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LEVELS OF TRACE ELEMENTS IN THE LIVER AND DIET OF FREE-LIVING KOALAS, *PHASCOLARCTOS CINEREUS* (GOLDFUSS)

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ABSTRACT: The mean liver concentrations of copper, manganese, zinc and cobalt were 0.25, 0.20, 2.97 mmol/kg and 2.81 μmol/kg respectively in free-living koalas in Victoria, Australia. The mean plasma copper concentration was 9.2 mmol/liter which was somewhat below the level in other hindgut fermenters. The mean concentrations of copper, manganese and zinc in their diet (*Eucalyptus* spp.) were 0.08, 4.46 and 0.27 mmol/kg respectively. Analysis of the data established a significant correlation between the age of the koalas and the inverse of the concentration of the copper ($r = -0.67$, $P < 0.001$) in the liver. There were no such correlations apparent for manganese, zinc or cobalt. The concentrations of trace metals in the *Eucalyptus* diet for the koala were comparable to those recommended in the diets for other hindgut fermenters such as horse, rabbits and rats. However there was evidence for suboptimal plasma copper levels (mean 9.2 mmol/liter) in some koalas, and reduced liver copper levels in older koalas. Liver histology revealed the presence of brown intracytoplasmic granules in hepatocytes. The size and number of these granules per cell was noted to increase with increasing age of the koala but the chemical nature and the role of the granules was not determined by the histochemical techniques used.

INTRODUCTION

Copper (Cu), manganese (Mn), zinc (Zn) and cobalt (Co) are recognized essential trace elements required for a range of mammalian body functions (Mertz, 1981). Although absorption of trace metals is regulated at the small intestinal mucosa (Reinhold, 1975) Co is absorbed as the biologically active compound cyanocobalamin (vitamin B12). This must be available in the diet or synthesized by enteric bacteria utilizing dietary Co. The absorbed elements are stored in hepatocytes and are incorporated into or complexed with specialized proteins e.g., Cu into ceruloplasmin. Almost all trace metals in plasma are protein bound and they are excreted usually in bile and feces (Smith and Marston, 1970; Miller, 1973; Cousins, 1979; Bremner, 1980).

While there are numerous publications on the metabolism and requirements of these trace elements in laboratory animals, ruminants and humans, there is a general lack of such information for koalas (Hume, 1982). There are no reported ranges of trace metal levels in the body tissues of free-living or captive koalas. Ullrey et al. (1981a) examined the levels of Cu, Zn, Fe and Se in leaves from 11 species of *Eucalyptus* grown in California, USA which were the diet for captive koalas. They concluded that in some of the samples Zn and possibly Cu were below the recommended dietary levels for sheep and horses. However, as the koalas' dietary trace metal requirements were unknown the authors were equivocal about the nutritional adequacy of the leaves for this species. As the koalas were in good health they suggested that their trace metal requirements were less than those of placental eutherians such as sheep and horses. The provision of lick supplements incorporating 0.03% Cu, 0.4% Zn, 0.3% Mn, 0.012% Co as well as some Fe and I were ignored by captive koalas (Ullrey et al., 1981b), however this may be related to the koala's selective eating habit rather than a lack of need for the supplement.

Free-ranging koalas in Victoria and South Australia appear to favor *Eucalyptus viminalis* Labillardiere and *E. ovata* Labillardiere (Dickens, 1975) but neither of these species were included in the study

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by Ullrey et al. (1981a) and the trace metal composition of leaves from these species has not been reported.

Knowledge of both the dietary trace metal requirement and a tissue reference range for the koala is important for both the adequate management of the species in captivity and interpretation of levels found in dead and diseased koalas found in the wild. It was the absence of reference data and the finding of low levels of Cu in the liver of koalas in the latter category which prompted this study.

MATERIALS AND METHODS

Twenty-eight free-living koalas (seven alive, 21 dead) were submitted from East Gippsland, Victoria (Study Area A) and French Island, Victoria (Study Area B) by wildlife officers for disease diagnosis purposes. They were examined using procedures described previously for koalas in Victorian veterinary laboratories (Obendorf, 1983). These procedures included a full post-mortem examination to establish the nature of any diseases present. The age class of the koalas was determined by examination of dental features and skull measurements (Martin, 1981). Using this method, age class 1 represented immature unweaned animals while those in class 6 or 7 were fully grown mature koalas.

For histological examination of the liver several 1 cm thick sections of the two major ventral lobes were fixed in 10% vol./vol. formol-saline. Selected sections were dehydrated in ethanol, embedded in paraffin and sectioned at 4 μm. In addition to routine hematoxylin and eosin staining (HE), other sections were stained for carbohydrate material by periodic acid Schiff reaction (PAS); acid fast material by Ziehl-Niehlsen (ZN) accumulated copper by rubeanic acid (RA); ferric iron by Perl's Prussian blue (PB) and bile by Hall's bilirubin method (HB) according to the procedures of Luna (1968). In addition, thin frozen sections of fresh liver were stained for lipid using Sudan black (SB) and oil red O (OR) stains, according to the procedures of Luna (1968). All sections were examined by light microscopy.

For trace metal analyses fresh portions of the ventral lobe of the liver were dried to constant weight at 105 C, and ground to a fine powder. Duplicate aliquots (0.5 g) were digested in an equivolume concentrated nitric, sulfuric and perchloric acid solution, heated in an aluminum mantle until digestion was complete and solutions were clear. Trace metals were measured by atomic absorption spectroscopy; Cu, Mn and Zn by flame atomization and Co by carbon furnace thermal atomization. Concentrations were determined from standard curves established by means of standards prepared from pure metals of Cu and Zn and standard solutions of manganous and cobalt chloride.

Blood samples were collected from six koalas prior to euthanasia into sterile glass tubes containing lithium heparin anti-coagulant. Plasma was separated by centrifugation and deproteinized with 0.5 ml 0.29 M strontium chloride solutions, centrifuged and the clear supernatant assayed for Cu using the above AAS method (Smeyers-Verbeke et al., 1973). Vitamin B12 levels were determined in three plasma samples by a competitive protein binding assay using radioactive cyanocobalamin tracer and fish serum binding protein (Ithakissios et al., 1977; Malecki, pers. comm.).

Composite virgin soil samples from the 0 to 10 cm layer were collected from within both study areas. The total Cu concentration in these samples was determined by atomic emission spectrographic methods (McKenzie, 1962).

Samples of mixed young and mature foliage were collected from *Eucalyptus* spp. trees in localized sites within the study areas where high populations of koalas were present. Samples were collected from 10 *E. viminalis* in each of the Study Areas A and B, five *E. ovata* in each of Study Areas A and B, and two *E. globulus* Labillardiere in Study Area B. No *E. globulus* were found in suitable sites of Study Area A. Data were analyzed for significance by Students *t*-test and by linear regression analysis.

RESULTS

Necropsies on the 28 koalas revealed skin ulcerations (39%), tick paralysis due to *Ixodes holocyclus* Newmann, 1899 (18%), road trauma injuries (14%) and no abnormalities in the remaining 29%. There was no clinical or pathological evidence of disease attributable to trace element deficiency in any of the koalas and none exhibited internal parasitism or liver disease.

Microscopic examination of livers showed the presence of granules within hepatocytes of mature koalas. The granules were intrinsically brown, intracytoplasmic and approximately 2 μm diameter. They did not stain with any of the

TABLE 1. Mean concentrations of copper, manganese, zinc and cobalt in dried koala liver. Values are means ± standard errors, in mmol/kg dry liver for copper, manganese and zinc, and in μmol/kg for cobalt.

Trace element	Mean concentration	Mean age class	Sample size
Copper	0.25 ± 0.03	3.9 ± 0.2	28
Manganese	0.20 ± 0.03	3.8 ± 0.3	17
Zinc	2.97 ± 0.09	3.6 ± 0.4	13
Cobalt	2.81 ± 0.57	3.8 ± 0.5	9

histochemical procedures performed being negative for carbohydrates (PAS), acid-fast material (ZN), Cu (RA), ferric iron (PB), bile (HB) and lipid (SB, OR). The granules maintained their brown color throughout these procedures. No granules were apparent in koalas of age class 1 or 2. The number of granules gradually increased, from approximately five per cell at age class 3, to approximately 30 per cell at age class 6. There was a slight increase in granule size over this period. The sample sizes for age classes 1, 2, 3, 4, 5 and 6 were 2, 2, 5, 12, 5 and 2, respectively.

The mean concentrations of Cu, Mn, Zn and Co in dried liver are shown in Table 1. Analysis of the Cu concentrations in liver tissues showed that they were comparable in male and female koalas, and in koalas from both study areas. The mean level of plasma Cu was 9.2 ± 2.3 μmol/liter ($n = 6$; range 4.9–16.6). The mean level of plasma vitamin B12 was 1,920 pg/ml ($n = 3$; range 1,120–2,720).

The pH of the grey sandy loam soil predominant in both areas was 5.3. The mean soil total Cu concentration was 0.08 mmol/kg and 0.12 mmol/kg for Study Areas A and B respectively.

Liver concentrations of Mn, Zn and Co were relatively constant, independent of the age class of the koala (Table 2). However, Cu concentrations in livers showed marked inverse associations with age class (correlation coefficient $r = -0.67$; $P < 0.001$) (Table 2).

The mean concentration of Cu, Mn and Zn in the 32 eucalypt foliage samples are shown in Table 3. The differences in levels between *E. viminalis* and *E. ovata* were not statistically significant. The levels of *E. globulus* appeared to be lower than that in *E. viminalis* and *E. ovata*, but as samples from this species were available only from Study Area B no statistical comparisons were made. The mean trace metal levels in all samples from Study Area A were compared with those from Study Area B. A lower mean level of Mn in foliage from Study Area A was the only statistically significant trend ($P < 0.01$).

DISCUSSION

The study established reference values for Cu, Mn, Zn and Co in the liver and

TABLE 2. Comparison of liver trace element concentrations in koalas from different age classes. Values are means ± standard errors, with sample sizes in parentheses.

Age class	Copper (mmol/kg)	Manganese (mmol/kg)	Zinc (mmol/kg)	Cobalt (μmol/kg)
1	0.61 ± 0.18 (2)	0.31 (1)	3.43 (1)	1.56 (1)
2	0.29 ± 0.08 (2)	0.18 (1)	3.01 (1)	6.55 (1)
3	0.25 ± 0.01 (5)	0.17 ± 0.05 (4)	2.95 ± 0.23 (4)	0.61 (1)
4	0.23 ± 0.02 (12)	0.13 ± 0.02 (7)	3.04 ± 0.06 (4)	3.01 ± 0.29 (3)
5	0.16 ± 0.03 (5)	0.33 ± 0.16 (3)	2.78 ± 0.36 (2)	2.50 ± 1.11 (2)
6	0.15 ± 0.01 (2)	0.37 (1)	2.68 (1)	2.55 (1)
Correlation coefficient r	−0.67	0.21	−0.43	−0.12
P (t-test)	<0.001	>0.1	>0.1	>0.1

TABLE 3. Comparison of mean copper, manganese and zinc concentrations in *Eucalyptus viminalis*, *E. ovata* and *E. globulus* foliage and the recommended concentrations in the diets of horses, rabbits and rats.

Diet	Trace metal concentration mmol/kg dried diet		
	Copper[a]	Manganese[a]	Zinc[a]
Eucalyptus viminalis	0.09 ± 0.01 (20)	4.12 ± 0.78 (6)	0.29 ± 0.03 (6)
Eucalyptus ovata	0.07 ± 0.01 (10)	5.62 ± 1.42 (4)	0.31 ± 0.09 (4)
Eucalyptus globulus	0.08 ± 0.02 (2)	3.19 ± 0.09 (2)	0.10 ± 0.02 (2)
Eucalyptus spp. combined	0.08 ± 0.01 (32)	4.46 ± 0.63 (12)	0.27 ± 0.04 (12)
Eucalyptus spp. Study Area A	0.09 ± 0.01 (16)	3.30 ± 0.64 (6)	0.24 ± 0.04 (6)
Eucalyptus spp. Study Area B	0.07 ± 0.01 (16)	6.84 ± 0.68 (4)	0.38 ± 0.05 (4)
Recommended horse rations	0.13	NA[b]	NA
Recommended rabbit rations	0.016	0.15	NA
Recommended rat rations	0.08	0.91	0.18

[a] Values are means ± standard errors, with sample size in parentheses. No significant differences were apparent in the trace element concentrations either between eucalypt species or study areas except that overall manganese levels were higher in leaves from Study Area B (P (t-test) < 0.01). The recommended trace metal concentrations in horse, rabbit and rat rations are taken from the National Academy of Sciences, 1973, 1977 and 1978, respectively.

[b] NA means information not available.

Cu in the plasma of free-living koalas in Victoria, Australia. Furthermore, the data established that the mean concentration of Cu in koala liver tissue was inversely related to the age of the koala while mean concentrations of Mn, Zn and Co were relatively stable with age.

The trace element levels reported were unlikely to have been affected by parasitism or intercurrent liver disease as neither were detected in the koala population studied. The liver levels of the trace elements in koalas are comparable to those in other hindgut fermenters such as the horse, rabbit and rat (see Table 4).

The established trend for decreasing liver Cu concentrations of the koalas of this study mimics the established trends in the livers of the rabbit: 0.58 mmol/kg newborn, 0.36 mmol/kg mature; and the rat: 0.9 mmol/kg newborn, 0.14 mmol/kg mature (Underwood, 1977).

However, the mean plasma Cu concentration is marginal compared to other hindgut fermenters (mean 9.2 ± 2.3; range 4.9–16.6 μmol/liter) (Table 4). This view that plasma Cu levels are marginal in the koalas studied is consistent with the observations made by Ullrey et al. (1981a) that the diets provided for captive koalas

TABLE 4. Comparison of mean copper, manganese and zinc concentrations in liver tissues and mean copper concentrations in plasma samples from koalas, horses, rabbits and rats.

Species	Liver[a]			Plasma[b]
	Copper	Manganese	Zinc	Copper
Koala	0.25	0.20	2.97	9.2
Horse	0.24[c]	NA[h]	NA	21.8[d]
Rabbit	0.36[e]	0.13[e]	NA	11.3[c]
Rat	0.33[f]	0.18[f]	1.44[f]	24.0[g]

[a] Values are in μmol/kg dry liver.

[b] Values are in μmol/liter.

[c] Established values from Institute of Medical and Veterinary Science, South Australia.

[d] From Egan et al., 1980.

[e] Values from Lorenzen and Smith, 1947.

[f] Values from Iyengar, 1980.

[g] From Linder et al., 1980.

[h] NA means information not available.

in the USA had concentrations of Cu which would be inadequate for sheep and horses. Ullrey et al. (1981a) reported the Cu concentration of *E. globulus* as 0.12 mmol/kg, but the same species growing in Study Area A contained only 0.08 mmol/kg. The mean Cu concentration of all species tested by Ullrey et al. (1981a) was 0.10 ± 0.01 mmol/kg. However, they did not test either *E. viminalis* or *E. ovata*, the major preferred species in Victoria. Our data showed that these species were again lower in Cu content (0.08 mmol/kg) than species that were grown in the USA and considered as possibly inadequate for koalas by Ullrey et al. (1981a). The lower Cu content of Victorian eucalypts may be related to the marginal soil Cu content found in both study areas. Although the levels of trace elements in the soil are generally considered to be a poor guide to tissue levels in associated herbivores (Underwood, 1981), lowered Cu content in pasture foliage has been recorded in the study areas of this investigation (McKenzie, 1966).

As koalas eat an average of 0.41 kg of leaves per day (Eberhard et al., 1975), the free-living koalas in this study would have consumed about 0.03 mmoles of Cu per day. Whether or not this amount is sufficient for these animals of known low metabolic rate (Degabriele and Dawson, 1979) remains equivocal. Measurement of the function of a physiological system for which Cu is essential would provide valuable understanding into the actual Cu status (Kirchgessner et al., 1977) of the koalas. In this regard plasma ceruloplasmin activity has been measured on five koalas, but the data are currently inconclusive (McOrist et al., unpubl. data).

Both Mn and Zn levels in the liver and diet of koalas in Gippsland, Victoria appear to be adequate in that they are comparable to or higher than the concentrations in the liver and diets recommended for horses, rabbits and rats (see Tables 3, 4). The plasma vitamin B12 levels suggest adequate dietary Co.

The presence of intracytoplasmic granules in hepatocytes of koalas has been noted by several authors (Backhouse and Bolliger, 1961; Dickens, 1975). Their nature and function have not been elucidated, although they were presumed to be bile in one study (Backhouse and Bolliger, 1961). Due to the accumulation of these granules with increasing age and their absence in unweaned and immature koalas (age classes 1 and 2), we suggest that they are more likely to be associated with the *Eucalyptus* spp. diet. A major component in the diet is the volatile essential oils, e.g., phellandrene, cineole (Eberhard et al., 1975). These oils are relatively toxic and we suggest that the hepatocyte maybe involved in detoxification of these oils, with in situ granule formation.

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