The influence of dietary crude protein intake on bone and mineral metabolism in sheep

T S Brand[a], Q Johnson[b], F Franck[a], W Veith[b], R Conradie[c] and F S Hough[c]

ABSTRACT

Increased dietary protein consumption is thought to cause calciuresis, a negative calcium balance and increased bone loss that may result in skeletal deformities and fracture. To explore this hypothesis, 40 approximately 100-day-old meat-type Merino ram lambs were fed, for 6 months, diets with an increasing crude protein (CP) content (114, 142, 171 and 190 g/kg DM) but approximately on an iso-nutrient basis with regard to metabolisable energy, calcium and phosphorus. Increased protein consumption modestly (NS) enhanced calciuresis and resulted in significant ($P \leq 0.01$) limb skewness. This could not, however, be ascribed to osteopaenic bones, and compared with animals consuming lower protein rations, the bone mineral density (BMD) and vertebral trabecular bone volume of animals fed high protein diets were significantly increased: the BMD of thoracic vertebrae was positively related to the CP intake ($r = 0.62$; $P \leq 0.001$). In animals consuming higher protein diets, skeletal radiology and quantitative bone histology revealed no evidence of increased bone turnover as would be expected in animals that are in negative calcium balance. No relationship existed between limb skewness and the growth rate of lambs. However, the ratio of Ca:P in the forelimb ($r = –0.98$), vertebrae ($r = –0.72$) and rib ($r = –0.42$) was found to be inversely correlated with increased protein intake and resulted from an increase in the phosphorus content of bone, while the amount of bone calcium was unaffected. We conclude that qualitative micro-architectural abnormalities, and not mere bone loss, may underlie the skeletal deformities induced by increased protein consumption in sheep.

Key words: bone loss, bone mineral density, calciuresis, limb skewness, sheep, skeletal deformities.

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INTRODUCTION

A number of reports have suggested an association between protein consumption and the incidence of skeletal fracture[2,11]. This has generally been ascribed to the fact that a high protein intake increases urinary calcium excretion, resulting in a negative calcium balance and bone loss[7,13].

Bone abnormalities also regularly develop in meat-type Merino ram lambs that are fed to induce a rapid growth rate. Such feeding strategies are normally associated with an increased dietary protein content. In a recent study spanning 8 years[20], it was reported that on average 8.6 % of ram lambs annually developed the bent-leg syndrome.

Despite the unequivocal impact of dietary protein on renal calcium handling and calcium balance, its long-term effects on bone and mineral metabolism remain controversial. Some studies have suggested that increased protein consumption only causes calciuresis in the short term and that urinary calcium excretion was not affected in long-term studies[18]. Furthermore, protein-induced alterations in urinary calcium excretion or calcium balance have not consistently resulted in the expected increased levels of serum parathyroid hormone (PTH), urinary cyclic AMP or biochemical markers of increased bone turnover[10]. In fact, it has often been difficult to demonstrate that high protein diets actually cause osteopaenia in experimental situations[22].

In this study, the influence of increasing crude protein, but fixed energy, calcium and phosphorus intake on bone and mineral metabolism in growing lambs was examined.

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MATERIALS AND METHODS

Animals and feeds

Four experimental diets (Table 1) were formulated with increasing crude protein (CP), but approximately iso-nitrogenous with regard to metabolisable energy (ME), calcium (Ca) and phosphorus (P). Blood-meal and fish-meal were used as protein sources (Table 1) and were bound to the whole grain by molasses powder, while the roughage components of the diets were hammer-milled (12 mm screen). The diets were thoroughly mixed in a feed mixer.

The dry matter (DM), organic matter (OM), crude protein (CP), Ca and P content of the experimental diets were determined according to standard AOAC procedures[1]. *In vitro* digestibility of OM[19] of the diets was also determined. The amino-acid composition of feed samples, after hydrolysis in a sealed tube, was determined using a Beckmann Model 7300 amino-acid analyser.

Forty meat-type Merino (SAMM) ram lambs, approximately 100 days of age with a mean (±SD) live mass of 32 ± 4 kg were randomly allotted to the 4 experimental diets, with 10 lambs per diet. Before the start of the study, lambs were drenched with a broad-spectrum anthelmintic, vaccinated against *Clostridium perfringens* Type D and adapted for 3 weeks to the experimental conditions and respective diets. Each ram was individually housed in an indoor pen equipped with feed and water trays. The lambs had free access to feed and water at all times. Feed intake and live mass were measured every 7 days and the dry matter intake, average daily mass gain and feed conversion ratios for the growth interval 30–70 kg live mass calculated.

Methods

During the last 10 days of the study, blood samples were obtained via the jugular vein from all animals, and 4 lambs per diet group were randomly selected for 24 h urine collections. Calcium and magnesium were determined using a Pye unicam SP9 atomic absorption spectrophotometer, and phosphate by the

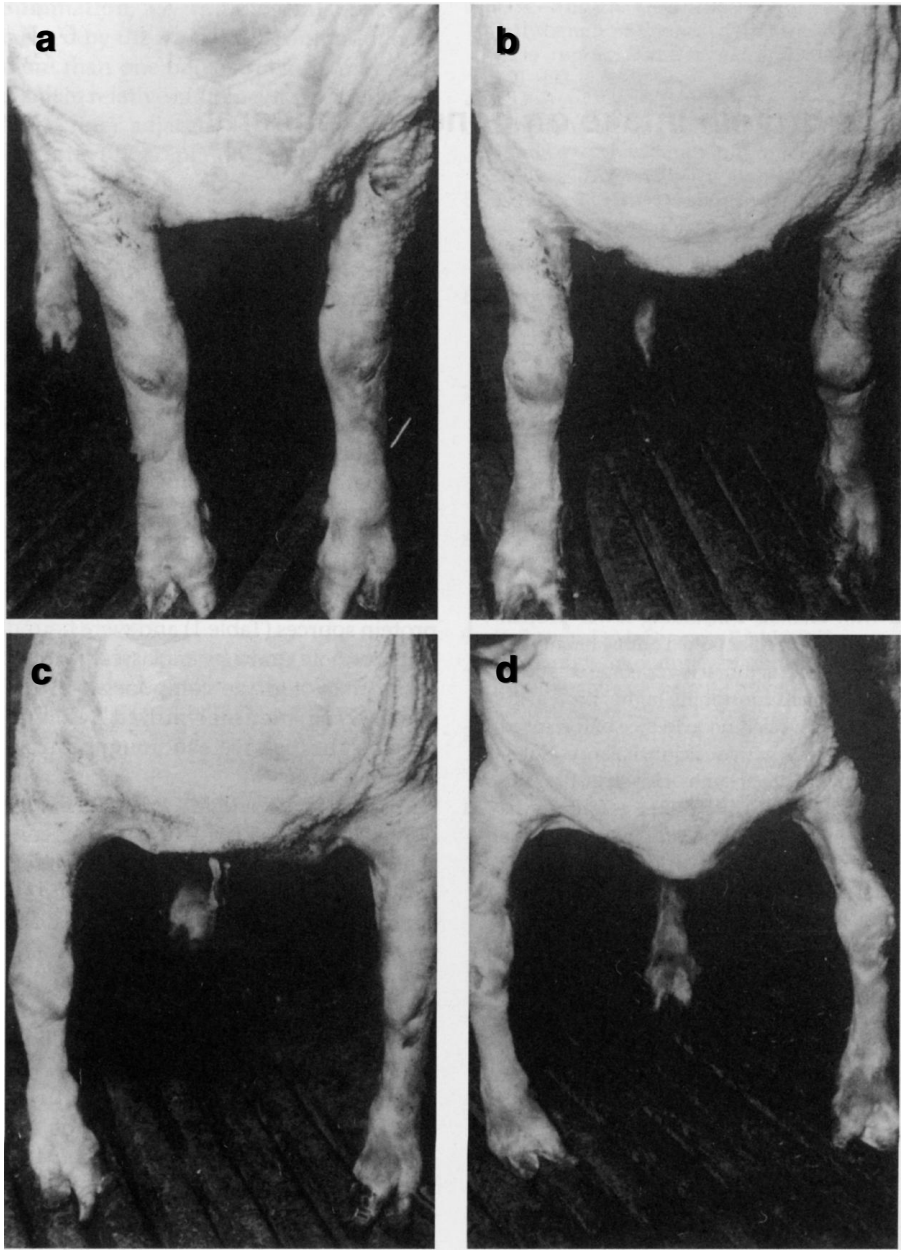

Fig. 1: **The scorecard used to judge the degree of skewness of the forelimbs of lambs. a = 25 %; b = 50 %; c = 75 %; d = 100 %.**

molybdenum blue method. The degree of limb skewness was scored independently by 2 observers at the end of the study period according to the arbitrary scale presented in Fig 1. The forelimbs of all the sheep were also radiographed with an Anodel R 35 X-ray unit at a focal spot-firm distance of 100 cm. Radiographs were examined for evidence of stress fractures, rickets or subperiosteal resorption. At the conclusion of the study, lambs were slaughtered at a mean (±SD) live mass of 79 ± 8 kg and approximately 300 days of age. Samples were taken from the forelimbs (5 cm from the fetlock joint), the 9th rib and the 13th thoracic vertebra of each carcass. Bone density (specific gravity), calcium, phosphate and ash content of bone samples were determined and expressed per unit volume of fresh bone[15].

Before sacrifice, all animals received 2 intramuscular injections of tetracycline hydrochloride (10 mg/kg body mass), as marker, 10 days apart. Animals were slaughtered 5 days after the 2nd dose, the 12th thoracic vertebra was removed, fixed in a Millonig's solution (3.7 % formaldehyde, 93 mM NaH_2PO_4, 105 mM NaOH and 14.6 mM sucrose) for 24 h, then embedded in methylmethacrylate, sectioned at 5 and 15 μm on a LKB sledge microtome, and stained by the Goldner Trichrome technique[16]. Histomorphometric analysis of vertebral sections, using a Merz-Schenk integrating eyepiece included: bone volume (BV/TV), osteoid volume (OV/TV; OV/BV), osteoid surface (OS/BS), osteoblast surface (Ob.S/BS), mineralising surface (MS/BS (d + s label); MS/BS (only d label), adjusted apposition rate (Aj.AR), eroded surface (ES/BS), and osteoclast surface (Oc.S/BS). Parameter terminology and definitions are those recommended by the ASBMR Histomorphometry Nomenclature Committee[14].

Statistics

Means and standard errors are presented for data to provide an indication of the variability between replicates. Differences between treatment means were tested for significance by 1-way analysis of variance, using the *F*-test to detect significance (Statgraphic Version 5.0, Statgraphics Corporation, Rockville, Maryland). One lamb was withdrawn from the study due to the development of a prolapse (diet 1), while another lamb died owing to *C. perfringens* Type D (diet 3), and their data were excluded from the statistical analysis. In scoring the degree of skewness of the legs, the average score of the 2 judges was used as an independent variable. Tendencies in the measured parameters due to different protein intake levels were derived by linear regression analysis. Procedures used were according to Snedecor and Cochran[17].

RESULTS

The chemical composition of the experimental diets is summarised in Table 1. The CP content increased by approximately 28.4 g CP/kg DM per diet (from 114 g CP/kg DM to 190.9 g/kg DM), but was approximately iso-nitrogenous in ME (11.37 MJ/kg DM), Ca (9.18 g/kg DM) and P (3.47 g/kg DM) content. The total sulphur amino-acid content of the diets increased by approximately 1.43 g/kg DM from diet 1 to diet 4.

The production data of the lambs on the different diets are presented in Table 2. The daily CP intake of the lambs increased by 50.3 ± 6.4 g CP per lamb from diet 1 to diet 4 ($P \leq 0.001$). The composition of the diets had no significant effect on DM, Ca or P intake. The growth rate of lambs on diets 2, 3 and 4 were similar, but significantly higher ($P \leq 0.005$) than that of lambs on diet 1. The lambs on diet 4 used protein approximately 39 % less efficiently ($P \leq 0.001$) in terms of live-mass gain than lambs on the other diets.

The degree of skewness of the forelimbs, the gravimetrically determined density of thoracic vertebrae (BMD), and the histomorphometric trabecular bone volume (TBV) are presented in Table 3. The limbs of lambs on the highest protein diet (diet 4) showed a significantly ($P \leq 0.01$) higher degree of skewness than the forelimbs of animals on the lower protein diets. Compared to animals consuming lower protein rations, the vertebral BMD

and TBV of animals fed high protein diets were significantly ($P \leq 0.05$) increased. The density of the thoracic vertebrae was positively related ($r = 0.62$; $P \leq 0.001$) to the individual daily crude protein intake of the lambs.

The Ca, P and ash content of long-bone (cannon), vertebral and rib samples are presented in Table 4. No significant effect of CP intake on the Ca content of bone was observed, although animals on the higher protein diets tended ($P \leq 0.10$) to have higher mean values. The Ca content in the ribs was positively related to CP intake ($r = 0.36$; $P \leq 0.03$). The CP intake of lambs significantly ($P \leq 0.05$) increased the P content in rib and vertebral samples. The protein intake was positively correlated ($r = 0.45$; $P \leq 0.01$) with the P content of the thoracic vertebrae. Owing to the sharp increase in the P content and the modest increase in bone Ca with increasing CP intakes, an inverse relationship between protein intake and skeletal Ca:P ratios was observed in all bone samples, reaching statistical significance ($P \leq 0.02$) in the cannon that became deformed (Table 4).

The mean plasma Ca (2.40 ± 0.04 mmol/ℓ), plasma P (1.55 ± 0.04 mmol/ℓ) and plasma Mg (1.18 ± 0.05 mmol/ℓ) did not differ significantly between treatments. Similarly no significant differences in the mean urinary excretion of Ca (3.7 ± 0.75 mmol/24 h) between treatments were detected, while urinary excretion of P tended ($P \leq 0.10$) to be higher in the high-protein group (mean 0.52 ± 0.06 mmol/24 h).

Skeletal radiology revealed no consistent evidence of stress fractures, rickets or subperiosteal resorption. Quantitative bone histomorphometry documented a significantly ($P \leq 0.05$) higher trabecular bone volume in animals consuming the higher protein diet (Table 3). Parameters of bone formation, including osteoid volumes and surfaces, osteoblast numbers and surfaces assuming a tetracycline label, were lower ($P \leq 0.05$) in animals consuming higher protein diets. Parameters of bone resorption were similar in all groups, without evidence of increased resorption in animals on the higher protein diets.

Table 1: **Ingredient** (air-dry basis) **and chemical composition** (dry-matter basis) **of the experimental diets**[a].

Composition	Experimental diets			
	Diet 1	Diet 2	Diet 3	Diet 4
Ingredient (kg/ton)				
Wheat grain	774	735	693	654
Wheat straw	100	100	100	100
Lucerne hay	50	50	50	50
Blood meal (883 g CP/kg DM)	—	26	54	80
Fish-meal (629 g CP/kg DM)	—	10	32	48
Molasses powder	20	20	20	20
Bicarbonate of soda	20	20	20	20
Calcium carbonate	14	13	13	12
Ammonium chloride	10	10	10	10
Dicalcium phosphate	6	4	2	0
Salt	4	4	4	4
Mineral and vitamin premix[b]	2	2	2	2
Composition				
Crude protein (g/kg DM)	114.0	141.8	171.0	198.9
Metabolisable energy (MJ/kg DM)[c]	11.88	11.00	11.59	11.02
Calcium (g/kg DM)	9.49	9.17	9.20	8.87
Phosphorus (g/kg DM)	3.52	3.4	3.53	3.41
Cystine (g/kg DM)	3.85	4.17	4.74	5.39
Methionine (g/kg DM)	2.64	2.74	4.63	5.06

[a]All diets contained 100 g/ton Thylan and 300 g/ton Tauratec.
[b]Supplied: 1500 g NaCl; 200 g MgO; 100 g $MnSO_4$ 4 H_2O; 45 g ZnO; 30 g S; 1 g KCl; 0.5 g $CoCO_3$; 5 g thiamine hydrochloride; 5000 IU Vitamin A; 1000 IU Vitamin D; 20 IU Vitamin E.
[c]Calculated from the formula: ME (MJ/kg DM) = 0.015 × digestible organic matter in dry matter[19].

Table 2: **Dry-matter intake** (DMI)**, crude protein intake** (CPI)**, Ca and P intakes, average daily gain** (ADG)**, feed conversion ratio** (FCR) **and protein efficiency ratio** (PER) **for lambs** (30–70 kg) **on diets with an increasing protein content***.

Parameter measured	Experimental diets			
	Diet 1** ($n = 9$)	Diet 2 ($n = 10$)	Diet 3 ($n = 9$)	Diet 4 ($n = 10$)
DMI, g/day	1560 ± 39	1669 ± 68	1577 ± 40	1700 ± 35
CPI, g/day	178 ± 4[a]	247 ± 10[b]	270 ± 7[c]	338 ± 7[d]
Ca intake, g/day	14.8 ± 0.4	15.3 ± 0.6	14.5 ± 0.4	15.1 ± 0.3
P intake, g/day	5.5 ± 0.1	5.7 ± 0.2	5.7 ± 0.1	5.8 ± 0.1
ADG, g/day	239 ± 18[a]	319 ± 14[b]	316 ± 19[b]	302 ± 13[b]
FCR, kg DMI/kg gain	6.83 ± 0.46[a]	5.29 ± 0.32[b]	5.15 ± 0.32[b]	5.15 ± 0.22[b]
PER, g CPI/g gain	0.78 ± 0.05[a]	0.78 ± 0.05[a]	0.88 ± 0.06[a]	1.13 ± 0.04[b]

*Data are presented as the mean ± standard error.
**Means with the same superscript letter are not significantly different ($P \leq 0.01$) between diets.

Table 3: **The degree of skewness of the forelimbs, gravimetrically determined density of thoratic vertebrae** (BMD) **and histomorphometric trabecular bone volume** (TBV) **of lambs on diets with an increased protein content.**

Measurement	Diet 1*	Diet 2	Diet 3	Diet 4
Degree of skewness, %	49.3[a] ± 0.9	49.3[a] ± 2.2	51.1[a] ± 1.2	60.8[b] ± 4.4
BMD, g/cm	1 .28[a] ± 0.02	1.32[a] ± 0.01	1.30[a] ± 0.02	1.37[b] ± 0.01
TBV, %	28.0[a] ± 4.0	28.6[a] ± 4.2	30.6[a] ± 4.6	34.7[b] ± 4.8

*Means with the same superscript letter are not significantly different ($P \leq 0.05$) between diets.

DISCUSSION

The lack of response in the average daily gain of lambs when CP intake exceeded 247 g per day (diets 2–4), as well as the poorer protein efficiency ratios of diets 3 (13 %) and 4 (45 %), compared with diets 1 and 2, is explained by their requirements at that growth stage. The crude protein requirements of a 50 kg lamb is 253 g per day[12]. Both daily Ca and P intakes were nearly equal for lambs on the different diets, and exceeded requirements for a 50 kg ram lamb (8 g Ca and 4 g P per lamb per day[12]). A previous study reported that the incidence of limb deformities in dogs increased with an increase in body size of the animal[5]. In agreement with the study of Van Niekerk *et al.*[19], no

Table 4: **The mineral content of ribs, cannons and thoracic vertebrae of lambs on diets with an increasing protein content***.

Component	Diet 1** (n = 9)	Diet 2 (n = 10)	Diet 3 (n = 9)	Diet 4 (n = 10)
Rib				
Calcium, mg/cm^3	203 ± 14	227 ± 8	225 ± 14	246 ± 14
Phosphorus, mg/cm^3	148 ± 19^a	124 ± 12^a	174 ± 22^{ab}	207 ± 20^b
Ash, %	36.8 ± 2.9	39.7 ± 0.6	38.7 ± 1.1	40.2 ± 1.9
Ca:P	1.18 ± 0.15	1.98 ± 0.20	1.55 ± 0.30	1.28 ± 0.14
Cannon				
Calcium, mg/cm^3	331 ± 17	320 ± 19	359 ± 13	373 ± 24
Phosphorus, mg/cm^3	230 ± 36	243 ± 39	282 ± 30	339 ± 29
Ash, %	47.8 ± 2.8	50.1 ± 1.7	49.3 ± 1.30	52.4 ± 3.8
Ca:P	1.86 ± 0.43	1.51 ± 0.15	1.38 ± 0.14	1.14 ± 0.09
Thoracic vertebrae				
Calcium, mg/cm^3	162 ± 10	165 ± 6	160 ± 8	178 ± 7
Phosphorus, mg/cm^3	140^a ± 12	149^a ± 10	196^b ± 9	187^b ± 14
Ash, %	27.3 ± 1.2	38.2 ± 5.3	28.9 ± 2.0	27.7 ± 1.5
Ca:P	1.20^a ± 0.10	1.15^a ± 0.09	0.83^b ± 0.05	1.03^{ab} ± 0.13

*Data are expressed as percentage of fresh bone and presented as the mean ± standard error.
**Means with the same superscript letter are not significantly different ($P \leq 0.05$) between diets.

relationship existed between limb skewness and the growth rate of lambs in our study.

The high intake of dietary proteins provided by most western diets is thought to predispose to the development of osteoporosis and skeletal fracture[2,11]. This is generally ascribed to the observation that high protein intake causes hypercalciuria and a negative calcium balance in experimental animals and in humans[7,13]. Numerous studies have suggested that dietary protein exerts a quantitatively greater effect on calcium balance and urinary excretion than does dietary calcium intake[9,23]. The mechanisms of protein-induced hypercalciuria are incompletely understood, but are thought to involve an increase in glomerular filtration rate and a decrease in fractional tubular reabsorption of calcium that may be related to the acid ash and sulphur amino-acid content of high-protein diets[21]. However, protein in the diet is generally accompanied by phosphorus, which is known to increase the renal tubular reabsorption of calcium[23] and may also impair intestinal calcium absorption[8]. It has therefore been proposed that the deleterious effects of a high protein diet on skeletal integrity may be offset by an increase in dietary phosphorus and calcium[6].

Despite the unequivocal impact of dietary protein on renal calcium handling and calcium balance, long-term studies have not consistently documented that a high protein intake results in secondary hyperparathyroidism, an increased bone turnover and bone loss. It has often been difficult to demonstrate that high protein diets cause osteopaenia[6,22].

The higher prevalence of bone abnormalities observed in animals consuming the high protein diet (diet 4) suggested that the deformity was causally related to the excessive protein and/or sulphur amino-acid intake, since the intake of other nutrients was nearly identical. In contradiction to earlier reports[7,13], which documented hypercalciuria with an increase in the intake of sulphur-containing amino-acids, urinary calcium excretion was only modestly and not significantly increased by a high protein diet in the present study. This may be a function of the small number of animals (n = 4/group) studied, but may also relate to the relatively high phosphorus content of the diet, which would tend to curb renal calcium wasting[8]. It is also possible that the animals were able to adjust to a certain extent to the high protein diets consumed during the experimental period. Moreover, although we did not measure PTH and biochemical parameters of bone turnover, quantitative bone histology revealed no evidence of a protein-induced increase in bone turnover. In fact, bone turnover tended to be higher in animals reared on a lower protein intake. Although Funaba *et al.*[7] have suggested that a high protein intake may lead to enhanced bone resorption in sheep, other studies have supported our observation that a high protein diet does not increase bone resorption when Ca and P intakes are adequate[3,4].

Although increased protein consumption resulted in significant ($P \leq 0.05$) limb skewness, this could not be ascribed to osteopaenic bones as suggested by previous studies[2,11]. Compared with animals consuming lower protein rations, gravimetrically determined bone density (BMD) and histological trabecular bone volume of animals fed high protein diets were significantly ($P \leq 0.05$) increased, while the BMD of thoracic vertebrae was positively related to the crude protein intake ($r = 0.62$; $P \leq 0.001$). The calcium content of bone samples was unaffected by higher protein intakes. The phosphorus content of bone, however, increased progressively with an increase in protein intake, resulting in a lower Ca:P ratio. The reason for the increase in the phosphorus content of the bone is, however, unclear. An inverse relationship between protein intake and skeletal Ca:P ratios was observed in all bone samples studied.

We conclude that in the present study high protein intake in sheep resulted in significant skeletal deformities that could not be ascribed to increased renal calcium wasting, a negative Ca balance, increased bone resorption or a decrease in bone density. High crude protein intake was associated with an increase in the phosphorus content and an impaired Ca:P ratio in the bone of lambs. These observations suggest that qualitative structural abnormalities and not mere bone loss may underlie the skeletal abnormalities induced by increased protein consumption in sheep.

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Book review — Boekresensie

Proceedings of a Symposium on Cheetahs as Game Ranch Animals

Edited by B L Penzhorn

1998. South African Veterinary Association Wildlife Group, Onderstepoort, South Africa, 209 pp. Price R120.00 (R140.00 elsewhere if paid by bank draft in South African rand); US$ 40.00. ISBN 1-875088-10-5.

A symposium entitled 'Cheetahs as Game Ranch Animals' was held at Onderstepoort on 23 and 24 October 1998 and the proceedings were made available at registration through the kind sponsorship of the De Wildt Cheetah and Wildlife Centre.

As quoted in the Foreword, 'This symposium is a further contribution to the SAVA Wildlife Group's series on various wildlife species as game ranch animals. The previous symposiums in this series were on African elephant (1991), sable antelope (1992), rhinos (1994), African buffalo (1996) and lions and leopards (1997). The aim of these symposiums is to bring together relevant information on the following topics: conservation status, ecology, behaviour, capture, handling facilities, management in captivity, reintroduction into conservation areas, reproduction, parasites, diseases and treatment, for each species.

'The objective is to have a manual available for field workers, biologists, game ranch managers, veterinarians and anybody interested in these species, thereby contributing to their conservation and utilisation on a sustained basis, not only in southern Africa, but throughout the African continent and wherever these animals are held in captivity.'

I believe that the proceedings of this cheetah symposium will achieve the above objectives, as the 57 carefully selected experts contributed 27 papers of a high standard to the proceedings. The papers cover a wide variety of issues relevant to cheetah, such as status in the wild on the African continent, ecology, behaviour, husbandry, nutrition in captivity, reproduction and breeding in captive breeding centres, relocations and introductions of wild cheetah, and their importance in conserving the species and for the tourism industry. Various diseases and veterinary issues specific to cheetahs are also covered in detail.

It is evident from the symposium that cheetah populations have rapidly dwindled in certain areas in Africa owing mainly to competition for space with man. In some north African countries the status of the cheetah is unknown. Cheetahs require large territories, especially where there is competition from other predators such as lion and hyena. There are therefore few privately-owned areas in South Africa large enough for cheetahs to co-exist naturally with other predators at viable population levels, and so most cheetahs on private land in South Africa are managed in intensive captive breeding programmes. This emphasises the need for large protected areas in southern Africa or in Africa in general, such as the 'conservancy' concept as a possible example for the long-term conservation of this species.

The proceedings contain a comprehensive 'Bibliography of the Cheetah' of 1033 references compiled by B L Penzhorn, D G A Meltzer and M S G Mülders. At the end of the book there is a useful index of key words for easy referencing to pages in the text.

This useful book is highly recommended to anybody interested in the conservation or captive management of cheetahs.

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