on a nutritional need. However, it still remains to be determined how giraffes and other ruminants are able to utilize this source of Ca and P.

4.6 FUTURE WORK

This project has provided valuable data for the study of osteophagia in giraffes and ruminants in general. However it has also raised many questions. The following aspects regarding giraffes would require further investigation namely whether:

- The giraffes' rumen biochemistry differs significantly in comparison to that of sheep,
- Provide giraffe with bones of known dry mass. Allow them to chew and collect to determine how much bone was lost through abrasion and, by implication, swallowed,
- Feed browse to giraffe in captivity and determine their P status, then supply bones for osteophagia, after which P status could be re-determined.

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Appendix A

Raw Data for Mass, Volume and Density

Treat.	Bone Type	Bone No.	Day	Initial Mass (g)	Dry Mass1(g)	Vol. b4 treat.(ml)	Dry Mass2(g)	Density (g/ml)	Dry Mass3(g)	Vol. after treat.(ml)	Dry Mass4(g)	Density (g/ml)
1	1	32	0	10.6	9.766	5.0	9.725	2.0	9.694	5.0	9.682	1.9
1	1	27	0	12.5	11.525	6.0	11.471	1.9	11.432	5.9	11.407	1.9
1	1	50	0	8.6	8.011	4.0	7.975	2.0	7.959	4.2	7.946	1.9
1	1	4	0	11.4	10.576	5.5	10.514	1.9	10.469	5.0	10.437	2.1
1	1	13	0	10.6	9.844	5.0	9.796	2.0	9.759	5.0	9.733	2.0
1	2	69	0	5.2	4.554	5.0	4.510	0.9	4.508	4.0	4.458	1.1
1	2	72	0	7.4	3.539	4.0	3.472	0.9	3.479	4.0	3.432	0.9
1	2	88	0	5.5	4.810	4.6	4.736	1.0	4.659	4.0	4.607	1.2
1	2	100	0	5.9	4.865	5.0	4.796	1.0	4.780	5.5	4.753	0.9
1	2	99	0	6.1	5.129	5.0	5.064	1.0	5.063	4.0	5.005	1.3
2	1	5	10	10.8	10.010	5.5	9.940	1.8	9.797	5.0	9.773	2.0
2	1	45	10	9.3	8.603	4.5	8.558	1.9	8.490	4.5	8.465	1.9
2	1	22	10	9.8	9.072	5.0	9.033	1.8	8.930	5.0	8.912	1.8
2	1	3	10	11.6	10.761	5.8	10.712	1.9	10.591	5.0	10.571	2.1
2	1	36	10	10.6	9.804	5.0	9.760	2.0	9.672	5.0	9.646	1.9
2	2	95	10	4.6	4.191	4.0	3.923	1.0	3.446	4.0	3.423	0.9
2	2	79	10	5.5	4.824	4.0	4.755	1.2	4.466	4.0	4.439	1.1
2	2	80	10	6.6	5.660	5.0	5.521	1.1	4.812	4.0	4.783	1.2
2	2	86	10	5.3	4.374	4.0	4.326	1.1	3.982	4.0	3.962	1.0
2	2	65	10	6.3	5.342	5.0	5.262	1.1	4.471	4.0	4.452	1.1
2	1	18	20	10.3	9.505	5.0	9.432	1.9	9.313	5.0	9.284	1.9
2	1	33	20	9.9	9.195	5.0	9.132	1.8	8.996	5.0	8.978	1.8
2	1	46	20	9.1	8.417	4.3	8.371	2.0	8.253	4.8	8.234	1.7
2	1	25	20	9.9	9.211	5.0	9.140	1.8	9.009	5.0	8.982	1.8
2	1	49	20	8.4	7.802	4.0	7.780	2.0	7.720	4.2	7.701	1.8
2	2	94	20	5.4	4.791	5.6	4.747	0.9	3.939	4.0	3.916	1.0
2	2	98	20	5.0	4.449	4.0	4.395	1.1	3.981	4.0	3.960	1.0
2	2	92	20	4.7	4.334	4.0	4.226	1.1	3.663	4.0	3.638	0.9
2	2	62	20	6.2	5.567	4.0	5.471	1.4	5.290	5.0	5.272	1.1
2	2	64	20	5.7	4.625	4.0	4.567	1.2	3.828	4.0	3.806	1.0
2	1	21	30	9.8	9.104	4.9	9.043	1.9	8.910	5.0	8.879	1.8
2	1	20	30	12.2	11.262	6.0	11.204	1.9	11.100	6.0	11.067	1.9
2	1	19	30	10.1	9.414	4.9	9.372	1.9	9.252	5.0	9.234	1.9
2	1	48	30	8.8	8.088	4.0	8.062	2.0	7.977	4.2	7.960	1.9
2	1	47	30	11.5	10.653	5.5	10.592	1.9	10.493	5.0	10.475	2.1
2	2	85	30	5.7	4.632	4.0	4.563	1.2	4.200	4.0	4.183	1.1

Treat.	Bone Type	Bone No.	Day	Initial Mass (g)	Dry Mass1(g)	Vol. b4 treat.(ml)	Dry Mass2(g)	Density (g/ml)	Dry Mass3(g)	Vol. after treat.(ml)	Dry Mass4(g)	Density (g/ml)
2	2	81	30	7.3	5.831	5.0	5.775	1.2	5.400	6.0	5.378	0.9
2	2	77	30	8.0	6.216	6.0	6.116	1.0	5.236	5.0	5.219	1.0
2	2	73	30	6.8	5.823	6.0	5.770	1.0	5.422	6.0	5.401	0.9
2	2	91	30	7.4	6.095	6.0	6.062	1.0	5.752	6.0	5.731	1.0
3	1	8	10	11.1	10.246	5.4	10.198	1.9	10.137	5.5	10.115	1.8
3	1	39	10	10.7	9.890	5.0	9.852	2.0	9.821	5.1	9.796	1.9
3	1	37	10	10.7	9.893	5.1	9.857	1.9	9.820	5.0	9.793	2.0
3	1	41	10	10.0	9.242	4.9	9.206	1.9	9.184	5.0	9.162	1.8
3	1	40	10	9.4	8.724	4.8	8.693	1.8	8.667	4.8	8.645	1.8
3	2	66	10	4.6	4.038	3.0	3.948	1.3	3.841	3.0	3.821	1.3
3	2	68	10	7.0	5.772	5.8	5.730	1.0	4.729	5.5	4.688	0.9
3	2	53	10	6.7	5.710	5.0	5.649	1.1	5.243	6.0	5.208	0.9
3	2	97	10	6.1	5.406	6.0	5.339	0.9	3.714	3.5	3.640	1.1
3	2	70	10	7.1	6.482	4.5	6.401	1.4	5.740	5.0	5.704	1.1
3	1	10	20	10.2	9.427	5.0	9.392	1.9	9.354	5.0	9.331	1.9
3	1	12	20	10.5	9.753	5.0	9.705	2.0	9.642	5.0	9.615	1.9
3	1	29	20	9.9	9.159	5.0	9.101	1.8	9.033	5.0	9.006	1.8
3	1	9	20	12.6	11.626	6.0	11.566	1.9	11.461	6.0	11.433	1.9
3	1	23	20	9.4	8.720	4.9	8.686	1.8	8.654	4.8	8.633	1.8
3	2	76	20	5.5	4.229	4.0	4.122	1.1	3.719	4.0	3.706	0.9
3	2	55	20	7.2	6.232	5.8	6.104	1.1	5.021	4.0	4.995	1.3
3	2	96	20	5.3	4.788	5.0	4.707	1.0	3.894	4.0	3.866	1.0
3	2	84	20	4.8	4.029	3.5	3.975	1.2	3.892	3.0	3.875	1.3
3	2	57	20	5.0	4.490	4.0	4.443	1.1	4.279	4.0	4.269	1.1
3	1	35	30	11.8	10.922	6.0	10.853	1.8	10.783	5.9	10.751	1.8
3	1	30	30	11.5	10.685	5.6	10.641	1.9	10.596	5.2	10.572	2.0
3	1	31	30	11.4	10.553	5.0	10.477	2.1	10.411	5.5	10.386	1.9
3	1	28	30	12.4	11.456	6.0	11.398	1.9	11.302	6.0	11.273	1.9
3	1	15	30	11.3	10.483	5.5	10.434	1.9	10.370	5.0	10.350	2.1
3	2	74	30	4.7	3.798	4.0	3.700	0.9	3.449	4.0	3.431	0.9
3	2	89	30	5.3	4.694	4.0	4.625	1.2	3.283	3.0	3.259	1.1
3	2	56	30	5.3	4.638	3.8	4.550	1.2	4.333	4.0	4.309	1.1
3	2	87	30	5.1	4.455	4.2	4.395	1.1	3.114	3.5	3.063	0.9
3	2	52	30	4.5	3.999	5.0	3.798	0.8	3.145	3.0	3.111	1.0
4	1	7	10	10.0	9.242	5.0	9.188	1.8	9.130	5.0	9.107	1.8
4	1	42	10	10.3	9.511	5.0	9.477	1.9	9.449	5.0	9.428	1.9
4	1	14	10	10.7	9.847	5.3	9.813	1.9	9.782	5.0	9.754	2.0
4	1	44	10	10.3	9.583	5.0	9.535	1.9	9.502	5.0	9.478	1.9
4	1	1	10	11.3	10.379	5.9	10.339	1.8	10.268	5.5	10.249	1.9
4	2	93	10	7.0	6.340	5.8	6.291	1.1	5.881	5.5	5.833	1.1
4	2	82	10	5.5	4.867	4.0	4.797	1.2	5.074	4.0	5.058	1.3
4	2	51	10	6.9	5.998	5.9	5.961	1.0	5.772	6.0	5.759	1.0
4	2	71	10	7.5	5.813	6.0	5.761	1.0	5.674	6.0	5.648	0.9

Treat.	Bone Type	Bone No.	Day	Initial Mass (g)	Dry Mass1(g)	Vol. b4 treat.(ml)	Dry Mass2(g)	Density (g/ml)	Dry Mass3(g)	Vol. after treat.(ml)	Dry Mass4(g)	Density (g/ml)
4	2	75	10	7.3	5.802	6.0	5.758	1.0	5.620	6.0	5.595	0.9
4	1	26	20	10.8	10.029	5.0	9.981	2.0	9.930	5.2	9.905	1.9
4	1	11	20	10.4	9.613	5.0	9.574	1.9	9.533	5.0	9.508	1.9
4	1	17	20	11.8	10.938	5.8	10.886	1.9	10.838	6.0	10.808	1.8
4	1	38	20	11.5	10.702	5.5	10.634	1.9	10.588	5.2	10.537	2.0
4	1	16	20	10.6	9.839	5.0	9.777	2.0	9.718	5.0	9.693	1.9
4	2	58	20	6.0	5.176	4.5	5.079	1.2	5.525	5.0	5.510	1.1
4	2	54	20	6.1	4.901	5.5	4.859	0.9	4.837	5.0	4.819	1.0
4	2	78	20	6.9	6.066	4.5	5.945	1.3	6.581	5.5	6.544	1.2
4	2	61	20	7.1	5.997	4.0	5.888	1.5	6.125	5.0	6.107	1.2
4	2	83	20	5.6	4.978	4.0	4.869	1.2	5.476	5.5	5.453	1.0
4	1	2	30	12.2	11.290	6.0	11.240	1.9	11.199	5.5	11.163	2.0
4	1	34	30	9.5	8.678	4.8	8.613	1.8	8.594	4.8	8.573	1.8
4	1	43	30	11.3	10.406	5.1	10.350	2.0	10.316	5.6	10.291	1.8
4	1	24	30	9.2	8.558	4.8	8.515	1.8	8.458	4.8	8.440	1.8
4	1	6	30	12.3	11.402	6.0	11.356	1.9	11.303	6.0	11.280	1.9
4	2	63	30	5.2	4.685	3.8	4.635	1.2	5.205	5.0	5.190	1.0
4	2	90	30	7.2	5.845	5.8	5.785	1.0	6.194	6.0	6.163	1.0
4	2	59	30	6.7	5.620	6.0	5.597	0.9	5.915	6.0	5.887	1.0
4	2	67	30	6.3	5.267	5.0	5.244	1.1	5.246	6.0	5.225	0.9
4	2	60	30	6.5	5.398	5.8	5.367	0.9	5.467	5.5	5.450	1.0

- Treatments: 1= control; 2= distilled water; 3= artificial saliva; 4= rumen fluid
- Bone Type: 1= metacarpus; 2= cervical vertebrae

Appendix B

Data for Ca and P determination

Bone No.	Sample Wt (g)	Sample Wt (g)	Dry Mass(g)	Dry Mass(g)	Ash (g)	Ash (g)	Ca Reading	Ca Reading	Avg. %Ca	P Reading	P Reading	Avg. %P
32	0.502	0.499	0.488	0.484	0.354	0.347	2.510	2.545	25.25	11.720	11.670	11.68
27	0.501	0.501	0.494	0.496	0.354	0.356	2.550	2.775	26.57	11.800	11.800	11.78
50	0.502	0.499	0.485	0.480	0.344	0.328	2.455	2.410	24.30	11.250	11.100	11.16
4	0.500	0.501	0.496	0.499	0.349	0.351	2.530	2.605	25.65	11.490	11.580	11.52
13	0.503	0.502	0.500	0.500	0.368	0.364	2.615	2.615	26.02	11.650	11.850	11.69
69	0.499	0.502	0.485	0.490	0.269	0.267	1.980	2.105	20.40	8.899	8.910	8.90
72	0.499	0.500	0.487	0.487	0.291	0.289	2.210	2.180	21.97	9.744	9.562	9.66
88	0.500	0.499	0.489	0.487	0.278	0.278	2.070	2.050	20.62	9.576	8.778	9.19
100	0.500	0.502	0.479	0.483	0.293	0.298	2.010	2.190	20.96	9.279	9.432	9.34
99	0.500	0.500	0.485	0.483	0.297	0.292	2.210	2.110	21.60	10.470	10.330	10.40
5	0.503	0.499	0.502	0.497	0.352	0.348	2.695	2.630	26.57	11.630	11.370	11.48
45	0.502	0.501	0.485	0.485	0.348	0.346	2.420	2.430	24.18	11.270	11.420	11.31
22	0.500	0.499	0.498	0.498	0.357	0.353	2.305	2.485	23.97	10.710	11.400	11.07
3	0.502	0.502	0.497	0.497	0.359	0.360	2.665	2.700	26.72	11.900	11.790	11.80
36	0.501	0.500	0.492	0.490	0.351	0.346	2.375	2.430	24.00	11.340	11.390	11.35
95	0.500	0.500	0.493	0.492	0.342	0.342	2.590	2.600	25.95	11.320	11.930	11.63
79	0.500	0.501	0.488	0.481	0.314	0.311	2.235	2.290	22.60	10.490	10.640	10.55
80	0.501	0.500	0.488	0.486	0.327	0.327	2.350	2.400	23.73	11.570	10.800	11.17
86	0.500	0.500	0.486	0.484	0.305	0.302	2.385	2.265	23.25	10.200	10.300	10.25
65	0.502	0.500	0.492	0.490	0.311	0.313	2.240	2.245	22.38	10.260	10.310	10.26
18	0.503	0.500	0.494	0.489	0.341	0.338	2.375	2.390	23.75	11.120	10.760	10.91
33	0.500	0.502	0.483	0.483	0.340	0.339	2.220	2.350	22.80	10.850	11.000	10.90
46	0.501	0.502	0.494	0.492	0.348	0.346	2.400	2.650	25.17	10.080	10.380	10.20
25	0.502	0.501	0.484	0.484	0.343	0.342	2.485	2.380	24.25	10.400	10.320	10.33
49	0.499	0.499	0.484	0.480	0.346	0.346	2.650	2.475	25.68	11.000	11.600	11.32
94	0.502	0.500	0.493	0.492	0.330	0.329	2.525	2.365	24.40	9.807	9.649	9.71
98	0.501	0.502	0.486	0.485	0.315	0.317	2.210	2.200	21.98	9.877	9.982	9.90
92	0.501	0.501	0.489	0.486	0.339	0.338	2.575	2.620	25.92	11.280	11.840	11.54
62	0.501	0.500	0.481	0.481	0.322	0.321	2.355	2.315	23.33	10.360	10.750	10.54
64	0.502	0.500	0.497	0.493	0.306	0.302	2.295	2.235	22.60	8.683	8.866	8.76
21	0.500	0.499	0.500	0.498	0.358	0.357	2.520	2.425	24.75	11.230	11.320	11.29
20	0.501	0.502	0.500	0.501	0.367	0.365	2.615	2.620	26.10	11.850	11.740	11.76
19	0.500	0.503	0.498	0.501	0.362	0.362	2.540	2.585	25.55	11.560	11.760	11.62
48	0.500	0.501	0.495	0.499	0.355	0.356	2.580	2.505	25.40	11.430	11.520	11.46
47	0.499	0.502	0.487	0.492	0.354	0.356	2.510	2.525	25.15	11.500	11.590	11.53

Bone No.	Sample Wt (g)	Sample Wt (g)	Dry Mass(g)	Dry Mass(g)	Ash (g)	Ash (g)	Ca Reading	Ca Reading	Avg. %Ca	P Reading	P Reading	Avg. %P
85	0.500	0.501	0.478	0.486	0.290	0.301	2.115	2.150	21.30	10.290	10.450	10.36
81	0.501	0.502	0.486	0.485	0.298	0.298	2.130	2.180	21.49	10.130	9.990	10.03
77	0.500	0.500	0.487	0.489	0.320	0.322	2.405	2.260	23.33	11.370	11.270	11.32
73	0.500	0.502	0.487	0.485	0.298	0.300	2.200	2.230	22.11	10.100	9.731	9.90
91	0.502	0.501	0.495	0.491	0.305	0.304	2.145	2.240	21.86	9.465	9.109	9.26
8	0.500	0.500	0.493	0.492	0.362	0.360	2.770	2.550	26.60	11.350	11.510	11.43
39	0.500	0.502	0.498	0.501	0.364	0.366	2.480	2.515	24.92	11.360	11.560	11.44
37	0.499	0.502	0.494	0.496	0.355	0.358	2.530	2.420	24.73	11.490	10.720	11.10
41	0.502	0.500	0.481	0.483	0.354	0.353	2.595	2.495	25.40	11.560	11.550	11.53
40	0.499	0.499	0.492	0.490	0.357	0.358	2.525	2.545	25.40	11.710	11.660	11.71
66	0.500	0.501	0.482	0.481	0.326	0.322	2.265	2.460	23.60	10.260	10.650	10.44
68	0.501	0.501	0.493	0.492	0.327	0.326	2.430	2.365	23.93	10.190	10.070	10.11
53	0.500	0.501	0.484	0.482	0.311	0.305	2.385	2.210	22.95	10.110	9.865	9.98
97	0.501	0.498	0.490	0.488	0.340	0.335	2.620	2.550	25.88	11.800	11.700	11.76
70	0.500	0.502	0.493	0.492	0.362	0.360	2.510	2.575	25.37	12.320	12.390	12.33
10	0.500	0.502	0.497	0.500	0.360	0.363	2.565	2.570	25.62	12.110	12.070	12.07
12	0.500	0.502	0.498	0.499	0.359	0.359	2.720	2.645	26.77	11.790	11.690	11.72
29	0.502	0.499	0.484	0.485	0.338	0.337	2.320	2.300	23.08	11.070	10.870	10.96
9	0.501	0.502	0.500	0.498	0.352	0.353	2.505	2.525	25.07	11.450	11.850	11.61
23	0.501	0.503	0.499	0.501	0.368	0.373	2.505	2.570	25.27	11.890	12.230	12.01
76	0.502	0.500	0.490	0.487	0.310	0.309	2.305	2.225	22.60	10.650	10.550	10.58
55	0.501	0.502	0.489	0.487	0.341	0.342	2.455	2.495	24.68	11.820	11.270	11.51
96	0.501	0.501	0.488	0.488	0.353	0.354	2.830	2.610	27.15	12.540	12.500	12.50
84	0.501	0.499	0.499	0.493	0.333	0.328	2.360	2.350	23.55	11.660	11.610	11.63
57	0.499	0.500	0.491	0.491	0.325	0.323	2.285	2.395	23.42	10.200	10.830	10.53
35	0.499	0.502	0.489	0.491	0.341	0.344	2.260	2.385	23.20	11.400	11.440	11.41
30	0.502	0.500	0.484	0.484	0.354	0.354	2.510	2.585	25.43	11.890	11.780	11.81
31	0.500	0.502	0.489	0.494	0.350	0.352	2.435	2.395	24.10	11.670	11.670	11.65
28	0.501	0.500	0.496	0.496	0.357	0.358	2.510	2.550	25.27	11.900	11.840	11.86
15	0.502	0.500	0.500	0.500	0.365	0.363	2.590	2.540	25.60	11.480	11.630	11.53
74	0.500	0.499	0.488	0.486	0.336	0.333	2.540	2.465	25.05	9.986	10.630	10.32
89	0.502	0.502	0.494	0.497	0.381	0.385	2.845	2.950	28.86	13.180	13.440	13.26
56	0.500	0.500	0.498	0.500	0.336	0.330	2.335	2.465	24.00	11.160	11.150	11.16
87	0.499	0.499	0.496	0.490	0.339	0.338	2.510	2.465	24.92	11.850	11.390	11.64
52	0.499	0.500	0.483	0.483	0.364	0.365	2.460	2.445	24.55	11.620	11.350	11.50
7	0.500	0.499	0.496	0.493	0.345	0.342	2.540	2.530	25.38	11.470	11.120	11.31
42	0.500	0.501	0.497	0.496	0.357	0.360	2.450	2.435	24.40	11.450	11.750	11.59
14	0.501	0.501	0.500	0.500	0.368	0.365	2.690	2.570	26.25	11.530	11.630	11.56
44	0.500	0.500	0.493	0.494	0.353	0.355	2.540	2.540	25.40	11.440	11.380	11.41
1	0.502	0.503	0.494	0.494	0.355	0.357	2.760	2.645	26.89	12.220	11.910	12.01
93	0.500	0.502	0.490	0.490	0.344	0.340	2.320	2.370	23.40	10.160	10.050	10.08
82	0.500	0.500	0.492	0.491	0.310	0.314	2.230	2.270	22.50	10.660	10.870	10.77
51	0.501	0.501	0.479	0.484	0.308	0.313	2.195	2.150	21.68	9.868	10.160	9.99

Bone No.	Sample Wt (g)	Sample Wt (g)	Dry Mass(g)	Dry Mass(g)	Ash (g)	Ash (g)	Ca Reading	Ca Reading	Avg. %Ca	P Reading	P Reading	Avg. %P
71	0.502	0.502	0.493	0.494	0.300	0.302	2.035	2.100	20.59	8.902	8.104	8.47
75	0.499	0.500	0.495	0.495	0.303	0.301	2.215	2.050	21.35	10.240	10.180	10.22
26	0.501	0.500	0.491	0.491	0.347	0.345	2.485	2.500	24.90	11.650	11.700	11.66
11	0.502	0.502	0.490	0.496	0.353	0.354	2.510	2.735	26.12	11.280	11.110	11.15
17	0.499	0.499	0.498	0.498	0.355	0.357	2.480	2.525	25.08	11.370	11.520	11.47
38	0.501	0.501	0.495	0.497	0.351	0.349	2.455	2.475	24.60	11.420	11.460	11.42
16	0.502	0.500	0.501	0.499	0.357	0.355	2.625	2.520	25.67	11.270	11.420	11.32
58	0.501	0.502	0.500	0.500	0.308	0.308	2.180	2.195	21.81	9.795	9.835	9.79
54	0.502	0.499	0.484	0.483	0.295	0.295	2.245	2.085	21.63	7.708	8.293	7.99
78	0.502	0.502	0.495	0.496	0.305	0.309	2.125	2.085	20.97	10.590	10.590	10.55
61	0.499	0.501	0.489	0.487	0.314	0.314	2.110	2.160	21.35	10.710	10.790	10.75
83	0.501	0.501	0.490	0.486	0.304	0.302	2.090	2.070	20.76	10.260	10.320	10.27
2	0.502	0.500	0.499	0.497	0.365	0.361	2.690	2.605	26.42	12.060	11.750	11.88
34	0.500	0.501	0.494	0.499	0.343	0.344	2.440	2.375	24.05	11.400	10.640	11.01
43	0.499	0.501	0.498	0.498	0.358	0.362	2.415	2.490	24.52	10.670	10.730	10.70
24	0.503	0.501	0.500	0.500	0.366	0.361	2.550	2.525	25.27	11.520	11.810	11.62
6	0.503	0.501	0.501	0.500	0.362	0.358	2.860	2.655	27.46	11.770	11.920	11.80
63	0.500	0.501	0.484	0.485	0.314	0.316	2.405	2.220	23.10	9.115	8.764	8.93
90	0.500	0.500	0.496	0.494	0.301	0.299	2.175	2.205	21.90	10.050	10.050	10.05
59	0.500	0.500	0.489	0.489	0.302	0.301	2.115	2.105	21.10	9.825	9.767	9.80
67	0.499	0.502	0.491	0.494	0.302	0.304	2.130	2.145	21.35	9.878	9.685	9.77
60	0.500	0.502	0.494	0.496	0.303	0.307	2.185	2.125	21.51	10.040	10.180	10.09