REFERENCE INTERVALS FOR HAEMATOLOGY, CLINICAL CHEMISTRY AND ACUTE PHASE REACTANTS IN FREE-RANGING AFRICAN ELEPHANTS (*LOXODONTA AFRICANA*)

By

Christine Steyrer

Submitted in fulfilment of the requirements for the degree Master of Science (Veterinary Science) in the Department of Companion Animal Clinical Studies, Faculty of Veterinary Science, University of Pretoria, South Africa

October 2020

DECLARATION OF ORIGINALITY

UNIVERSITY OF PRETORIA

The Department of Companion Animal Clinical studies places great emphasis upon integrity and ethical conduct in the preparation of all written work submitted for academic evaluation.

While academic staff teach you about referencing techniques and how to avoid plagiarism, you too have a responsibility in this regard. If you are at any stage uncertain as to what is required, you should speak to your lecturer before any written work is submitted.

You are guilty of plagiarism if you copy something from another author's work (eg a book, an article or a website) without acknowledging the source and pass it off as your own. In effect you are stealing something that belongs to someone else. This is not only the case when you copy work word-for-word (verbatim), but also when you submit someone else's work in a slightly altered form (paraphrase) or use a line of argument without acknowledging it. You are not allowed to use work previously produced by another student. You are also not allowed to let anybody copy your work with the intention of passing if off as his/her work.

Students who commit plagiarism will not be given any credit for plagiarised work. The matter may also be referred to the Disciplinary Committee (Students) for a ruling. Plagiarism is regarded as a serious contravention of the University's rules and can lead to expulsion from the University.

The declaration which follows must accompany all written work submitted while you are a student of the Department of Companion Animal Clinical Studies. No written work will be accepted unless the declaration has been completed and attached.

Full names of student: Christine Steyrer

Student number: u25934651

Topic of work: Reference intervals for haematology, clinical chemistry and acute phase reactants in African elephants *(Loxodonta africana)*

Declaration

1. I understand what plagiarism is and am aware of the University's policy in this regard.
2. I declare that this dissertation (eg essay, report, project, assignment, dissertation, thesis, etc) is my own original work. Where other people's work has been used (either from a printed source, Internet or any other source), this has been properly acknowledged and referenced in accordance with departmental requirements.
3. I have not used work previously produced by another student or any other person to hand in as my own.
4. I have not allowed, and will not allow, anyone to copy my work with the intention of passing it off as his or her own work.

SIGNATURE 

Table of Contents

ACKNOWLEDGEMENTS

This study was funded with a Research and Development Grant from the University of Pretoria (UP) and the African Wildlife Health and Management Research Theme Fund of the Faculty of Veterinary Science UP, as well as dnata4good-UP's Wild over Wildlife program.

I am grateful to the University of Pretoria for the financial support granted for my MSc; in particular to the University of Pretoria Library Services and the Department of Companion Animal Clinical Studies for their contribution towards the publication of my manuscript (Frontiers article processing charge). It is greatly appreciated.

I would like to express my gratitude to the following people:

Prof Emma H. Hooijberg, my supervisor; for initiating this important and long overdue project, for sharing her passion and knowledge and guidance during the whole process. Thank you for teaching me and providing me with new skills – be it in front of the microscope or the computer. I've learned so much during this time; for ex I enjoy cytology now, especially if it includes bilobed monocytes and I am ok with statistics (apologies I am not going as far as enjoying it (yet)). 😊 Thank you for your time shared, professionally, and privately, for car rides, runs and laughs and so much more. Thank you for having me as your student and everything else far beyond that.

Prof Michele Miller, my co-supervisor, for her shared knowledge, passion, and professionalism. Thank you for your continuous work towards saving wildlife and for all your time and effort put into this project and thank you for your valuable comments, advices and motivational words along the way.

Dr Jennie Hewlett, my co-worker, for all her knowledge, passion and stories shared, as well as the pharmacological and anaesthesia related mental input provided. Thank you for your hours shared and spent in front of the microscope, debates about lymphocytes vs monocytes and your passion shared for game runs and excellent coffee 😊

Dr Peter Buss, my co-worker, for sharing his knowledge and passion working with wildlife. Thank you for your never-ending efforts saving injured animals in the Kruger National Park, for all the samples supplied, and for being part of this project.

Thank you to the four of you, I feel honoured to have been working with you and could have not asked for a better team.

Furthermore, I would like to thank the technologists from the Veterinary Wildlife Services Laboratory, SANParks, Skukuza and the Veterinary Clinical Pathology Laboratory, Faculty of Veterinary Science, University of Pretoria for assistance with sample analysis.

I would also like to thank my colleagues and friends, for their support along the way, their many positive words, and the shared laughs and tears, as well as skills and advices given to help me survive in South Africa – until now, they have worked.

I am also grateful to my siblings for always having an open ear; and Nikolaus, for being there, for your love and support, and of course for your computer skills which saved (not just) me. :-)

LIST OF ABBREVIATIONS

ALP	Alkaline phosphatase
APP	Acute phase protein
APR	Acute phase reactant
AST	Aspartate aminotransferase
ASVCP	American Society for Veterinary Clinical Pathology
BUN	Blood urea nitrogen
CI	Confidence interval
CITES	Convention on International Trade in Endangered Species of Wild Fauna and Flora
CK	Creatine kinase
CLSI	Clinical and Laboratory Standards Institute
CP	Ceruloplasmin
CRP	C-reactive protein
CV	Coefficient of variation
DALRRD	Department of Agriculture, Land Reform and Rural Development
EEHV	Elephant Endotheliotropic Herpes Virus
G	Gaussian
GGT	Gamma-glutamyl transferase
HP	Haptoglobin
HGB	Haemoglobin
HCT	Haematocrit
IFCC	International Federation of Clinical Chemistry
IUCN	International Union for Conservation of Nature
KNP	Kruger National Park
LRL	Lower reference limit
MCH	Mean corpuscular haemoglobin
MCHC	Mean corpuscular haemoglobin concentration
MCV	Mean corpuscular volume
MG	Magnesium
Mtb	*Mycobacterium tuberculosis*
NG	Non-Gaussian

NP	Non-parametric method
OVAH	Onderstepoort Veterinary Academic Hospital
P	Parametric method
PCV	Packed cell volume
Phos	Phosphorus
PLT	Platelet
QC	Quality control
r	Correlation coefficient
RBC	Red blood cell
RI	Reference interval
SAA	Serum amyloid A
SANParks	South African National Parks
SD	Standard deviation
SOP	Standard operating procedure
TB	Tuberculosis
TP	Total Protein
URL	Upper reference limit
VWS	Veterinary Wildlife Services
WBC	White blood cell

LIST OF FIGURES

LIST OF TABLES

SUMMARY

Reference intervals for haematology, clinical chemistry and acute phase reactants in free-ranging African elephants (*Loxodonta africana*)

By

Christine Steyrer

Supervisor:	Prof EH Hooijberg (University of Pretoria)
Co-supervisor:	Prof M Miller (University of Stellenbosch)
Department:	Companion Animal Clinical Studies
Degree:	MSc

The African elephant (*Loxodonta africana*) is listed as Vulnerable on the Red List of the International Union for Conservation of Nature. Populations on the African continent have been declining for decades, through loss of habitat, human-elephant conflict, and hunting and poaching for ivory and bushmeat. Elephants are very popular zoo animals and numbers held in captivity across the globe are well within the thousands. Reference intervals (RI) are a valuable tool in monitoring health and disease status of populations and provide assistance with making diagnoses and determining prognosis in diseased individuals. Most publications reporting reference values for elephants provide information on limited measurands or have used small sample sizes, were conducted with outdated methods, or sampled either culled or captive animals. Moreover, no RI study has been performed for the African elephant according to the guidelines for RI generation provided by the American Society for Veterinary Clinical Pathology (ASVCP).

The objectives of this study were therefore 1) to generate RIs for haematology, clinical chemistry and acute phase reactants (APR) in free-ranging African elephants, 2) to compare the established RIs to those already published, and 3) to assess changes in

clinical chemistry of diseased animals, using these RIs. The ASVCP recommendations were followed throughout the process of RI generation.

The reference sample population initially consisted of 79 apparently healthy free-ranging elephants from the Kruger National Park. Samples were collected from these elephants prior to the start of this study. Clinical chemistry analysis was performed on stored serum using the Large Animal Rotor on an Abaxis Vetscan VS2. Measurement of the acute phase reactants in serum was performed on the Roche Cobas Integra 400 Plus, using a colorimetric peroxidase assay for haptoglobin, the ferrozine zinc method for iron and an immunoturbidometric assay for serum amyloid A (SAA). Haematology analysis was performed at the time of sample collection using EDTA whole blood. Haematology samples were analysed with a Scil Vet ABC or a Horiba ABX Micros ESV 60 using the domestic horse setting. A manual packed cell volume was also performed. Blood smears were made for all animals, and these were examined, and a manual differential count performed, for this study.

Statistical analysis was performed with the RefVal Advisor add on for Excel. Strict outlier identification and elimination was applied as the samples originated from a wild animal population and clinical examination was limited. Either parametric or non-parametric methods were used to generate the 95% reference intervals for the population, depending on the data distribution. The 90% confidence intervals (CI) of the lower and upper reference limits were calculated using a bootstrap method.

An additional 17 samples from injured (all snare-related) animals were selected and analysed for the same clinical chemistry and APR measurands as described above and blood smears were evaluated for 200-cell differential count and morphological changes.

After outlier exclusion, results from 50 clinical chemistry profiles, 43 APR profiles, 51 blood smears, 48 packed cell volumes and 23 haematology analyses were included. The following RIs were generated: PCV 34-49%, RBC 2.80-3.96 $x10^{12}$/L, HGB 116-163 g/L, MCV 112-134 fL, MCH 35.5-45.2 pg, MCHC 314-364 g/L, PLT 182-386 $x10^9$/L, WBC 7.5-15.2 $x10^9$/L, segmented heterophils 1.5-4.0 $x10^9$/L, band heterophils 0.0-0.2 $x10^9$/L, total monocytes 3.6-7.6 $x10^9$/L (means for “normal” were 35.2%, bilobed 8.6%, round 3.9% of total leukocytes), lymphocytes 1.1-5.5 $x10^9$/L, eosinophils 0.0-0.9 $x10^9$/L, basophils 0.0-0.1 $x10^9$/L. Clinical chemistry RIs were: albumin 41-55

g/L, ALP 30-122 U/L, AST 9-34 U/L, calcium 2.56-3.02 mmol/L, CK 85-322 U/L, GGT 7-16 U/L, globulin 30-59 g/L, magnesium 1.15-1.70 mmol/L, phosphorus 1.28-2.31 mmol/L, total protein 77-109 g/L, urea 1.2-4.6 mmol/L. Acute phase reactants RIs were: HP 0.16-3.51 g/L, SAA < 10 mg/L and iron 8.60-16.99 µmol/L.

Clinical pathology changes in the injured animals were mostly characterised by a left shift heterophilia. Morphologically, a higher number and severity grade of toxic changes in the heterophils were observed as well as an increased number of active monocytes. For the clinical chemistry analytes, the biggest changes were seen in albumin, magnesium and GGT (10 out of 17 animals had a hypoalbuminaemia and hypomagnesaemia and increased GGT); and calcium (8 out of 17 had hypocalcaemia), when compared to our RIs. Changes in APRs were apparent in all injured animals, but the magnitude thereof varied greatly. Iron was within the RIs in one animal only, two animals had physiological SAA values and four animals' HP was considered physiological.

The RIs in this study were found to be grossly comparable to what has been published in the literature previously, with some variations for geographical locations. Generally, our generated RIs have narrower ranges than other studies' results and hence will provide clinicians working with the species with a valuable diagnostic tool. This study also showed that some clinical chemistry measurands and APRs do show changes related to injury in this species and that APRs should therefore be added more frequently to clinical pathology profiles, especially in injured animals. Further studies will be needed to investigate their diagnostic and possibly prognostic value.

CHAPTER 1: LITERATURE OVERVIEW

THE AFRICAN ELEPHANT

The African elephant (*Loxodonta africana)* is the largest terrestrial mammal – a megaherbivore - weighing up to seven tons and reaching 3.3 m in shoulder height, with a life span of up to 70 years, - and belongs to the order Proboscidea, named after their distinct anatomical feature, the trunk. This order also includes the Asian elephant (*Elephas maximus*) with its three subspecies: *E. m. maximus* (Sri Lanka), *E. m. indicus* (mainland Asia) and *E. m. sumatranus* (Sumatra). The African elephant was divided into two subspecies - *L. a. africana* and *L. a. cyclotis* - which were re-classified some years ago as two different species: *Loxodonta africana* – the savannah or bush elephant and *Loxodonta cyclotis* – the forest elephant (1). Both species are currently listed as "vulnerable" on the International Union for Conservation of Nature (IUCN) Red List as populations are declining (2). The next closest relatives to elephants are Sirenia (dugongs and manatees) and Hyracoidae (hyraxes), who also belong to the superorder Afrotheriae. About 10 million African elephants used to roam around almost the whole of the sub-Saharan African continent in the 1930's (WWF). Since then, populations have declined drastically, mainly due to habitat loss and fragmentation, conflicts between elephants and humans, and poaching for ivory. Since 1979, elephant numbers have declined by over 50% (3) - many populations were reduced by drastic poaching due to the immense increase in the ivory price (4). It is estimated that there were roughly 415 000 individuals on the African continent in 2015, whereas in 2006 more than 508 000 elephants were counted (5). According to the Great Elephant Census, populations in Tanzania and Mozambique are suffering the most due to poaching, whereas numbers in Kenya, Zambia and South Africa remained stable or even increased slightly (6).

THREATS TO WILD POPULATIONS – IVORY AND BUSHMEAT

Ivory trade has taken place since the time of the Roman Empire, when elephants were mainly imported from North Africa and were used as fighting animals in the arenas and for transport (7). Throughout history, ivory has been appreciated for its appearance and easy-to-carve attributes and has therefore been used for buttons, handles, billiard

balls, relics and piano keys. Slaughter of elephants for ivory drove the species towards extinction until trade was prohibited in 1989, when the elephant was listed on Appendix I of CITES (Convention on International Trade in Endangered Species of Fauna and Flora). In order to facilitate the sale of ivory stock pile sales to Asia (once off ivory sales in 1999 and in 2008), southern African countries had their elephant populations moved to Appendix II. This means that trade is allowed but limited and permit controlled. In the years following the ivory trade ban, illegal poaching has increased continuously, linked to rising prices and a growing demand for ivory, especially in the markets of China and Thailand (8, 9). Markets in these two countries are graded as white, grey or black markets according to the legal status of the ivory sold in them, which was traded at prices of $2.15/gram on the online black market in 2014 (10). According to CITES, close to 600 000 kg of ivory was seized between 1991 and 2016, which is equivalent to about 50 000 elephants per year.

In order to track the poaching incidents and the illegal ivory trade, especially the hardest hit poaching areas and the locations to which the ivory is shipped, a daughter organisation, MIKE (Monitoring of Illegal Killing of Elephants), was introduced by CITES in 2002. Eastern and southern Africa, specifically Tanzania and Mozambique, have been identified as poaching hot spots. Most of the ivory seized during 2006 and 2014 intended for the Asian and south east Asian black market originated from two bordering game reserves: the Selous Game Reserve in south-eastern Tanzania and the Niassa Game Reserve in northern Mozambique (8, 11). For example, an estimated 41 tons of ivory was seized in 2011 and 51 tons in 2013, driving the number of illegally killed elephants to above 90 000 animals in just two years (11). The poaching peak was identified as occurring in 2011, thereafter poaching decreased, but not enough to sustain the populations (12).

The question of how to reduce poaching comes down to reducing demand and supply. Since the ban was implemented, more than 260 tons of ivory have been burnt to prevent it from re-entering the market (13). On the supply side, the main focuses are on increased patrolling as well as better law enforcement to reduce illegal activities not just limited to ivory, but also applicable to rhino horn or more general bushmeat poaching (14, 15). Hunting for, and harvesting of bushmeat from elephants is usually only a secondary motivation (after ivory). A survey, conducted by the IUCN in Central

Africa, showed that in most kills some meat is taken, but usually not even half of what is available, mainly due to logistics and safety (16). As elephants are usually not killed next to the road, too much manpower would be needed to carry out all the meat. Smoking it beforehand is a common procedure as it reduces weight, but also brings more risk, due to the time needed, which increases the risk of discovery and arrest.(16). Poaching is mainly poverty driven – as in especially poor financial situations the possible benefit outweighs the risk (12, 17). One part of the solution needs to be the integration of community-based conservation programs, to generate fixed incomes for hunters, middle men or women and vendors, which would help the community and protect the wildlife (12, 18, 19).

Southern Africa, including South Africa, Namibia, Botswana, Mozambique, Zambia, Angola, and Zimbabwe, is home to the highest numbers of elephants on the continent, 70% of Africa's population (20). In Angola, the population declined by about 20% between 2005 and 2015, due to poaching but also as a result of human settlement (21). Botswana's elephants have also become more prone to poaching incidents. An aerial survey conducted in 2018 revealed 385 +/- 58 carcasses of poaching victims, with this tendency rising in recent years (22). Botswana hosts about a third of the total elephant population of southern Africa. Within South Africa itself, the estimated total number of individuals is 18 841, living in an area of roughly 30 600 square kilometres. Ninety-three percent of this habitat consists of protected areas such as National Parks, privately owned land and private game reserves, of which the largest area is the Kruger National Park (KNP) (20). In 2015, at least 17 086 elephants were counted within this 19 845 km^2 large park (23). The population of KNP is currently increasing; nevertheless, poaching is becoming a problem. Seventy-one elephants were poached within the park in 2018, and about 31 in 2019 (24, 25).

The African elephant is currently still listed under CITES Appendix II for these countries: South Africa, Namibia, Botswana and Zimbabwe, where limited trade and hunting is controlled but allowed. For the rest of the world, the African elephant is listed under Appendix I.

ELEPHANTS IN CAPTIVITY

Elephants are commonly kept in captive settings such as zoos and circuses, including 78 zoos in North America and 114 in Europe (26). They are considered a flagship species; the animal a visitor would want to see in a zoo. According to the Elephant database, close to 9 000 individuals are recorded in captivity; this number does not distinguish between Asian and African elephants (27). There is no actual number available on individuals held in circuses. With regards to zoological facilities within Europe, there are more than 40 institutions listed as keeping African elephants. Globally, the number of elephants held in captivity (all species) is estimated to be about 16 000. In South Africa, around 120 African elephants are held in captive or semi-captive facilities, of which many are used for human-animal interactions (28).

Elephants in captivity face various health problems, often related to their environment. In free-ranging populations, elephants have complex social structures, which are not feasible to maintain in captivity, mostly due to small herd sizes and limited space. Musculoskeletal and especially foot problems occur frequently in zoos (29). These conditions are often associated with concrete surfaces and little exercise. Free-ranging elephants walk between 30 and 50 miles almost daily and the ground surface walked upon varies greatly. Pododermatitis has, however, been reported in free-ranging elephants in the KNP, mainly affecting mature bulls, and was associated with the hard mopane shrubs, creating lesions and facilitating the entry of bacterial pathogens (30). Some zoos are attempting to mimic the animals' natural behaviour by walking their animals. Other common conditions of captive elephants include but are not limited to gastrointestinal disorders and skin lesions (31). Infectious diseases such as Elephant Endotheliotropic Herpesvirus (EEHV) and Tuberculosis (TB) occur more commonly in Asian elephants but have been reported in African elephants as well (32, 33). Currently seven serotypes of EEHV are known (EEHV1 - EEHV7), some found in the Asian elephant and some in the African elephant. The clinical signs vary from virus-associated skin nodules to the haemorrhagic disease, which is often lethal (32). Elephants are susceptible to *Mycobacterium tuberculosis* (Mtb), the human-associated strain of TB, which commonly affects the Asian elephant as this species is often held in close proximity to humans (34, 35). *Mycobacterium tuberculosis* has also been

reported in a free-ranging African elephant from the KNP and the seroprevalence of Mtb complex within the park's population is believed to be between 6 and 9% (36, 37).

REFERENCE INTERVALS

What is normal? – a question that has been asked numerous times in multiple contexts and still it is not always easy to answer.

"To present a compilation of values which may be termed normal" (38) was the description of a book's content in the 1950's which could be considered as an early approach to reference intervals nowadays. Medicine is based on comparisons (39); clinicians compare daily – how is my patient compared to yesterday or to a week ago? Breaths or beats per minute, blood or urine values, and other variables where we need to know and define what is normal, so that we can distinguish the abnormal or diseased, especially when it is not clinically obvious.

Reference intervals (RIs) are a highly important tool in the decision-making process for any clinician as they define where disease and health start or stop. They are the basis of what comes next for the patient, which further diagnostic tests to perform, a treatment plan or no action at all. With regards to reference intervals or "normal ranges", their generation depends on definitions and agreements, which lead to a need for the standardisation and unification of these ranges and methods (40). Gräsbeck and Saris (1969) were probably some of the first authors to publish standardised suggestions for generating normal ranges in human medicine (41). Later these methods were adapted and described for veterinary medicine, with dogs, cattle and horses being the first species to contribute (42, 43). The International Federation of Clinical Chemistry (IFCC) published guidelines on how to produce standardised reference values in six parts from 1986 – 1988 (44-49). The described principles are still applicable today but were simplified by the Clinical and Laboratory Standards Institute (CLSI) in 2008 in their approved guideline (50). This document also contains the definitions of reference intervals and related terms as well as their interaction and relation to each other as can be seen in an adapted version in Figure 1 below (44, 51).

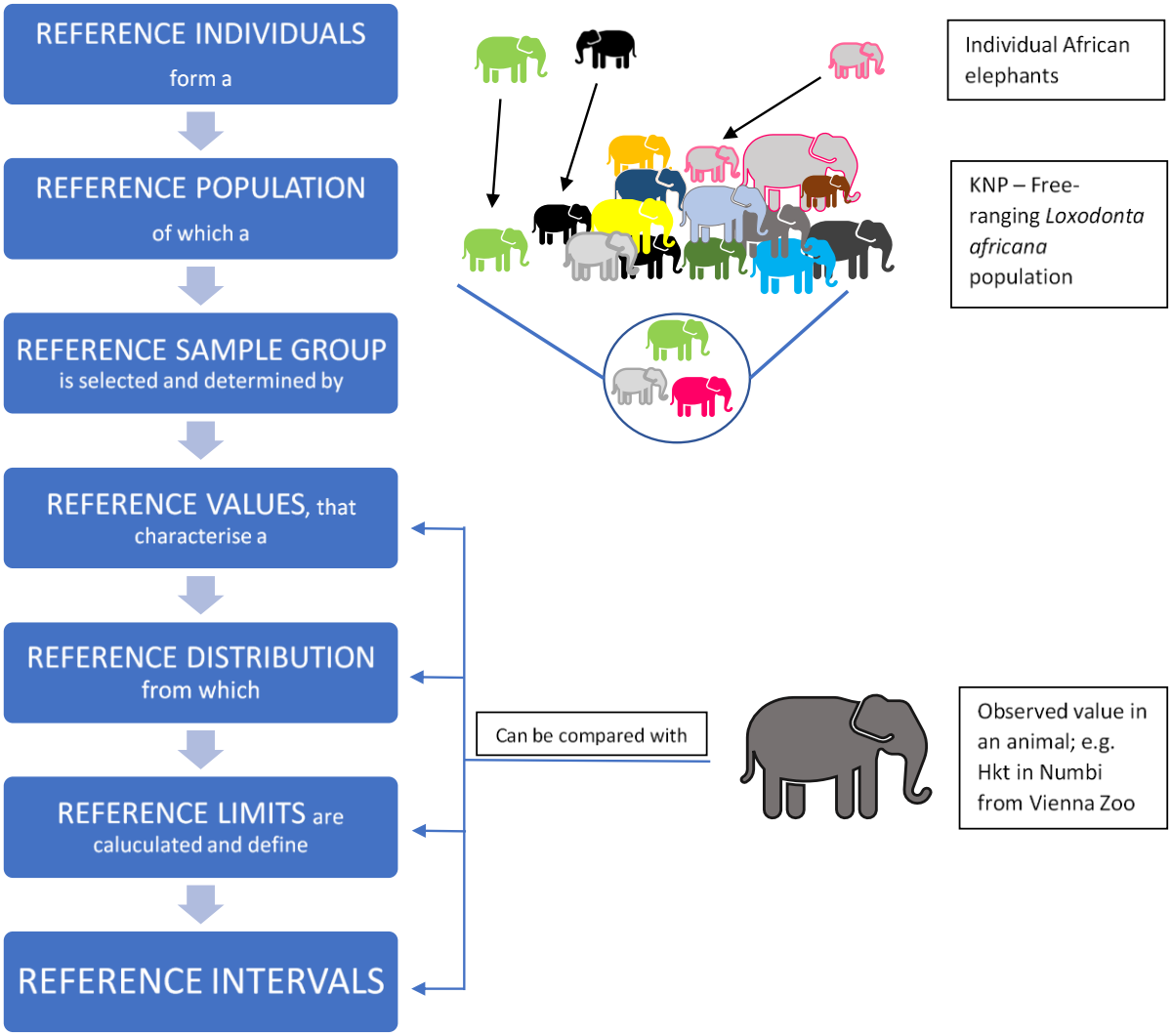


Figure 1: IFCC/CLSI recommended terms related to reference values and the relationship between them; derived from (44) and (51). KNP; Kruger National Park, Hkt; haematocrit

The guidelines have been extensively reviewed and adapted for use in veterinary medicine by the American Society for Veterinary Clinical Pathology (ASVCP) (51, 52). Before starting the analysis for a reference interval study, methods - with regards to sample collection, handling, transportation and storage - need to be standardized and unified (49, 52). Some preanalytical factors that should be considered for RI studies in veterinary medicine include the preparation and handling of the animal, for example:

- Was it physically or chemically restrained?

- If anaesthetized, with which protocol, and does this have an influence on the analytes?
- Method of the sample collection (needle, vacutainer system)?
- Venepuncture site (jugular, saphenal, coccygeal)?
- Use of an anticoagulant, transportation and storage of the sample, storage duration and temperature?

These are just some of the questions which need to be answered beforehand. Analytical methods should be standardised, meaning that strict quality control is in place and the same analysers are used for the selected measurands, leaving no room for variations other than within the individuals themselves (52, 53). The necessary procedures and steps are summarized in Table 1 (52):

Table 1: Procedural steps on how to generate *de novo* reference intervals in veterinary medicine

	Procedural step	Example/comment
1)	Literature research about the measurands.	Which measurands will be used, provide appropriate information, etc.
2)	Reference population needs to be selected, with inclusion and exclusion criteria.	Free-ranging elephants from KNP, juveniles are excluded, haemolytic and lipaemic samples are excluded.
3)	Questionnaire for caretakers/owners where applicable, to see if the animal fits the study or needs to be in a subgroup.	Health status, specific age or sex group.
4)	Number of samples/individuals available (availability of 90% confidence interval calculations).	If possible, select 10-20% more than the desired number, to leave room for analytical re-runs, etc.
5)	Reference individual selection.	
6)	Standardized sampling and handling.	Same tubes, same storage process, etc.
7)	Analysis of the samples.	Implemented quality control, standardized analysers.
8)	Histogram.	Visual inspection of data distribution, outliers can easily be identified.
9)	Outlier identification, usually repetition is necessary.	Method needs to be included into publication.
10)	Data distribution definition – Gaussian or non-Gaussian.	Depending on distribution, parametric, robust or non-parametric method will be applied.
11)	Calculation of upper and lower reference limits, and confidence intervals.	
12)	Partitioning if needed and applicable.	Only possible if sample size is big enough, e.g. males vs females.
13)	Documentation of all previous steps.	Raw and retired data should also be kept.

The first step for RI studies is to decide on the RI population, the limits thereof, inclusion and exclusion criteria of the population (for example, age or sex) and of the samples

(e.g. haemolysis) in order to create a “clean” sample pool (52). One of the most important statistical considerations when creating RI is outlier identification; the most common tests used are the Dixon Reed and the Tukey test. The former calculates the ratio of the difference between the outermost to the next value compared to the range; if the result is more than one third, this measurement is marked as an outlier (54). The latter divides all measurements in quartiles and uses 25 and 75 percentile fences to identify values above and below these fences as outliers (54). Both are automatically performed when using the statistical freeware add-on for Excel, RefVal Advisor (55), for calculating RI. Outliers can also often easily be detected when inspecting the histogram. Once identified, a decision needs to be made on how to proceed with them. The general approach is to retain them if possible, in particular if the sample numbers are limited. In domestic animals one can usually review details about the animal and its medical history which is generally not possible for wild animal populations. Certain species and non-domesticated animals tend to show signs of illnesses or injuries much later in the disease progress. The assumption is that in any population a certain percentage of animals has an underlying condition, such as parasite infestation, infectious disease or another abnormality which is masked on visual inspection, especially as many species need to be captured and either physically or chemically restrained in order to obtain the samples in the first place (56). In addition, the nutritional status is not always known, nor is their environmental exposure, for example to temperature and climate, predominantly in migratory species (56). Clinical abnormalities which would be identified in domestic species, will be missed much of the time in wild animals. As a result of the difficulty in determining health in wild animals, all data from an individual sample containing an outlier should be eliminated completely from the reference sample set, not just the outlying measurand (56).

Most of the published studies concerning African, but also Asian elephant blood measurands, took place before these guidelines existed. Particularly in earlier publications, the analytical methods vary, or they are not properly described or not given at all. In the more recent papers, the technical and analytical methods (see Table 2) are usually explained but they rarely fulfil the criteria for recommended sample sizes, which are ideally 120 (or above) but at least 40 for the generation of RI (52).

Table 2: Overview of reference value haematology and clinical chemistry studies on *Loxodonta africana*, including methods, animals and geographical location.

No of animals	Sample material	Status of animal	Blood collection site	Analytical methods	Geographical location	Reference	Year
23	Blood in heparin	Free-ranging, shot	Jugular vein free flow	Giemsa stain 100-cell differential count; WBC, improved Neubauer chamber	Mkomasi Game Reserve, Tanzania	Debbie and Claussen (57)	1975
37-141	Serum and plasma	Free-ranging, shot	Auricular vein	Improved Neubauer chamber, Standard Technicon AAII	Rwenzori NP, Kabalega Falls NP, Uganda	White and Brown (58-62)	1976-1980
47	Plasma	Various groups, culled and sedated	Not given	Biuret method (TP)	KNP, SA	Hattingh (63)	1984
23-31	Serum	Captive, immobilised	Auricular or medial saphenous vein	Technicon SMA Autoanalyser	Not given, maybe US?	Allen (64)	1985
92	Serum	Free-ranging, culled	Jugular vein	Automated analyser, Electro Nucleonics, Biuret and modified Bromocresol method	Hwange NP, Zimbabwe	Hill (65)	1990
12-15	Serum, Plasma	Free-ranging, immobilised	Auricular vein	Automated analyser, Electro Nucloenics	Sengwa Wildlife Research area, Zimbabwe	Kock (66)	1993

A particular feature of the older studies concerning haematology and clinical chemistry of the African elephant is that sampling was opportunistic, meaning that if elephants were culled for management purposes, samples were obtained once the elephants were killed (57, 67). The health status of those animals is not always mentioned and for haematological analyses, mostly manual methods were used.

HAEMATOLOGY AND MORPHOLOGY OF CELLS

Elephants have the largest erythrocytes of all terrestrial mammals. The average red cell volume is 125 fL and the mean diameter is 9.3 µm (+/- 0.7) (68, 69). In comparison to this, the human erythrocyte (the second largest) diameter is 7.5-8.7 µm. Other domestic species range between 5-6 µm for cattle and horses and around 7 µm

diameter for dogs, which have a 60-77 fL mean corpuscular volume. Manatees (105-140 fL, Florida manatee, *Tricheus manatus*) (70) which are close relatives to the African elephant, and some pinniped species have comparable red cell volumes (104-129 fL, grey seal, *Halichoerus grypus*), whereas the erythrocytes of hyraxes (another relative of the elephant) are much smaller (69 fL, rock hyrax, *Procavia capensis*) (71). Rouleaux formation is commonly present in elephant blood smears, similar to horses. Red blood cell count is lower than in domestic species but varies amongst studies. Published haematology data are presented in Table 3.

Another species-specific detail is that elephants, like manatees, have heterophils instead of neutrophils, according to the staining attributes of these cells with the Wright-Giemsa stain. Elephant heterophils are between 12 and 16 µm in diameter (69). Granules and vacuoles are present within the heterophils, the latter increase with toxic changes. Some authors state that band heterophils are not present in clinically healthy elephants, and that basophils occur in very low numbers and not in all animals (64, 69). Monocytes are present in three different forms (69). The most common form is a classic monocyte with a kidney- to horseshoe-shaped nucleus, comparable to the same cell in other species. The second form is a bilobed monocyte, with a nucleus with two lobes present, either connected by a thin isthmus, or “free floating”. Sometimes the nucleus of this cell type contains three lobes. The third monocyte fraction consists of cells that are round with almost round nuclei; they are smaller in size and their chromatin is denser. In the literature, monocytes have been previously confused with lymphocytes and were counted differently by some authors (69). Lymphocytes are mostly round but do vary in size with a round to oval nucleus.

Table 3: Haematology reference values from different publications for African and Asian elephants. Means are given with (ranges) or (+/- SD). Results have been converted to SI units where necessary.

Reference	Number of animals	RBC (10^{12}/L)	PCV (%)	Hb (g/L)	MCV (fL)	MCH (pg)	MCHC (g/L)	WBC (10^6/L)	Lymphocytes	Heterophils	Eosinophils	Basophils	Monocytes
African Elephants													
Bartels, 1963 (72)	1	3.2		124									
Young & Lombard, 1967 (73)	11	5.0 (4.2-6.6)	48 (44-51)	145 (115-180)				10.4 (6.8-14.3)	58% (44-69)	38% (27-54)	2% (0-6)	0%	2% (0-4)
Debbie & Clausen, 1975 (57)	8-23	3.77 1.92-6.74)		71				11 (5.7-21.0)	69.1% (56-85)	20% (7-33)	1.33% (0-4.0)	0.11% (0-1.0)	8.17% (2.0-17.0)
White & Brown, 1978 (61)	99-173	3.9 (1.4-6.0)	49 (18-80)	162 (56-259)				10.4 (1.75-38.0)	76% (46-39)	21% (7-46)	2% (0-6)	0% (0-2)	0% (0-5)
Woodford 1979 (74)	5	2.85 (2.32-3.14)	34 (29.8-36.1)	113 (104-120)	120.0	39.6	33.1	6.81					

Reference	Number of animals	RBC (10^{12}/L)	PCV (%)	Hb (g/L)	MCV (fL)	MCH (pg)	MCHC (g/L)	WBC (10^6/L)	Lymphocytes	Heterophils	Eosinophils	Basophils	Monocytes
Allen et al., 1985 (64)	31	3.0 (2.1-4.2)		118 (91-169)				13.6 (7.0-22.0)	6 x10^6/L (3-9)	3 x10^6/L (0-5)	0 x10^6/L (0-1)	0 x10^6/L (0-1)	3 x10^6/L (0-7)
Species 360	244-7410	2.87 (2.06-3.91)	35.8 (25.5-47.5)	126 (98-160)	125.7 (101.3-144.0)	43.9 (36.2-51.7)	351 (301-403)	9.6 (5.3-15.7)	39.8% (9.0-74.0)	25.4% (10.1-48.0)	1.4% (0.0-4.0)	0.4% (0.0-3.0)	23.0% (0.6-58.0)
Asian Elephants													
Lewis, 1974 (75)	6	2.88 (2.13-3.85)	36 (33-40)	122 (97-164)	127 (129-136)	42.5 (40.0-45.5)	32.3 (27.7-34.4)	12.7 (11.1-16.1)	73% (71-74)	10% (4-14)	13% (8-16)	Not reported	3% (0-11)
Gromadzka-Ostrowska, 1986 (76)	10	2.66 (+/- 0.32)	39.2 (+/- 2.36)		131.3 (+/- 9.05)	33.6 (+/- 1.86)	25.2 (+/- 1.38)	5.43 (+/- 0.48)	67% (+/- 1.59)	25.8% (+/- 2.17)	4.28% (+/- 0.73)	1.14% (+/- 0.36)	1.78% (+/- 0.29)
Niemueller, 1989 (77)	108 (4 animals only)	3.13 (+/- 0.16)	36 (+/- 1)	136.30 (+/- 4.93)	117.20 (+/- 3.67)	43.50 (+/- 2.17)	370.62 (+/- 6.67)	15.96 (+/- 2.20)					
Silva, 1993 (78)(domesticated)	43-108	3.6 (1.7-5.0)	36.0 (25.0-45.0)	117 (74-154)	105 (81-160)	34.0 (24-56)	325 (230-390)	11.5 (4.0-21.0)	38% (10-70)	27% (10-50)	5% (0-20)	0.31% (0-3)	29% (1-60)

Reference	Number of animals	RBC (10^{12}/L)	PCV (%)	Hb (g/L)	MCV (fL)	MCH (pg)	MCHC (g/L)	WBC (10^{6}/L)	Lymphocytes	Heterophils	Eosinophils	Basophils	Monocytes
Silva 1993 (79) free-ranging	37	3.2 (2.3-5.4)	38.0 (31.0-44.0)	127 (98-158)	118 (81-158)	41 (30-50)	338 (280-400)	18.0 (8.0-26.0)	44% (15-68)	23% (3-46)	5% (0-11)	0.5% (0-6)	27% (4-60)
Salakij, 2005 (80)	14	2.66 (+/- 0.08)	32.2 (+/- 0.7)	115 (+/- 3)	121.8 (+/- 1.6)	43.4 (+/- 0.5)	356 (+/-4)	15.0 (+/- 0.89)	27.3% (+/-3)	28.8% (+/- 2.0)	4.9% (+/- 1.0)	0.1% (+/- 0.05)	37.3% (+/- 2.5)
Janyamethakul, 2017 (81)	41 (male)	1.9-3.2	29.4-40.7	98-152	104-132.8	Not given	299-38.9	7.92-21.89	1.67-11.18 x10^{9}/L	0.96-13.42 x10^{9}/L	0-0.86 x10^{9}/L	0-0.14 x10^{9}/L	0-2.39 x10^{9}/L
	108 (female)	1.9-3.1	27.8-43	101-156	105.7-127.3	Not given	32.1-38.7	7.2-23.2	1.05-12.03 x10^{9}/L	0.82-13.51 x10^{9}/L	0-1.17 x10^{9}/L	0-0.036 x10^{9}/L	0-3.29 x10^{9}/L
DosSantos, 2020 (82)	603-765	Not given	34 (29-42)	117 (95-143)				15.8 (9.5-24.6)	30% (16-46)	27% (14-44)	4% (0-11)	Not given	38% (21-55)

PCV, packed cell volume; RBC, red blood cell concentration; HGB, haemoglobin concentration; MCV, mean cell volume; MCH, mean cell haemoglobin; MCHC, mean cell haemoglobin concentration; PLT, platelet concentration; WBC, white blood cell concentration;

CLINICAL CHEMISTRY

Clinical chemistry has been commonly used as an important diagnostic tool in human and veterinary medicine; nowadays, it is more often used in the health screening process. Geriatric screenings or preanesthetic profiles are well established in modern veterinary practices, especially for pets such as cats and dogs. In livestock medicine, subclinical conditions can be detected more easily (than in non-domestic species) and the use of metabolic or nutritional profiles can increase production and improve herd health. In many modern zoos, regular, or at least annual, screening of animals is common practice. This is important as non-domestic animals often show clinical signs much later in the process of a disease, compared to domestic animals. Panels and measurements often vary, depending on the animal species and the zoological institution. Many zoo animals, elephants in particular, are trained to allow venepuncture on a regular basis, reducing the need for general anaesthesia and allowing closer monitoring of metabolic or hormonal changes, thus contributing to animal welfare (83).

Clinical chemistry measurands that are useful in elephants are more comparable with those used for horses and ruminants rather than for small animals (84). An overview on clinical chemistry measurand reference values, from published studies on the African elephant, can be found in Table 4. Some changes occur with musth, where elevations could be seen in adult bulls for gamma-glutamyl transferase (GGT), alkaline phosphatase (ALP), and creatinine during those periods (77, 84). Alkaline phosphatase is also higher in young elephants, as observed in other species, due to increased bone metabolism (59). Some seasonal changes have also been reported in free-ranging African elephants, with phosphorus, albumin, potassium and urea lower during the dry season, and sodium, creatinine and globulin higher (59, 62). These changes may be linked to different feeding preferences of free-ranging elephants which adapt to their environment and geographical as well as seasonal changes. For example, in the KNP, elephants in the northern part of the park feed on significantly more grass during the dry season, compared to the animals in the southern part (85). During the wet season, grass is about 50% of all elephants' diets and contains more protein than during the dry season (85, 86). For additional supplementation, geophagy has been observed in elephants in the Mount Elgon region in Kenya, where the animals lick cave salts, containing minerals and trace elements like calcium, phosphorus,

magnesium, selenium, and iodine (87). In captivity, where the animals are dependent on the diet provided, it seems particularly important to include mineral measurands into routine screenings, to investigate whether nutritional needs are met and to have a baseline for each individual. Some health conditions have been reported in captive elephants in connection with suboptimal nutrition, for example, metabolic bone disease in elephant calves (88). The likelihood of prolonged parturition and dystocia can also be a result of underlying subclinical hypocalcaemia (89). With regards to this condition, the importance of reference values (especially for calcium) has been emphasized (90).

Measurements of enzymes are based on their ability to catalyse a reaction – most assays make use of this principle and measure how fast a substrate is used or a new compound forms. The majority of assays function spectrophotometrically, with the initial reaction coupled to a colour indicator, which can appear or disappear and then is measured (91).

The Abaxis Vet Scan V2 is a fully automated clinical chemistry analyser, which analyses fixed chemistry panels compiled using liquid reagents within cuvettes combined in a plastic rotor, made for use in veterinary medicine (92). Whole blood, plasma or serum can be used, as the machine is fully capable of separating blood. A minimum volume of 100 µl is needed for the full analysis. Twelve different profile rotors, containing different combinations of measurands, are available. Automated quality control is performed whenever the analyser is switched on, before analysis. The Large Animal Panel was designed to suit ruminant herd health needs and consists of the following measurands: albumin, ALP, aspartate aminotransferase (AST), calcium, creatine kinase (CK), GGT, globulin, magnesium, phosphorus, total protein (TP) and urea.

This rotor is applicable and useful for the African elephant as it is comparable to a suggested elephant panel which includes all the above except magnesium (84). Additional measurands that have been suggested for use in elephants are: creatinine, bile acids, total bilirubin, lactate dehydrogenase (LDH), Na, Cl and fibrinogen (84). The RIs given for this panel by the manufacturer are for bovids. No RIs are available for the African elephant.

Table 4: Clinical chemistry measurands reference values for the African elephant. Means are given with (ranges) or (+/- SD). Results have been converted to international units where necessary and into units/liter (U/L) for easier comparison

Reference	Number of animals	Albumin (g/L)	ALP (U/L)	AST (U/L)	Calcium (mmol/L)	CK (U/L)	GGT (U/L)	Globulin (g/L)	Magnesium (mmol/L)	Phosphorus (mmol/L)	Total Protein (g/L)	Urea (mmol/L)
White & Brown, 1976-80 (58, 59)	37-141	37.9 (+/- 5.5)		19.7 (3-117)	2.81 (2.35-3.28)	152.9 (0-324)			1.81 (1.07-2.55)	2.78 (1.26 - 4.30)	87.2 (86-107)	4.24 (+/- 1.07)
Hattingh, 1984 (63)	47	39-44									99-117	
Allen, 1985 (64)	23-31	31 (23-37)	210 (71-478)	28.6 (15-54)	2.6 (2.28-30)			39 (27-62)		1.84 (1.42-2.39)	70 (57-83)	2.82 (1.79-4.64) [BUN]
Hill & Dale, 1990 (65)	92	39.6 (+/- 6.69			2.66 (1.30-3.37)				1.25 (0.76-1.43)	1.59 (0.85-2.99)	81.0 (47-105)	2.58 (0.8-4.8)
Kock, 1993 (66)	15	42.5 (+/- 1.4)	147.7 (+/- 16.2)	26.2 (+/-1.7)	2.5 (+/- 0.04)	285.6 (+/-45	8.6 (+/- 0.77)	44.3 (+/- 1.4)	1.24 (+/-0.03)	1.18 (+/-0.04)	86.9 (+/- 1.4)	1.34 (+/- 0.13) [BUN]
Kock, 1993 (66)	12	37.0 (+/- 0.85	108.7 (+/- 13.2)	16.8 (+/- 0.73)	2.2 (+/- 0.02)	269.5 (+/- 29)	8.25 (+/-0.5)	42.5 (+/- 1.3)	1.11 (+/-0.04)	1.45 (+/- 0.06)	79.6 (+/- 1.3)	4.3 (+/- 0.37) [BUN]
Species 360 (93)	Given above, 20-242	n=20, 30 (23-35)	n= 234, 91 (33-221)	n=236, 20 (9-40)	n= 242, 2.7 (2.4-3.0)	n= 215, 226 (90-502)	n=211, 10 (1-18)	n=36, 43 (27-62)	n= 162, 0.97 (0.36-1.32)	n= 242, 1.59 (1.03-2.29)	n=240, 77 (65-89)	n= 234, 3.1 (0.7-5.7)

ALP, alkaline phosphatase; AST, aspartate aminotransferase; BUN, blood urea nitrogen; CK, creatine kinase; GGT, gamma-glutamyltransferase;

ACUTE PHASE RESPONSE

The acute phase response occurs in the body immediately after tissue has been damaged (94). It is a non-specific reaction in an animal or human in response to trauma or infection with the purpose of removing the cause of tissue damage and to restore homeostasis (94, 95). Fever, leukocytosis and elevated cortisol levels are observed along with decreased thyroxine concentrations and decreased serum iron and zinc levels (96). Certain plasma proteins also belong to the acute phase response – called acute phase proteins (APP). These can be divided into positive and negative-acting proteins, depending on their increase or decrease in reaction to injury (94). Most of them are synthesized mainly in the liver. C-reactive protein (CRP), serum amyloid A (SAA), haptoglobin (HP), alpha-1-glycoprotein (AGP), ceruloplasmin (CP) and fibrinogen are positive APPs, whereas albumin and transferrin are negative APPs (97-99). In human medicine, APPs have been used as markers of inflammation for decades and were first described in pneumonia patients in 1930, with CRP being the first recognized APP. It was initially named complement reactive substance, based on its ability to bind c-polysaccharides of bacteria (100). Roughly ten years later, the c-reactive substance was identified as a protein in ill humans and monkeys and the term acute-phase was introduced (101). C-reactive protein was identified as a part of the non-specific immune reaction to inflammatory conditions – originating from infectious and non-infectious stimuli and also malignant neoplasia (102). Currently, acute phase reactants (APRs) serve as prognostic indicators for various conditions and diseases, such as impaired lung function, inflammatory bowel disease, cancer, and cardiovascular disease (103-106). The latter is one of the most common causes of death in the Western world, and contributing factors, such as lack of physical activity and a high body mass index can be linked to elevated CRP serum levels (107). In contrast, a Mediterranean-style diet has been found to help decrease CRP levels (108).

In veterinary medicine, APRs are relatively new diagnostic tools. Some APPs are more likely to change in an acute event, and others are more likely to increase with chronic tissue damage (109). They can also be classified into major, moderate and minor APPs, depending on the degree of increases during the acute phase response (94). A major APP is characterized by a 10-100-fold increase, moderate by 2-10-fold increase,

and minor less than that (94). An APP can be categorized as major in one species, whereas in another species it might only act as a minor APP and vice versa. For example, in nonhuman primates as well as in dogs, CRP is a major APP, whereas CP and HP act as moderate APPs in dogs and fibrinogen and SAA as moderate APPs in monkeys (98). In cattle, goats and sheep, SAA and HP are major APPs and AGP, CRP and fibrinogen are minor APPs (98, 110). In cats and horses, SAA is the major APP (111, 112). More than 200 other proteins are known to react during the acute phase response, with most of them elevating, except for albumin which acts as negative APP in almost all animals (97, 98).

Iron also acts as a negative APR as it decreases with inflammatory processes (97). It is an essential nutrient and plays an important role in many metabolic processes, oxygen transport, DNA synthesis and enzymatic reactions within the citric cycle (113). It is absorbed via the small intestine from the diet, into the plasma, where it is transported bound to transferrin. About 75% will reach the bone marrow for incorporation into haemoglobin and remaining iron will be stored in the liver as well as macrophages in the form of haemosiderin or ferritin. Macrophages will also take up iron present in haptoglobin-haemoglobin complexes, resulting from intravascular haemolysis. A shift from serum iron to storage iron happens during inflammatory processes. Hypoferraemia is the body's iron-withholding defence mechanism – as iron is a requirement for microbes as well as neoplastic cells for reproduction and growth (113). Serum iron is bound to transferrin if the sample is not haemolysed. For the analysis, Fe^{3+} needs to be separated from transferrin and reduced to Fe^{2+} via ascorbic acid, and then forms a complex with a dye which is detected photometrically (91).

Serum Amyloid A is a small hydrophobic protein, synthesized mainly, but not exclusively, in the liver, and can be found in serum of humans and animals. It plays a role in immune responses as well as in platelet activation and transportation of cholesterol from the tissue into the hepatocytes (114). SAA is measured with an immunoturbidometric method; the assay has been found to cross-react with SAA from multiple wildlife species samples such as cheetah, lion, red deer, some macaque species, zebra and the Asian elephant (115).

Haptoglobin is a protein, synthesized in the liver, which binds and removes any free haemoglobin (97). Therefore, if free haemoglobin is increased in serum, HP levels

have been shown to decrease (114). It also acts as a bacteriostat by building haptoglobin-haemoglobin complexes and restricting iron availability for bacteria (114). Measurement is based on the preservation of peroxidase activity of haemoglobin which is directly proportional to the haptoglobin present in serum.

Diseases in which acute phase reactions have been identified are comparable to those of humans. In dogs, CRP levels elevate with pneumonia, pyometra, polyarthritis, pancreatitis, inflammatory bowel disease, haemoparasitic diseases such as babesiosis, and lymphoma (94). Similarities have been seen in feline medicine, even though the acute phase response has not been researched as extensively as in dogs. Changes in APRs have been reported in connection with chronic kidney disease, infectious diseases such as feline infectious peritonitis and cancerous illnesses (116, 117). The role of SAA has been investigated as an inflammatory marker in cats as high levels were found after injury as well as urinary tract inflammation (118). In cattle, SAA, HP and fibrinogen are the most studied APRs. Significant elevations were found associated with enteritis, endometritis and endocarditis, to name just a few diseases (110, 114). Fibrinogen was found to be a useful indicator for the presence of bacteria in mastitis cases, and SAA and HP levels increase with moderate and even mild mastitis cases (119). SAA is also synthesized extrahepatically within the gland cells of the mammae – so called mammary serum amyloid A (M-SAA) which can be measured from the milk and be used to diagnose mastitis-affected quarters (120). Furthermore, the diagnostic usefulness of APRs in claw diseases has been studied (121), and some authors suggest that their validity can be expanded to measure herd health and stress after transportation in calves as well as the infectious pressure the animals will likely undergo (114, 122, 123).

In non-domestic species, APRs and their possible value have not been studied as extensively as in ruminants or other domestic species, as diagnostic needs differ and access to samples is not as easy. In addition, detection methods and their analytical and diagnostic performance for most species still need to be evaluated. Acute phase proteins have been detected and described in some non-domestic ruminant species, cheetahs, sharks and turtles (124-127). As in domestic equines, SAA was identified as the major APP in zebra (128). A HP assay was used in loggerhead sea turtles undergoing rehabilitation, and showed potential for prognostic use (129). Acute phase reactants have also been studied in other animals undergoing rescue and

rehabilitation, like harbor seals or other pinnipeds, where it is especially important to monitor progress and health status to determine a release prognosis (130). For harbor seals, ringed seals and Steller sea lions, HP has been suggested as an additional diagnostic tool for the evaluation of stress (capture) and inflammatory processes (131-133). In manatees, SAA has been reported to be the most sensitive and specific marker for inflammation, whereas HP is useful for prognosis of the rehabilitation process, for example after boat strike injuries (134, 135). In white rhinoceros, APPs have been assessed and RIs have been published (136). Increased HP and decreased iron were found to be the best combination to identify inflammatory processes in this species (136). Table 5 contains RIs for APRs in selected non-domestic mammals.

Serum Amyloid A is a major APP in Asian elephants, with a significant increase measured in animals undergoing viraemia due to EEHV (137). One recent study suggests that APPs react with different intensity to various EEHV strains (138). Reference intervals have been established for captive Asian elephants for SAA, HP, CRP, albumin and globulin, and APRs showed significant changes in animals with some pathological conditions such as active pododermatitis (139). Acute phase reactants are currently also being assessed in connection with Mtb infections in Asian and African elephants (138). Reference intervals were generated for captive Asian elephants in one study and data for captive African elephants are available from the Species 360 data collection (139).

Table 5: Reference intervals for acute phase reactants (APRs) in selected non-domestic mammals

APR	White rhinoceros (136) (n = 23-48)	Manatee (140) (n =71)	Asian elephant (139) (n=35)	African elephant (93) (n = 68-407)
SAA (mg/L)	<20 (n = 23)	< 10-50	0-47.5	0.0-18.6
HP (g/L)	1.0-4.3 (n = 47)	0.4-2.5	0-1.10	0.08-2.02
Iron (µmol/L)	9.7-35 (n = 48)			3.1-25
Albumin (g/L)	18-31 (n =48)		29.2-46.9	23-35

APR, Acute phase reactants; SAA, Serum Amyloid A; HP, Haptoglobin; n, number of individuals

CHAPTER 2: RESEARCH OBJECTIVES

RESEARCH AIM

The aim of the research project is to construct reference intervals for haematology and clinical chemistry measurands, as well as assess acute phase reactants, for the African elephant (*Loxodonta africana*) using samples from free-ranging animals from the Kruger National Park.

OBJECTIVES

Objective 1: To generate reference intervals for the following:

Haematology: RBC, HCT/ PCV, Hb, MCV, MCHC, PLT, WBC, heterophils, lymphocytes, monocytes, eosinophils, basophils

Clinical chemistry: Alb, ALP, AST, Calcium, CK, Glob, GGT, Magnesium, Phosphorus, TP, Urea (BUN)

Acute phase reactants: SAA, HP, serum iron

Objective 2: To investigate whether there are differences between the generated RIs in this study, and those already published.

Objective 3: To investigate the changes in clinical chemistry measurands and APR in injured elephants.

CHAPTER 3: MATERIAL AND METHODS

ANIMALS AND SAMPLES

The samples for this study originate from the free-ranging African elephant population from the KNP, South Africa. The animals were immobilised for park management purposes or other unrelated studies. Immobilisation was performed by Veterinary Wildlife Service (VWS) veterinarians according to SANParks Standard Operating Procedures (SOP). Elephants were darted from a helicopter using an air-pressurised dart (3 mL Dan Inject plastic dart; DAN-INJECT, International S.A., Skukuza 1350, South Africa) propelled by a carbon dioxide powered rifle (DAN-INJECT JM-special, Skukuza 1350, South Africa). Darts were loaded with etorphine (Novartis, Kempton Park 1619, South Africa), azaperone (Janssen Pharmaceutical Ltd., Halfway House 1685, South Africa), and hyaluronidase (Kyron Laboratories, Benrose 2011, South Africa), with dose ranges based on subjective weight and age estimation by the same veterinarian. Once the elephant was recumbent, the ground crew approached and assisted the elephant into lateral recumbency, if required. At the end of the procedure naltrexone (Kyron Laboratories, Benrose 2011, South Africa) was administered intravenously at 20 times the etorphine dose (mg), and the animal observed until it had fully recovered. Sample collection proceeded according to a standardised protocol as follows: blood was taken via an 18G needle and direct vacutainer collection from an auricular vein at first handling. Whole blood was collected in sealed EDTA and serum vacutainers (BD Biosciences, Franklin Lakes, New Jersey, USA). Serum samples were left to clot for at least 30 minutes standing upright in a cooler box. Samples were transported cooled, until they were processed in the laboratory within six hours of collection. EDTA whole blood was analysed with the scil Vet abc (scil Animal Care Company, Ontario, Canada) for the first period and later with the Horiba ABX Micro ESV60 (Horiba ABX SAS, Kyoto, Japan) haematology analyser, its replacement. Blood smears were made using a standard pushing technique and stained with a commercial eosin-methylene blue stain (Kyro-Quick stain, Kyron Laboratories) for manual differential counts. Microhaematocrit tubes for determination of packed cell volume (PCV) were prepared using a microhaematocrit centrifuge (Model HKT-400, Gemmy Industrial Corporation, Taipei, Taiwan; 15,000*g*, 5 min). Serum tubes were centrifuged

at 1300 *g* for 10 minutes, and serum aliquoted into cryotubes (Greiner Bio-One, Lasec S.A., PTY LTD Cape Town, 7405, South Africa) and frozen at -80°C.

At the time of immobilization, a physical examination was performed. Animals without injuries and free of clinical abnormalities were considered healthy. All data for the animal, including the sex, general condition, age and weight estimation, microchip number and geographical location of the immobilization site were recorded in Excel spreadsheets. Notes were added for abnormal clinical findings or injuries, if present. Sample selection was made according to this information, with only the clinically healthy animals included for the RI generation sample pool. The injured animals were kept and recorded on a separate list. All selected samples were collected between October 2014 and August 2019, meaning they were stored no longer than five years. This threshold was chosen as no studies could be found on stability beyond this time (141). Samples and data from a total of 79 apparently clinically healthy animals were selected for the reference interval study.

HAEMATOLOGY

Data from the original haematology analysis were reviewed. Analysis was performed using EDTA whole blood, with a scil Vet abc (scil Animal Care Company, Ontario, Canada) (first 25 results) and a Horiba ABX Micro ESV60 (Horiba ABX SAS, Kyoto, Japan) (last 11 results), using the horse setting on both analysers. These analysers and settings have not been validated for elephant blood. Internal quality control using manufacturer-supplied quality control material was performed every day before analysis. Firstly, the automated calculated haematocrit (HCT) was compared to a manual PCV performed at the same time. Only automated results with a HCT within 3% of the PCV were included in this study. The white blood cell count (WBC), red blood cell count (RBC) and platelet count (PLT) as measured by impedance (both analysers), and the haemoglobin concentration (HGB), as measured by a cyanide-free photometric method (both analysers) (142) were considered accurate enough to be used for our study. The erythrocyte indices mean cell volume (MCV), mean cell haemoglobin (MCH) and mean cell haemoglobin concentration (MCHC) were calculated using the following standard equations (91):

$$MCV = \frac{[PCV \times 10]}{RBC}; MCH = \frac{Hb}{RBC}; MCHC = \frac{[Hb \times 100]}{PCV}$$

(Units: Hb g/L RBC $x10^{12}$/L, PCV %; MCV fL, MCH pg, MCHC g/L)

A 200-cell leukocyte differential count was performed on the available corresponding blood smears. Two smears could not be evaluated due to poor quality. Morphology of the erythron, leukon and thrombon were recorded. The amount of rouleaux formation was graded from 0-4, where 0 was no rouleaux, 1 was the extension of rouleaux to 25% of the smear from the droplet end, 2 was extension to 50%, 3 was extension to 75% and 4 was extension of rouleaux all the way to the feather edge. The number of heterophils with toxic changes was recorded (few, moderate, severe) as well as the severity thereof (1+ to 4+) according to a standardised grading system described for domestic species (143). Morphology of other leukocytes was recorded based on the descriptions for elephants found in Schalm's Veterinary Hematology (144) and the morphological changes reported by Stacy et al. (2017) (145). Monocytes were subclassified as normal monocytes, bilobed monocytes, and round monocytes, and each type was recorded separately. Monocytes were counted as bilobed when either two clearly separated nuclear lobes were visible or the isthmus connecting them was less than a third of the nucleus diameter. Characteristics used to define round monocytes were the almost complete roundness of their nuclei, a nucleus to cytoplasm ratio of 1:1, and coarser chromatin, compared to those of lymphocytes. Active lymphocytes (enlarged cells, increased amounts of cytoplasm, perinuclear clear zones) were also recorded if there were more than 5% on a slide.

CLINICAL CHEMISTRY

Analysis was performed using the Abaxis Vetscan VS2 (Abaxis, Union City, California, USA) according to the manufacturer's instructions. The Large Animal rotor (Abaxis, catalogue number 500-0023-12) was used and included the following measurands: albumin, ALP, AST, calcium, CK, GGT, globulin, magnesium, phosphorus, TP and urea. The analytical methods for these measurands are shown in Table 6. This profile was considered by veterinarians experienced in elephant medicine to be the most valuable for this species. Serum samples were thawed overnight at 4°C, then mixed using a vortex machine for 15-30 seconds, and thereafter pipetted into the sampling area of the rotor and analysed. An automatic electronic quality control check is performed on each rotor before analysis. In order to obtain an estimate of analytical precision specifically for elephant serum, remaining serum was pooled, and this pool

was measured twenty times (on twenty rotors) in one day. The imprecision, represented by the coefficient of variation (CV), was calculated from the mean and standard deviation (SD) (CV%=SD/mean x 100).

To examine the potential effect of storage time on clinical chemistry measurands, the number of days from storage to analysis was enumerated, and the correlation between days in storage and measurand concentration or activity calculated using Pearson's correlation coefficient, *r* ($p < 0.05$ considered significant).

Table 6: Assay methods utilised by the Abaxis Vetscan VS2 (146)

Measurand	**Method**
Albumin	Bromocresol green
Alkaline phosphatase	Kinetic (p-nitrophenol phosphate)
Aspartate aminotransferase	Kinetic (L-aspartate and α-ketoglutarate)
Calcium	Arsenazo III method
Creatine kinase	Kinetic (creatine phosphate and ADP)
Gamma glutamyltransferase	Kinetic (L-γ-glutamyl-3-carboxy-4-nitroanilide and glycylglycine)
Globulin	Calculated (total protein minus albumin)
Magnesium	Enzymatic (hexokinase)
Phosphorus	Enzymatic (glucose-6-phosphate dehydrogenase)
Total protein	Biuret method

Urea	Enzymatic (urease)

ACUTE PHASE REACTANTS

Measurements of iron, HP and SAA were performed using a wet chemistry analyser, the Cobas Integra 400 Plus (Roche Products (Pty) Ltd, Basel, Switzerland) as per manufacturer's instructions. Methods and standards were: HP, colorimetric peroxidase assay with a modified serum calibrator (species unknown) (PHASE Haptoglobin Assay, Tridelta, Maynooth, Ireland); iron, ferrozine zinc with a modified human serum calibrator (Roche Products (Pty) Ltd, Basel, Switzerland); SAA, immunoturbidometric assay (LZ-SAA Assay, Eiken Chemical Co., Japan) with a modified serum calibrator (containing polyclonal and monoclonal anti-human SAA antibodies). For all three methods, assay performance was monitored by daily internal quality control procedures according to laboratory protocols and performance goals published for veterinary species (147). Frozen serum samples were thawed to room temperature, gently mixed and measured over the course of one day, in a batch.

Partial analytical validation was carried out for HP and SAA. Intra-assay imprecision and linearity were determined using recommended protocols as previously described (148). For the intra-assay imprecision study two pools of sera were created: a low pool from the group of healthy elephants and one from the injured group with expected high concentrations. These were each measured 20 times over the course of one day. An inter-assay imprecision study was not performed as reagents were not available for longer than two days. The performance goals for maximum imprecision were set at 8.5% for HP and 20% for SAA (136). For the linearity experiments, an eight-step dilution was performed, and triplicate measurements were made. Linearity was evaluated using Spearman's correlation coefficient (r) and linear regression analysis.

STATISTICAL ANALYSES

Statistical analyses were performed with MedCalc software version 19.1.7 (MedCalc Software, Ostend, Belgium) and the Excel add-on Reference Value (RefVal) Advisor version 2.1 (55) according to published guidelines from the ASVCP. For RI

determination histograms were inspected visually, and Dixon and Tukey tests were used to identify outliers. If any measurand was identified as an outlier, the whole data set of this sample was excluded, and the statistical analysis rerun. This procedure was repeated until no more outliers remained, for any measurands. Normality was assessed using a Shapiro-Wilk test. Based on ASVCP guidelines for RI determination (52), as well as recommendations from recent RI modelling studies (149, 150), the following strategies were then followed:

For sample sizes of 40-80, a cut-off p-value of > 0.2 for the Shapiro-Wilk test, not > 0.05, was used to define a Gaussian distribution (149). If $p > 0.2$, then the parametric method was used to generate the 95% reference limits. If $p \leq 0.2$, the non-parametric method was used (52, 150). The 90% confidence intervals (CI) of the lower and upper reference limits were calculated using a bootstrap method (52). The ratio of the upper or lower CI to the RI was calculated by dividing the former by the latter (52).

Goodness-of-fit tests have been shown to be inaccurate for identifying a normal distribution at small sample sizes (149). The non-parametric method (i.e. minimum-maximum) was subsequently used for all sample sizes of 20-40 (150). The 90% CI of the reference limits (RL) could not be calculated using this method with a sample number of < 40.

INJURED ANIMALS

Samples from an additional 15 animals which were recorded as having injuries, were included for blood smear evaluation as well as clinical chemistry analyses and APR assays. Sample selection was based on the availability and age of the samples, ranging from October 2014 until August 2019. Two results were removed from the reference sample group and added to the injured group after sample analyses were performed, as additional information was received that indicated that these two animals were in fact injured. Anaesthetic protocol, sampling materials, technique, handling and storage as well as analytical methods used, were the same as described above. All information on the injured animals was recorded in a separate Excel spreadsheet. Information on the injury as well as additional comments on the prognosis (where available) was included. Due to the small sample size, no statistical analysis was performed. All results are presented in separate tables for haematology, clinical

chemistry and APRs and were compared to the generated RIs. Outliers were marked in orange if below the RI, and in blue if above the RI.

ETHICS APPROVAL AND PERMITS

Ethical approval for this study was obtained from the University of Pretoria Faculty of Veterinary Science Research Ethics Committee and Animal Ethics Committees (certificate number REC 132-19).

A Section 20 permit was obtained from the Department of Agriculture, Land Reform and Rural Development (DALRRD) which is needed to perform research on animals or animal specimens in South Africa.

A Biomaterial Transfer Agreement (BMTA) was issued by the Veterinary Wildlife Services, Kruger National Park and specimens were transported with the necessary permits to the Clinical Pathology Laboratory at the Faculty of Veterinary Science for further analyses. The Faculty of Veterinary Science has a standing permit (TOPS) to work with and store specimens from threatened and protected species.

CHAPTER 4: RESULTS

REFERENCE INTERVALS

Samples for the RI study were selected from free-ranging elephants, with no overt clinical abnormalities, living in the KNP in South Africa and were collected between October 2014 and August 2019. Of the original 79 selected samples, two were excluded after further detailed analysis of their capture records, as one was found deceased subsequent to a road traffic accident, and one had a snare injury. Results from these two elephants were included in the injured group. After statistical analysis and removal of 28 complete data sets with outliers, 51 samples from apparently healthy animals remained. These consisted of 42 males and 9 females. All were sub-adults or adults according to the original selection datasheet, except for one male calf which was estimated to be four years old. Results for PCV were available for 48 of these animals. Only 23 samples from the automated haematology analysis were finally included, after applying the described screening procedures. For these, RI and CI are not reported, and the results presented are minimum- maximum range, shown in Table 7 (including manually derived absolute leukocyte differential counts). Results for all manual relative differential leukocyte counts (n=51) are also presented in Table 8. Clinical chemistry RI were established from 50 individuals (one serum sample was missing) and are shown in Table 9. Mean, median, standard deviation as well as the distribution and chosen statistical method for all measurands are presented.

The CVs obtained for the clinical chemistry measurands from the repeatability study with elephant serum on the VS2 were: albumin 1.1%, ALP 3.1%, AST 4.7%, calcium 0.8%, CK 5.2%, GGT 6.6%, globulin 3.7%, magnesium 0.9%, phosphorous 1.0%, TP 1.4%, urea 3.9%. Imprecision of all measurands was considered acceptable, when compared to total allowable analytical error guidelines for veterinary species (147).

There was no significant correlation between storage time and measurand concentration/activity found for any measurand apart from AST and CK (AST r=0.69, p<0.001; CK r=0.50, p<0.001). In other words, older samples appeared to have a decreased AST and CK activity compared to more recent samples, which may indicate a storage effect.

Morphology of cells from the microscopic blood smear evaluation was recorded, with examples shown in Figures 2 and 3. Rouleaux ranged from 2+ in 22 blood smears to 4+ in one blood smear. Mild anisocytosis was seen in over 90% of the evaluated smears. Band heterophils were seen in 31 of 51 blood smears. The number of heterophils showing toxic changes was categorised as few to moderate in 20 blood smears and high in 6 blood smears. In 16 of the 26 smears with toxic heterophils, dark blue-grey cytoplasm with vacuoles or toxic granulation was noted (3+) (Figure 3) and karyolysis was seen in one (4+ toxicity grade). Monocytes were divided into three categories: normal, bilobed and round (Figure 2). Normal monocytes were observed most commonly and round monocytes least commonly (Table 3 and Figure 2). Occasionally, trilobed monocytes were seen, which were counted as bilobed.

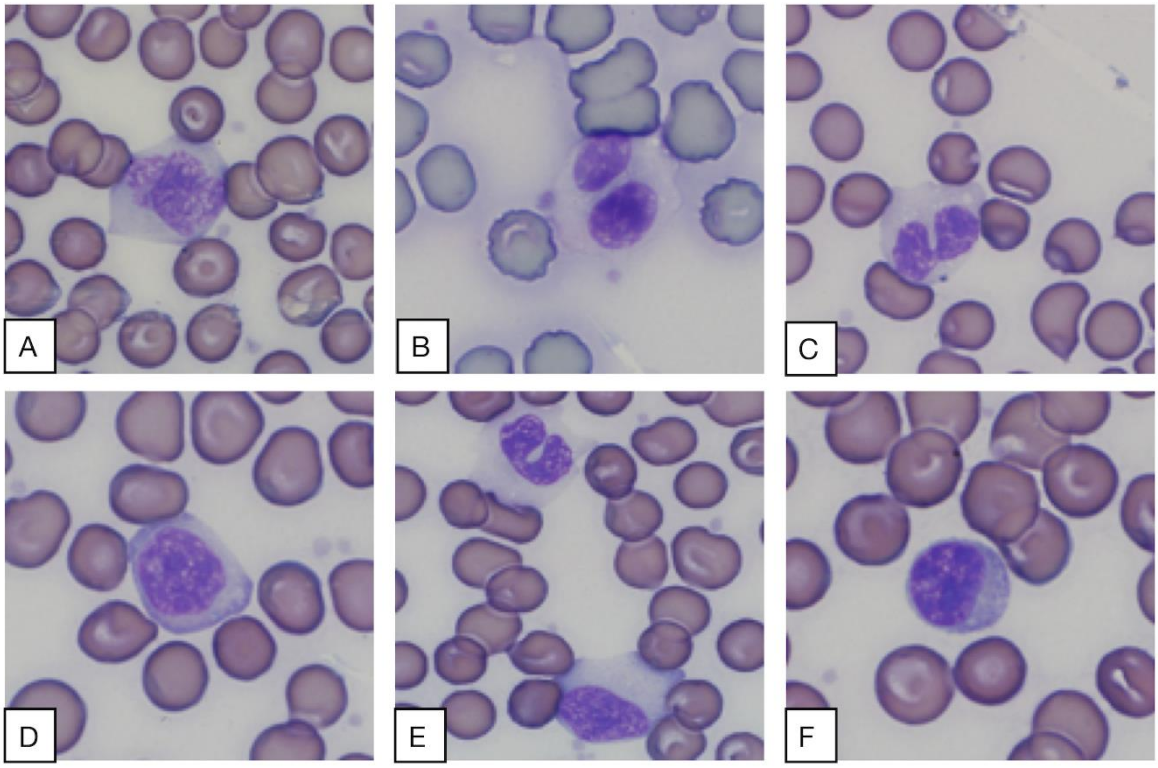


Figure 2: Images of monocytes (A-E) and lymphocyte (F) in blood smears from an African elephant (*Loxodonta africana*). A: Normal monocyte; B and C: Bilobed monocytes; D: Round monocyte; E: Normal and bilobed monocyte; F: Lymphocyte; Wright Giemsa stain; 1000x magnification

Monocyte activity, characterised by the number of vacuoles, was mild in 41 smears and recorded as moderate in 6 smears. More than 5% of lymphocytes were reactive in 26 smears. Eosinophils (Figure 3) were characterised by bright red granules and were present in every slide. Basophils had a light blue to transparent cytoplasm with non-staining granules and were detected in seven smears only (Figure 3).

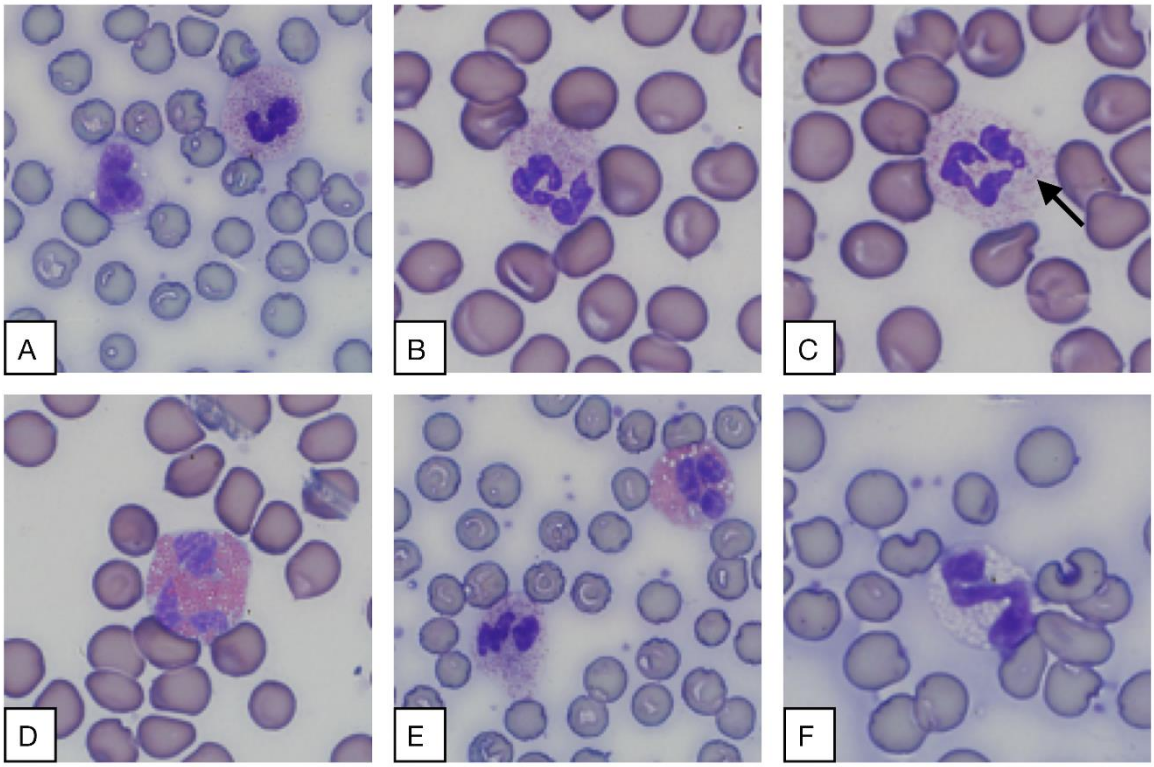


Figure 3: Images of granulocytes in blood smears from an African elephant (*Loxodonta africana*). A: Heterophil and normal monocyte; B: Heterophil, C: Heterophil with toxic changes (Dohle body, arrow), D: Eosinophil, E: Heterophil and eosinophil, F: Basophil. Wright-Giemsa Stain, 1000x magnification

Table 7: Haematology reference intervals from free-ranging African elephants (*Loxodonta africana*) in the Kruger National Park, South Africa. PCV was measured by manual methods; WBC, RBC HGB and PLT were measured using a scil Vet abc or Horiba ABX Micro ESV60; MCV, MCV, MCH and MCHC were calculated. Absolute leukocyte numbers were derived from WBC and 200-cell manual leukocyte differential counts.

Measurand (unit)	n	Mean	SD	Median	Min	Max	RI	LRL	URL	Distribution	Method
PCV (%)	48	41	4	41	33	49	34-49	32-35	47-50	G	P
RBC (x10^{12}/L)	23	3.44	0.30	3.48	2.80	3.96					NP
HGB (g/L)	23	141	13	143	116	163					NP
MCV (fL)	23	121	6	121	112	134					NP
MCH (pg)	23	40.9	2.6	41.4	35.5	45.2					NP
MCHC (g/L)	23	338	15	337	314	364					NP
PLT (x10^{9}/L)	23	284	54	281	182	386					NP
WBC (x10^{9}/L)	23	11.2	2.2	11.3	7.5	15.2					NP
Segmented heterophils (x10^{9}/L)	23	2.8	0.7	2.9	1.5	4.0					NP
Band heterophils (x10^{9}/L)	23	0.1	0.1	0.1	0.0	0.2					NP
Total monocytes (x10^{9}/L)	23	5.2	1.1	5.0	3.6	7.6					NP

Measurand (unit)	n	Mean	SD	Median	Min	Max	RI	LRL	URL	Distribution	Method
Monocytes normal (x10^9/L)	23	3.8	0.9	3.7	2.7	6.1					NP
Monocytes bilobed (x10^9/L)	23	1.1	0.5	0.9	0.3	2.3					NP
Monocytes round (x10^9/L)	23	0.3	0.3	0.3	0.0	0.9					NP
Lymphocytes (x10^9/L)	23	2.7	1.3	2.3	1.1	5.5					NP
Eosinophils (x10^9/L)	23	0.4	0.2	0.3	0.0	0.9					NP
Basophils (x10^9/L)	23	0.0	0.0	0.0	0.0	0.1					NP

PCV, packed cell volume; RBC, red blood cell concentration; HGB, haemoglobin concentration; MCV, mean cell volume; MCH, mean cell haemoglobin; MCHC, mean cell haemoglobin concentration; PLT, platelet concentration; WBC, white blood cell concentration; n, number of individuals; SD, standard deviation; RI, reference interval; LRL 90% confidence interval of the lower reference limit; URL, 90% confidence interval of the upper reference limit; G, Gaussian; NG, non-Gaussian; P, parametric; NP, non-parametric

Table 8: Reference intervals for relative leukocyte differential counts for African elephants (*Loxodonta africana)* in the Kruger National Park, South Africa. 200-cell manual differential counts were performed.

Leukocyte	n	Mean	SD	Median	Min	Max	RI	LRL	URL	Distribution	Method
Segmented heterophils (%)	51	25.6	4.9	25.5	15.5	36.0	15.6-35.5	13.8-17.5	33.6-37.4	G	P
Band heterophils (%)	51	1.0	1.0	1.0	0.0	3.5	0.0-3.5	0.0-0.0	3.0-3.5	NG	NP
Total monocytes (%)	51	47.6	8.0	48.0	31.0	63.5	31.5-63.8	28.5-34.5	60.6-66.9	G	P
Monocytes normal (%)	51	35.2	6.5	35.0	24.0	49.0	22.1-48.3	19.7-24.5	45.7-50.8	G	P
Monocytes bilobed (%)	51	8.6	4.0	8.5	1.0	19.5	0.4-16.8	0.0**-1.9	15.1-18.3	G	P
Monocytes round (%)	51	3.9	2.9	3.5	0.0	9.5	0.0-9.4	0.0-0.0	8.2-9.5	NG	NP
Lymphocytes (%)	51	22.2	8.9	23.0	6.0	40.0	7.4-39.9	6.0-10.7	36.5-40.0	NG	NP
Eosinophils (%)	51	3.5	1.4	3.5	1	7.0	1.2-6.7	1.0-1.7	5.7-7.0*	NG	NP
Basophils (%)	51	0.1	0.3	0.0	0.0	1.0	0.0-1.0	0.0-0.0	0.8-1.0	NG	NP

*The CI (confidence interval of the upper or lower reference limit) to RI ratio exceeded 20%. The calculated lower lower limit of the CI was a negative result (-1.1) and this has been replaced by 0.0.

n, number of individuals; SD, standard deviation; RI, reference interval, LRL, 90% confidence interval of the lower reference limit; URL, 90% confidence interval of the upper reference limit; G, Gaussian; NG, non-Gaussian; P, parametric; NP, non-parametric

Table 9: Serum clinical chemistry reference intervals from free-ranging elephants in the Kruger National Park, South Africa, using the Abaxis Vetscan VS2.

Measurand (unit)	n	Mean	SD	Median	Min	Max	RI	LRL	URL	Distribution	Method
Albumin (g/L)	50	48	3	48	41	56	41-55	41-44*	52-56*	NG	NP
ALP (U/L)	50	68	23	64	28	124	30-122	28-38	115-124	NG	NP
AST (U/L)	50	21	6	21	9	36	9-34	6-11	31-36	G	P
Calcium (mmol/L)	50	2.79	0.11	2.78	2.55	3.04	2.56-3.02	2.52-2.61	2.98-3.07	G	P
CK (U/L)	50	203	58	200	86	341	85-322	63-107	298-345	G	P
GGT (U/L)	50	11	2	11	7	16	7-16	7-8	14-16*	NG	NP
Globulin (g/L)	50	45	7	44	31	60	30-59	28-33	56-62	G	P
Magnesium (mmol/L)	50	1.41	0.15	1.41	1.14	1.70	1.15-1.70	1.14-1.22	1.68-1.70	NG	NP
Phosphorus (mmol/L)	50	1.82	0.23	1.82	1.27	2.31	1.28-2.31	1.27-1.45	2.23-2.31	NG	NP
TP (g/L)	50	93	8	93	78	112	77-109	74-80	106-112	G	P
Urea (mmol/L)	50	2.9	0.8	3.0	1.0	4.8	1.2-4.6	0.9-1.	4.3-5.0	G	P

*The CI (confidence interval of the upper or lower reference limit) to RI ratio exceeded 20%.

ALP, alkaline phosphatase; AST, aspartate aminotransferase; CK, creatine kinase; GGT, gamma-glutamyltransferase; n, number of individuals; SD, standard deviation; RI, reference interval; LRL,90% confidence interval of the lower reference limit; URL, 90% confidence interval of the upper reference limit; G, Gaussian; NG, non-Gaussian; P, parametric; NP, non-parametric

ACUTE PHASE REACTANTS

Results for the imprecision experiment are presented in Table 10. Imprecision was acceptable for both HP and SAA assays.

Table 10: Intra-assay imprecision for the Tridelta PHASE haptoglobin and Eiken LZ-SAA assays using African elephant serum pools.

	Low pool	**High pool**
Haptoglobin mean	0.18 g/L	2.33 g/L
Haptoglobin CV	2.7%	0.4%
SAA mean	11 mg/L	137 mg/L
SAA CV	4.1%	1.3%

The haptoglobin assay was found to be linear up to 2.5 g/L (which is also the concentration of the highest calibrator). The SAA assay showed acceptable linearity using elephant serum up to 155 mg/L. All samples with results greater than the upper linearity limits were diluted 1:2 (haptoglobin) or 1:5 or 1:10 (SAA) to obtain the final results.

Initially 50 serum samples were analysed from healthy elephants. Seven samples were identified as outliers and eliminated. Reference intervals were calculated using the same methods as for haematology and clinical chemistry measurands and are presented in Table 11.

Table 11: Reference intervals for serum acute phase reactants from free-ranging elephants in the Kruger National Park, South Africa, using the Tridelta PHASE haptoglobin, Eiken LZ-SAA assays, and ferrozine zinc method for iron on a Roche Cobas Integra 400 Plus chemistry analyser.

Measurand (unit)	n	Mean	SD	Median	Min	Max	RI	LRL	URL	Distribution	Method
Iron (µmol/L)	43	12.09	2.55	11.70	8.6	17	8.60-16.99	8.60-8.91	16.42-17.00	NG	NP
Haptoglobin (g/L)	43	1.34	0.81	1.16	0.16	3.54	0.16-3.51	0.16-0.22	2.63-3.54*	NG	NP
SAA (mg/L)	43	All results < 10 mg/L					<10				

SAA, Serum amyloid A; n, number of individuals; SD, standard deviation; RI, reference interval; LRL,90% confidence interval of the lower reference limit; URL, 90% confidence interval of the upper reference limit; NG, non-Gaussian; NP, non-parametric

INJURED ELEPHANTS

Fifteen samples of injured animals were initially selected, with two elephants added later, as explained above. Details of the demographics and clinical findings of the injured elephant group are presented in Table 12.

Table 12: List of injured elephants, with demographic and clinical information

Elephant Number	Sex	Life Stage	Injury	Comment
1	Female	Prime adult	Snare removal, right hind leg, soft tissue defect, infected	
2	Female	Young adult	Snare removal, around thorax, just behind mammary glands, deep tissue cut	
3	Female	Young adult	Snare removal (below carpus), infected tissue, severe necrosis	Guarded prognosis
4	Female	Young adult	Snare around right front distal of carpus, infected wound, necrosis, swollen distal limb	Snare was present for at least 4 months
5	Male	Prime adult	Cellulitis of left front leg, severe non weight bearing	Euthanasia was advised
6	Female	Old	Snare removal right front	Poor body condition
7	Male	Young adult	Snare around left carpus, swollen, no skin cut	Ticks +++
8	Male	Young adult	Snare around the trunk, struggling to breath	
9	Female	Not available	Snared	
10	Female	Calf	Cable snare around left hind foot	
11	Male	Young adult	Snare wound on left hind foot, wounded	
12	Male	Juvenile (6y)	Cable snare around left hind foot, wounded	
13	Male	Prime adult	Cable snare, left hind foot	
14	Male	Calf	Snare around the head	Ear faces forward now
15	Male	Young adult	Snare around right front leg, wounded	
16	Male	Young adult	Snare, no tissue injury	
17	Male	Sub adult	Snare wound around front leg, cutting into tissue	

Blood smear and haematology results are presented in Table 13, clinical chemistry results in Table 14 and APR results in Table 15. Results above the RIs generated in this study are marked blue; results below the RIs are marked orange. Prominent changes were seen in the blood cell morphology; the most common of which was a left shift heterophilia. Nine smears had band heterophils above 3.5%, with the highest result of 16%. In 12 smears the amount of toxic changes in the heterophils was moderate to many, and the severity was graded 3+ and 4+ in 10 smears. In terms of the clinical chemistry measurands, there was a hypoalbuminaemia in ten animals, CK was elevated in four injured animals and GGT was increased in ten. Changes were also seen in minerals; a hypomagnesaemia was present in ten animals and eight showed hypocalcaemia (Table 14).

Sixteen serum samples from injured animals were analysed for SAA, HP and iron. Most of the injured animals showed changes in the APRs. Only one of the injured animals had an iron concentration within the RI, one had a hyperferraemia, while 14 elephants had a hypoferraemia. Haptoglobin was elevated in 12 animals and SAA was elevated in 14 elephants. Results for SAA differed greatly (<10-920.25 mg/L), and the highest result was above 920 mg/L. Only one animal did not show increases in HP or SAA, and had hyperferraemia. This animal was the only calf and had a snare around its neck; no information was available about the degree or chronicity of the tissue damage (Table 12).

Table 13: Haematology and morphological changes of WBC in injured African elephants.

Elephant Number	Rouleaux	Segmented heterophils (%)	Band heterophils (%)	Toxic change – heterophils affected	Toxic change - grade	Eosinophils %	Normal monocytes (%)	Bilobed monocytes (%)	Round monocytes (%)	Monocyte activity	Lymphocytes (%)	Reactive lymphocytes
1	1+	19.5	8,5	moderate	1+	6.5	19.0	5.0	7.0	1+	34.5	>5%
2	3+	9.0	19.0	many	3+	0.5	19.5	26.5	0.5	2+	25.0	>5%
3	3+	17.0	11.5	many	3+	3.0	33.5	15.5	0	1+	19.5	>5%
4	3+	13.0	16.0	many	3+	4.5	23.5	6.0	0	1+	37.0	<5%
5	2+	27.0	24.0	many	1+	0	31.5	5.0	2.0	1	10.5	>5%
6	2+	35.0	7.0	few	1+	2.5	24.5	12.5	2.5	0	15.5	<5%
7	3+	21.0	0.5	moderate	3+	3.0	36.5	19.5	0	1+	18.5	>5%
8	3+	37.0	7.0	moderate	1+	0	22.0	14.5	0.5	1+	19.0	<5%
9	3+	38.5	1.5	many	3+	1.5	27.5	8.0	4.0	1+	18.0	<5%
10	3+	15.0	2.5	0	0	0	39.0	42.0	0.5	1+	0.5	<5%
11	3+	35.5	0.5	0	0	2.0	23.0	16.5	0	1+	22.5	<5%
12	2+	14.5	0.5	moderate	3+	0	47.0	21.0	0	1+	17.0	<5%
13	2+	30.5	3.0	moderate	4+	4.5	40.5	10.5	2.5	0	8.5	>5%
14	2+	24.5	2.0	moderate	3+	5.5	29.0	21.5	8.0	2+	9.5	<5%
15	3+	32.5	4.5	moderate	3+	3.5	27.0	14.0	5.0	1+	13.5	>5%
16	2+	26.5	1.0	0	0	1.5	26.0	16.0	2.5	0	26.5	<5%
17	3+	37.5	7.0	few	3+	2.0	28.0	11.5	3.0	1+	11.0	<5%

Results above the RIs are marked in blue, results below the RIs are marked in orange.

Table 14: Clinical chemistry results of the injured African elephants. Measured results above the generated RI are marked red and below the generated RI are marked orange

Elephant Number	Albumin (g/L)	ALP (U/L)	AST (U/L)	Calcium (mmol/L)	CK (U/L)	GGT (U/L)	Globulin (g/L)	Magnesium (mmol/L)	Phosphorus (mmol/L)	TP (g/L)	Urea (mmol/L)
1	29	59	16	2.52	176	10	38	1.07	1.75	67	1.6
2	38	90	17	2.83	214	16	46	1.21	1.83	84	1.6
3	35	63	15	2.47	285	11	52	1.09	1.26	87	1.6
4	29	142	22	2.48	204	25	37	1.44	1.27	67	1.4
5	37	90	10	2.30	224	17	62	0.81	1.94	99	5.4
6	41	174	17	2.50	282	25	45	0.90	1.30	85	1.2
7	45	115	19	2.64	1182	20	39	1.20	2.31	84	1.5
8	53	104	19	2.58	1404	14	27	0.95	1.81	79	3.2
9	37	114	17	2.63	188	20	39	1.01	1.38	76	2.9
10	46	253	20	2.78	659	26	34	0.99	2.38	80	1.9
11	43	139	28	2.73	260	19	52	1.15	2.05	95	2.8
12	30	115	25	2.35	349	16	36	0.85	1.84	66	2.4
13	48	147	22	2.69	235	14	38	1.15	1.93	85	3.2
14	38	120	20	2.65	306	23	40	1.21	1.51	78	1.0
15	30	125	28	2.48	198	25	48	1.01	1.23	78	1.4
16	50	87	13	2.74	196	13	33	1.53	2.09	83	2.5
17	40	138	13	2.54	138	27	52	1.07	1.41	92	1.2

ALP, alkaline phosphatase; AST, aspartate aminotransferase; CK, creatine kinase; GGT, gamma-glutamyltransferase; TP, total protein; Results above the RI are marked in blue, results below the RI are marked in orange.

Table 15: Acute phase reactants of injured free-ranging African elephants, and (available) comments on the injury.

Elephant Number	Iron (µmol/L)	HP (g/L)	SAA (mg/L)	Injury mentioned, comments
1	5.1	6.39	80.30	Snare wound on right hind leg, marked soft tissue damage.
2	5.3	7.23	423.10	Snare wound around thorax, (behind mammary glands), deep cut, soft tissue damage.
3	5.7	3.72	361.05	Snare wound around right front limb (just below carpus), necrotic and infected tissue (guarded prognosis given).
4	4.8	6.09	385.75	Snare wound, necrotic and infected tissue, (snare was known to be there at least 4 months).
5	4.1	7.41	300.70	Severe subacute cellulitis on left front leg, non-weight bearing.
6	4.5	6.72	555.30	Snare on right front.
7	7.0	3.60	920.25	Snare on left front, swollen carpus, not tissue cutting, ticks+++.
8	5.1	0.39	<10	Snare around trunk, struggling to breath.
9	5.6	5.61	99.05	Snared.
10	4.1	9.85	443.35	Snare left hind.
11	9.0	6.03	128.85	Snare wound left hind, clean wound.
12	3.4	6.06	401.60	Snare wound left hind.
13	7.5	3.33	29.45	Snare on left hind.
14	19.7	3.24	<10	Snare around head/neck (calf, 5 years).
15	5.0	2.94	33.95	Snare wound right front leg.
17	4.5	6.39	135.99	Snare wound leg, deep tissue cut.

HP, Haptoglobin; SAA, Serum Amyloid A; Results above the RI are marked in blue, results below the RI are marked in orange.

CHAPTER 5: DISCUSSION

REFERENCE INTERVALS

This study presents RIs for haematology and clinical chemistry values for the free-ranging African elephant, with the intent of providing valuable guideline data for clinicians working with the species. Haematology RIs were generated from automated analysis as well as from manual counts; the latter supplying useful data for situations where resources are limited. The selected clinical chemistry panel includes measurands that are applicable and useful for this species. For all RIs a very strict outlier elimination approach was followed, as the samples originated from wild animals whose healthy status was presumed, based on a cursory examination but could not be confirmed. It can be assumed that in the wild, a certain proportion of a population has an underlying subclinical disease, and strict outlier exclusion ensures that the resultant reference intervals are as representative of a truly healthy population as possible.

Reference interval studies for African elephants have been performed and described previously, albeit with various limitations in materials and methods. To our knowledge, this is the second study for this specific geographic location and population. A previous study, published in 1984, generated data for some haematology and clinical chemistry measurands from African elephants in KNP, but most of the animals were shot prior to blood collection (63). Culled animals were similarly used in studies with larger sample sizes (n = up to 141) published between 1977 and 1980; all these data originate from the same elephant population in East Africa (59-62, 67). The same blood collection method was performed in a smaller Tanzanian study (n = 18-23), where on later examination, infestation with the bile duct hookworm (*Grammocephalus sp.*) and other parasites was discovered (57). Post mortem findings or coproscopic examinations were either not performed or reported for any of the other studies involving culled animals (58, 61, 63, 65). Two RI studies were performed on captive animals which were either chemically immobilised or trained to stand for blood collection (64, 74). In both, blood was collected via venepuncture from either the auricular or the saphenal vein and animals were reported to be clinically healthy. The limitations of the first of these studies are the analysis of a limited range of measurands, small sample size (n = 5) and a different (sub)species of African elephant (*Loxodonta (africana) cyclotis*)

(74). Allen et al. (1985) report data from 31 animals for comparable measurands to our study, but all their elephants were young, between four and eight years old and the immobilisation protocol varied amongst individuals (64). Most of the aforementioned studies used manual analytical methods for haematology, when methods were reported (74). Data were presented as mean or median with the standard deviation (SD) or range; outlier identification and exclusion were not performed and RI and CI of the RLs were not calculated.

Studies using Asian elephants are more common and involve both captive and free-ranging animals. Captive elephants often reside in tourist camps or are working elephants from the timber industry, from various locations in South East Asia, including India, Sri Lanka, Thailand and Myanmar (78, 79, 82, 151). Blood was collected from the auricular vein without immobilisation, except for one study on free-ranging elephants where a combination of etorphine and acepromazine was used (79). All blood samples were obtained from auricular veins with either 14G needles or vacutainer systems, but variation in sample handling, storage and transportation was described and analytical methods range from manual to completely automated. In most of the studies, reference data for haematology and at least some clinical chemistry measurands were presented, apart from one of the earliest studies, from elephants in India (151). Another study using samples from zoo Asian elephants focused on blood cell morphology using conventional light and electron microscopy and also described cytochemical staining characteristics (80).

In all the above mentioned studies on Asian elephants, the animals were described as clinically healthy or apparently healthy. Sample sizes used varied greatly; the smallest one was performed on six individuals with longitudinal samples (77), with the largest sample size up to 765, although some of the animals were sampled two or three times (82). Silva et. al. (1993) were able to compare domesticated with free-ranging animals, all originating from one geographical region and using the same study methods (78, 79). Most published results have been reported as means or medians, SDs and ranges. More recent studies include outlier detection and calculation of 90% CI (81, 82). Only the most recent publication using Asian elephants generated RI in accordance to the ASVCP guidelines and performed partitioning for sexes and age

groups (82). A few studies have also investigated the influence of musth on clinical chemistry measurands and hormone levels (77, 81).

Haematologic values have been shown to vary by geographical location. The means for RBC (3.9 $x10^{12}$) and PCV (49%) described in the East African study, were higher than those determined in our study and the minimum-maximum (1.4-6.0 $x10^{12}$/L and 18-80%) ranges much wider, in comparison to our RIs (67). Reasons for this could include the presence of underlying illnesses or dehydration as no health assessment was performed prior to collection, differing blood collection methods and preanalytical errors, and the lack of outlier identification and subsequent exclusion of samples. Haematocrits (39.3-47.7%) of KNP elephants, sampled from seven groups with different restraint methods (immobilized to culled), are comparable to our PCV findings, however other haematology RIs were not given (63). Immobilised captive African elephants showed lower mean RBC (3.0 $x10^{12}$/L) and PCV (35.1%); MCV range (106-122 fL) was slightly narrower and MCHC range (310-390 g/L) wider but comparable to our RIs (64). Asian elephants tend to have lower PCVs with lower haemoglobin concentrations and lower RBC counts compared to African elephants (78, 79, 82). However, our established means of MCV (121 fL), MCHC (338 g/L) and MCH (41 pg) are almost the same as those reported in immobilised Asian elephants in Sri Lanka (79).

The WBC RIs generated in this study are found to be well within the middle of the ranges of data presented in other studies, where the lowest WBC count is 6.83 ($x10^9$ /L), reported in the African forest elephant (n = 5) (74) and the highest is 13.6 ($x10^9$ /L) from captive and immobilised African elephants (64). Asian elephant WBC counts varied at least as widely amongst various studies and ranged from 8.8 ($x10^9$/L) in tuskers (151) to 18.0 ($x10^9$/L) in free-ranging elephants in Sri Lanka, the latter being the highest reported WBC count in apparently healthy elephants (79). The percentages described for leukocyte subpopulations vary greatly between studies, especially for monocytes and lymphocytes, due to discrepancies in the identification and classification of bilobed and round monocytes. The morphology of these monocytes is unique to the Paenungulata clade, which also includes the Procaviidae (hyraxes) and the Sirenia families (dugongs and manatees) (71). Bilobed and round monocytes have been confirmed to be of monocytic origin based on cytochemical staining (64, 69). In

our study, the three types of monocytes were identified using morphological characteristics described in the more recent literature (69, 145). Presumably bilobed as well as round monocytes have been counted as lymphocytes in multiple other studies, which resulted in lymphocyte percentages above 50%, up to 77% (57, 67, 151). Two studies involving captive and free-ranging Asian elephants identified the bilobed monocyte as such, but the reported lymphocyte counts still appeared to be high, with means of 38% for captive, and 44% for free-ranging elephants (78, 79). Exclusion of samples and handling of outliers were not described. Possible reasons for the high proportion of lymphocytes are that round monocytes were counted as lymphocytes, and that animals with subclinical illnesses were included.

Elephants share another haematological characteristic with sirenians, in that these species have heterophils rather than neutrophils (70). Band heterophils have been described in low numbers in Asian elephants (78-80), but there is disagreement as to whether they occur in healthy animals (69). They have only been described in one study on African elephants (1.17%), but these animals were known to have parasites (57). The presence of band heterophils, and heterophils with toxic change, in over half of the elephants in this study indicates that inflammatory disease, not apparent during clinical examination, was possibly present in many of our reference individuals. Our findings indicate that band heterophils of up to 3.5% can be expected for apparently healthy KNP elephants. The potential causes of this tendency towards a left shift need to be investigated further.

Eosinophils and basophils have been reported by most authors in low and comparable numbers to those described here except that eosinophil concentrations from captive African elephants (eosinophils 0.05 $x10^9$/L) were lower than those in this study (0.4 $x10^9$/L) (64). This could reflect a higher parasite load in the free-ranging, compared to the captive population. Similar percentages of eosinophils and basophils were described for free-ranging and domesticated Asian elephants with 5% for eosinophils and 0.03% for basophils (78, 79). Interestingly, the most recent study on elephants in Myanmar does not report findings on either basophils or thrombocytes (82).

Platelets were the cell fraction with the widest ranges, as described in most studies where platelets were assessed (77-79, 81). They occurred in large numbers but were

very small in size and often found as clumped aggregates, making it difficult to identify single cells. Our range determined for the KNP elephant population is narrower than for other African elephants (294-455 $x10^9$/L) (69). The lowest mean number for platelets was found for domestic Asian elephants in Sri Lanka (215 $x10^9$/L), (78) which still falls within the range established for elephants in Thailand (101-590 $x10^9$/L) (81). The highest reported count is 719 $x10^9$/L, also found in the Asian elephant (69). The wider ranges reported in other studies may be due to the presence of platelet clumping, differing analytical methods and the lack of outlier exclusion.

Most of the results for clinical chemistry measurands reported in other studies of comparable geographical region were similar to our measurements, even though analytical methods differed (61-63, 66). Reported means/medians were mostly within our RIs. Seasonal changes were seen for some measurands, other authors reported variations between age groups, especially for minerals (62, 65). Differences were observed in data published from captive African elephants, although sample size and age (4-8 years) need to be considered when making direct comparisons (64). Variations between sexes seem more prominent in the Asian elephant than in the African elephant, especially for serum activities of ALP and GGT, which are much higher during musth periods (77, 82). The majority of measurands included in our panel have also been studied in the Asian elephant: total protein or protein fractions tend to be included in different studies as well as selected enzymes, while magnesium and GGT have not been widely investigated (78, 81, 82). Veterinarians with clinical experience in these species, advised the inclusion of fibrinogen, creatinine, LDH and electrolytes (Na, K, Cl) in clinical chemistry panels for elephants, in addition to our measurands (84). These were not included in our study due to the set profile of the VetScan VS2 rotor system.

Serum mineral levels in the African elephant vary by geographical location. The means of calcium, magnesium and phosphorus in our study were higher compared to those from elephants at Sengwa Wildlife Research, Zimbabwe, but lower in comparison to elephants from Ruwenzori National Park in Uganda (59, 63). Firstly, a contributing factor that may explain the higher mineral levels (at least phosphorus) in the Ruwenzori study could be haemolysis, as these elephants were bled after being culled. Secondly, nutrition may play a role; it has been shown previously that season and location have

an influence on mineral and protein concentrations in blood (59, 62, 66). The blood samples in our study were collected almost all year round, except during the months of January and February. One study showed that calcium and phosphorus were higher in male elephants and phosphorus was higher in young animals compared to animals above five years (65). In semi-captive Asian elephants in Myanmar, calcium was lower (2.15-2.75 mmol/L), means of phosphorus and magnesium of captive Asian elephants were also lower than our established means (82, 151). Blood mineral concentration data collected from zoo-kept African elephants also showed lower means than the KNP population (93). Seasonal and geographical factors, and therefore nutritional variation, need to be taken into consideration when using the RIs in the future, as elephants tend to graze more during the wet season, when grassland is lush (152). In zoos, this information could be used as an aid for evaluating nutritional status and preventing mineral deficiencies which could influence bone health.

Determination of AST and CK activity is especially important for captured animals, as increases in these enzymes are associated with muscle injury, intramuscular injection, trauma and capture stress in domestic and wild animals such as dogs, horses, some ruminants and rhinoceros (153-155). Animals known to be injured were excluded from the RI calculation of our study. Kock et al. (1993) described a difference in the activities of AST and CK depending on etorphine dosages and the time to recumbency that elephants experienced during capture (66). The capture protocol with the higher etorphine dosages led to quicker recumbencies and lower AST and CK, compared to a lower dose protocol with longer times to recumbency. Although with both protocols, measurements were still within our RIs, AST and CK levels for the higher dosed group were visibly lower; duration of chasing and capture must be considered as both enzymes rise within hours of the insult. Creatine kinase reaches its peak after 6 to 24 hours and declines thereafter (in domestic animals) (156, 157). As blood samples from our study elephants were drawn immediately after the animals were recumbent, results reflect CK activity well before its peak. Distinct increases in activities of both enzymes are usually seen after long transports or exhaustion, exercise and rhabdomyolysis (153). Reference intervals generated for AST from captive African elephants are very similar to ours, while other enzymes (ALP, GGT and CK) show much broader RIs (93). Young animals, also true for elephants, have higher ALP levels, due to increased osteoblast activity. In male Asian elephants, ALP is especially elevated during musth

periods (59, 77). This will need to be evaluated in future studies to determine if this also occurs in the African elephant. Unfortunately, the presence of musth in the male elephants in our population was not recorded.

Published means of TP and albumin were consistently lower than those found in our study, for wild African elephants from various geographical locations (62, 66). Results from captive *Loxodonta africana* were even lower, whereas urea and globulin means were higher than our results (64, 93). Total protein reported in the older study of elephants from KNP was most similar to our findings (63). Seasonal and geographical changes have been recorded for all protein constituents, pointing to nutrition as the biggest variable (62). This needs to be considered when comparing results to established RIs. Total protein values from Asian elephant populations were similar to each other (65-93 g/L) and generally lower than TP in African elephants (78, 79, 81, 82).

Anaesthetic drugs, stress, capture and transport or a combination thereof can lead to PCV and TP changes in various species, and thus influence RIs (158-160). In other animal species, stress during capture can lead to an increase in PCV and HGB via catecholamine release and contraction of the spleen (161). In the adult African elephant, the spleen acts mostly as a filtering organ and not as blood storage organ (capsule with muscle tissue) as is the case in animals which flee if they encounter enemies or hazards, like equids (84, 162). The elephant's splenic capsule consists of connective tissue, therefore contraction during a stress response seems unlikely (84). The anaesthetic drugs etorphine and azaperone or combinations thereof have been commonly used for the African elephant as well as other wildlife species (163-166). Cardiopulmonary measurands, such as heart and respiratory rate, blood pressure, blood gases and haemoglobin in arterial blood have been described for the African elephant anaesthetised with these drugs (167). The influence of these agents on clinical pathology has not been evaluated in this species so far. In one small antelope study, where etorphine was a component (but not used on its own or with azaperone), a decrease in RBC, WBC, PCV and HGB was found (168). A RI study on African buffalo (*Syncerus caffer*) from the same geographical location (KNP), using the same drug combination and clinical chemistry panel, did not consider the influence of the anaesthetics relevant (169). One reason for this could be that blood is collected very

soon after recumbency which does not allow enough time for measurands to react. This is a general assumption which needs to be studied further, for example by comparing results from samples taken under similar conditions and analysed using the same methods, from non-immobilized and immobilized captive elephants. As most captive elephants are not immobilised for blood collection, the possible influence of drugs on the RIs generated in this study needs to be considered if these RIs are used to evaluate results from captive individuals.

ACUTE PHASE REACTANTS

Reference intervals for the APR in the African elephant have only been measured and described in captive animals so far. In our study, we assessed the APRs and calculated RIs, for the free-ranging African elephant population from the KNP. As with haematology and clinical chemistry measurands, they have been generated according to the AVSCP guidelines for RIs. The described assays and methods have been used for other wildlife species, including Asian elephants (134, 136, 170). Serum Amyloid A (SAA ELISA assay) was identified to be a valuable marker of inflammation in the Florida manatee, which also belongs to the superorder of the Afrotheriae (134, 135). Reference intervals for SAA in manatees (n=71) were reported as <10-50 mg/L. These results are very similar to the RIs given for the Asian elephant (0-47.5 mg/L) (immunoturbidometric assay) calculated from 35 samples (139). Serum Amyloid A is of special interest as a diagnostic marker for EEHV, as elevations are seen, before viraemia is present (137). Our generated RIs for the African elephant are lower (<10 mg/L) and can be compared with results for captive African elephants (0.10-6.914 mg/L) (138) and data available via Species 360 (also captive animals), where SAA RIs are 0-20 mg/L, calculated from a larger sample size (71), but from fewer animals (n=25).

Haptoglobin results of the African elephant (0.16-3.51 g/L) can be compared to results from other non-domestic mammals species, like the Florida manatee (0.4-2.5 g/L) white rhinoceros (1.0-4.3 g/L) and the Asian elephant (0-1.10 g/L) (colorimetric assay), which has the narrowest RIs (134, 136, 139). Results presented by Edwards et al. (138) for captive Asian elephants (0.024-3.99 g/L) were more similar to ours, than the ones for the captive African elephant (0.21-2.35 g/L). Similar results were established

in the Species360 database (0.08-2.02 g/L) (colorimetric assay) for the African elephant. The methods used for the manatee values differed from ours, as an immunoturbidometric assay was used. Methods used for the Asian elephant for HP and SAA were comparable to the methods in the current study (139).

Reference Intervals for serum iron are 8.6-16.99 µmol/L which is very similar to results published in 1985 from 31 captive elephants (7.52-16.65 µmol/L) (64). Another study, also on elephants from the KNP, found serum iron levels were lower in culled animals (mean: 14.5 µmol/L (12.7 -19.5) in comparison to anaesthetized animals (mean: 18.5 µmol/L (11.5-25.8)); both mean and ranges were within our RIs or above, possibly indicating, that some elevation in serum iron levels is not clinically relevant for this species. Reference intervals for iron available via Species 360 have a much lower LRL (3.1-25.3 µmol/L). These RIs are given for the automated method, whereas RIs generated for serum iron measured with mass spectrometry start at almost the same level as ours (8.7-24.8 µmol/L). These differences demonstrate the importance of analytical method, when interpreting results against published RIs. Interestingly, although the analytical method used in our study is comparable to automated methods used in other studies, the results differ greatly. One reason for this could be that the data presented in other studies were generated from captive populations, and the nutritional plane of captive animals, in terms of iron, is different to free-ranging animals.

INJURED ANIMALS

All of the 17 animals had injuries that were snare-related, with great variations in the severity of tissue damage. Some of the snares were observed on the animals for weeks or months before removal and the clinical outcome after the treatment and removal is unknown due to the free-ranging nature of these elephants. The most common observed changes in serum chemistry included hypocalcaemia and hypomagnesaemia, probably associated with the hypoproteinaemia and hypoalbuminaemia, since a component of the minerals is bound in circulating protein complexes. About 40% of the total serum calcium is bound to proteins (80% to albumin), and 30% of total serum magnesium is protein-bound (91). In domestic animals, the most common cause for a decrease in total calcium and magnesium is hypoproteinaemia. Hypocalcaemia and hypomagnesaemia may also occur with renal

disease. Decreases in both minerals have also been reported with protein-losing enteropathy in dogs due to hypoalbuminaemia (171). Furthermore, hypomagnesaemia and hypocalcaemia are a common finding in horses with gastrointestinal diseases, especially in colic cases (172, 173). In ruminants, these changes are most commonly described in the peri- and post-partum periods, due to excessive needs from muscle activity and later on, milk production. In human medicine, decreases in serum mineral levels have been described in patients with severe burn wounds due to loss of albumin (174). Hypomagnesemia has also been reported in human patients suffering from stress and trauma, and is postulated to be related to the release of adrenaline (175). In our injured elephants more than one factor may be involved as hypomagnesaemia and hypocalcaemia were found in animals with and without hypoalbuminaemia. Most certainly the animals caught in snares would experience a certain level of stress and trauma, and loss of albumin was more severe in some animals than in others. Whether snare-injured animals also had a concurrent decrease in food intake would have been dependant on restriction of movement and severity of injury and is unknown.

More than half of our injured animals (10 out of 17) showed elevated GGT levels. In domestic animals GGT is linked to cell membranes and found in many cells such as renal tubular epithelium, biliary epithelial cells, pancreatic acinar cells and the mammary gland cells (157). Most commonly, GGT serum activity is increased with cholestasis and biliary hyperplasia and is known as a more sensitive indicator of these conditions in ruminants and horses than cats and dogs. For elephants, very limited information is available on the possible reasons for a GGT increase; musth periods seem to influence GGT increase in the Asian elephant (77, 84). Other reasons for increased GGT activity in domestic animals include some plant toxicities, and drugs like phenobarbital as well as exogenous or endogenous corticosteroids (176). The latter, as would be the case with chronic stress, could serve as an explanation for the elevated GGT activity in our injured animals, but additional examinations and follow-up analyses would be needed for confirmation.

Changes in the APRs were present in all injured/snared animals to different degrees, except for one animal. To the author's knowledge this is the first report of APR changes in injured animals in this species. In one conference presentation, elevations of APPs were described with EEHV infections, in both Asian and African elephants (138). Acute

phase reactant expression has been documented for the Asian elephant during EEHV1 infections in particular, but is also present with other EEHV strains (137, 138) One study, which described the detection of APR in a variety of non-domestic animals, reported changes in SAA in two injured Asian elephants (skin wounds and trauma) (170). Elevated SAA levels were also reported in Asian elephants with active pododermatitis, traumatic injuries and an infected tusk, while HP levels were raised in only some cases, and to a lesser extent (139). This study's findings are mostly in line with what has been reported in previous studies and provides further evidence that SAA is a major APP not only in the Asian, but also the African elephant. Haptoglobin was elevated in most samples of injured elephants, but to a lesser extent and is most likely a minor to moderate APP, as is the case in most animal species (98). An increase in HP is also linked to more chronic conditions and has also been proposed as an indicator of animal welfare in relation to stress (98, 177). In the elephants in this study, it is difficult to determine the chronicity of the injury as in most elephants the presence of a snare was not observed over time.

The most common causes for decreased serum iron include blood loss (especially chronic blood loss), reduced intake or malabsorption, as well as acute and chronic inflammation. In human medicine, iron has been known as a negative acute phase reactant for almost 40 years and hypoferraemia has been linked to a variety of stressful events such as infections, trauma or myocardial infarction (178). Reductions of iron happen within 12-24 hours after the initial trigger but can remain low for several weeks before returning to physiological levels. It has been proposed as a marker of inflammation for conditions like mastitis or reticuloperitonitis in cattle, and has been used to monitor recovery from surgical trauma in equines (179, 180). In dogs and cats, serum iron levels were observed to decrease in up to 90% of patients with inflammatory diseases (181). In white rhinoceros injured by poaching events, serum iron was decreased in approximately 70% of affected individuals (136). Our findings for serum iron in the African elephant seem to be in line with these studies, as only two snared animals did not have decreased serum iron, and one of these individuals not showing any other changes in APRs. Unfortunately, at this stage it was not possible to link the severity or chronicity of the injury to the elevation or decrease in APRs. To the author's knowledge, this is the first report on serum iron changes in African elephants as a negative acute phase reaction to injury.

LIMITATIONS

These RIs were generated from a free-ranging elephant population, meaning that most likely not all reference individuals were truly healthy, as indicated by the presence of band heterophils and some toxic changes. It is difficult to determine health under field conditions as only certain parameters are assessable and the animals are chemically immobilized. Above all, wild animals show signs of illness very late in the progression of disease. Parasitic loads will likely also differ from captive animals, as faecal examinations and prophylactic deworming schedules are common practice in captive animals (182). The above-described strict outlier elimination was performed to exclude most of the potentially "unhealthy" animals and gain the cleanest possible data set.

Another limitation of our study could be the age of the bio-banked samples, which were stored for up to five years at -80°C. The literature suggests that human serum samples can be stored for at least 13 months and plasma for up to 5 years at the same temperature (-80°C) without significant changes in the measurands (183, 184). To support these findings, the number of days from storage to analysis was calculated as well as the correlation between days in storage and measurand concentration or activity (Pearson's correlation coefficient, r). Apart from AST and CK (AST $r=0.69$, $p<0.001$; CK $r=0.50$, $p<0.001$), there was no correlation between storage time and measurand concentration/activity found for the other measurands. As these two enzymes were lower in the older samples, this could indicate a storage effect. This would have influenced the RIs by resulting in falsely lower LRLs. However, as low activity of these enzymes is not considered clinically relevant, this limitation is not likely to have an effect on the clinical utility of the RIs.

CHAPTER 6: CONCLUSIONS AND FUTURE DIRECTIONS

Reference intervals for haematology, clinical chemistry and APRs were established for the free-ranging African elephant, using appropriate statistical methods and a strict outlier approach. Therefore, our generated RIs are narrower than the ones currently available in the literature and will provide any researcher or clinician working with this species with a valuable diagnostic tool. Additionally, APRs were assessed in snared African elephants and we could show that all three APRs show distinctive changes in serum of free-ranging *Loxodonta africana* with tissue damages.

For future studies, the sample size will need to be increased, especially for the automated haematology analyses, as our sample size was relatively small, and the analysers were not validated for this species. Ideally, the same machine should be used for all samples, which in the best case is validated for the species analysed. Unfortunately, this was not possible for this study due to resource restrictions by the laboratory. The desirable sample size for all measurands would be 120, but above 39 is considered reasonable for RI studies, which we were able to achieve for blood smear analyses and WBC counts, as well as chemistry measurands and APRs. Larger sample sizes would allow partitioning of sexes, which was not possible in this study. Male samples were overly represented, since most samples were obtained opportunistically.

Further investigations in changes in serum chemistry and APRs are needed to improve assessments of injuries, as all injured elephants showed changes, except one. SAA could be identified as a major APR in the African elephant, HP as a moderate APP and iron as a negative APR. Ideally, these changes will be linked to the type, severity and chronicity of injury for better evaluation and prognosis. Therefore, follow up examinations should be emphasized wherever possible, especially in severely injured animals. Measurement of SAA or HP might not be possible for every animal in a zoo or under field conditions, but even adding serum iron measurement to the routine panel may provide valuable additional information and is usually available from a standard laboratory.

More than half of the injured animals also showed changes in clinical chemistry measurands, especially hypoalbuminaemia, hypomagnesaemia and hypocalcaemia. The same recommendation is applicable here: that for accurate grading, more studies will be needed, which should include follow-up measurements on the injured animals. In captive or rehabilitation settings, the presence of hypomagnesaemia, hypocalcaemia and hypoproteinaemia could be treated with adequate nutrition and supplementation of minerals. The assumed benefit to recovery could then be verified with repeated measurements.

CHAPTER 7: REFERENCES

1. Roca AL, Georgiadis N, Pecon-Slattery J, O'Brien SJ. Genetic evidence for two species of elephant in Africa. Science. 2001;293(5534):1473-7.

2. Eggert LS, Rasner CA, Woodruff DS. The evolution and phylogeography of the African elephant inferred from mitochondrial DNA sequence and nuclear microsatellite markers. Proc: Biol Sci. 2002;269(1504):1993-2006.

3. Blanc J. *Loxodonta africana*. The IUCN Red List of Threatened Species 2008. 2018 [accessed on: 10 August 2019] [Available from: https://dx.doi.org/10.2305/IUCN.UK.2008.RTLS.T12392A3339343.en].

4. Douglas-Hamilton I. African elephants: population trends and their causes. Oryx. 1987;21(1):11-24.

5. The status of the African Elephants 2018. Winter 2018 [accessed on 13 February 2019] [Available from: https://www.worldwildlife.org/magazine/issues/winter-2018/articles/the-status-of-african-elephants].

6. Chase MJ, Schlossberg S, Griffin CR, Bouché PJC, Djene SW, Elkan PW, et al. Continent-wide survey reveals massive decline in African savannah elephants. PeerJ. 2016;4:e2354-e.

7. Hughes JD. Europe as consumer of exotic biodiversity: Greek and Roman times. Landsc Res. 2003;28(1):21-31.

8. Wittemyer G, Northrup JM, Blanc J, Douglas-Hamilton I, Omondi P, Burnham KP. Illegal killing for ivory drives global decline in African elephants. Proc Natl Acad Sci USA. 2014;111(36):13117-21.

9. Sosnowski MC, Knowles TG, Takahashi T, Rooney NJ. Global ivory market prices since the 1989 CITES ban. Biol Conserv. 2019;237:392-9.

10. Gao Y, Clark SG. Elephant ivory trade in China: trends and drivers. Biol Conserv. 2014;180:23-30.

11. Wasser SK, Brown L, Mailand C, Mondol S, Clark W, Laurie C, et al. Genetic assignment of large seizures of elephant ivory reveals Africa's major poaching hotspots. Science 2015;349(6243):84-7.

12. Hauenstein S, Kshatriya M, Blanc J, Dormann C, Beale C. African elephant poaching rates correlate with local poverty, national corruption and global ivory price. Nat Commun. 2019;10:2242.

13. Biggs D, Holden M, Braczkowski A, Possingham H. Track the impact of Kenya's ivory burn. Nature. 2016;534:179.

14. Hilborn R, Arcese P, Borner M, Hando J, Hopcraft JGC, Loibooki M, et al. Effective enforcement in a conservation area. Science. 2006;314:1266.

15. Keane A, Jones J, Edwards-Jones G, Milner-Gulland E. The sleeping policeman: understanding issues of enforcement and compliance in conservation. Anim Conserv. 2008;11:75-82.

16. Stiles D. Elephant meat and ivory trade in central Africa. Pachyderm. 2011;50(1):26-36.

17. Adams W, Aveling R, Brockington D, Dickson B, Elliott J, Hutton J, et al. Biodiversity conservation and the eradication of poverty. Science 2004;306:1146-9.

18. Naidoo R, Weaver C, Diggle R, Matongo G, Stuart-Hill G, Thouless C. Complementary benefits of tourism and hunting to communal conservancies in Namibia. Conserv Biol. 2015;30.

19. Brooks JS. Design features and project age contribute to joint success in social, ecological, and economic outcomes of community-based conservation projects. Conserv Lett. 2017;10(1):23-32.

20. Thouless CR, Dublin HT, Blanc JJ, Skinner DP, Daniel TE, Taylor RD, et al. African Elephant Status Report 2016: an update from the African Elephant Database. Occasional Paper Series of the IUCN Species Survival Commission. 2016;No. 60IUCN/SSC Africa Elephant Specialist Group:vi+309pp.

21. Schlossberg S, Chase MJ, Griffin CR. Poaching and human encroachment reverse recovery of African savannah elephants in south-east Angola despite 14 years of peace. PLoS One. 2018;13(3):e0193469.

22. Schlossberg S, Chase MJ, Sutcliffe R. Evidence of a growing elephant poaching problem in Botswana. Curr Biol. 2019;29(13):2222-8e4.

23. Ferreira S, Greaver C, Simms C. Elephant population growth in Kruger National Park, South Africa, under a landscape management approach. Koedoe 2017;59(1):1-6.

24. Isilow H. Rhino, elephant poaching declines in South Africa. 2020.[accessed on 22 September 2020] [available from: https://www.aa.com.tr/en/africa/rhino-elephant-poaching-declines-in-south-africa/1723436].

25. Modise A. Minister Nomvula Mokonyane highlights progress on the implementation of the Integrated Strategic Management of Rhinoceros and other associated endangered species in SA. 2019 [accessed 13 February 2019] [Available from: https://www.environment.gov.za/mediarelease/mokonyane_2018integratedstrategic_managementofrhinoceros_2019feb_].

26. Graf R, Pfleiderer J, Fritsche M, Schmidt J, Mantei R, Peter S, et al. Zootierliste. 2019. [accessed 25 April 2020] [Available from: www.zootierliste.de].

27. Koehl D. The Elephant Database. 1995 [accessed 24 March 2019]. [Available from: https://www.elephant.se/].

28. de Mori B, Stagni E, Ferrante L, Vogt G, Ramsay KA, Normando S. Scientific and ethical issues in exporting welfare findings to different animal subpopulations: The case of semi-captive elephants involved in animal-visitor interactions (AVI) in South Africa. Animals. 2019;9(10):831.

29. Fowler ME. Foot disorders. In: Fowler ME Mikota S, editor. Biology, Medicine and Surgery of Elephants. Ames, Iowa: Blackwell Publishing; 2006. p. 271-90.

30. Keet D, Grobler D, Raath J, Gouws J, Carstens J, Nesbit J. Ulcerative pododermatitis in free-ranging African elephant (*Loxodonta africana*) in the Kruger National Park. Onderstepoort J Vet Res. 1997;64(1):25-32.

31. Edwards KL, Miller MA, Carlstead K, Brown JL. Relationships between housing and management factors and clinical health events in elephants in North American zoos. PloS one. 2019;14(6): e0217774.

32. Long SY, Latimer EM, Hayward GS. Review of Elephant Endotheliotropic Herpesviruses and Acute Hemorrhagic Disease. ILAR J. 2016;56(3):283-96.

33. Mikota SK, Peddie L, Peddie J, Isaza R, Dunker F, West G, et al. Epidemiology and diagnosis of *Mycobacterium tuberculosis* in captive Asian elephants. J Zoo Wildl Med. 2001;32(1):1-16, .

34. Zachariah A, Pandiyan J, Madhavilatha GK, Mundayoor S, Chandramohan B, Sajesh PK, et al. *Mycobacterium tuberculosis* in wild Asian elephants, Southern India. Emerg Infect Dis. 2017;23(3):504-6.

35. Simpson G, Zimmerman R, Shashkina E, Chen L, Richard M, Bradford CM, et al. *Mycobacterium tuberculosis* infection among Asian elephants in captivity. Emerg Infect Dis. 2017;23(3):513-6.

36. Miller MA, Buss P, Roos EO, Hausler G, Dippenaar A, Mitchell E, et al. Fatal Tuberculosis in a free-ranging African elephant and One Health implications of human pathogens in wildlife. Front Vet Sci. 2019;6:18-.

37. Kerr TJ, Waal CRd, Buss PE, Hofmeyr J, Lyashchenko KP, Miller MA. Seroprevalence of *Mycobacterium tuberculosis* complex in free-ranging African elephants (*Loxodonta africana*) in Kruger National Park, South Africa. J Wildl Dis. 2019;55(4):923-7.

38. Sunderman FW, Boerner F. Normal values in clinical medicine. Saunders and Co. Philadelphia and London: 1950.

39. Schneider AJ. Some thoughts on normal, or standard, values in clinical medicine. Pediatrics. 1960;26:973-84.

40. Gräsbeck R, Fellman J. Normal values and statistics. Scand J ClinLab Invest. 1968;21(3):193-5.

41. Gräsbeck R, Saris NE. Establishment and use of normal values. Scand J Clin Invest. 1969;Suppl 110:62-3.

42. Lumsden JH, Mullen K, McSherry BJ. Canine hematology and biochemistry reference values. Can J Comp Med. 1979;43(2):125-31.

43. Lumsden JH, Mullen K, Rowe R. Hematology and biochemistry reference values for female Holstein cattle. Can J Comp Med. 1980;44(1):24-31.

44. Solberg H. Approved recommendation (1986) on the theory of reference values. Part 1. The concept of reference values. Clin Chim Acta. 1987;165(1):111-8.

45. HE S, PetitClerc C. Approved recommendation (1987) on the theory of reference values. Part 2. Selection of individuals for the production of reference values. Clin Chim Acta. 1987;139(2-3):S1-S11.

46. Solberg HE. Approved recommendation (1987) on the theory of reference values. Part 5. Statistical treatment of collected reference values. Determination of reference limits. Clin Chim Acta. 1987;170(2-3):S13-S32.

47. Dybkœr R, Solberg HE. Approved recommendation (1987) on the theory of reference values. Part 6. Presentation of observed values related to reference values. Clin Chim Acta. 1987;170(2-3):S33-S41.

48. Solberg HE, Stamm D. IFCC recommendation: - theory of reference values. Part 4. Control of analytical variation in the production, transfer and application of reference values. Clin Chim Acta 1991;202(1-2):S5-S11.

49. Solberg HE, PetitClerc C. Approved recommendation (1988) on the theory of reference values. Part 3. Preparation of individuals and collection of specimens for the production of reference values. Clin Chim Acta. 1988;177(3):S3-S11.

50. Horowitz G. Defining, Establishing, and Verifying Reference Intervals in the Clinical Laboratory; Approved Guidelines, CLSI document C28-A3, Vol. 28, No. 3. 3rd ed. Wayne, PA: Clinical and Laboratory Standards Institute; 2008. 61 p.

51. Geffre A, Friedrichs K, Harr K, Concordet D, Trumel C, Braun JP. Reference values: a review. Vet Clin Pathol. 2009;38(3):288-98.

52. Friedrichs KR, Harr KE, Freeman KP, Szladovits B, Walton RM, Barnhart KF, et al. ASVCP reference interval guidelines: determination of *de novo* reference intervals in veterinary species and other related topics. Vet Clin Pathol. 2012;41(4):441-53.

53. Flatland B, Freeman KP, Friedrichs KR, Vap LM, Getzy KM, Evans EW, et al. ASVCP quality assurance guidelines: control of general analytical factors in veterinary laboratories. Vet Clin Pathol. 2010;39(3):264-77.

54. Solberg HE, Lahti A. Detection of outliers in reference distributions: Performance of Horn's algorithm. Clin Chem. 2005;51(12):2326-32.

55. Geffre A, Concordet D, Braun JP, Trumel C. Reference Value Advisor: a new freeware set of macroinstructions to calculate reference intervals with Microsoft Excel. Vet Clin Pathol. 2011;40(1):107-12.

56. Harr K, Deak K, Murawski S, Reavill D, Takeshita R. Generation of red drum (*Sciaenops ocellatus*) hematology reference intervals with a focus on identified outliers. Vet Clin Pathol. 2018;47(1):22-8.

57. Debbie JG, Clausen B. Some hematological values of free-ranging African elephants. J Wildl Dis. 1975;11(1):79-82.

58. Brown IR, White PT. Serum enzyme activities in the African elephant (*Loxodonta africana*). Experientia. 1976;32(8):980-2.

59. Brown IRF, White PT. Serum calcium, magnesium, phosphorus and alkaline phosphatase in the african elephant, *Loxodonta africana*. Comp Biochem Phys B. 1977;56(2):159-62.

60. Brown IRF, White PT. Serum electrolytes, lipids and cortisol in the African elephant, *Loxodonta africana*. Comp Biochem Physi A. 1979;62(4):899-901.

61. Brown IRF, White PT. Elephant blood haematology and chemistry. Comp Biochem Phys B. 1980;65(1):1-12.

62. Brown IRF, White PT, Malpas RC. Proteins and other nitrogenous constituents in the blood serum of the African elephant, *Loxodonta africana*. Comp Biochem Phys A. 1978;59(3):267-70.

63. Hattingh J, Wright PG, de Vos V, McNairn IS, Ganhao MF, Silove M, et al. Blood composition in culled elephants and buffaloes. J S Afr Vet Assoc. 1984;55(4):157-64.

64. Allen JL, Jacobson ER, Harvey JW, Boyce W. Hematologic and serum chemical values for young African elephants (*Loxodonta africana*) with variations for sex and age. J Zoo Anim Med. 1985;16(3):98-101.

65. Hill F, Smith D. Clinical chemistry values for free-ranging elephants (*Loxodonta africana*) in Hwange National Park, Zimbabwe. Zimb Vet J. 1990;21(1):33-42.

66. Kock MD, Martin RB, Kock N. Chemical immobilization of free-ranging African elephants (*Loxodonta africana*) in Zimbabwe, using etorphine (M99) mixed with hyaluronidase, and evaluation of biological data collected soon after immobilization. J Zoo Wildl Med. 1993;24(1):1-10.

67. White1* P, Brown1 IF. Haematological studies on wild African elephants (*Loxodonta africana*). J Zool. 1978;185(4):491-503.

68. Benga G, Kuchel PW, Chapman BE, Cox GC, Ghiran I, Gallagher CH. Comparative cell shape and diffusional water permeability of red blood cells from Indian elephant (*Elephas maximus*) and man (*Homo sapiens*). Comp Haematol Int. 2000;10(1):1-8.

69. Harr KE, Isaza R, Blue JT. Hematology of Elephants. In: Weiss DJ, Wardrop KJ, Schalm OW editors. Schalm's Veterinary Hematology. 6th ed. Ames, Iowa: Wiley-Blackwell; 2010:p942-9.

70. Harvey JW, Harr KE, Murphy D, Walsh MT, Nolan EC, Bonde RK, et al. Hematology of healthy Florida manatees (*Trichechus manatus*). Vet Clin Pathol. 2009;38(2):183-93.

71. Aroch I, King R, Baneth G. Hematology and serum biochemistry values of trapped, healthy, free-ranging rock hyraxes (*Procavia capensis*) and their association with age, sex, and gestational status. Vet Clinical Pathol. 2007;36(1):40-8.

72. Bartels H, Hilpert P, Barbey K, Betke K, Riegel K, Lang EM, Metcalfe J. Respiratory functions of blood of the yak, lama, camel, Dybowski deer and African elephant. Am J Physiol. 1963;205:331-6.

73. Young E LC. Physiological values of the African elephant (*Loxodonta africana*). The Veterinarian. 1967;4:169-72.

74. Woodford MH. Blood characteristics of the African elephant (*Loxodonta africana cyclotis*). J Wildl Dis. 1979;15(1):111-3.

75. Lewis JH. Comparative hematology: studies on elephants, *Elephas maximus*. Comp Biochem Physiol A. 1974;49(1):175-81.

76. Gromadzka-Ostrowska J, Jakubów K, Zalewska B, Krzywicki Z. Haematological and blood biochemical studies in female domesticated Indian elephants (*Elaphas maximus I*.). Comp Biochem Physiol A. 1988;89(3):313-5.

77. Niemuller C, Gentry PA, Liptrap RM. Longitudinal study of haematological and biochemical constituents in blood of the Asian elephant (*Elephas maximus*). Comp Biochem Physiol A 1990;96(1):131-4.

78. Silva ID, Kuruwita VY. Hematology, plasma, and serum biochemistry values in domesticated elephants (*Elephas maximus ceylonicus*) in Sri Lanka. J Zoo Wildl Med. 1993;24(4):440-4.

79. Silva ID, Kuruwita VY. Hematology, plasma, and serum biochemistry values in free-ranging elephants (*Elephas maximus ceylonicus*) in Sri Lanka. J Zoo Wildl Med. 1993;24(4):434-9.

80. Salakij J, Salakij C, Narkkong N-a, Apibal S, Suthunmapinuntra P, Rattanakukuprakarn J, et al. Hematology, cytochemistry and ultrastructure of blood cells from Asian elephant (*Elephas maximus*). Witthayasan Kasetsat. 2004;39:482-93.

81. Janyamethakul T SS, Somgird C, Pongsopawijit P, Panyapornwithaya P, Klinhom S, Loythong J, Thitaram C. Hematologic and biochemical reference intervals for captive Asian elephants (*Elephas maximus*) in Thailand. Kafkas Univ Vet Fak Derg. 2017;23(4):665-8.

82. Santos DJFd, Jackson J, Phil M, Aung HH, Nyein UK, Htut W, et al. Sex differences in the reference intervals of health parameters in semicaptive Asian elephants (*Elephas maximus*) from Myanmar. J Zoo Wildl Med. 2020;51(1):25-38, 14.

83. Morfeld KA, Brown JL. Metabolic health assessment of zoo elephants: Management factors predicting leptin levels and the glucose-to-insulin ratio and their associations with health parameters. PloS one. 2017;12(11):e0188701-e.

84. Mikota SK. Hemolymphatic System. In: Fowler ME, Mikota SK, editor. Biology, Medicine, and Surgery of Elephants. Ames, Iowa: Blackwell Publishing; 2006. p. 325-45.

85. Codron J, Lee-Thorp JA, Sponheimer M, Codron D, Grant RC, de Ruiter DJ. Elephant (Loxodonta africana) diets in Kruger National Park, South Africa: spatial and landscape differences. Journal of mammalogy. 2006;87(1):27-34.

86. Sach F, Dierenfeld ES, Langley-Evans SC, Watts MJ, Yon L. African savanna elephants (*Loxodonta africana*) as an example of a herbivore making movement choices based on nutritional needs. PeerJ. 2019;7:e6260.

87. Bowell R, Warren A, Redmond I. Formation of cave salts and utilization by elephants in the Mount Elgon region, Kenya. Geol Soc Spec Publ. 1996;113(1):63-79.

88. Dierenfeld E. Nutrition. In: Fowler ME, Mikota SK, editors. Biology, Medicine, and Surgery of Elephants. Ames, Iowa: Wiley-Blackwell Publishing; 2006. p. 57-65.

89. van der Kolk JH, van Leeuwen JP, van den Belt AJ, van Schaik RH, Schaftenaar W. Subclinical hypocalcaemia in captive Asian elephants (*Elephas maximus*). Vet Rec. 2008;162(15):475-9.

90. Schaftenaar W. The challenge of obtaining reference values for use in captive animals like elephants. Vet Q. 2020;40(1):115-7.

91. Stockham SL, Scott MA. Fundamentals of veterinary clinical pathology. Ames, Iowa: Blackwell Publishing; 2008.

92. Vetscan VS2 Chemistry Analyzer USA. c2020 [accessed 29 September 2020] [Available from: https://www2.zoetisus.com/diagnostics/instruments/vetscan-vs2].

93. *Loxodonta africana* No_selection_by_gender_All_ages_combined_Standard_International_Units_2013_CD. Bloomington: Species360; 2013. p. Species360.

94. Ceron JJ, Eckersall PD, Martynez-Subiela S. Acute phase proteins in dogs and cats: current knowledge and future perspectives. Vet Clin Pathol. 2005;34(2):85-99.

95. Baumann H, Gauldie J. The acute phase response. Immunol Today. 1994;15(2):74-80.

96. Eckersall D. Acute phase proteins as markers of inflammatory lesions. Comp Haematol Int. 1995;5:93-7.

97. Kaneko JJ. Chapter 5 - Serum proteins and the dysproteinemias. In: Kaneko JJ, Harvey JW, Bruss ML, editors. Clinical biochemistry of domestic animals 5th edition. San Diego, California: Academic Press; 1997. p. 117-38.

98. Cray C, Zaias J, Altman NH. Acute phase response in animals: a review. Comp Med. 2009;59(6):517-26.

99. Jain S, Gautam V, Naseem S. Acute-phase proteins: as diagnostic tool. J Pharm Bioallied Sci. 2011;3(1):118-27.

100. Tillett WS, Francis T. Serological reactions in pneumonia with a non-protein somatic fraction of pneumococcus. J Exp Med. 1930;52(4):561-71.

101. Abernethy TJ, Avery OT. The occurrence during acute infections of a protein not normally present in the blood: I. Distribution of the reactive protein in patients'

sera and the effect of calcium on the flocculation reaction with polysaccaride of phneumococcus. J Exp Med. 1941;73(2):173-82.

102. Pepys MB. C-reactive protein fifty years on. Lancet. 1981;317(8221):653-7.

103. Dahl M, Vestbo J, Lange P, Bojesen SE, Tybjærg-Hansen A, Nordestgaard BG. C-reactive protein as a predictor of prognosis in chronic obstructive pulmonary disease. Am J Respir Crit Care med. 2007;175(3):250-5.

104. Jin X, Beguerie JR, Zhang W, Blizzard L, Otahal P, Jones G, et al. Circulating C reactive protein in osteoarthritis: a systematic review and meta-analysis. Ann Rheum Dis. 2015;74(4):703-10.

105. Magro F, Sousa P, Ministro P. C-reactive protein in Crohn's disease: how informative is it? Expert Rev Gastroenterol Hepatol. 2014;8(4):393-408.

106. Mahmoud FA, Rivera NI. The role of C-reactive protein as a prognostic indicator in advanced cancer. Curr Oncol Rep. 2002;4(3):250-5.

107. Kao PC, Shiesh SC, Wu TJ. Serum C-reactive protein as a marker for wellness assessment. Ann Clin Lab Sci. 2006;36(2):163-9.

108. Esposito K, Marfella R, Ciotola M, Di Palo C, Giugliano F, Giugliano G, et al. Effect of a mediterranean-style diet on endothelial dysfunction and markers of vascular inflammation in the metabolic syndrome: a randomized trial. JAMA. 2004;292(12):1440-6.

109. Horadagoda NU, Knox KM, Gibbs HA, Reid SW, Horadagoda A, Edwards S, et al. Acute phase proteins in cattle: discrimination between acute and chronic inflammation. Vet Rec. 1999;144(16):437-41.

110. Murata H, Shimada N, Yoshioka M. Current research on acute phase proteins in veterinary diagnosis: an overview. Vet J. 2004;168(1):28-40.

111. Jacobsen S, Andersen PH. The acute phase protein serum amyloid A (SAA) as a marker of inflammation in horses. Equine Vet Educ. 2007;19(1):38-46.

112. Eckersall PD, Bell R. Acute phase proteins: Biomarkers of infection and inflammation in veterinary medicine. Vet J. 2010;185(1):23-7.

113. Kontoghiorghes G, Weinberg E. Iron: mammalian defense systems, mechanisms of disease, and chelation therapy approaches. Blood Rev. 1995;9(1):33-45.

114. Petersen HH, Nielsen JP, Heegaard PM. Application of acute phase protein measurements in veterinary clinical chemistry. Vet Res. 2004;35(2):163-87.

115. Eiken Chemical Co. L. Serum amyloid A (SAA) assay for multispecies. c2020 [accessed 29 September 2020] [Available from: https://www.eiken.co.jp/en/products/vet_saa/].

116. Javard R, Grimes C, Bau-Gaudreault L, Dunn M. Acute-phase proteins and iron status in cats with chronic kidney disease. J Vet Intern Med. 2017;31(2):457-64.

117. Selting K, Ogilvie G, Lana S, Fettman M, Mitchener K, Hansen RA, et al. Serum alpha 1-acid glycoprotein concentrations in healthy and tumor bearing cats. J Vet Intern Med. 2000;14:503-6.

118. Sasaki K, Ma Z, Khatlani TS, Okuda M, Inokuma H, Onishi T. Evaluation of feline serum amyloid A (SAA) as an inflammatory marker. J Vet Med Sci. 2003;65(4):545-8.

119. Eckersall PD, Young FJ, McComb C, Hogarth CJ, Safi S, Weber A, et al. Acute phase proteins in serum and milk from dairy cows with clinical mastitis. Vet Rec. 2001;148(2):35-41.

120. Kováč G, Tóthová C, Nagy O, Seidel H. Milk amyloid A and selected serum proteins in cows suffering from mastitis. Acta Vet Brno. 2011;80(1):3-9.

121. Ilievska K, Atanasov B, Dovenski T, Smolac O, Stojanov B, Trojachanec P. Acute phase proteins – as indicators of claw diseases in dairy cattle. Maced Vet Rev. 2019;42(1):i-vi.

122. Murata H. Stress and acute phase protein response: an inconspicuous but essential linkage. Vet J. 2007;173(3):473-4.

123. Gånheim C, Alenius S, Persson Waller K. Acute phase proteins as indicators of calf herd health. Vet J. 2007;173(3):645-51.

124. Depauw S, Delanghe J, Whitehouse-Tedd K, Kjelgaard-Hansen M, Christensen M, Hesta M, et al. Serum protein capillary electrophoresis and measurement of acute phase proteins in a captive cheetah (*Acinonyx jubatus*) population. J Zoo Wildl Med. 2014;45(3):497-506.

125. Cray C, Dickey M, Norton T, Murray M, Barysauskas C, Arheart K, et al. Assessment of hemoglobin binding protein in loggerhead sea turtles (*Caretta caretta*) undergoing rehabilitation. J Zoo Wildl Med. 2014;45(3):700-3.

126. Krol L, Allender M, Cray C, George R. Plasma proteins and selected acute-phase proteins in the whitespotted bamboo shark (*Chiloscyllium plagisoum*). J Zoo Wildl Med. 2014;45(4):782-6.

127. Baldrey V, Verghese R, Wernery U, Bailey T. Acute phase proteins in three healthy antelope species. Vet Rec. 2011;170:54.

128. Cray C, Hammond E, Haefele H. Acute phase protein and protein electrophoresis values for captive Grant's zebra (*Equus burchelli*). J Zoo Wildl Med. 2013;44(4):1107-10.

129. Cray C, Dickey M, Norton T, Murray M, Barysauskas C, Arheart K, et al. Assessment of hemoglobin binding protein in loggerhead sea turtles (*Caretta caretta*) undergoing rehabilitation. J Zoo Wildl Med. 2014;45:700-3.

130. Gelain ME, Bonsembiante F. Acute phase proteins in marine mammals: state of art, perspectives and challenges. Front Immunol 2019;10(1220).

131. Kakuschke A, Erbsloeh HB, Griesel S, Prange A. Acute phase protein haptoglobin in blood plasma samples of harbour seals (*Phoca vitulina*) of the Wadden Sea and of the isle Helgoland. Comp Biochem Physiol B: Biochem Mol Biol. 2010;155(1):67-71.

132. Thomton JD, Mellish JA. Haptoglobin concentrations in free-range and temporarily captive juvenile steller sea lions. J Wildl Dis. 2007;43(2):258-61.

133. Krafft BA, Lydersen C, Kovacs KM. Serum haptoglobin concentrations in ringed seals (*Pusa hispida*) from Svalbard, Norway. J Wildl Dis. 2006;42(2):442-6.

134. Harr K, Harvey J, Bonde R, Murphy D, Lowe M, Menchaca M, et al. Comparison of methods used to diagnose gneralized inflammatory disease in manatees (*Trichechus manatus latirostris*). J Zoo Wildl Med. 2006;37(2):151-9.

135. Harr KE, Rember R, Ginn PE, Lightsey J, Keller M, Reid J, et al. Serum amyloid A (SAA) as a biomarker of chronic infection due to boat strike trauma in a free-ranging Florida manatee *(Trichechus manatus latirostris*) with incidental polycystic kidneys. J Wildl Dis. 2011;47(4):1026-31.

136. Hooijberg EH, Cray C, Steenkamp G, Buss P, Goddard A, Miller M. Assessment of the acute phase response in healthy and injured southern white rhinoceros (*Ceratotherium simum simum*). Front Vet Sci. 2020;6:475.

137. Stanton JJ, Cray C, Rodriguez M, Arheart KL, Ling PD, Herron A. Acute phase protein expression during elephant endotheliotropic herpesvirus-1 viremia in Asian elephants (*Elephas maximus*). J Zoo Wildl Med. 2013;44(3):605-12.

138. Edwards KL, Latimer EM, Siegel-Willot JL, Glaeser SS, JL B, editors. Acute phase proteins as indicators of the elephant immune response to infectious disease. 16th International Elephant Conservation & Research Symposium; 2019; South Africa;p.471-486

139. Isaza R, Wiedner E, Hiser S, Cray C. Reference intervals for acute phase protein and serum protein electrophoresis values in captive Asian elephants (*Elephas maximus*). J Vet Diag Invest. 2014;26(5):616-21.

140. Harr K, Harvey J, Bonde R, Murphy D, Lowe M, Menchaca M, et al. Comparison of methods used to diagnose generalized inflammatory disease in manatees (*Trichechus manatus latirostris*). J Zoo Wildl Med. 2006;37(2):151-9.

141. Cray C, Rodriguez M, Zaias J, Altman NH. Effects of storage temperature and time on clinical biochemical parameters from rat serum. J Am Assoc Lab Anim Sci. 2009;48(2):202-4.

142. Horiba. ABX Micros ESV 60, Hematology Analyzer. Oberursel, Germany; c2020 [accessed on 22 September 2020] [Available from:

https://www.horiba.com/en_en/products/detail/action/show/Product/abx-micros-esv-60-506/].

143. Harvey JW. Chapter 1 - Examination of Blood Samples. In: Harvey JW, editor. Atlas of veterinary hematology. Philadelphia: W.B. Saunders; 2001. p. 3-20.

144. Weiss DJ, Wardrop KJ. Atlas of veterinary hematology. 6th ed. Ames, Iowa: Wiley-Blackwell; 2011.

145. Stacy NI, Isaza R, Wiedner E. First report of changes in leukocyte morphology in response to inflammatory conditions in Asian and African elephants (*Elephas maximus* and *Loxodonta africana)*. Plos One. 2017;12(9):e0185277.

146. Abaxis I. VetScan operator's manual Union City, CA; 2013 [accessed 22 September 2020] [Available from: https://www.zoetisus.com/products/diagnostics/vetscan/product-data/vs2/technical-papers/vetscan-chemistry-system-methods-of-performance.pdf].

147. Harr KE, Flatland B, Nabity M, Freeman KP. ASVCP guidelines: allowable total error guidelines for biochemistry. Vet Clinl Pathol. 2013;42(4):424-36.

148. Arnold JE, Camus MS, Freeman KP, Giori L, Hooijberg EH, Jeffery U, et al. ASVCP guidelines: Principles of quality assurance and standards for veterinary clinical pathology (version 3.0). Vet Clin Pathol. 2019;48(4):542-618.

149. Le Boedec K. Sensitivity and specificity of normality tests and consequences on reference interval accuracy at small sample size: a computer-simulation study. Vet Clin Pathol. 2016;45(4):648-56.

150. Le Boedec K. Reference interval estimation of small sample sizes: A methodologic comparison using a computer-simulation study. Vet Clin Pathol. 2019;48(2):335-46.

151. Nirmalan G, Nair SG, Simon KJ. Hematology of the Indian elephant (*Elephas maximus*). Can J Physiol Pharmacol. 1967; 45(6):985-91

152. Codron J, Lee-Thorp JA, Sponheimer M, Codron D, Grant RC, de Ruiter DJ. Elephant (*Loxodonta africana*) diets in Kruger National Park, South Africa: Spatial and landscape differences. J Mammal. 2006;87(1):27-34.

153. Kaneko J, Harvey J, Bruss M. Clinical biochemistry of domestic animals. San Diego, California: Academy Press; 1997.

154. Marco I, Vinas L, Velarde R, Pastor J, Lavin S. Effects of capture and transport on blood parameters in free-ranging mouflon (*Ovis ammon*). J Zoo Wildl Med. 1997;28(4):428-33.

155. Kock MD, du Toit R, Kock N, Morton D, Foggin C, Paul B. Effects of capture and translocation on biological parameters in free-ranging black rhinoceroses (*Diceros bicornis*) in Zimbabwe. J Zoo Wildl Med. 1990;21(4):414-24.

156. Aktas M, Auguste D, Lefebvre H, Toutain P-L, Braun J-P. Creatine kinase in the dog: a review. Vet Res Commun. 1993;17:353-69.

157. Hoffmann WE, Solter PF. Chapter 12 - Diagnostic enzymology of domestic animals. In: Kaneko JJ, Harvey JW, Bruss ML, editors. Clinical biochemistry of domestic animals 6th. San Diego: Academic Press; 2008. p. 351-78.

158. Szabo MP, Matushima ER, de Castro MB, Santana DA, de Paula CD, Duarte JM. Hematology of free-living marsh deer (*Blastocerus dichotomus*) from southeast Brazil. J Zoo Wildl Med. 2005;36(3):463-9.

159. Presidente PJA, Lumsden JH, Presnell KR, Rapley WA, McCraw BM. Combination of etorphine and xylazine in captive white-tailed deer: II. Effects of hematologic, serum biochemical and blood gas values. J Wildl Dis. 1973;9(4):342-8.

160. Knowles TG, Warriss PD, Brown SN, Edwards JE, Watkins PE, Phillips AJ. Effects on calves less than one month old of feeding or not feeding them during road transport of up to 24 hours. Vet Rec. 1997;140(5):116-24.

161. Marco I, LavÍN S. Effect of the method of capture on the haematology and blood chemistry of red deer (*Cervus elaphus*). Res Vet Sci. 1999;66(2):81-4.

162. Valli VE, Jacobs RM. Structure and function of the hemopoietic system. In: Feldmann BF, Zinkl JG, Jain NC, Schalm OW editor. Schalm's Veterinary Hematology. 5th. Philadelphia, Lippincott: Williams and Wilkins; 2000. p. 225-39.

163. Still J, Raath JP, Matzner L. Respiratory and circulatory parameters of African elephants (*Loxodonta africana*) anaesthetised with etorphine and azaperone. J S Afr Vet Assoc. 1996;67(3):123-7.

164. Gaudio E, Laubscher LL, Pfitzer S, Raath JP, Hoffman LC, De Benedictis GM. Immobilisation quality and cardiopulmonary effects of etorphine alone compared with etorphine-azaperone in blesbok (*Damaliscus pygargus phillipsi*). Vet Anaesth Anal. 2020;47(4):528-36.

165. Vitali F, Kariuki EK, Mijele D, Kaitho T, Faustini M, Preziosi R, et al. Etorphine-azaperone immobilisation for translocation of free-ranging Masai giraffes (*Giraffa Camelopardalis Tippelskirchi*): a pilot study. Animals. 2020;10(2):322.

166. Buss P, Miller M, Fuller A, Haw A, Wanty R, Olea-Popelka F, et al. Cardiovascular effects of etorphine, azaperone and butorphanol combinations in chemically immobilized captive white rhinoceros (*Ceratotherium simum*) J Zoo Wildl Med. 2016;47(3):834-43.

167. Schumacher J, Heard DJ, Caligiuri R, Norton T, Jacobson ER. Comparative effects of etorphine and carfentanil on cardiopulmonary parameters in juvenile African elephants (*Loxodonta africana*). J Zoo Wildl Med. 1995;26(4):503-7.

168. Drevemo S, Karstad L. The effect of xylazine and xylazine-etorphine-acepromazine combination on some clinical and haematological parameters in impala and eland. J Wildl Dis. 1974;10(4):377-83.

169. Couch CE, Movius MA, Jolles AE, Gorman ME, Rigas JD, Beechler BR. Serum biochemistry panels in African buffalo: defining reference intervals and assessing variability across season, age and sex. PloS one. 2017;12(5):e0176830-e.

170. Bertelsen MF, Kjelgaard-Hansen M, Grøndahl C, Heegaard PM, Jacobsen S. Identification of acute phase proteins and assays applicable in nondomesticated mammals. J Zoo Wildl Med. 2009;40(1):199-203.

171. Kimmel SE, Waddell LS, Michel KE. Hypomagnesemia and hypocalcemia associated with protein-losing enteropathy in Yorkshire terriers: five cases (1992-1998). J Am Vet Med Assoc. 2000;217(5):703-6.

172. Coelho C, Fonseca L, Souza V. Prevalence of hypocalcemia and hypomagnesemia in equines with gastrointestinal diseases. Arq Bras Med Vet Zootec. 2012;64(3):755-8.

173. Garcia-Lopez JM, Provost PJ, Rush JE, Zicker SC, Burmaster H, Freeman LM. Prevalence and prognostic importance of hypomagnesemia and hypocalcemia in horses that have colic surgery. Am J Vet Res. 2001;62(1):7-12.

174. Berger MM, Rothen C, Cavadini C, Chiolero RL. Exudative mineral losses after serious burns: a clue to the alterations of magnesium and phosphate metabolism. Am J Clin Nutr. 1997;65(5):1473-81.

175. Tso EL, Barish RA. Magnesium: clinical considerations. J Emerg Med. 1992;10(6):735-45.

176. Stockham SL, Scott MA. Chapter 12 - Enzymes. In: Stockham SL, Scott MA editor. Fundamentals of veterinary clinical pathology. 2nd ed: Wiley Blackwell; 2008. p. 639-674

177. Gymnich S, Knura-Deszczka S, Wimmers K, Bidlingmaier M, Schellander K, Petersen B. Haptoglobin as an indicator for animal welfare: Effects of different hygienic conditions and transport stress on haptoglobin plasma concentration. Acta Vet Scand. 2003;44(Suppl1).

178. Fitzsimons EJ, Levine SR. Rapid drop in serum iron concentration associated with stress. Am J Med Sci. 1983;285(2):12-7.

179. Baydar E, Dabak M. Serum iron as an indicator of acute inflammation in cattle. J Dairy Sci. 2013;97(1):222-8.

180. Jacobsen S, Nielsen JV, Kjelgaard-Hansen M, Toelboell T, Fjeldborg J, Halling-Thomsen M, et al. Acute phase response to surgery of varying intensity in horses: A preliminary study. Vet Surg 2009;38(6):762-9.

181. Neumann S. Serum iron level as an indicator for inflammation in dogs and cats. Comp Clin Pathol. 2003;12(2):90-4.

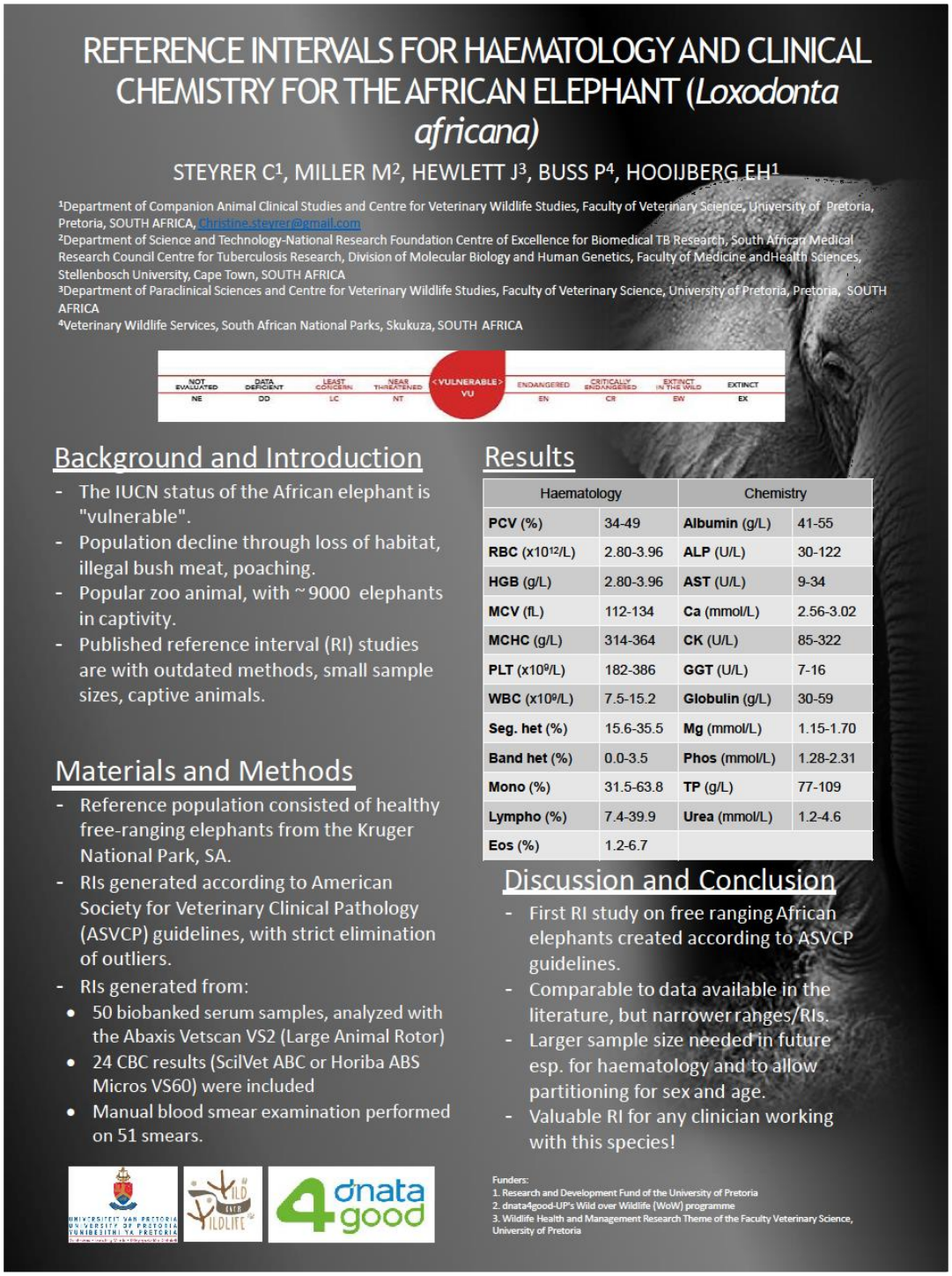
182. Mikota S. Preventive health care and physical examination. In: Mikota S, Fowler ME, editors. Biology, medicine, and surgery of elephants. Ames, Iowa: Wiley-Blackwell; 2006. p. 67-73.

183. Brinc D, Chan MK, Venner AA, Pasic MD, Colantonio D, Kyriakopolou L, et al. Long-term stability of biochemical markers in pediatric serum specimens stored at -80 °C: a CALIPER Substudy. Clin Biochem. 2012;45(10-11):816-26.

184. Clark S, Youngman LD, Palmer A, Parish S, Peto R, Collins R. Stability of plasma analytes after delayed separation of whole blood: implications for epidemiological studies. Int J Epidemiol. 2003;32(1):125-30.

APPENDICES

Poster presentation related to this thesis: Reference intervals for haematology and clinical chemistry for the African elephant *(Loxodonta africana);* European Association of Zoo and Wildlife Veterinarians; Zoo and Wildlife Health Online Conference, July 2020



UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Veterinary Science
Animal Ethics Committee

3 September 2019

Approval Certificate
New Application

AEC Reference No.: REC132-19
Title: Reference intervals for haematology, clinical chemistry and acute phase reactants in the African Elephant, Loxodonta africana
Researcher: Dr C Steyrer
Student's Supervisor: Prof EH Hooijberg

Dear Dr C Steyrer,

The **New Application** as supported by documents received between 2019-07-02 and 2019-08-26 for your research, was approved by the Animal Ethics Committee on its quorate meeting of 2019-08-26.

Please note the following about your ethics approval:

1. The use of species is approved:

Species and Samples	Number
Elephants (Loxodonta africana)	(collected from immobilized elephants)
Blood smears (stored samples)	140
Serum (stored samples only)	140

2. Ethics Approval is valid for 1 year and needs to be renewed annually by 2020-09-03.
3. Please remember to use your protocol number (REC132-19) on any documents or correspondence with the AEC regarding your research.
4. Please note that the AEC may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.

Ethics approval is subject to the following:

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.
Yours sincerely

Prof V Naidoo
CHAIRMAN: UP-Animal Ethics Committee

Room 6-13, Arnold Theiler Building, Onderstepoort
Private Bag X04, Onderstepoort 0110, South Africa
Tel: +27 (12) 529 8403
Fax +27 (12) 529 8323
Email [illegible]
www.up.ac.za

Fakulteit Veeartsenykunde
Lefapha la Disearse tša Bongakadiruiwa