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CERULOPLASMIN AS AN INDICATOR OF COPPER RESERVES IN WILD RUMINANTS AT HIGH LATITUDES

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ABSTRACT: Northern ungulates must establish trace mineral reserves when forage is available in spring and summer to sustain biochemical activities through the long winter. Copper (Cu), zinc (Zn) and iron (Fe) reserves were measured in the serum, digestive tract, liver, and kidney of six male caribou and reindeer (*Rangifer tarandus*) fed a complete pelleted ration. Dry matter content and absolute amounts of Cu, Zn and Fe were highest in the liver. Digesta contents of Cu and Zn were greatest in the rumen but dry matter concentrations were greatest in the cecum reflecting the high levels of Cu and Zn in the diet. Serum ceruloplasmin (an oxidase containing Cu) activity was related to liver copper in captive reindeer and caribou ($P < 0.05$, $r^2 = 0.82$) during spring and winter but not during the rut. Michaelis-Menten kinetics of ceruloplasmin were measured in sera from captive reindeer, muskox (*Ovibos moschatus*) and moose (*Alces alces*) ($n = 3$/species). Maximum velocities (V_{MAX}) were 42, 20 and 9 ($IU \cdot L^{-1}$); k_M were 0.38, 0.55 and 0.62 (mM) for muskox, reindeer and moose respectively. Wild caribou ($n = 3$) from the Teshekpuk herd and moose ($n = 3$) from the Colville River had lower V_{MAX} (7 $IU \cdot L^{-1}$) and higher k_M (1.9 mM) than their captive conspecifics. These kinetic parameters probably reflect differences in ceruloplasmin structure between species as well as differences in tissue reserves between populations within each species. Serum ceruloplasmin activity and kinetics can provide a non-lethal alternative to direct measures of hepatic Cu reserves in wild and captive populations. However, the method requires validation for the effects of sex, season, development and disease in each species.

Key words: *Alces alces*, ceruloplasmin, copper, iron, metalloenzymes, *Ovibos moschatus*, *Rangifer tarandus*, trace minerals, zinc.

INTRODUCTION

Trace minerals have long been known to limit reproduction and growth of domestic ruminants selected for high production of meat or milk (Underwood, 1977; National Research Council, 2000). Similar limitations have been demonstrated in nondomesticated ruminants under captive conditions (Gogan et al., 1989; Blakley et al., 1998). However, there is a growing awareness that trace minerals also may limit production of ruminant populations in the wild. For example, Flueck et al. (1994) found increased calf survivorship in wild black-tailed deer (*Odocoileus hemionus*) supplemented with selenium (Se), whereas Flynn et al. (1977) and Frank et al. (1994) reported population declines of moose (*Alces alces*) associated with low copper (Cu) status.

The short growing season for plants at high latitudes provides a concomitantly brief season for production in moose, muskox (*Ovibos moschatus*), reindeer and caribou (*Rangifer tarandus*; White et al., 1987; Schwartz and Renecker, 1997). Summer production includes deposition of both lean and fat tissues in growing calves and in adult males before rut. Most adult females produce milk during summer while accruing body reserves to sustain pregnancy during winter when forages may be limiting in both quantity and quality (Barboza and Bowyer, 2000). Although these species deposit fat to provide both insulation and an energy reserve (Gerhart et al., 1996; Stephenson et al., 1998), nitrogen and minerals in lean tissue must also provide a reserve for sustaining biochemical function through winter (Hyvärinen et al., 1977).

Copper, zinc (Zn) and iron (Fe) comprise the catalytic centers of numerous metalloenzymes for extracellular, cytoplasmic and nuclear functions in blood, skin and reproductive organs (Brody, 1999).

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Aberrant status of these minerals impairs gas transport, oxidative defense, immune function, cell development and gametogenesis to affect pregnancy, lactation, growth and maintenance (Saker et al., 1998; Grace and Clark, 1991). These divalent cations interact through absorption and transport in the body (Van Soest, 1994) to produce several secondary conditions. For example, high levels of Zn can limit Cu absorption to produce a secondary deficiency of Cu (Cousins, 1985; Van Soest, 1994). Similarly, Cu can interact with molybdenum (Mo) and sulfur (S) to produce insoluble compounds that are unavailable for absorption (Suttle, 1991; Ward and Spears, 1997).

Copper metabolism involves carrier proteins such as ceruloplasmin and metallothionein (Cousins, 1985). Metallothionien is an intracellular protein that binds both Zn and Cu as well as several other minerals. Dietary copper is transported on albumin protein but is rapidly transferred to ceruloplasmin as it enters the liver for recirculation to other organs (Linder et al., 1998). Ceruloplasmin is an enzyme with multiple domains: 6 to 8 atoms of Cu are bound at sites with high affinity while a further 10 sites with low affinity bind Cu, Zn and other metals (Owen, 1982; Urich, 1994). Although ceruloplasmin accounts for most of the Cu in blood, it is not only a transport molecule but also an oxidase that allows Fe to be stored in the protein ferritin (Reilly et al., 1998). Therefore, Cu and ceruloplasmin provide a nexus for the evaluation of trace mineral status in northern ruminants.

We report the distribution of Cu, Zn and Fe in tissues of captive reindeer and caribou, and compare hepatic Cu reserves with two serum parameters (total Cu and ceruloplasmin activity). The kinetics of ceruloplasmin activity were compared in captive moose, muskox, reindeer and caribou. Kinetics were also measured in caribou and moose from two wild populations with suspected aberrations of trace mineral status.

MATERIALS AND METHODS

Distribution of trace minerals

Reindeer and caribou were selected from a herd reared in captivity (Large Animal Research Station, Fairbanks, Alaska, USA; 65°N 146°W). Six adult males (two caribou, two reindeer, and two castrated reindeer) were studied in October and November 1997 during the end of the rut (mean ± SD; 155 ± 27 kg). Animals were maintained on seasonally available forages (browse and pasture) and a pelleted supplement based on barley and corn with mineral and vitamin premixes (Textured Reindeer Ration; Alaska Pet and Garden, Anchorage Alaska). The supplement contained 88% dry matter which was comprised of 18% neutral detergent fiber, 7% acid detergent fiber, 19% crude protein, 3% crude fat, 8% ash, 1.4% Ca, 1.1% P, 343 ppm Fe, 39 ppm Cu, 144 ppm Zn. Animals were held in a common pen provided with supplement ad libitum since forage availability was limited by snow cover. Water was provided ad libitum as free water or snow.

Deer were physically restrained in an hydraulic chute for sampling blood and for injection of immobilants. For euthanasia, animals were immobilized with a combination of xylazine (0.5 mg/kg; Xyla-ject, 100 mg/ml, Phoenix Pharmaceutical, St. Joseph, Missouri, USA) and ketamine (5.5 mg/kg; Ketaject, 100 mg/ml, Phoenix Pharmaceutical) or with a mixture of tiletamine and zolazepam (3 mg/kg; Telazol 100 mg/ml, Fort Dodge Animal Health, Fort Dodge, Iowa, USA). Blood was collected from the jugular vein into evacuated tubes (Vacutainer Systems, Becton Dickinson, Franklin Lakes, New Jersey, USA) without additive for serum. Serum was separated at 1000×*g* for 10 min on a bench-top centrifuge. Animals were euthanized by barbituate overdose (12 ml containing 390 mg/ml pentobarbital sodium and 50 mg/ml phenytoin sodium; Euthasol, Delmarva Laboratories, Midlothian, Virginia, USA) or by application of a captive-bolt to the head.

Liver was dissected, weighed and sampled at the caudate lobe. Kidney was dissected from surrounding fat, weighed and sampled as a cross-section at the mid-point of the organ. The digestive tract was ligated to isolate the reticulo-rumen, the abomasum, the small intestine to the ileocecal junction, and the cecum-proximal colon to the descending colon where fecal pellets begin to form. Segments were weighed and emptied to collect digesta samples. The mass of digesta was determined as the difference in mass between intact and empty segments. Tissues and digesta were sampled from the following segments: ventral sac of the rumen, along the greater curvature of the ab-

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omasum, along the proximal 1 m of the small intestine, and from the apex of the cecum.

All samples were stored in sealed plastic bags or vials at −20 C. Frozen tissues and serum were shipped to the Analytical Sciences Laboratory (University of Idaho, Boise, Idaho, USA) for assays of trace minerals by inductively coupled plasma atomic emission (Moller et al., 1990; Anderson, 1996).

Digesta was dried at 55 C in a fan-forced oven before grinding to pass through #20 mesh in a bench-top Wiley mill (Wiley, Philadelphia, Pennsylvania, USA). Milled samples were assayed in duplicate by digestion of 0.2 g in a mixture of 70% v/v HNO_3 (1000 ml), 32M H_2SO_4 (200 ml), 70% v/v $HCLO_4$ (343 ml) and water (57 ml). Digestions were performed at 165 C for 15 min followed by 315 C for 35 min. Digests were diluted to 75 ml with distilled, deionized water and assayed by absorbance of direct current plasma (Spectrascan Spectrometer, Applied Research Laboratories, Brea, California, USA) for Cu (λ 327 nm), Zn (λ 206 nm), and Fe (λ 260 nm).

Ceruloplasmin assay

Ceruloplasmin was assayed from the oxidation of *o*-dianisidine by modification of the method of Schosinsky et al. (1974). Blood serum (0.05 ml) was incubated with acetate buffer (0.75 ml) at pH 5. The reaction was started with the addition of 7.88 mM *o*-dianisidine substrate (0.1 mL) and stopped by addition of 9M H_2SO_4 (2 ml). The oxidized substrate was measured by absorbance at 534 nm in 1 cm^2 cuvettes (Beckman Spectrophotometer Model DU 530, Fullerton California). Oxidation rates were determined in duplicate as the difference in absorbance between 15 min and 25 min. Activity was calculated from the molar absorptivity of the oxidized substrates (9.6 $\mu mol^{-1} \cdot cm^{-1}$) and expressed as the rate of oxidation ($\mu mol \cdot min^{-1} \cdot L^{-1}$ or $IU \cdot L^{-1}$). This unit of activity is equivalent to 0.2863 $mg \cdot dL^{-1}$ of ceruloplasmin in human serum (Schosinsky et al., 1974). Enzyme kinetics were measured in three samples selected at random from each group of animals. Reaction velocities were measured with duplicate assays at 15 and 25 min in a series of substrate concentrations declining from 7.88 mM *o*-dianisidine (3.53, 1.95, 1.28, 0.96 mM).

Comparisons between species and within species

Serum ceruloplasmin activity and serum copper were compared to liver copper in captive reindeer during summer, winter and the period of rut from late August through October. These animals had been euthanized for clinical or experimental purposes within the last four years. Serum samples were stored at −80 C during this period. Trace mineral assays of serum and liver were performed within two weeks of necropsy at the University of Idaho as above.

We also used archived (stored at −80 C) serum sampled from captive muskox under physical restraint for routine clinical or experimental purposes. Blood was also sampled from captive moose immobilized with carfentanil citrate and xylazine (Stephenson et al., 1998). Moose sera were stored at −20 C. Ceruloplasmin is stable in frozen plasma and serum for long periods at these temperatures (Owen, 1982). Muskox were held at the University of Alaska Fairbanks and provided with seasonally available forages, grass hay (*Bromus* sp.) and the pelleted supplement used for reindeer and caribou (see above). Moose were maintained at the Moose Research Center (Soldotna, Alaska) on a diet of fresh browse and a pelleted supplement based on wood fiber with cereal byproducts and premixes of minerals and vitamins (Schwartz et al., 1985; Alaska Pet and Garden, Anchorage, Alaska).

Two sets of blood samples were drawn from wild populations on the North Slope of Alaska: moose from the Colville River drainage during 1996 and 1997; and caribou near Teshekpuk Lake from 1995 to 1998. Blood was sampled from the jugular vein of animals immobilized with carfentanil citrate (Wildnil 3 mg/ml, Wildlife Pharmaceuticals, Fort Collins, Colorado, USA) and xylazine (Sedazine, 100 mg/ml, Wildlife Pharmaceuticals). Low copper status was suspected in both wild populations because Cu concentrations in liver and hair were low compared to those of domestic species and other wild and captive populations of the same species (O'Hara et al., 1999; T. O'Hara, unpubl. data).

Calculations and statistics

Mineral concentrations of digesta were expressed on the basis of dry matter whereas tissue minerals were calculated on the basis of fresh mass. Total contents of trace minerals were calculated as the product of the concentration and the total mass of tissue or digesta. Serum content was estimated with the assumption that blood volume was 7% of body mass and hematocrit was 50%. Distribution of dry matter and minerals in the digestive tract were expressed as the percentage of the sum of ligated segments.

Means are reported ± one standard deviation. Percentages were arcsine transformed for ANOVA (Zar, 1974). Tissues and digesta segments were compared by ANOVA followed by

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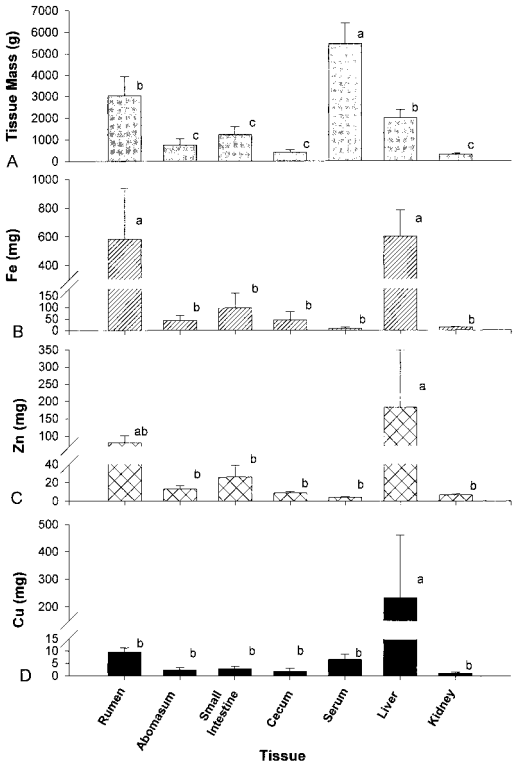

FIGURE 1. Fresh mass of tissues (A) and their total content (mg) of Fe (B), Zn (C) and Cu (D) in male reindeer and caribou ($n = 4$ for all tissues except serum and kidney where $n = 6$). Letters indicate significant differences between tissues within each measure ($P < 0.05$).

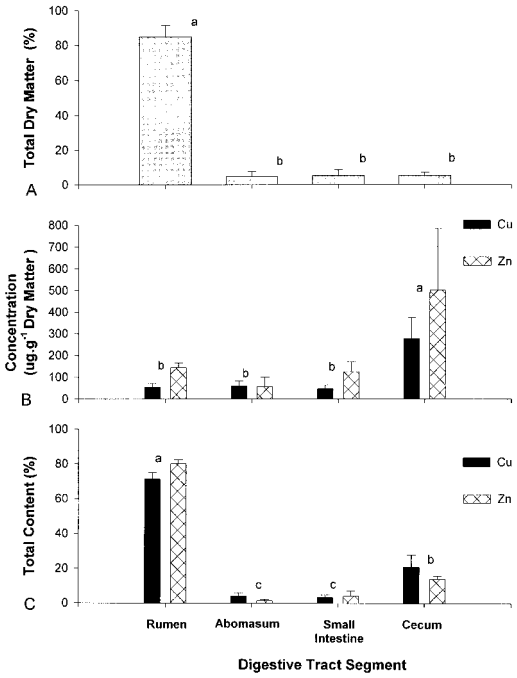

FIGURE 2. Distribution along the digestive tract of dry matter in digesta contents (%) (A), Zn and Cu concentration ($\mu g \cdot g^{-1}$ dry matter) (B), and total content (%) of Zn and Cu in digesta (C). Letters indicate significant differences between segments within each measure ($P < 0.05$).

pair-wise comparisons of means with Bonferroni's adjustments (Wilkinson and Coward, 1998a). Relationships between liver Cu, serum Cu and ceruloplasmin activity were tested by least-squares linear regression (Wilkinson and Coward 1998b). Michaelis-Menten parameters of maximum velocity (V_{MAX}) and substrate affinity (k_M) were derived from the least-squares regression of the Lineweaver-Burke plot where the intercept of the y axis is $1/V_{MAX}$ and the intercept of the x axis is $-1/k_M$ (Matthews and Van Holde, 1996).

RESULTS

Tissue concentrations of Cu (127 ± 103 ppm), Fe (452 ± 233 ppm) and Zn (91 ± 84 ppm) were highest in liver. Consequently, liver provided the greatest reserve of these trace minerals in tissues (Fig. 1). The large mass of rumen tissue contained similar amounts of Fe and Zn to the liver but relatively small amounts of Cu. Low serum concentrations of Cu (1.2 ± 0.3 ppm), Fe (1.4 ± 0.8 ppm) and Zn (0.7 ± 0.1 ppm) subtended small reserves of trace minerals despite the relatively large estimate of serum mass (Fig. 1).

The large volume of the rumen contained the largest amount of digesta dry matter, Cu and Zn in the digestive tract even though concentrations of these minerals were low and similar to levels in the abomasum and small intestine (Fig. 2). The highest concentrations of Cu and Zn in digesta were present in the cecum.

Copper concentration in the liver was significantly related to both serum Cu and ceruloplasmin activity (Fig. 3) in reindeer and caribou sampled during summer or winter. These relationships were not significant for samples from both males and females collected during the period of rut.

Oxidation of the substrate *o*-dianisidine

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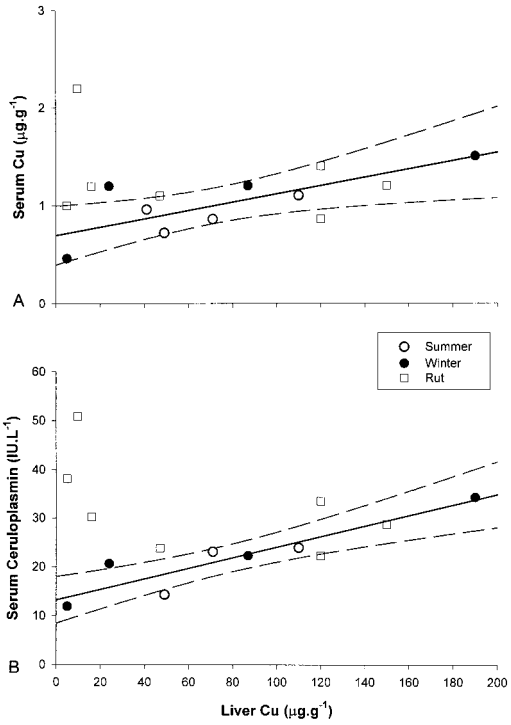

FIGURE 3. Relationships between liver Cu concentration ($\mu g \cdot g^{-1}$ wet mass) and serum Cu concentration (A) or serum ceruloplasmin activity (B) in reindeer and caribou. Solid lines are regression relationships for summer (open circles) and winter (closed circles) combined without data for rut (open squares). Dashed lines are 95% confidence intervals for the regression. Serum Cu concentration = 0.697 + 0.004 × Liver Cu concentration; $P < 0.05$; r^2 = 0.504; SEE = 0.228 Serum Ceruloplasmin = 13.197 + 0.107 × Liver Cu concentration; $P < 0.05$; r^2 = 0.816; SEE = 2.933.

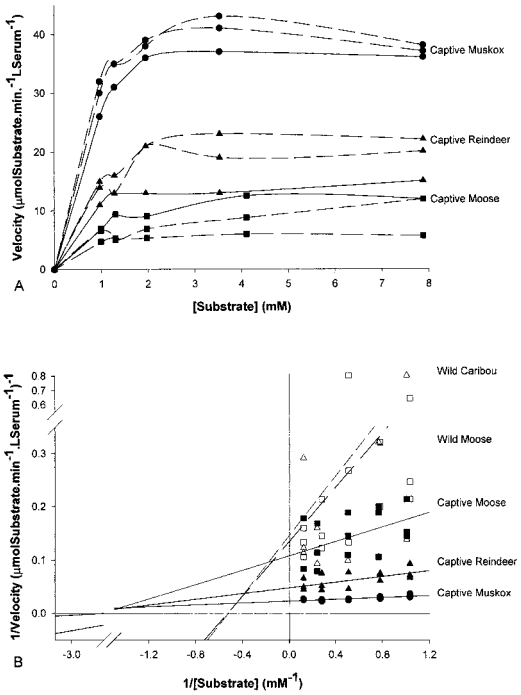

FIGURE 4. (A) Michaelis-Menten plot of ceruloplasmin oxidase reaction velocity against concentration of the substrate o-dianisidine in sera of captive moose (closed squares), muskox (closed circles) and reindeer (closed triangles). Lines are spline curves for individual data sets (n = 3 per species). (B) Lineweaver-Burk Plot for captive moose, muskox and reindeer with moose from Colville River (open squares) and caribou from Teshekpuk Lake (open triangles) on the North Slope of Alaska. Lines are least-squares regressions for each group: solid lines for each species in captivity; dashed lines for wild populations.

was linear from 15 to 30 min over the range of substrate concentrations (7.88–0.99 mM). The hyperbolic relationship between reaction velocity and substrate concentration indicated that the enzyme obeyed Michaelis-Menten kinetics (Fig. 4a). Lineweaver-Burk plots provided estimates of V_{MAX} that were higher for muskox (42 $IU \cdot L^{-1}$) than for reindeer (20 $IU \cdot L^{-1}$) and moose (9 $IU \cdot L^{-1}$) in captivity (Fig. 4b). Substrate affinities indicated by k_M (the substrate concentration at $V_{MAX}/2$) were also greater for muskox (0.38 mM) than for reindeer (0.55 mM) and moose (0.62 mM) in captivity. Enzyme kinetics in wild caribou and moose were much more variable than in their captive conspecifics (Fig. 4b). Wild animals were low in maximum activity (V_{MAX} 7 $IU \cdot L^{-1}$) and substrate affinity (k_M 191 mM) when compared to captive deer.

DISCUSSION

Liver is the greatest tissue reserve of labile Cu, Fe and Zn in reindeer and caribou (Fig. 1). However, large proportions of body mineral may be committed to intracellular or extracellular protein complexes. Sheep (*Ovis aries*) raised for wool production contain 38% of body Fe in hemoglobin within blood cells, whereas in-

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tracellular proteins in muscle contain 32% body Zn, 25% body Fe, and 12% body Cu, with a further 46% body Zn, 5% body Fe and 14% body Cu in the fleece (Grace and Clark, 1991).

Digesta pools of Cu and Zn were highest in the rumen and in the cecum-proximal colon of reindeer (Fig. 2). This distribution is similar to those of macro-minerals (Ca, Mg, K and Na) in captive reindeer and in wild muskox (Staaland and Thing, 1991, Staaland et al., 1986) with net absorption of macrominerals centered on the small intestine (White et al., 1984). The concentration of trace minerals in digesta from reindeer suggests similar uptake from the small intestine even though most digestion of dry matter is achieved in the rumen (Fig. 2). Biliary secretions contribute to mineral concentration in digesta along the small intestine (White et al., 1984; Linder et al., 1998). Although these secretions can be absorbed at the ileum and along the hindgut, excess absorbed Cu and Zn are eliminated through bile. The high concentrations of Cu and Zn in cecal digesta of reindeer and caribou probably reflect the combined effects of biliary excretion and low absorption commensurate with a high intake of Cu and Zn from the pelleted ration.

Biliary excretion is consistent with regulation of hepatic reserves of Cu in reindeer and caribou. Although serum Cu levels were significantly related to hepatic Cu concentration (Fig 3.), the predictive value of this relationship is mitigated by the shallow slope of the regression. Serum Cu is considered a poor indicator of hepatic reserves and thus liver status in domestic sheep (Underwood, 1977; Grace and Clark, 1991) and wild muskox (Blakley et al., 2000). The relationship between serum ceruloplasmin and liver concentration of Cu provides a better predictive measure in reindeer and caribou. However, this relationship is restricted to measures of animals outside the rut. This discrepancy could be due to vigorous exertion, fasting, and acute phase responses to infection all of which are known to elevate serum ceruloplasmin activity (Urich, 1994; Owen, 1982). Furthermore, serum ceruloplasmin may not indicate hepatic reserves of Cu in animals with chronic conditions such as heart disease (Klipstein-Grobusch, et al. 1999) or prolonged protein malnutrition (Owen, 1982). Developmental changes in Cu transport should also be considered in evaluating status because ceruloplasmin activities are low in neonates (Owen, 1982).

The activity of ceruloplasmin also varies with species, for example V_{MAX} of captive muskox was double that of reindeer held in the same facilities with similar supplemental rations. Blakley and Hamilton (1985) reported a significant difference in serum ceruloplasmin activity between domestic sheep and cattle that was probably independent of Cu status. These differences may be related to small variations in the sequence of the protein or its associated polysaccharide chain (Urich, 1994). Although some difference in activity is probably related to the amount of enzyme in circulation, the substrate affinity (k_M) is independent of enzyme concentration. The low k_M in muskox reflects a greater affinity for a common substrate when compared with reindeer/caribou or moose indicating a difference in enzyme kinetics between the species. These kinetic differences were also evident between populations within species and could be related to Cu status. Wild caribou and moose with low Cu in liver and hair (T. O'Hara, unpubl. data) had lower activities and substrate affinities than their captive conspecifics. The suppression of affinity may be associated with depletion of Cu at the active sites of the enzyme. This hypothesis is supported by the restoration of serum ceruloplasmin activity following administration of Cu to Cu-deficient rats (Linder et al., 1979) presumably through restored synthesis of the enzyme with fully functional active sites containing Cu. Restoration of ceruloplasmin activity and kinetics

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in Cu deficient ungulates awaits direct confirmation.

Management implications

Serum ceruloplasmin activity and kinetics provide a non-lethal alternative to direct measures of hepatic Cu reserves in wild and captive populations within a species. However, application of this method requires further validation for the effects of season, development and disease within species. The method may be particularly useful for evaluating northern ruminants during periods of metabolic Cu demand (the last trimester of gestation, lactation and during molt of the winter coat) and in relation to seasonal patterns of feed intake and body mass. Intracellular Cu proteins such as superoxide dismutase can also indicate Cu status (Milne, 1998) but these assays require isolation of intact cells that may be difficult to achieve under field conditions during investigations of wild animals.

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