Reference intervals for selected haematological and clinical biochemistry measurands in Temminck's ground pangolin (*Smutsia temminckii*)

by

Dr Karin Lourens

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Supervisor: Prof Leith Meyer

Department of Paraclinical Sciences,

Faculty of Veterinary Science, University of Pretoria

Co-supervisor: Prof Emma Hooijberg

Dept of Companion Animal Clinical Sciences

Faculty of Veterinary Science, University of Pretoria

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Table of contents

List of tables

List of figures

Figure 6: Blood smear images from Temminck's ground pangolin. A, neutrophil; B, neutrophil (right) and eosinophil (left); C, eosinophil; D, monocytes; E, activated monocyte with vacuoles; F, basophil; G, small lymphocyte; H, reactive lymphocyte; I, granular lymphocyte. Platelets are visible in E and H (arrowheads). Wright-Giemsa, x100 objective. 34

List of abbreviations

ALB	-	albumin
ALP	-	alkaline phosphatase
ALT	-	alanine aminotransferase
Ca	-	total calcium
CI	-	confidence interval
Crea	-	creatinine
EDTA	-	ethylenediamine tetraacetic acid
G	-	gaussian
Glob	-	globulin
Glu	-	glucose
HCT	-	haematocrit
HGB	-	haemoglobin
K	-	potassium
LRL	-	lower reference limit
MCH	-	mean corpuscular haemoglobin
MCHC	-	mean corpuscular haemoglobin concentration
MCV	-	mean corpuscular volume
N	-	number of individuals
Na	-	sodium

NG	-	non-gaussian
P	-	parametric
Phos	-	inorganic phosphorus
R	-	robust method
RBC	-	red blood cell count
SD	-	standard deviation
T	-	box-cox transformed data
Tbil	-	total bilirubin
TP	-	total protein
URL	-	upper reference limit
WBC	-	white blood cell count

Abstract

Reference intervals for selected haematological and clinical biochemistry measurands in Temminck's ground pangolin (*Smutsia temminckii*)

K Lourens[1], EH Hooijberg[2&3], L Meyer[1&3]

1 Department of Paraclinical Sciences & Centre for Veterinary Wildlife Studies, Faculty of Veterinary Science, University of Pretoria, Johannesburg Wildlife Veterinary Hospital, South Africa. info@jwvh.org.za
2 Department of Companion Animal Clinical Studies & Centre for Veterinary Wildlife Studies, Faculty of Veterinary Science, University of Pretoria, South Africa
3 Centre for Veterinary Wildlife Studies, Faculty of Veterinary Science, University of Pretoria, South Africa

An alarming number of pangolins are currently illegally traded for their scales and meat. Many pangolins confiscated from the trade are severely clinically compromised. Unfortunately, little is known about the physiology and normal health of pangolin, making it difficult to identify disease processes and treat them. The purpose of this study was to establish reference intervals (RIs) for haematology and plasma clinical chemistry in the Temminck's ground pangolin. Blood samples were collected from 27 healthy free-living or rehabilitated pangolins and reference intervals were generated according to international guidelines. Clinical chemistry analysis was performed using the Abaxis VetScan VS2 and Cobas Integra 400 Plus analyser and haematology was performed using the Abaxis VetScan HM5 analyser. Vetscan VS2 plasma clinical chemistry RIs were: albumin 26-41 g/L, amylase 316-1014 U/L, ALP 29-153 U/L, ALT 25-307 U/L, bilirubin 1.5-10.8 μmol/L, calcium 1.8-2.5 mmol/L, creatinine 9.7-46.3 μmol/L, glucose 3.8-10.0 mmol/L, phosphate 1.3-2.6 mmol/L, total protein 53-84 g/L, and urea 5.6-19.9 mmol/L. Cobas plasma clinical chemistry RIs were: albumin 19-33 g/L, amylase 396-1669 U/L, ALP 25-301 U/L, ALT 17-291 U/L, bilirubin 1.5-18.3 μmol/L, calcium 1.8-2.4 mmol/L, creatinine <58 μmol/L, glucose 3.6-10.1 mmol/L, phosphate 0.9-2.3 mmol/L, total protein 48-74 g/L, and urea 6.2-20.4 mmol/L. Haematology RIs

were: WBC 1.8-10.71 x10^9/L, RBC 3.88-8.31 x10^{12}/L, HGB 73-150 g/L, HCT 26-51%, MCV 55-72 fL, MCH 15.6-21.4 pg, MCHC 242-332 g/L, and RDW 14.3-19.1%. The Wilcoxon test revealed significant differences between results for the following measurands for the Cobas versus the Abaxis Vetscan VS2: albumin ($p = <0.0001$); ALT ($p = <0.0001$); amylase ($p= <0.0001$); bilirubin ($p= 0.038$); calcium ($p= <0.0001$); phosphate ($p= <0.0001$); total protein ($p= <0.0001$); urea ($p= <0.0001$). RIs for some measurands were wide, probably due to the small sample size. Nevertheless, these are the first RIs generated for the Temminck's ground pangolin and the results presented here will allow veterinarians to better determine the health status of pangolin patients, thus enabling them to formulate optimal treatment plans in the hope of increasing patient survival rates of this endangered species.

Chapter 1

1. Literature review

1.1 Background

Pangolins are small- to medium-sized, scale-covered mammals found in parts of Asia and Africa.(1) Pangolins are unique amongst mammals as they are covered by a layer of imbricated scales - these scales are their primary defence mechanism; they roll up into a tight ball when threatened.(2) They are myrmecophagious – feeding only on formicide ants and termites (3,4) – shy, mostly nocturnal animals, and are rarely encountered in the wild.(5) These animals are solitary, only pairing up briefly for a few days at a time to mate, and the female will give birth to a single young after a gestation period of approximately 135 days.(6–8) Other than this, very little is known about pangolin reproduction, with only a few studies published for the Formosan (Chinese) pangolin (*Manis pentadactyla pentadactyla)* (9) and the Sunda pangolin (*Manis javanica*).(10) No published data exist for the Temminck's ground pangolin (*Smutsia temminckii*).

Figure 1: Temminck's ground pangolin (Image credit: Dr Karin Lourens)

There are eight species of pangolin worldwide: four species in Asia and four in Africa. Only one of these African species resides in South Africa, namely the Ground pangolin, also known as the Temminck's ground pangolin or Cape pangolin. (**Figure 1**). The eight pangolin species fall under the order Pholidota and are grouped in the family Manidae.(11) Du Toit *et al.* (2014) published a study sequencing the whole mtDNA of the Temminck's ground pangolin and determined the phylogenetic position of Pholidota within Eutheria.(12) Pangolins are unique in that they are myrmecophagious, they are covered by a layer of scales, are fossorial, edentate (they have no teeth), and have a very long tongue (almost as long as their bodies) attached to a modified xiphoid process (xiphisternum). Although pangolins share many traits with the order Xenarthra (South-American anteaters, sloths, and armadillos) through convergent evolution, molecular phylogenies have revealed that they are actually a sister-group to Carnivora. (12)

Pangolins are highly coveted for their meat and scales.(8,13,14) In many Asian cultures the scales are considered a cure for a variety of ailments, including skin and liver diseases.(15) In traditional Chinese medicine, pangolin body parts and blood are used to treat ailments such as cancer, asthma, and reproductive problems. The meat is also seen as a delicacy in these same cultures and thousands of dollars are spent on a single dish – pangolin foetuses in particular are believed to enhance virility and are therefore in high demand. Pangolin populations in China and Vietnam have been decimated by poaching and, in some areas, are considered all but extinct. In response to dwindling numbers, smugglers now source animals from other parts of Asia and the Indian subcontinent to satisfy demand in China and Vietnam.(16) Shipments containing tons of frozen meat and scales are regularly seized by customs authorities. As Asian pangolin populations continue to dwindle, demand has shifted towards African species. In the last few years, seizures of large shipments of pangolin scales originating in Africa and destined for Asia have become a regular occurrence.(7,13,17) In Africa, pangolins were mostly hunted for bushmeat and used locally for traditional

medicine.(14,17,18) This trade was sustainable until the scales became a commodity (19,20), and now poverty is driving the trade in African pangolins for Asian markets.(21)

Pangolins are now the most illegally-traded mammal in the world, with a staggering one million pangolins believed to have been taken from the wild in the past decade.(8,16,22). Pangolins are slow-growing mammals and have a generation length of 15-18 years. (2) They produce only one offspring at a time, once every 12-18 months, after a gestation period of 105-140 days. The rate at which they are being killed is therefore wholly unsustainable and could result in the loss of an entire species within the next decade.

A positive move is that all eight species have now been upgraded to Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) Appendix I at the 17th meeting of The Conference of the Parties (Cop17) (1) – they were previously listed as Appendix II. Appendix I lists species that are the most endangered among the animals and plants listed on CITES. These species are threatened with extinction. When listed on Appendix I, international trade in specimens of these species is prohibited, except for exceptional cases when the purpose of the import is not commercial, for instance for scientific research. In these cases, trade may take place provided authorisation is given, and import and export permits need to be granted.

However, in spite of these interventions, the illegal trade in pangolins has not declined. In fact, it has increased and the illegal wildlife trade is booming despite efforts by law enforcement.(23) The driving force behind the trade is the continuing demand for pangolin meat and products, which has not been addressed.(21)

Many studies have focused on the illegal trade and poaching of pangolins, but very little is known about their biology. A few studies have been published,

mainly on Asian species, most of which have involved captive animals. To date, most research has focused primarily on behaviour, eating habits, and husbandry, but little has been done in terms of their physiology.(3,24,25) A few studies have been published where reference intervals were generated for clinical chemistry and haematology in two Asian species and one African species. These were for the Chinese pangolin, where two studies were published (the Formosan pangolin is the same species as the Chinese pangolin) (26) (27), the Sunda pangolin (28), and the White-bellied pangolin (29) respectively. However, the Asian species are different to the Temminck's pangolin. Research on their diet, biology and behaviour indicates that the Asian and African species and intra-African species are vastly different (11), and therefore the data from Asian pangolins may not necessarily be applicable to the African species. Additionally, each of these published studies used different analytical methods which would potentially have yielded different results.

As a direct result of the illegal trade, an increasing number of these animals find their way to wildlife rehabilitation centres or veterinary facilities. Some data have been published about the treatment and care of Asian pangolins in captivity, including information about wound management in the Sunda pangolin (*Manis javanica*) and aspects of digestive anatomy and physiology in Chinese pangolins (*Manis pentadactyla*).(30,31) From the latter study it has become clear that feeding pangolins in captivity (zoo or wildlife rehabilitation centres) is very difficult.(31) In terms of the Temminck's pangolin, there is one existing publication describing the osteology of the forelimb; apart from this no studies have been published in the areas of physiology or veterinary medicine.(32)

With more and more Temminck's ground pangolins being treated in South Africa post-confiscation from illegal traders, the need for more research on this particular species has become a matter of urgency. For domestic animal species, full publications, and in some cases textbooks, have been written on their normal physiology, which enables clinicians to effectively diagnose and treat patients from these species. The trade in pangolins has left veterinarians scrambling for treatment options, and this void in knowledge must be filled to

enable more evidence-based treatment options and therefore more successful outcomes – and we can only do that once we know what their normal physiology is.

1.2 Reference Intervals

Reference values are an essential part of laboratory testing and are used to describe the distribution of physiological variables in healthy adult animals. They are usually reported as population-based reference intervals (RIs) which represent values for 95% of a healthy population.(33) These RIs are often used to define disease status in an animal and could significantly influence how a patient is treated. For this reason, researching and defining appropriate reference values for a specific species are of utmost importance.(37)

The most critical steps in the determination of reference values are the selection of reference individuals based on pre-determined inclusion criteria (e.g. sex, age, reproductive, and health status) and the use of quality-controlled analytical procedures.(33) The dilemma in wild versus captive animals is that values obtained may vary significantly, even when using samples from 'normal' healthy animals.(34) International recommendations state the preferred method as *a priori* nonparametric determination from at least 120 reference individuals, but acceptable alternative methods include transference or validation from previously established RIs. When only small numbers of values are available, RIs can be estimated by other methods, but reference limits thus obtained may be imprecise. These recommendations are a challenge in veterinary clinical pathology, especially when only small numbers of reference individuals are available. (35-38)

To produce RIs to support a reasonable comparison of patient results, certain principles are crucial. Firstly, as much detail as possible must be collected from each reference individual. There are pre-determined data (in this study there were many exclusion criteria such as lactating animals and neonates) as well as concerns which may include stress, medication, wounds, exercise, and so on. The reference individuals should be selected using a strictly controlled process to optimise the use of the reference intervals. Secondly,

sample collection should be as per routine clinical practice, and the conditions the samples are collected and processed in should be near identical for every sample. Thirdly, all samples must be analysed using standardised methods, and the statistical methods used should be appropriate for the sample size.(35)

In veterinary medicine, as in human medicine, reference values are necessary for useful interpretation of laboratory results. Without suitable reference values, the sensitivity and specificity of tests are questionable, which can result in inaccurate diagnoses of disease and poor treatment protocols, which may subsequently cause harm to a patient or, at worst, lead to their death. Reference values specific to the species tested and the equipment and reagents used are essential for accurate interpretation, and limited availability of sufficient numbers of many species, especially threatened or endangered wild animal species, makes getting enough samples for a non-parametric study (120 samples) unfeasible.(36) However, standard techniques are well described and recommendations have been made for generating these intervals in wild animals where it is difficult to collect a large number of samples.(33,36–39)

The paper published on reference intervals for Chinese pangolins in Taiwan (26) used 100 free-ranging animals as reference individuals. All animals were captured and temporarily removed from their natural environment to a research facility, where they were kept for three days and two nights. Blood samples were taken on day two. Animals were anaesthetised using inhalation gas anaesthesia, sexed, and weighed, and heart rate measurement was performed. They were then released on day three. This study found that reference values were similar in males and females, but variations occurred in heart rate, body temperature, serum alanine aminotransferase (ALT) and lipase activities and phosphate concentrations with the change in season. There were also significant differences in the reference intervals between adult and sub-adult Chinese pangolins. The authors advised that seasonal and age group differences should be taken into account when using the reference values documented in their study.(26)

The paper published on reference intervals for Sunda pangolins in Singapore (28) used 58 rescued animals as reference individuals. The animals were rescued either by government agencies, non-government organisations, or members of the public. Each animal was transported in a vehicle to the Singapore Zoological Gardens within a few hours of capture. The animals were all anaesthetised the day after their arrival for a health assessment including physical examination, radiography, blood and urine sample collection, and faecal parasite screening. The animals deemed healthy and stable enough were then released on day three. This study selected 51 clinically normal Sunda pangolins to generate haematology and clinical chemistry reference intervals. No sex-related differences were noted in this study, but age-related differences were observed: adult Sunda pangolins had a significantly higher mean corpuscular volume (MCV) than juveniles, and juvenile Sunda pangolins had significantly higher red blood cell counts and haemoglobin levels than those of the adults ($P<0.05$). Age-related differences were also noted in several serum biochemistry parameters: alkaline phosphatase (ALP) was significantly higher in juveniles, and total protein was significantly higher in adult Sunda pangolins.

The study published on reference intervals for Formosan pangolins in Taiwan (27) used 51 apparently healthy animals that were rescued and brought to the Taipei Zoo. These animals were kept in isolation for four weeks and blood was collected during the 3rd week of this quarantine period. Samples were also taken six months after quarantine and were included in the study only if the animal survived for more than six months after sample collection. Every pangolin included in the study had survived for over one year in captivity after capture. Sex-related differences were observed in platelet count, ALT, mean corpuscular haemoglobin concentration (MCHC), and total protein. Age-related differences were also noted: juveniles had significantly higher platelet counts and ALP reference intervals than their adult counterparts.

The study published on reference intervals for White-bellied pangolins used nine wild-collected animals.(29) These animals were transported to the United States, except for one that was born at the Brookfield Zoo. On arrival all the animals were anaesthetised for a full clinical examination, and blood gas and

select biochemical analyses were performed. The authors advised that clinicians be aware that this species may hypoventilate while anaesthetised and that concomitant administration of respiratory depressants could compound this effect in a species where endotracheal intubation has not been described.

1.3 Study objectives

The study objectives were to establish reference intervals for haematology and clinical chemistry for the Temminck's ground pangolin and to compare results obtained from two different chemistry analysers.

1.4 Benefits arising from the project

The primary goal of this study is to generate reference intervals to which veterinarians can compare results from compromised or sick animals. This comparison will enable the formulation of specific treatment plans for each individual animal and, in turn, hopefully increase survival rates in a species poached to near extinction. The reference intervals generated could also be compared to the results found in the other four studies, which may reveal species differences.

Chapter 2

2. Materials and Methods

2.1 Reference sample population

The animals (n = 27) sampled in this study were either wild, free-ranging (n = 18) Temminck's ground pangolin from Tswalu Kalahari Reserve (Northern Cape, South Africa) (TKR), or confiscated (from the illegal trade) wild animals (n = 9) that were treated at the Johannesburg Wildlife Veterinary Hospital (JWVH), and subsequently released in good health. Animals at the JWVH were kept in "pangolin-boxes" during the day. These boxes were specifically designed for pangolins; they are made from wood and reinforced with steel bars – this is a dark box when closed and therefore simulates being in a burrow during the day. They usually sleep up to 14 hours during the day and normally woke up at around 17:00 in summer. They were then taken out to forage (each animal had a dedicated walker) at a location with all the correct ant and termite species; foraging normally took four to six hours. If a pangolin had been treated with an antibiotic or non-steroidal anti-inflammatory, these drugs would have been discontinued two weeks prior to their release, and therefore blood collection. Only healthy, adult animals (animals > 3kg) of both sexes were included in the study. Health of free-ranging animals was determined by body condition score and the absence of external wounds. Rehabilitated animals were considered ready for release using the following criteria: 1) sufficient weight gain during the rehabilitation period and ability to successfully forage for ants and termites, 2) normal or stable albumin levels (as tested at JWVH), 3) normal or stable blood glucose levels (as tested at JWVH), 4) normal blood smear – meaning assumed normal white cell count, sufficient platelets, and normal red cells, 5) no external wounds, and 6) no outward signs of disease. Samples were taken from these animals on the day of release. Lactating females and females with a pup at foot were excluded

from this study. The number of animals sampled was limited by the rarity of this species and the difficulty in finding them in their natural environment.

2.2 Experimental procedures

For data collection in free-ranging animals in the field, each animal was tracked on foot and anaesthetised using isoflurane (Isofor Inhalation Anaesthetic, Piramal Healthcare).(40,41) Pangolins cannot be unrolled while fully conscious and blood collection is only possible in anaesthetised animals. Anaesthesia was achieved by using a modified induction chamber – placing the animal inside a plastic container with a sealable lid and then adding a piece of cotton wool soaked in isoflurane. This approach achieved an acceptable level of anaesthesia in order to actively work with the animals. The pangolins received isoflurane until handling was possible and were not maintained on anaesthesia for the duration of the procedure. The same procedure was used on the animals from the JWVH. Once an animal was sufficiently anaesthetised, it was taken out of the container and placed on a blanket in dorsal recumbency. Heating was supplied during winter months using a hot water bottle. Heart rate and respiratory rate were measured, and each animal was weighed, measured (nose to tail tip), and sexed, had ears and eyes checked, and external parasite type and load were noted. To ensure an open airway the head was kept flexed towards the chest of the animal. The area caudal to the anus was cleaned using a skin disinfectant (F10 Skin Prep RTU, Health and Hygiene (Pty) Ltd, South-Africa) so that blood could be collected from the coccygeal (tail) vein. To achieve this, a 21 G needle and a 10 mL heparinised syringe was used to collect the blood sample by inserting the needle approximately 1 cm caudal to the anus in the midline at a 45° downward angle (**Figure 2**). 10 mL of blood was collected from each animal; 4 mL placed into a heparin tube, 4 mL into a serum tube, and 2 mL into a paediatric Ethylenediamine tetraacetic acid (EDTA) tube (Vacutainers: BD vacutainer and BD microtainer, Becton and Dickinson, Plymouth, United Kingdom). In addition, two blood smears were made for each animal. Once the animal had sufficiently recovered from anaesthesia, it was released

immediately. The time taken for data collection did not exceed 15 minutes and all the animals were tracked post release to ensure that they were behaving and foraging normally. They were marked on their scales to ensure no animals were sampled more than once. Animals released from JWVH all had microchips, VHF (very high frequency) and satellite trackers placed and were monitored for more than two months post release to ensure they remained healthy. In-field collected samples were taken to a field research facility where they were processed, and haematological analysis was run within 3 hours of collection. Serum and plasma samples were centrifuged (3000 rpm x 5 minutes) and the plasma and serum were frozen (initially at -20°C at the research facility and within 5 days at -80°C) and the plasma analysed in two batches at a later date. The serum was collected for long-term archiving. Samples collected at the JWVH were taken to the Clinical Pathology Laboratory at the Faculty of Veterinary Science in Pretoria, which is a 45-minute drive away. All plasma and serum samples were kept for more than 90 days as sample collection was done over a period of two years.

Figure 2: Blood collection from Temminck's ground pangolin. (Image credit: Dr Karin Lourens)

2.3 Laboratory analysis

We used the Vetscan HM5 (Abaxis Europe GmbH, Griesheim, Germany), which is a portable bench top unit, to determine haematological variables. This analyser uses impedance technology for cell counting and cell volume measurement, and a cyanide-free photometric method to determine haemoglobin concentration. In a pilot study, the pangolins' MCV was similar to that of cattle, so the bovine setting was used for this analysis, which included a white blood cell count (WBC), red blood cell count (RBC), haemoglobin (HB), haematocrit (HT), MCV, MCHC, and platelet count (PLT). All samples were analysed within 3-8 hours post collection, both from in the field and from samples collected at JWVH. One level of the manufacturer-supplied quality control material was analysed before each pangolin sample and results evaluated against the manufacturer's target ranges.

Blood smears were stained with a Wright-Giemsa stain on an automated stainer (HemaTek 2000, Siemens Healthcare, Erlangen, Germany). A 200-cell manual leukocyte differential count was performed by a board-certified clinical pathologist. Observations on morphological characteristics of the erythron, leukon, and thrombon were also recorded.

Clinical chemistry analysis was performed using the Vetscan VS2 (Abaxis Europe GmbH, Griesheim, Germany), a portable benchtop analyser, which uses pre-packaged rotors with liquid reagents (Comprehensive Diagnostic Profile rotor, Abaxis Europe GmbH, Griesheim, Germany). Albumin, alkaline ALP, ALT, amylase, creatinine, calcium (Ca), globulin, glucose, phosphate, total bilirubin, total protein, and urea were measured. Analytical methods are further detailed in **Table 1**. An internal quality-control procedure was run before each analysis.

All chemistry samples were concurrently run on a Cobas Integra 400 Plus analyser (Roche Products [Pty] Ltd., Basel, Switzerland - hereafter referred to as the Cobas). This device is an automated wet chemistry analyser (see **Table 1** for analysis methods) and daily internal and monthly external quality control was performed according to laboratory protocols.

2.4 Data and statistical analysis

The statistical analysis for the generation of RIs was performed according to the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) and the American Society for Veterinary Clinical Pathology (ASVCP) guidelines, using Reference Value Advisor for Excel.(33)(42)

Firstly, data were examined visually using histograms and descriptive statistics were performed. Outliers were identified using Dixon and Tukey tests. The Anderson-Darling and McWilliams runs test were used to assess normality and symmetry, respectively. In order to increase specificity for the small data set, a p-value of < 0.27 was used with the Anderson-Darling test; P was set at < 0.05 for the runs test. (39) Non-Gaussian data were Box-Cox transformed. The 95% reference intervals were calculated using parametric (Gaussian data) or robust (non-Gaussian data) methods. The 90% confidence interval of the lower and upper reference limits was calculated with a bootstrap method. Results for each measurand on the Abaxis Vetscan VS2 and Cobas were compared using a Wilcoxon test for paired samples; a p-value of < 0.05 was considered significant.

Table 1: Assay methods utilised by the Cobas Integra and Abaxis Vetscan VS2 for the analysis of Temminck's ground pangolin clinical chemistry.

	Cobas Integra 400 plus	Abaxis Vetscan VS2
Albumin	Bromocresol green dye-binding method	As for Cobas Integra
ALT	Kinetic (L-alanine and 2-oxyglutarate)	As for Cobas Integra
ALP	Kinetic (p-nitrophenol phosphate))	As for Cobas Integra
Amylase	Enzymatic (ethylidene-G7PNP)	Enzymatic (2-chloro-p-nitrophenyl-D-maltotrioside)
Bilirubin	Diazo Method	Enzymatic (bilirubin oxidase)
Calcium	Cresophthalein complexone method	Arsenazo III method
Creatinine	Modified Jaffe reaction	Enzymatic (creatinine amidohydrolase)
Globulin	Calculated	Calculated
Glucose	Hexokinase method	As for Cobas Integra
Phosphate	Phosphomolybdate method	Enzymatic (glucose-6-phosphate dehydrogenase)
Potassium	Ion-selective electrode	Enzymatic (pyruvate kinase)
Sodium	Ion-selective electrode	Enzymatic (beta-galactosidase)
Total protein	Biuret Method	As for Cobas Integra
Urea	Enzymatic (urease)	As for Cobas Integra

ALP, alkaline phosphatase; ALT, alanine aminotransferase; K, potassium; Na, sodium

Chapter 3

3. Results

3.1 Study population and samples

The study population consisted of 27 adult pangolins – 18 free-ranging animals from TKR and nine rehabilitated animals from the JWVH. Of the TKR pangolins five were male and 13 female, and of the JWVH pangolins six were male and three were female. Partitioning based on sex and location could not be done because of the low numbers of these animals. Animals ranged in weight from 3.4 kg to 15.1 kg, and lengths (nose to tail tip) from 53 cm to 114 cm. All of the sampled animals fulfilled the inclusion and exclusion criteria. Clinical chemistry was assessed in all 27 animals, but haematology was only assessed from 25 animals because samples from two animals could not be analysed within 24 hours. Blood smears were assessed only from 23 animals as smears from four animals were damaged in the transport process and could not be included in the data set. No haemolysis, icterus, or lipemia was seen in any of the plasma samples.

3.2 Clinical chemistry

The results for reference intervals generated for plasma clinical chemistry measurands for the Abaxis Vetscan VS2 and Cobas are presented in **Table 2**, and histograms are presented in **Figures 3 and 4**. Reference intervals were generated for Na and K on the Vetscan VS2, but as the method is not recommended by the IFCC, these results were not reported. Data distribution and the statistical methods used for each measurand are also included in **Table 2**.

Table 2: Plasma clinical chemistry reference intervals for Temminck's ground pangolin for the Abaxis Vetscan VS2 and Cobas Integra 400 Plus chemistry analysers

Measurand	N	Mean	Median	SD	Min	Max	RI	LRL 90% CI	URL 90% CI	Distribution	Method
Albumin (g/L)											
Abaxis	27	35	34	4	27	41	26-43	24-28*	41-46*	NG	T, R
Cobas	27	27	27	3	18	32	19-33	14-22*	31-34	NG	T, R
ALP (U/L)											
Abaxis	27	86	69	66	23	327	29-340	25-37	157-733*	NG	T, R
Cobas	27	83	69	60	19	275	25-301	21-32	160-553*	NG	P, R
ALT (U/L)											
Abaxis	27	82	72	50	31	198	25-307	21-31	184-475*	NG	T, R
Cobas	27	74	59	51	22	192	17-291	14-23	173-464*	NG	T, R
Amylase (U/L)											
Abaxis	27	567	530	169	317	1106	316-1014	288-360	856-1227*	NG	T, R
Cobas	27	1033	979	304	533	1904	396-1669	242-562*	1495-1836	G	P
Bilirubin (µmol/L)											
Abaxis	27	6	6	2	4	14	4.0-14.0	Data not Gaussian or symmetrical, even after transformation. Min-max used as RI			
Cobas	27	5.2	3.8	3.5	1.3	16.4	1.5-18.3	1.3-1.8	10.5-30.0*	NG	T, R
Calcium (mmol/L)											
Abaxis	27	2.3	2.3	0.2	1.4	2.6	1.8-2.5	1.4-2.1*	2.5-2.6	NG	T, R
Cobas	27	2.2	2.2	0.2	1.4	2.4	1.8-2.4	0.0-2.0*	2.3-2.4	NG	T, R
Creatinine (µmol/L)											
Abaxis	27	28	27	9	<18	47	<47	Seven results < 18 µmol/L, unable to compute a RI. < max used as RI			
Cobas	27	31	31	12	<18	58	<58	Four results < 18 µmol/L, unable to compute a RI. <max used as RI			
Glucose (mmol/L)											
Abaxis	27	6.9	6.8	1.5	3.1	9.4	3.8-10	2.9-4.6*	9.1-10.8*	G	P
Cobas	27	6.9	6.7	1.6	3.0	9.8	3.6-10.1	2.8-4.5*	9.2-11.0*	G	P
K (mmol/L)											
Abaxis	NR										
Cobas	27	4.1	4.1	0.6	3.0	6.0	3.1-5.8	2.9-3.4	5.2-6.5*	NG	T, R
Na (mmol/L)											
Abaxis	NR										
Cobas	27	144	144	3	137	149	137-150	135-139*	149-152*	G	P
Phosphate (mmol/L)											
Abaxis	27	1.8	1.7	0.3	1.4	2.5	1.3-2.6	1.3-1.4	2.3-2.9*	NG	T, R
Cobas	27	1.3	1.3	0.3	1.0	2.2	0.9-2.3	0.9-1.0	1.8-3.0*	NG	T, R
Total protein (g/L)											
Abaxis	27	69	68	8	52	84	53-84	49-57*	80-88*	NG	T, R
Cobas	27	62	62	6	47	72	48-74	45-52*	70-78*	NG	T, R
Urea (mmol/L)											
Abaxis	27	9.5	8.9	3.1	5.2	18.2	5.6-19.9	5.1-6.2	14.2-28.2*	NG	T, R
Cobas	27	10.4	9.9	3.1	6.0	19.4	6.2-20.4	5.6-6.9	15.1-28.3*	NG	T, R

ALP, alkaline phosphatase; ALT, alanine aminotransferase; CI, confidence interval; G, gaussian; LRL, lower reference limit; N. number of individuals; NG, non-gaussian; NR, no reported; R, robust method; SD, standard deviation; T, box-cox transformed data; URL, upper reference limit, * the CI to RI ration exceeded 20%

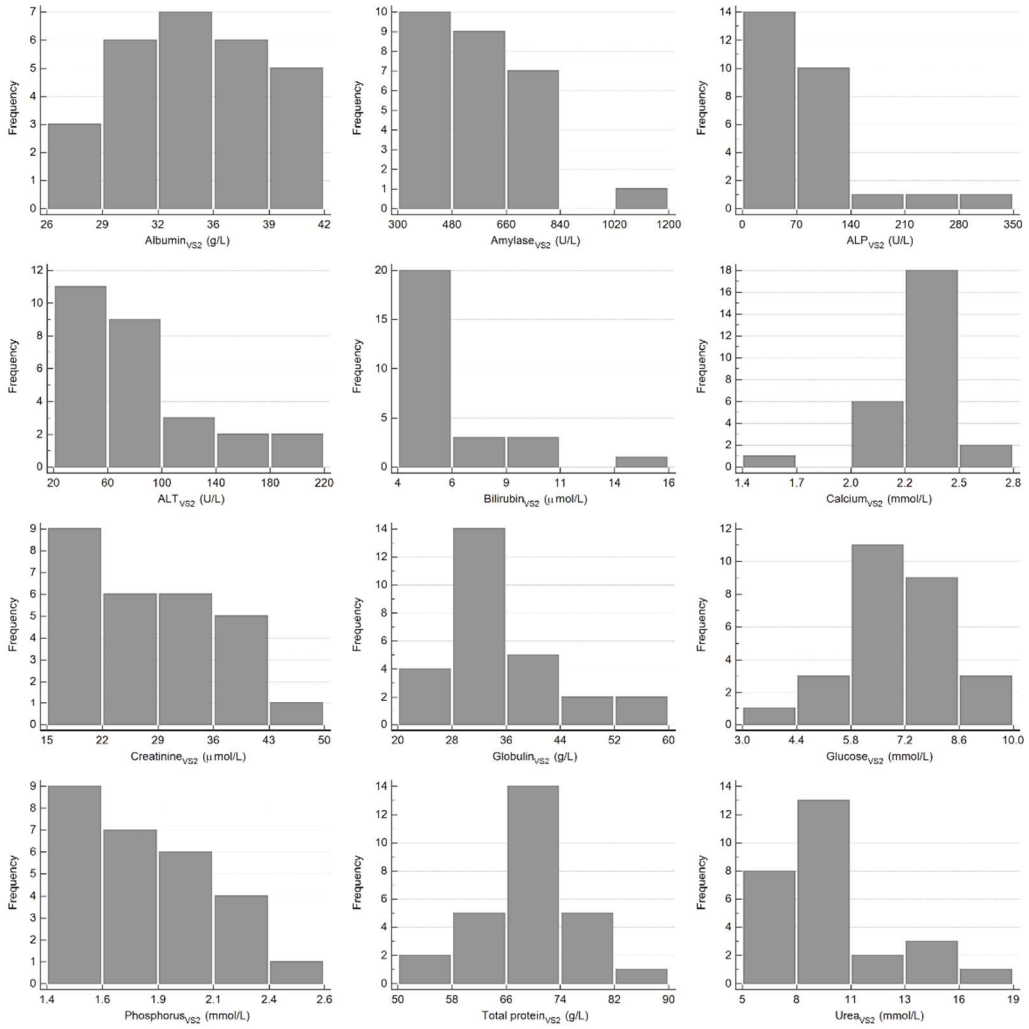

Figure 3: Histograms showing the distribution of results for plasma clinical chemistry measurands for the Abaxis Vetscan VS2. The x-axis represents the measurands and the y-axis represents the frequency of these values occurring.

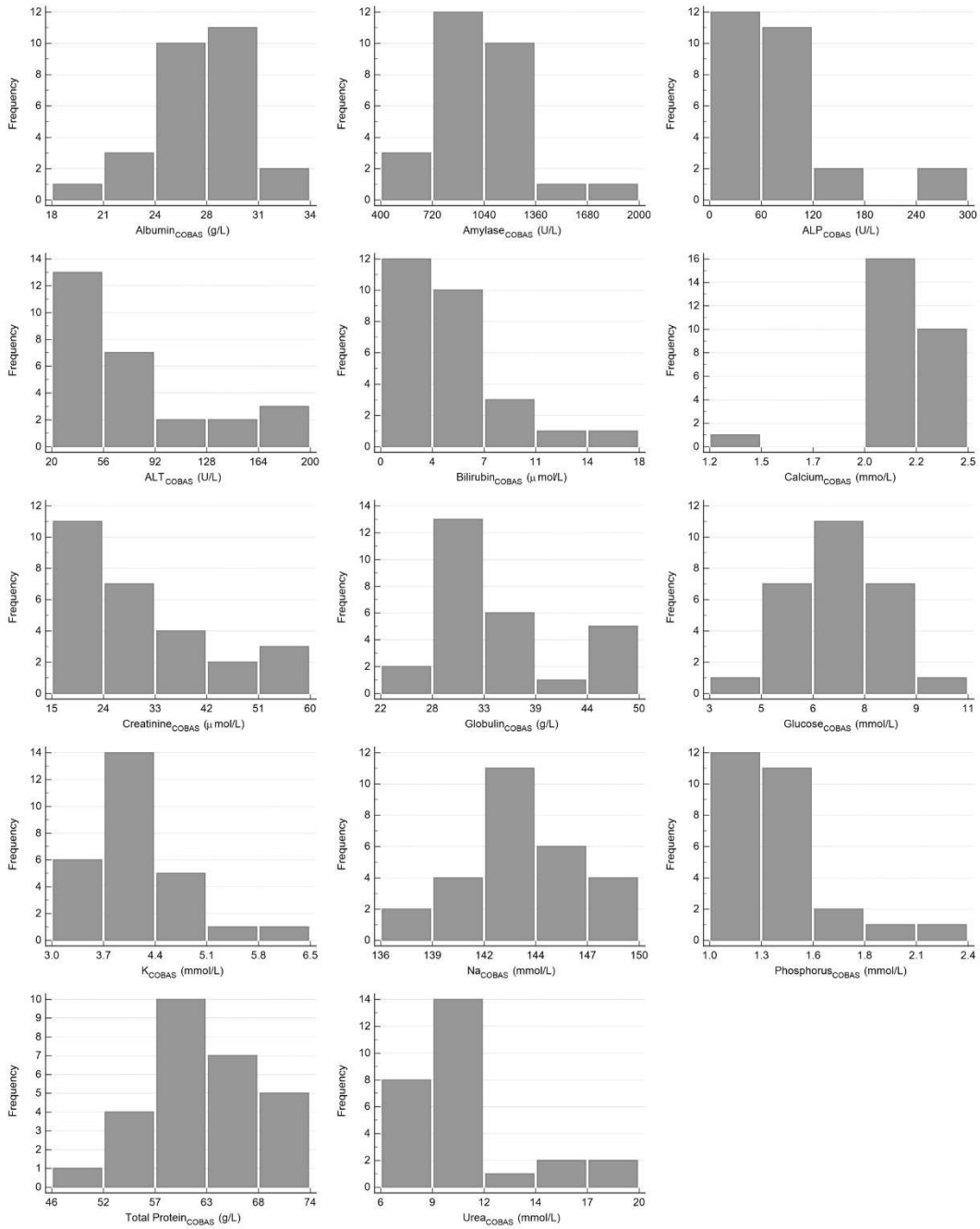

Figure 4: Histograms showing the distribution of results for plasma clinical chemistry measurands for the Cobas Integra 400 Plus. The x-axis represents the measurands and the y-axis represents the frequency of these values occurring.

For ALP results, two high outliers, from both analysers, were identified using the Tukey test. These outliers were from apparently healthy pangolins from TKR. Similarly, from a healthy female pangolin from TKR, a calcium result was identified as a low outlier from both analysers. For urea results, two high outliers, from both analysers, were identified from two confiscated adult males who were deemed clinically healthy after they had been treated at JWVH for dehydration, malnutrition, and minor injuries. No post-release survival information is available for one of these individuals, but the second died three days post-release from exposure to very low temperatures. One low outlier was identified for one albumin result from the Cobas analyser. The result came from a young female from TKR that had a good body condition score and showed no obvious abnormalities. Two high outliers were identified for amylase results from both analysers. These results came from one of the previously described rehabilitated male pangolins with a high outlier for urea. One high outlier was identified in the bilirubin data sets from both analysers, from a JWVH-rehabilitated pangolin. This pangolin was still doing well three months post-release. All of these outliers were retained due to the small sample size and lack of a clinical or analytical reason to discard them. (33) Vetscan VS2 bilirubin data did not attain a Gaussian or symmetrical distribution after transformation; therefore, the minimum to maximum range was used as the reference interval. Seven of the 27 Vetscan results and four of the 27 Cobas results for creatinine fell below the detection limit of the analysers (≤ 18 μmol/L); therefore, it was not possible to determine a reference interval, so the minimum to maximum range was reported.

The Wilcoxon test revealed significant differences between results for the following measurands for the Cobas versus the Abaxis Vetscan VS2: albumin ($p = <0.0001$); ALT ($p = <0.0001$); amylase ($p= <0.0001$); bilirubin ($p= 0.038$); calcium ($p= <0.0001$); phosphate ($p= <0.0001$); total protein ($p= <0.0001$); urea ($p= <0.0001$).

3.3 Haematology

The results for reference intervals generated for haematology are presented in **Table 3** and histograms are presented in **Figure 5**.

Table 3: Haematology reference intervals for Temminck's ground pangolin for the Abaxis HM5 haematology analyser and manual leukocyte differentials (200 cell count)

Measured	N	Mean	Median	SD	Min	Max	RI	LRL 90% CI	URL 90%CI	Distribution	Method
WBC ($x10^9$/L)	25	6.25	6.33	2.12	2.81	9.63	1.8-10.71	0.68-3.00*	9.44-11.92*	G	P
RBC ($x10^{12}$/L)	25	6.1	6.22	1.05	3.78	8.48	3.88-8.31	3.33-4.58*	7.62-8.84*	G	P
HGB (g/L)	25	111	111	18	66	146	73-150	62-84*	139-160*	G	P
HCT (%)	25	39	39	6	26	54	26-51	23-30*	48-55*	G	P
MCV (fL)	25	64	63	4	59	72	55-72	53-57	69-74*	NG	R
MCH (pg)	25	18.3	18.1	1.4	15.8	21.8	15.6-21.4	15.1-16.4	20.4-22.5*	NG	T, R
MCHC (g/L)	25	287	291	21	257	325	242-332	229-257*	321-346*	NG	R
RDWc (%)	25	16.7	16.8	1.1	14.7	18.5	14.3-19.1	13.7-15.0*	18.4-19.7*	G	P
Neutrophil ($x10^9$/l)	23	3.33	3.22	1.32	1.59	5.61	1.19-7.04	0.9-1.62	5.83-8.26*	NG	T, R
Neutrophil (%)	23	53	50	12	33	77	27-79	20-35*	72-87*	G	P
Lymphocyte ($x10^9$/l)	23	1.94	1.61	0.97	0.71	4.51	0.65-5.16	0.51-0.84	3.59-7.34*	NG	T, R
Lymphocyte (%)	23	31	30	12	15	57	13-63	11.0-16.0	52-74*	NG	T, R
Monocyte ($x10^9$/l)	23	0.31	0.18	0.25	0.03	1.02	0.03-1.26	0.02-0.06	0.72-1.80*	NG	T, R
Monocyte (%)	23	5	4	3	1	14	1.0.14.0	0.0-1.0	11-18*	NG	T, R
Eosinophil ($x10^9$/l)	23	0.65	0.59	0.6	0.05	2.6	0.00-2.74	0.00-0.07	1.71-3.73*	NG	T, R
Eosinophil (%)	23	10	7	7	1	27	0.0-28.0	0.0-1.0	20-34*	NG	T, R
Basophils ($x10^9$/L)	23	0.03	0.00	0.07	0.00	0.34	0.07-2.82	0.04-0.14	1.75-3.87*	NG	T, R
Basophil (%)	23	1	0	1	0	5	0-3	0.0-0.0	2.0-4.0*	NG	T

G, gaussian; HCT, haematocrit; HGB, haemoglobin; LRL, lower reference limit; MCH, mean corpuscular haemoglobin; MCHC, mean corpuscular haemoglobin concentration; MCV, mean corpuscular volume; N, number of individuals; NG, non-gaussian; P, parametric; R, robust method; RBC, red blood cells; SD, standard deviation; T, box-cox transformed data; URL, upper reference limit; WBC, white blood cells, * the CI to RI ratio exceeds 20%

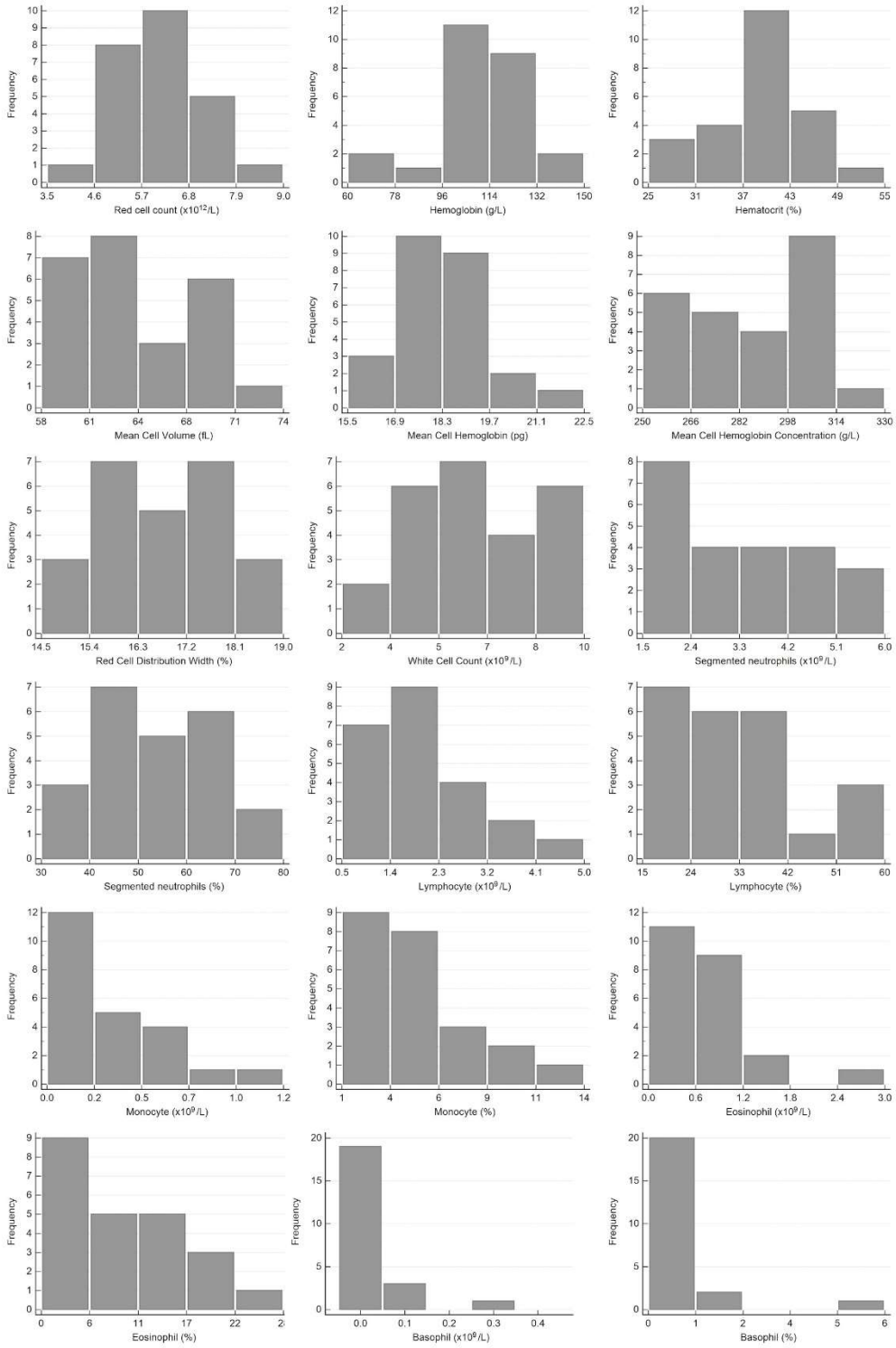

Figure 5: Histograms showing the distribution of results for haematology measurands for the Abaxis Vetscan HM5 and manual leukocyte differential counts. The x-axis represents the measurands and the y-axis represents the frequency of these values occurring.

One high outlier was identified using the Turkey test for MCH, from an adult male released from the JVWH that was not monitored post-release. Several outliers were identified for leukocyte subpopulations by the Tukey test; one high basophil (percentage and absolute count), one high eosinophil (percentage and absolute count), and two high monocyte (one percentage and one absolute count). All these outliers were from different, apparently healthy pangolins from TKR. One high outlier was identified for lymphocyte absolute count, from a male pangolin treated at JWVH that survived at least two months post-release. One high outlier was identified for HCT, from a mature adult male released from the JWVH that was monitored post-release. No other outliers (chemistry or hematology) were identified for this animal and no reason for the high HCT could be identified.
As for the clinical chemistry results, all outliers were retained.

Reference intervals were not generated for platelets due to the high prevalence of clumping. Five of the samples had platelet counts of less than 100 $x10^9$/L. Numerous small platelet clumps were present on most of the blood smears, including from these five samples, and the platelet counts were considered inaccurate. Reference intervals were therefore not generated. The automated platelet counts ranged from 16 – 318 $x10^9$/L with a median of 197 $x10^9$/L.

3.4 Morphological features of the erythrocytes

In WG-stained films, the erythrocytes were round in shape (diameter 7-9 µm) with a mild central pallor or lacking central pallor. (**Figure 6**) Some of the smears showed mild rouleaux formation. Occasional schistocytes and acanthocytes were seen (<1/1000x high power field). Many crenated erythrocytes were present, presumably as a preparation artefact.

3.5 Morphological features of the leukocytes

The neutrophils were round (diameter 12-15 µm) with a moderate amount of clear to light blue cytoplasm containing fine, pale pink granules and segmented nuclei with two to five lobes with coarse chromatin patterns. (**Figure 6A, 6B**)

The eosinophils were round (diameter 16-17 µm) with a moderate amount of clear to light blue cytoplasm with prominent small, round, orange-pink granules. The nuclei were segmented with two lobes and coarse chromatin patterns. (**Figure 6B, 6C**)

The basophils were round (diameter 13-15 µm) with a moderate amount of clear cytoplasm containing numerous purple granules. The nuclei had two lobes with finely stippled chromatin. (**Figure 6F**)

The monocytes had round to pleomorphic shapes (diameter14-18 µm) with moderate to abundant light blue-grey cytoplasm. Occasionally they had cytoplasmic vacuoles. The nuclei were irregularly round, oval, or bean-shaped, with finely stippled chromatin. (**Figure 6D, 6E**)

The lymphocytes were round (diameter 9-11 µm) with round to slightly oval nuclei. They had a scant amount of blue cytoplasm with dense- to coarsely clumped cytoplasm. (**Figure 6G**) A few reactive lymphocytes were also noted: these were 1.5 times larger, with an increased amount of cytoplasm, sometimes darker blue. (**Figure 6H**) Occasional lymphocytes with this morphology also had magenta-staining cytoplasmic granules – consistent with a large granular lymphocyte morphology. (**Figure 6I**)

3.6 Morphological features of the platelets

The platelets were round or oval shaped with occasionally very elongated forms (diameter 2-5 µm). The cytoplasms were pale pink with centrally located purple granules. Small to large platelet aggregates were seen on most smears. (**Figure 6E, 6G, 6H**)

3.7 Other features seen

Piroplasms were present (ranging from rare to a few) in six smears in the red blood cells. These could not be identified based on their morphology, and further molecular diagnostic investigation is currently underway. The red cell parameters of these six animals were not affected by the parasites.

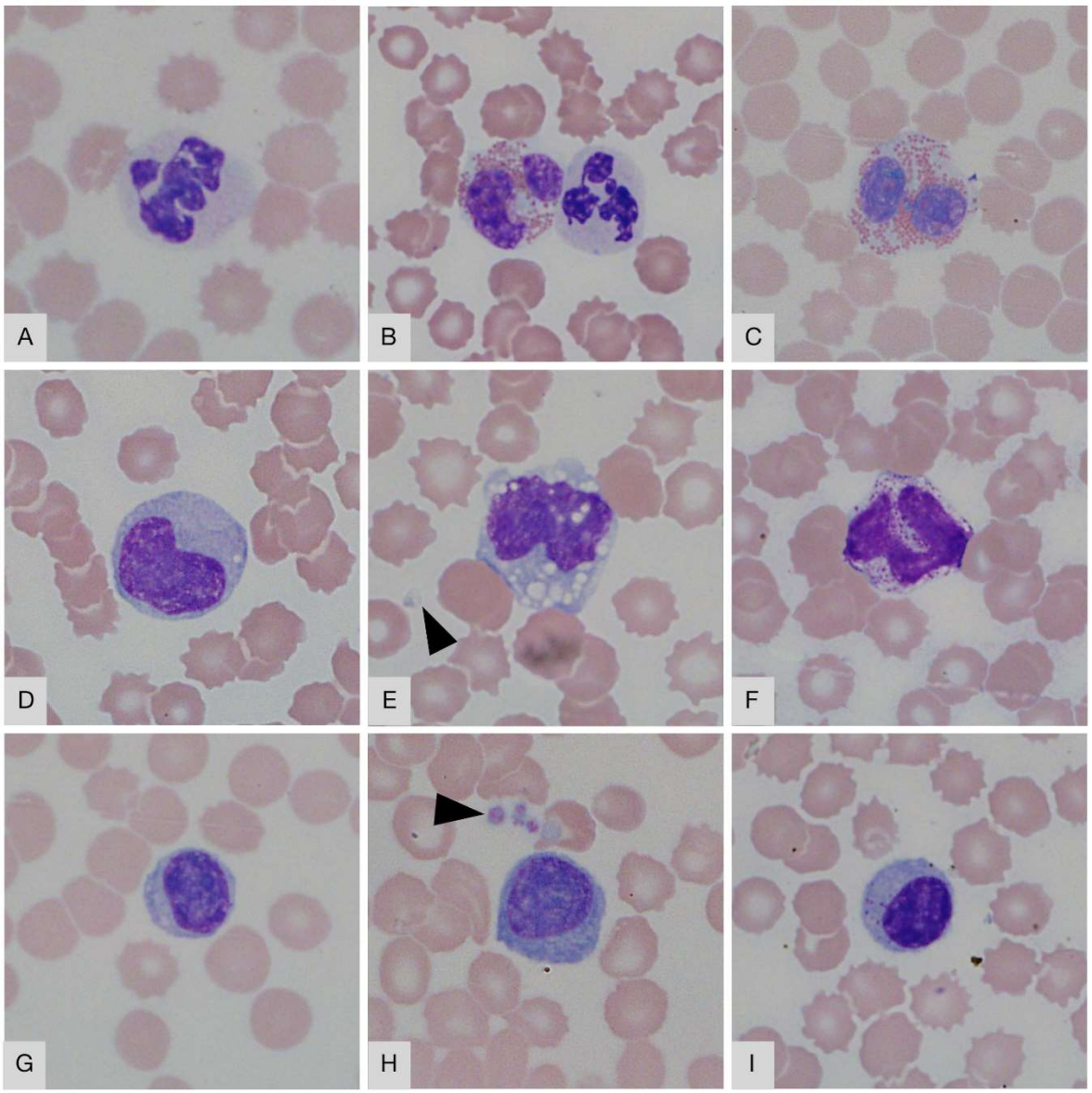

Figure 6: Blood smear images from Temminck's ground pangolin. A, neutrophil; B, neutrophil (right) and eosinophil (left); C, eosinophil; D, monocyte; E, activated monocyte with vacuoles; F, basophil; G, small lymphocyte; H, reactive lymphocyte; I, granular lymphocyte. Platelets are visible in E and H (arrowheads). Wright-Giemsa, x100 objective.

Chapter 4

4. Discussion

This study reports reference intervals for haematology and clinical chemistry derived from a small reference sample group of Temminck's ground pangolin. These reference intervals provide novel clinical pathology data for this species and are intended to assist in the health assessment and treatment of individual pangolins, particularly those confiscated from the illegal wildlife trade.

Reference intervals should preferably be calculated using the largest sample size to provide for accurate statistical analysis – ideally a minimum of 120 individuals per study.(33) This number proved difficult for this study as the Temminck's ground pangolin is a rare species and, due to its nocturnal and shy behaviour, is difficult to track. Therefore, the sample size used was the minimum number needed for generating accurate RIs; the minimum number advised is 20, and this study sampled 27 animals.(33,38,39,43) For larger sample sizes outliers would be eliminated, but in this study it was decided to keep all these results (43,44) as eliminating them would have decreased the already-small sample size. When generating RIs, especially in smaller sample sizes (<40) whether or not to retain outliers becomes an important consideration. It is best practice to eliminate outliers if the sample size allows for this, but in the case of rare species where sample collection is difficult, outliers (with certain considerations) should be retained. These considerations should include only clinically healthy animals, only one clinical chemistry or haematology outlier per sample animal and no other tested abnormalities. Outliers may skew data but when considered in conjunction with all the other results generated, these outliers should be included. ASVCP guidelines also states (33): "When reference individuals are selected randomly from well-defined populations and health is confidently established, retention of all reference values is favored. However, when reference individuals are

selected by convenience, health is not readily confirmed (e.g., wild-caught species), or field methods introduce higher levels of inaccuracy and imprecision, reference values located at the extremities should be examined more rigorously for possible exclusion."

The outliers were examined rigorously and all animals with outliers, except one, had only one outlier per animal sampled and for each of these animals all haematology values were within normal limits. Other than the one outlier, no other indication of disease or illness was noted. The only animal with two outliers had been released from JWVH and had vhf and satellite trackers placed and was followed for more than three months post release. During this time, he had shown normal behavior and for the first month post release he was weighed first daily (the first week) then weekly and he had shown consistent weight gain. For the following months he was weighed once a month and was maintaining a healthy weight for his size, therefore I believe this animal was in good health and his data was not excluded based on these outliers.

For this study rehabilitated animals, treated at JWVH, were included as sufficient numbers of wild, free-roaming pangolins could not be found. (43) Using rehabilitated animals might have created some biases in the data, but we are of the opinion that our health assessment before release would have minimised these potential biases. For future studies a larger sample size would be ideal, especially if the study is done over a longer period, which means that more animals can be sampled. Another option would be to sample rehabilitated pangolins 3-6 months post release to ensure that their data matched those of other free-roaming pangolins.

Another limitation to this study was that the use of the bovine setting on the Abaxis HM5 haematology analyser was not fully validated – for example, by comparing the analyser-calculated HCT to a spun a PCV for each pangolin. PCV determined by centrifugation of microhematocrit tubes is the optimum method to evaluate the proportion of blood volume taken up by erythrocytes. (45) The spun PCV and the analyser calculated HCT values should not differ

by more than 3%. Manual-spun HCTs (PCVs) were not performed as the necessary equipment was not available in the field at the time of sampling. It would be advised that this be included in all future haematology studies. The reference data presented here should therefore ideally only be used when comparing pangolin patient results determined on the Abaxis HM5 using the bovine setting.

All plasma samples were kept stored and frozen for more than 90 days. This practice was due to the difficulty in finding animals in the wild and led to the in-field collection taking nearly two years. Although chemistry analytes can degrade over time the samples were kept in a -80°freezer for this whole time and samples were all analysed at the same time to minimise any variability that may be introduced by using different batches of rotors or reagents, or different procedural approaches.

For this study RIs were generated on two different chemistry analysers, a point-of care and a reference laboratory analyser. This was done in order to increase the options of using different analysers for clinical sample analysis.(46) (47)

Results for the following clinical chemistry measurands were similar for both the Abaxis VS2 and the Cobas analysers: ALP, creatinine, globulin, and glucose. Results from all the other measurands differed significantly. Method-dependant differences in clinical chemistry measurands have reported in several other studies. (47,48)

To our knowledge these are the first RIs for the Temminck's ground pangolin. Four other publications report RIs for three other pangolin species: the Sunda pangolin (*Manis javanica*), (28) White-bellied pangolin (*Phataginus tricuspis*), (29) and two studies for the Chinese pangolin (*Manis pentadactyla*) (26) and the Formosan pangolin (*Manis pentadactyla pentadactyla*) (27) (the *Manis pentadactyla and Manis pentadactyla pentadactyla* are the same species). Results are not directly comparable, as in some cases different analytical methods and statistical procedures for the generation of RIs were used (see **Table 4** and **Table 5**).

Table 4: Clinical chemistry analysers used in each study with comparative reference intervals

Clinical chemistry	Study 1	Study 2	Study 3	Study 4	Temminck's this study	Temminck's this Study
Species	Chinese pangolin	Formosan (Chinese) pangolin	Sunda pangolin	White-bellied pangolin	Temminck's ground pangolin	Temminck's ground pangolin
Analyser	FUJI DRI- CHEM 4000i	ADVIA 1800 Chemistry System	Vetscan 2 Analyser	i-STAT clinical blood gas analyser	Vetscan VS2	Cobas Integra 400 Plus
Distributor	FUJIFILM Co., Minato-ku, Tokyo, Japan	Siemens Medical Solutions Diagnostics, Tarrytown, New York 10591, USA	Abaxis, Union City, CA, USA	Abaxis Inc., Union City, CA 94587, USA	Abaxis Europe GmbH, Griesheim, Germany	Roche Products [Pty] Ltd Basel, Switzerland
Measurand	Reference intervals					
Albumin (g/L)	27.00-47.95	28-45	27-63	Not reported	26-43	19-33
ALP (U/L)	Not analysed	69-308	156-903	Not reported	29-340	25-301
ALT (U/L)	48.05-395.83	46-308	71-569	Not reported	25-307	17-291
Amylase (U/L)	50.50-475.00	256-588	114-653	Not reported	316-1014	396-1669
Bilirubin (µmol/L)	3.42-30.69	1.17-30.78	6.00-22.00	Not reported	1.5-10.8	1.5-18.3
Calcium (mmol/L)	1.96-3.10	2.04-3.09	1.96-2.78	Not reported	1.8-2.5	1.8-2.4
Creatinine (µmol/L)	8.84-48.4	8.84-114.92	4-104	Not reported	<45	<58
Glucose (mmol/L)	2.30-8.63	1.88-9.99	2.6-9.7	4.60-6.93	3.8-10	3.6-10.1
Phosphate (mmol/L)	1.18-2.84	1.32-2.35	1.52-4.23	Not reported	1.3-2.6	0.9-2.3
Total Protein (g/L)	49.50-74.00	52-96	50-93	Not reported	53-84	48-74
Urea (mmol/L)	7.38-23.73	5.89-31.05	3.7-20.6	Not reported	5.6-19.9	6.2-20.4

ALP, alkaline phosphatase; ALT, alanine aminotransferase

Table 5: Haematology analysers used in each study with comparative reference intervals

Haematology	Study 1	Study 2	Study 3	Study 4	Temminck's this study
Species	Chinese pangolin	Formosan (Chinese) pangolin	Sunda pangolin	White-bellied pangolin	Temminck's ground pangolin
Analyser	Hemavet 950 Analyzer	Heska Vet abc-diff haematology analyser	Vetscan HM5	i-STAT clinical blood gas analyser	Vetscan HM5
Distributor	Erba Diagnostics, Inc., Miami, FL, USA	Love- land, Colorado 80538, USA	Abaxis, San Francisco, CA, U.S.A.	Abaxis Inc.	Abaxis Europe GmbH, Germany
Species setting	Not specified	Not specified	Canine	Not specified	Bovine
Manual Diff Count	No	No	Yes	No	Yes
Measurand			Reference intervals		
WBC (x10^9/L)	2.34-8.58	3.30-13.2	1.86-17.86	Not reported	1.8-10.7
RBC (x10^{12}/L)	2.66-7.73	3.50-8.62	1.92-9.65	Not reported	3.88-8.31
HGB (g/L)	81.83-179.88	83-186	61-194	Not reported	73-150
HCT (%)	18-53	23.5-55.3	25-55	38-53	26-51
MCV (fL)	58.5-83.59	58.6-82.3	56-75	Not reported	55-72
MCH (pg)	18.36-31.61	20.1-28.9	17.3-29.5	Not reported	15.6-21.4
MCHC (g/L)	245.35-463.2	31.3-38.6 **	289-426	Not reported	242-332
RDWc (%)	Not reported	Not reported	Not reported	Not reported	14.3-19.1
Neutrophil (x10^9/l)	Not reported	Not reported	1.29-13.16	Not reported	1.19-7.04
Neutrophil (%)	Not reported	Not reported	Not reported	Not reported	27-79
Lymphocyte (x10^9/l)	Not reported	Not reported	0.3-3.0	Not reported	0.65-5.16
Lymphocyte (%)	Not reported	Not reported	Not reported	Not reported	13-63
Monocyte (x10^9/l)	Not reported	Not reported	0.01-2.50	Not reported	0.03-1.26
Monocyte (%)	Not reported	Not reported	Not reported	Not reported	1.0.14.0
Eosinophil (x10^9/l)	Not reported	Not reported	0.00-0.97	Not reported	0.00-2.74
Eosinophil (%)	Not reported	Not reported	Not reported	Not reported	0-28
Basophils (x10^9/L)	Not reported	Not reported	0.00-0.08	Not reported	0.07-2.82
Basophil (%)	Not reported	Not reported	Not reported	Not reported	0.00-0.34

HCT, haematocrit; HGB, haemoglobin; MCH, mean corpuscular haemoglobin; MCHC, mean corpuscular haemoglobin concentration; MCV, mean corpuscular volume; RBC, red blood cells; WBC, white blood cells

Amylase was only measured in the Chinese pangolin and the values differ remarkably from the Temminck's: see **Table 4**. The reason for the differences is most likely contrasting analytical methods in the chemistry analysers used in each study. Both analysers use an enzymatic method, but with different substrates (Vetscan VS2: 2-chloro-p-nitrophenyl-D-maltotrioside; FUJI DRI-CHEM 4000i: 4,6- ethylidene-4-nitrophenyl-a-D- maltoheptaoside). The Vetscan VS2 uses liquid reagents while the FUJI DRICHEM operates with dry slides. Other components of the reaction system like reaction times and pH likely also differ, but this information is proprietary.

ALP values were generated for the Sunda pangolin, Formosan pangolin, and the Temminck's (**Table 4**). The Sunda pangolin and the Temminck's studies both used the same chemistry analysers and therefore direct comparisons can be made. ALP RIs in the Sunda pangolin had higher URLs than in the Temminck's. The main difference between the Sunda pangolin study compared to the Temminck's, is the inclusion of juvenile animals. ALP activity is known to be higher in juvenile animals, which may explain why the values for the Sunda pangolin study were higher, as juvenile animals were excluded from our Temminck's study.(45) The higher ALP values in juveniles are caused by increased osteoblastic activity in growing animals, with a resultant increase in bone ALP activity, which is measured as part of total plasma ALP activity.(45)

Reference interval values for ALT were similar two pangolin species (Sunda pangolin and Chinese pangolin). (**Table 4**) Similarly, bilirubin values were similar three species (Sunda, Chinese and Formosan pangolin), despite these studies being performed on different analysers. However, in the Temminck's the upper reference limit was much lower than in these three species. For a direct comparison (same analysers used), the Sunda pangolin had greater upper reference limits compared to the Temminck's study. This finding might be due to the fact that in the Sunda pangolin study the animals were fasted before blood collection, which could falsely elevate total bilirubin levels.(61) Fasting bilirubinaemia is best described in equids (48), but can also occur in other species.(49) In the Temminck's study none of the animals were intentionally starved.

Total calcium was also similar in three species: Sunda pangolin, Formosan/Chinese pangolin, and Temminck's (**Table 4**). In the White-bellied pangolin, ionized calcium was measured, rendering these results not comparable with those from the other species, as total calcium was measured in them (**Table 4**).

Creatinine values were similar in Sunda pangolin and Formosan pangolin, with much higher upper reference limits compared to the Chinese pangolin and Temminck's. When the Sunda pangolin study's creatinine RIs are

compared to the Temminck's RIs, the Sunda pangolin study's upper reference limit was much higher. The reason for this is unknown. Creatinine is most commonly increased by pathological processes that cause decreased glomerular filtration rate (GFR), the initiating process being pre-renal, renal, or post-renal.(45) In contrast, a decreased creatinine is not clinically recognised or clinically significant.(45) Animals with a decreased muscle mass would tend to have a lower creatinine. In most species, the lower reference limit for serum creatinine is near the detection limit of creatinine assays, so documenting a true decrease would be difficult. However, further studies are needed to ascertain the reason for low creatinine levels in the Temminck's.

Blood glucose levels had similar upper reference limits in the different species: Sunda pangolin; Formosan pangolin; White-bellied pangolin and Temminck's. (**Table 4**) Relatively high concentrations of glucose may be seen in healthy animals due to excitement or fright. Catecholamines (adrenaline and noradrenaline) stimulate glycogenolysis and promote growth-hormone release.(45) When results for the same analysers were compared, the Sunda pangolins and Temminck's RIs were similar. The RIs for K, Na and phosphate were similar in all five species.

Total protein RIs were generated for all but the White-bellied pangolin, and in the three species, the reference intervals were very similar. Urea reference intervals were generated for all but the White-bellied pangolin and in these three species (Sunda, Formosan/Chinese and Temminck's) the intervals were also similar.

The haematology RIs of the Temminck's pangolin revealed several similarities to the other four pangolin papers, with a few important differences. For direct comparison, the Sunda pangolin study was used. (**Table 5**). These differences could be accounted for by a number of reasons, but age of the animals sampled could again have played a role. The majority of animals sampled in the Sunda pangolin study were juveniles – 35 out of the 51 (68%). Many studies have been published looking at age-related variations in haematological and clinical chemistry test results.(50–52) These studies indicate that the WBC count decreases over time and with age, and RBC

count and HGB concentration increase with age. This seems to be true for most mammals; however, further age-related studies on the Temminck's ground pangolin are needed to verify this.

The eosinophil and basophil counts had markedly higher upper reference limits in the Temminck's study compared to the Sunda pangolin study. Eosinophilia can suggest the possibility of many disease states such as hypersensitivity (allergic disorders), parasitism (ectoparasites; tissue nematodes, trematodes, and protozoa), hypoadrenocorticism, mast cell degranulation caused by inflammation, and idiopathic eosinophilic conditions. Persistent mild eosinophilia can be normal in apparently healthy mammals.(62) The aforementioned conditions could not be excluded in the animals sampled, but all study subjects were clinically healthy at the time of sampling. A possible reason for the high upper reference limit could be parasitism. A number of parasites have been described to occur in both Asian and African pangolins. Papers have been published on parasites in pangolins. One such study by Mohapatra, Rajesh Kumar *et al*. (2014) produced a checklist of 34 genera of parasites and bacteria, including four genera of protozoan, 13 genera of helminths, eight genera of ticks, two genera of mites, and seven genera of bacteria reported in seven of the eight pangolin species. The Temminck's pangolin was included in this study.(53) Causes of basophilia may not always be apparent, but can be linked to allergic, neoplastic, and parasitic (especially fleas and gastrointestinal parasites such as nematodes) states.(63) The most likely cause of basophilia in apparently healthy animals would be parasites and would correlate with the eosinophilia observed. Further studies are necessary to validate these deductions, especially where repeat complete blood counts can be performed on the same animals over time.

Cell morphology was very similar to that of other mammalian species. A number of the blood smears (10) showed platelet clumping, which in turn led to low automated platelet counts (PLT<100 $x10^9$/L) in five of these ten samples. Platelet counts in these five samples ranged from 16-58 $x10^9$/L with corresponding HCTs within the reference interval (28-33 %), making a true

thrombocytopenia unlikely. Platelet clumping can be caused by a variety of factors, the most common being as a result of anti-coagulants in the sampling tube and blood collection technique leading to platelet activation and aggregation.(54)(55)

Species-specific reference intervals are an invaluable tool for veterinarians. They aid in diagnosing and treating patients successfully. In wildlife patients a full medical work-up is not always possible, but with technological advances in the development of point-of-care analysers, portable units are now available to practitioners in the field.(56–59) The point-of-care Vetscan analysers used in this study are convenient for wildlife veterinary work as they are portable units that can be used in the field. Research into reference intervals for high value and commonly treated wildlife species is warranted, to improve the quality of veterinary care provided to them and rehabilitation success. In an online article published in February 2018 by *Dan Challender, Chair of the IUCN SSC Pangolin Specialist Group,* "Scaling up pangolin conservation like never before", the author states that, according to best available knowledge, all eight pangolin species are now considered threatened with extinction on The IUCN Red List of Threatened Species™, where they are categorised as Critically Endangered, Endangered, or Vulnerable.(6) These previously understudied animals have now forced the world to look more closely at their veterinary care. This study will hopefully build a base for more in-depth research into these animals. Veterinarians and wildlife rehabilitation facilities worldwide dealing with pangolins need all the tools available to them in order to save the order Pholidota from extinction. This study has added a valuable tool to the treatment of Temminck's ground pangolin post confiscation from illegal traders in South-Africa and will hopefully lead to more research as the number of traded pangolins becomes ever greater.

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Appendices

Ethics approval

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

PROJECT TITLE	Reference intervals for haematology and serum biochemistry in free-ranging Temminck's Ground Pangolin (*Smutsia temminckii*)
PROJECT NUMBER	V069-17
RESEARCHER/PRINCIPAL INVESTIGATOR	Dr. K Laurens

STUDENT NUMBER (where applicable)	U_97041387
DISSERTATION/THESIS SUBMITTED FOR	BSc

ANIMAL SPECIES	Temminck's Ground Pangolin (*Smutsia temminckii*)	
NUMBER OF SAMPLES	45	
Approval period to use animals for research/testing purposes		June 2017- June 2018
SUPERVISOR	Prof. L Mayer	

KINDLY NOTE:

Should there be a change in the species or number of animal/s required, or the experimental procedure/s - please submit an amendment form to the UP Animal Ethics Committee for approval before commencing with the experiment

APPROVED	Date 26 June 2017
CHAIRMAN: UP Animal Ethics Committee	Signature

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

Extension No. 1

PROJECT TITLE	Reference intervals for haematology and serum biochemistry in free-ranging Temminck's Ground Pangolin (*Smutsia temminckii*)
PROJECT NUMBER	V069-17
RESEARCHER/PRINCIPAL INVESTIGATOR	Dr. K Laurens

STUDENT NUMBER (where applicable)	U_97041387
DISSERTATION/THESIS SUBMITTED FOR	MSc

ANIMAL SPECIES	Temminck's Ground Pangolin (*Smutsia temminckii*)	
NUMBER OF SAMPLES	45	
Approval period to use animals for research/testing purposes		March 2019 – March 2020
SUPERVISOR	Prof. L Meyer	

KINDLY NOTE:

Should there be a change in the species or number of animal/s required, or the experimental procedure/s - please submit an amendment form to the UP Animal Ethics Committee for approval before commencing with the experiment

APPROVED	Date 23 April 2019
CHAIRMAN: UP Animal Ethics Committee	Signature

Poster for:

Onderstepoort faculty day: University of Pretoria, Faculty of Veterinary Science

10th Oppenheimer Research Conference, October 2019