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Title: Haematological and serum biochemical reference intervals of free-ranging Lumholtz's tree-kangaroos (*Dendrolagus lumholtzi*)

Running title: Tree-kangaroo blood reference intervals

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Abstract Reference intervals for haematology and serum biochemistry parameters were developed for free-ranging Lumholtz's tree-kangaroo (*Dendrolagus lumholtzi*) using 35 samples from 12 female and 15 male free-ranging animals. Captive tree-kangaroos (n=12) were also sampled for comparison. Differences were found between free-ranging and captive animals in white blood cell and neutrophil counts, and levels of aspartate aminotransferase, alkaline phosphatase, bilirubin, creatine kinase, phosphate, triglycerides and lipase. These differences may be attributed to diet, activity, capture methods or age group. Reference intervals generated may be used for both free-ranging and captive Lumholtz's tree-kangaroos. This study provides a valuable tool for the assessment of health in rescued and

captive tree-kangaroos and will aid in investigations into population health and disease in free-ranging Lumholtz's tree-kangaroos.

Objective To develop reference intervals (RIs) for haematology and serum biochemistry parameters in Lumholtz's tree-kangaroos.

Methods Haematological and serum biochemical RIs were determined using 35 samples from 27 clinically healthy Lumholtz's tree-kangaroos from the Atherton Tablelands region of Queensland examined between 2014-2019. Haematology and serum biochemistry parameters were measured from 16 samples from 12 captive animals for comparison.

Results Reference intervals based on 35 samples from free-ranging animals showed higher mean and standard deviation values for white blood cell and neutrophil counts, and levels of aspartate aminotransferase, alkaline phosphatase, bilirubin, creatine kinase, phosphate, triglycerides and lipase than results for 16 samples from captive animals. Captive individuals showed higher mean values than free-ranging individuals for albumin, protein, creatinine as well as Hb, MCV, MCH and MCHC.

Conclusion The haematological and serum biochemistry RIs developed for Lumholtz's tree-kangaroos in this study will provide a valuable tool during clinical examination and investigations into disease and population health by veterinarians and researchers. The differences in parameters between free-ranging and captive animals are consistent with differences in diet, age cohort, activity or capture methods. Reference intervals generated from free-ranging animals should also be valid for captive Lumholtz's tree-kangaroos.

Keywords health assessment, marsupial, wildlife, serology, clinical pathology

Abbreviations RBC, red blood cell count; Hct, haematocrit; Hb, haemoglobin; MCH, mean cell haemoglobin; MCHC, mean cell haemoglobin concentration, MCV, mean cell volume; WBC, white blood cell; neut, neutrophil; lymph, lymphocytes; mono, monocytes; eosin, eosinophils; baso, basophils; Na, sodium; K, potassium; Cl, chloride; P, phosphate; Ca, calcium; AST, aspartate aminotransferase, ALT, alanine aminotransferase; ALP, alkaline phosphatase; CK, creatine kinase; TP, total protein; glu, glucose; LTK, Lumholtz's tree-kangaroo; RI, reference intervals, CI, confidence intervals.

INTRODUCTION

Lumholtz's tree-kangaroo (*Dendrolagus lumholtzi*) inhabits rainforest in far north Queensland and is one of two species of tree-kangaroo found in Australia. Captive collections exist in several wildlife parks and zoos. As with many free-ranging Australian fauna, little information on baseline health data is available for this species.[1, 2] To date, published information about haematology in this species consists of morphological descriptions of white blood cells and haematologic reference range intervals based on seven captive animals, and no blood biochemical studies have been reported.[3, 4] Established reference intervals provide a range of haematologic and serum biochemical values for making health assessments and diagnoses, and parameters may differ between captive and free-ranging wildlife.[2, 5] This study sought to determine whether there are any significant differences in haematologic and serum biochemical parameters between free-ranging and captive Lumholtz's tree-kangaroos. Using recommendations for determination of de novo reference intervals [6] a set of reference intervals was established for 12 haematological (RBC, WBC, neutrophils, lymphocytes, monocytes, eosinophils, basophils, Hct, Hb, MCV,MCH,

MCHC) and 20 biochemical parameters (Na, K, Cl, P, Ca, bicarbonate, TP, albumin, globulin, urea, creatinine, glucose, bilirubin, AST, ALT, ALP, CK, cholesterol, triglycerides and lipase) in free-ranging Lumholtz's tree-kangaroos.

MATERIALS AND METHODS

Blood samples were collected as part of a study on the population and health of Lumholtz's tree-kangaroo (LTK) on the Atherton Tablelands, Queensland which involved the chemical immobilization of a total of 44 individuals over a period from 2014-2019. Samples were excluded from animals with signs of active infection, injury or other conditions considered likely to alter blood chemistry or haematology, or if samples appeared compromised e.g. marked haemolysis. Fifty-one samples from 39 individuals (27 free-ranging and 12 captive) were included in the study. Multiple samples collected from seven free-ranging individuals were regarded as independent measures as physiological parameters of wild animals change over time in relation to environmental change such as seasonal changes in food sources and changes in host phenology such as breeding.[7, 8] These samples were compared to ensure that they varied and were a valuable contribution towards understanding normal physiology despite the difficulty in collecting them. Three free-ranging females were sampled at two times at intervals from initial sampling ranging from 105-229 days and one female was sampled three times at intervals of 122 and 480 days from initial sampling resulting in a total of 17 samples. Three of the 15 free-ranging males were sampled two times at intervals ranging from 78 to 456 days from initial sampling resulting in a total of 18 samples. As healthy adult free-ranging females of reproductive age usually had either pouch young or young at foot, lactating females were not excluded from the sample population. Seven free-ranging animals (n=4 females, n=3 males) weighing less than 4.5 kg were designated as

juveniles. Additional samples (taken between 85 and 996 days apart) obtained from each of four captive animals were included in the analysis.

Free-ranging Lumholtz's tree-kangaroo were located, observed and captured at various times of day and locations throughout the Atherton Tablelands, Queensland Australia. Locations were primarily in higher altitude (700-1100m) rainforest and fragmented patches of trees, shrubs and vines associated with agriculture or residential areas. Animals were observed using binoculars (CL 8x30, Swarovski Optik KG, Absam Tyrol, 6067 Austria). At night, animals were observed under illumination provided by headlamp (Model H8, LEDLenser™ West Ryde, New South Wales 2214 Australia) and/or a hand-held thermal imaging device (Pulsar XD19S, Yukon Advanced Optics, Vilnius LT 06326, Lithuania) prior to capture. An animal was deemed to be in a position appropriate for capture attempt if, using a range finder (Bushnell Sport 850, Bushnell Corporation, Overland Park, Kansas USA) it was sitting no more than 15 meters above ground level, darting access was unobstructed by vegetation, body was oriented to allow darting targeted at hindquarters, and 'fall zone' beneath animal was clear of dangerous obstacles. Animals were darted with tiletamine-zolazepam (Zoletil®, Virbac Australia Pty. Ltd., Milperra, New South Wales 2214 Australia) at a concentration of 200 mg/ml, dosed at 5-10 mg/kg estimated body weight using a 0.25 ml Minidart fitted with a 12 mm X 1.1 mm barbless needle fired through a 6.3 mm calibre barrel from a CO_2 powered Taipan 2000 remote projector (Tranquil Arms Company/R.W. Martin, Tarzali, QLD 4885 Australia). In general, 3-12 minutes after darting, animals were sufficiently sedated to fall from the tree. Animals were caught in a net positioned under their location in the tree then placed in an open weave cloth bag and transported to an area for examination and collection of samples. Captive animals were caught via capture net and/or manual restraint within an enclosure then sedated via intramuscular injection with tiletamine-zolazepam at 3-5

mg/kg with examinations performed in outdoor, enclosed holding areas during daylight hours. With the exception of one captive bred male, captive animals had been wild-caught from various locations on the Atherton Tablelands and were being held in wildlife parks in Queensland. All capture and sampling protocols were as per James Cook University animal ethics approval (Animal Ethics permits A1927/A2283) and QPWS/DEHP scientific research permits (WISP 1374213/17705716 and WITK 13724113/17705816).

Blood was collected via jugular or cephalic venipuncture using a 21 or 20 gauge needle on a 5 or 10 ml syringe within 30 minutes of the animal being sufficiently sedated to permit safe handling. Samples were placed into serum separator and EDTA vacuum tubes which were either refrigerated (4°C) or placed in an insulated container containing ice packs within 15 minutes of collection. Blood samples for serum collection were held for a minimum of 30 minutes before centrifugation at 3200 rpm for 25 minutes. Whole blood in EDTA and an aliquot of serum were stored at 4°C for up to 12 hours until they could be sent to a commercial laboratory for processing. Complete blood count (CBC) and serum biochemical analysis using a commercially available uninterpreted canine panel, was performed by a commercial laboratory (QML Pathology/Vetnostics, Murarrie QLD 4172). Chemistry and haematology analyzers at this laboratory were updated during the course of this study. Serum biochemistry samples were run on Integra 800 (Roche Diagnostics International Ltd CH6343 Rotkreuz Switzerland) until April 2016 when samples were run on a Cobas 8000 (Roche Diagnostics International Ltd). A Coulter AcT Diff (Beckman Coulter Australia Pty Ltd, 23-27 Chaplin Drive, Land Cove NSW 2066 Sydney, Australia) was used for haematology until December 2018 when the laboratory changed over to Roche Sysmex XN-1000 (Roche) Diagnostics International Ltd). The laboratory's quality control measures ensured standards

used for calibration of samples remained consistent over time and between equipment used to ensure comparability of data.

Data was analysed using MedCalc Statistical Software (MedCalc Version 19.2, MedCalc Software, Ostend, Belgium). For 35 samples from free-ranging animals, reference intervals were generated for data having normal distribution, calculated as 95%, double sided with 90 % confidence interval (CI), upper and lower CI, bootstrap CI 10000 iterations, random number seed 978. Box-Cox transformation was used on data not having normal distribution then back-transformed to generate reference intervals. If normality couldn't be established for data, then the Robust method (CLSI C28-A3) was used to calculate the reference interval. The Mann-Whitney test was used to determine whether there were significant differences ($p<0.05$) between data from free-ranging and captive animals. Summary statistics (range, mean±SD, median) for 16 samples from captive animals were also generated.

RESULTS

Reference intervals for haematology and serum biochemical parameters were generated from a total of 35 samples from free-ranging animals (Table 1). A one-sided upper reference value using the percentile method was calculated for alanine aminotransferase (ALT) as a reference interval was unable to be calculated due to the high number (17/35) of samples with the same value (<10 U/L). Basophils were seen in only six of the total 51 blood samples, with value ranging from 0.05-0.15 X 10^9/L and were not included in the analyses. Summary statistics for haematology and serum biochemistry parameters were generated for 16 samples from 12 captive animals (Table 2).

Free-ranging animals showed higher values ($p < 0.05$ on Mann-Whitney test) compared with captive animals for ALP, AST, bilirubin, CK, lipase, phosphate, triglycerides, WBC and neutrophils. (Table 3). Captive animals showed higher values ($p < 0.05$ on Mann Whitney test) for Hb, MCH, MCV, MCHC, albumin, protein, and creatinine (Table 3). While there were significant differences in parameters between free-ranging and captive animals, there was considerable overlap in ranges between the two cohorts.

DISCUSSION

Overall, many blood parameters differed between free-ranging and captive Lumholtz's tree-kangaroos. However, the values were similar enough to propose that the reference intervals generated will be useful for either cohort.

Free-ranging LTK showed significantly higher levels of CK which may be attributed to muscle trauma associated with life in the wild (eg. impact from jumping between branches or falls from trees) and/or with mild trauma associated with darting, fall and capture. Creatine kinase (CK), found in skeletal muscle, cardiac and smooth muscle, brain and, to a lesser extent in liver, intestine and spleen is a muscle specific leakage enzyme which rises rapidly following skeletal muscle injury or trauma.[9] Captive animals lead much more sedentary lives than their free-ranging counterparts and it may be that the trauma of being netted and manually restrained for hand-injection would be a source of muscle trauma roughly equivalent to being darted under free-ranging conditions. While CK levels rise quickly (within 5 hours) and return to normal within 24-48 hours after acute transient muscle injury in horses, persistent or ongoing muscle injury will maintain high CK activity.[10] Aspartate aminotransferase (AST) levels increase more gradually after muscle injury (in dogs and horses) and stay increased for longer periods of time, hence are a better indicator of long term

muscle conditions .[10] As levels of AST were also significantly higher in free-ranging tree-kangaroos this suggests that they routinely experience more muscle trauma than their captive counterparts.

Differences in diet may explain the finding that liver enzymes (AST, ALP, bilirubin**)** were significantly higher in free-ranging animals. Captive tree-kangaroos are fed a more limited range of plant material and items not encountered by free-ranging animals .[11] The varied diet of free-ranging tree-kangaroos includes plants containing a broad range of toxins and secondary plant metabolites. These compounds are produced as chemical defences against herbivores and are found in numerous plants known to be consumed by free-ranging tree-kangaroos.[12] Examples include: phenolic compounds in privet (*Ligustrum* species)[13]; alkaloids, terpenes, lignans and essential oils in laurel (*Litsea* species)[14]; saponins and triterpenes in sarsaparilla (*Alphitonia* spp.)[15]; pyrrolizidine alkaloids in silk pod vine (*Parsonsia straminea*)[16]; alkaloids and glycosides in lantana (*Lantana camara*)[17]; terpenes and essential oils in camphor laurel (*Cinnamomum camphora*)[18] and alkaloids and glycosides in tobacco bush (*Solanum mauritianum*)[19]. Hepatic microsomal enzymes enable generalized detoxification pathways and specifically oxidation, reduction, hydrolysis, esterification, N-dealkylation and conjugation of toxicants .[20, 21] Detoxification, as well as sublethal injury to hepatocytes caused by these plant metabolites may explain the elevated liver enzyme values seen in free-ranging Lumholtz's tree-kangaroos. [20, 21]

WBC and neutrophil counts were significantly higher in free-ranging compared with captive animals (Table 3). This may reflect higher stress associated with being observed prior to capture, mild skin infections and inflammation associated with minor traumatic injuries (cuts, scrapes, insect bites) seen in free-ranging animals that navigate varied habitat.

Most of the free-ranging animals were captured while actively feeding whereas captive animals were fasted prior to sedation so the higher levels of serum phosphate in the free-ranging animals may be due to mild post-prandial elevation of phosphate.[22] Higher phosphate levels occur in young growing animals.[23] Although all seven juveniles tested were free-ranging, when juveniles were excluded from analysis phosphate levels were still significantly higher in free-ranging LTK (Table 3).

Alanine aminotransferase (ALT) is considered a useful indicator of hepatocellular injury in dogs, cats, rats and primates but, as in large animals, may not be useful in *Dendrolagus*.[24] The low range (<9–104 U/L) of ALT found in *D. lumholtzi* is consistent with values reported for *D. matschiei* (0-22 I/L) and *D. goodfellowi* (0-17 U/L).[25]

Alkaline phosphatase (ALP) is known to be elevated in young, growing animals due to bone deposition, but in dogs, an increase can indicate cholestasis.[26] Hence elevations in ALP in free-ranging tree kangaroos may be partly due to the seven juveniles included in the free-ranging cohort. However, after excluding juveniles from the analysis, levels in free-ranging animals remained significantly higher ($p<0.05$) than their captive counterparts (Table 3) so we propose the elevated levels may be attributable to cholestasis, caused by hepatotoxic compounds in food plants.

Lipase levels in juveniles were significantly higher when compared to all adults (n=10 lactating; n=3 non-lactating adult females; n=30 adult males). The cause and importance of increased lipase in juveniles is unknown.

Our results may be influenced by a range of factors that make it difficult to obtain consistent haematological measurements from free-ranging and captive wildlife. Differences between parameters in free-ranging and captive animals may be due the small sample size of captive individuals. We recommend that more samples be obtained from individuals held in captivity. A commercial laboratory was used for analysis of blood samples as this most accurately reflects how samples from captive animals would be analysed. In the interest of safety and reducing handling stress, samples were obtained from animals sedated using chemical immobilization. Some stress associated with capture, handling and sedation is unavoidable but by utilizing the same protocols for capture and sedation for all animals, the effects on physiological parameters may be minimized and should be comparable across all animals sampled. Capturing an arboreal animal in uneven terrain and dense forest habitat was challenging and inhibited collection of many samples.

When compared to published RI for the two species of tree-kangaroos most commonly held in zoos, Matschie's tree-kangaroo (*D. matschiei*) and Goodfellow's tree-kangaroo (*D. goodfellowi*) apparent differences were noted in WBC, creatine kinase, bilirubin, AST, ALT and ALP.[25] These differences may be attributed to samples being predominantly from captive animals for Matschie's and Goodfellow's tree-kangaroo but may also reflect differences in diet and habitat as both these species are from Papua New Guinea and can occur at elevations up to around 3000 metres. Some species of rock-wallabies (considered to be the closest extant relative to tree-kangaroos) show similar blood RI to those found in this study.[25] The RIs generated in this study can be used when assessing captive LTK. This study provides a valuable resource for health assessment of captive and free-ranging Lumholtz's tree-kangaroos by establishing baseline reference intervals for haematologic and biochemical parameters.

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