Cardiac troponin I immunoassay validation, reference interval determination and serum cardiac troponin I changes in translocated southern-central black rhinoceros (*Diceros bicornis minor*) and southern white rhinoceros (*Ceratotherium simum simum*)

by

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DECLARATION

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ETHICS STATEMENT

The author, Yolandi Rautenbach, has obtained the required ethics approvals for the research described in this thesis. These approvals were granted by the Research Ethics Committee of the Faculty of Veterinary Science and Animal Ethics Committee of the University of Pretoria (REC205-21 and REC057-23).

The author declares that she has observed the ethical standards required in terms of the University of Pretoria's code of ethics for researchers and the policy guidelines for responsible research.

DEDICATION

G.J. & M. van Tonder

Genesis 2:15
Die Here God het die mens geneem en hom in die tuin van Eden geplaas om dit te bewerk en op te pas.

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LIST OF ABBREVIATIONS

AAB	American Association of Bioanalysts
Abs	Antibodies
AST	Aspartate aminotransferase
ASVCP	American Society for Veterinary Clinical Pathology
BHB	β-hydroxybutyrate
BLAST	Basic logical alignment search tool
bp	Base pair
BV	Biological variation
cDNA	Complementary DNA
CDS	Coding sequence
CI	Confidence interval
CITES	Convention on International Trade in Endangered Species of Wild Fauna and Flora
CK	Creatine kinase
CLIA	Clinical Laboratory Improvement Amendment ’88 Proficiency Testing Limits, US Federal Register
CLSI	Clinical and Laboratory Standards Institute
cm	Centimeters
CM	Capture myopathy
cTn	Cardiac troponin
cTnI	Cardiac troponin I

cTnT	Cardiac troponin T
CV	Coefficient of variation
DNA	Deoxyribonucleic acid
EAzaB	Etorphine and azaperone intramuscular, then butorphanol intravenous
EB	Etorphine intramuscular, then butorphanol intravenous
EDTA	Ethylenediaminetetraacetic acid
EMedB	Etorphine and medetomidine intramuscular, then butorphanol intravenous
EMidB	Etorphine and midazolam intramuscular, then butorphanol intravenous
hs-cTnI	High-sensitivity cardiac troponin I
FDA	United States of America Food and Drug Administration
FVS	Faculty of Veterinary Science
G	Gaussian
GABA	Gamma-aminobutyric acid
IFCC	International Federation of Clinical Chemistry
IM	Intramuscular
IQR	Interquartile range
IUCN	International Union for Conservation of Nature
IV	Intravenous
kDa	Kilodalton
km	Kilometre
LoB	Limit of blank
LoD	Limit of detection

LoQ	Limit of quantification
LRL	Lower reference limit
Ma	Mega annum/ 1 million years
MEGA	Molecular Evolutionary Genetics Analysis
MI	Myocardial infarction
min	Minutes
mRNA	Messenger RNA
MSIMI	Mental stress-induced myocardial ischaemia
N* or *n	Number of levels of quality control material
NCBI	National Centre for Biotechnology Information
NG	Non-Gaussian
NP	Non-parametric method
O_2	Oxygen
OD	Optical density
OPSpec	Operating process specifications
P	Parametric method
PaO_2	Partial pressure of oxygen
PCR	Polymerase chain reaction
P_{ed}	Probability of error detection
P_{fr}	Probability of false rejection
POC	Point-of-care
QA	Quality assurance
QC	Quality control

QCM	Quality control material
r	Correlation coefficient
r^2	Coefficient of determination
RE	Random error
REC	University of Pretoria Animal Research and Ethic Committee
Rho	Spearman's rank correlation coefficient
RI	Reference interval
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT-PCR	Reverse transcription PCR
s	Seconds
SD	Standard deviation
SE	Systematic error
skTnI	Skeletal troponin I
TE_a	Total allowable error
TE_{obs}	Total observed error
TF-CB	Task Force on Clinical Applications of Biomarkers
URL	Upper reference limit
99^{th} URLs	99^{th} percentile upper reference limits
US	United States of America
V/Q	Ventilation-perfusion
α	Alpha
σ	Sigma metric

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SUMMARY

Cardiac troponin I immunoassay validation, reference interval determination and serum cardiac troponin I changes in translocated southern-central black rhinoceros (*Diceros bicornis minor*) and southern white rhinoceros (*Ceratotherium simum simum*)

By

Yolandi Rautenbach

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Co-promoters:	Prof. Leith C.R. Meyer (University of Pretoria)
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Department:	Companion Animal Clinical Studies
Degree:	PhD

The abundance and range of occurrence of the two rhinoceros species in sub-Saharan Africa, the southern-central black (*Diceros bicornis minor*) and southern white (*Ceratotherium simum simum*) rhinoceros, have decreased dramatically. Common threats to rhinoceros conservation include poaching, habitat fragmentation and loss,

international trade in illegal rhinoceros products, and reduced financial resources due to global financial recessions and pandemics. Translocation of wildlife species is a commonly used tool for the conservation of threatened and endangered animals, with a focus on restoring and enhancing populations. It plays an integral part in national and international conservation plans for African rhinoceros. Chemical immobilisation is often used during translocation, with etorphine forming the basis of most drug combinations used. Ensuring animal welfare during wildlife transport is critical and dehydration, electrolyte imbalances, a negative energy balance, muscle damage, protein catabolism, stress-induced immunomodulation, and oxidative stress are the main pathophysiological findings reported in rhinoceros translocated over long distances. Investigation into possible cardiomyopathy in transported rhinoceros has been hampered by the lack of validated immunoassays to measure serum biomarkers, specifically cardiac troponin I (cTnI), in rhinoceros.

The broad objectives of this study were therefore to 1) sequence the cTnI gene in both rhinoceros species, to obtain the inferred amino acid sequences from the messenger ribonucleic acid (mRNA) transcript sequences, and assess the potential affinity of several commercial cTnI immunoassays for detecting cTnI in African rhinoceros; 2) validate two point-of-care (POC) cTnI immunoassays for use in African rhinoceros; 3) to generate cTnI reference intervals (RIs) on the high-sensitivity (hs)-cTnI immunoassay in both rhinoceros species and apply subset partitioning in white rhinoceros that were chased by helicopter during capture versus those that were captured in a boma and therefore not chased; and 4) investigate the serum cTnI changes in long-distance translocated rhinoceros and in rhinoceros chemically immobilised with different drug

protocols during capture. Best practice guidelines for method validation, quality control (QC) and RI generation as published by the American Society for Veterinary Clinical Pathology were followed. Expert consensus recommendations on the clinical laboratory practice for hs-cTnI assays as advised by the International Federation of Clinical Chemistry Task Force on Clinical Applications of Biomarkers (IFCC TF-CB) were also complied with.

The mRNA cTnI transcript sequences were obtained by RNA extraction from myocardium of deceased rhinoceros followed by primer design, complementary deoxyribonucleic acid (cDNA) synthesis using reverse transcription polymerase chain reaction, and Sanger sequencing. The percentage identity between black and white rhinoceros cDNA nucleotide sequences was 99%, while inferred amino acid sequences were identical. There were five amino acid differences between humans and rhinoceros in the epitope binding sites of immunoassay antibodies and five assays contained antibodies against epitopes that were not conserved. Nevertheless, only one assay was deemed unlikely to cross-react with rhinoceros cTnI and five assays were found to be suitable for further investigation into cTnI measurement in African rhinoceros.

The Siemens Stratus CS Acute Care troponin I cTnI and Siemens Atellica VTLi hs-cTnI were the two POC cTnI immunoassays selected for analytical method validation. Validation experiments included precision studies, reportable range, haemoglobin interference studies, recovery studies, and detection limit studies with results assessed against two total allowable error (TE_a) performance goals, namely 30% and 70%. Imprecision was acceptable and met low cTnI concentration performance goals. The

reportable ranges were similar to the manufacturer's specifications. For the Stratus CS, high haemoglobin concentrations in white rhinoceros resulted in bias. The QC validation results showed that a simple 1_{3s} QC rule using two levels of QC material and a TE_a of 70% could be used in both analysers, except at very low cTnI concentrations in the Atellica VTLi. This study showed that both cTnI POC analysers are suitable for use in African rhinoceros and analytical performance goals for low cTnI concentrations in hs-cTnI assays were met.

To allow for the identification of cardiomyocyte injury in African rhinoceros, RIs were established for both species of rhinoceros using the validated hs-cTnI assay. Reference intervals were generated from 62 and 87 apparently healthy, free-living immobilised black and white rhinoceros, respectively. Additionally, the 99th percentile upper reference limits were also determined. Of interest is that subclass partitioning was valid for white rhinoceros based on whether they were immobilised in a boma or chased by a helicopter before being immobilised.

Although chemical capture and translocation (involving capture and long-distance transport) in African rhinoceros are essential components of conservation strategies aimed at improving the conservation status of the species, several adverse pathophysiological effects, specifically hypoxaemia, acidosis and muscle damage, are associated with these processes which negatively impact rhinoceros' welfare. Serum cTnI concentration was measured using the Atellica VTLI hs-cTnI assay on stored serum samples collected during four long-distance translocation studies in black and white rhinoceros and in one chemical immobilisation study in white rhinoceros. Measurement

of serum cTnI concentration in rhinoceros translocated over long distances showed significantly increased cTnI concentrations during transportation and at release when compared to concentrations at capture. The degree of cTnI increase was more significant in cohorts chased and darted from helicopters. Concurrent skeletal and cardiac muscle damage was demonstrated in transported black and white rhinoceros, indicative of capture myopathy (CM) in these animals. Furthermore, hypoxaemia, acidosis and a negative energy balance were correlated with elevated cTnI concentrations, highlighting specific areas in procedures involving chemical immobilisation, capture, and transport that need to be addressed to mitigate these adverse effects.

The results of this study will allow wildlife veterinarians involved in African rhinoceros conservation procedures, and in the treatment of injured animals, to assess if cardiomyocyte damage is present. This assessment will allow for cardioprotective adjustments to be implemented in these procedures, resulting in improved animal welfare. Demonstration of concurrent elevated skeletal and cardiac muscle biomarkers in translocated rhinoceros will assist wildlife veterinarians in identifying animals at risk of developing CM that should be kept in confinement (boma) at the receiving end of the journey for monitoring and reduction in stress before release.

CHAPTER 1: BACKGROUND

Conservation Strategies for African Rhinoceros

Rhinocerotidae, Tapiridae and Equidae are included within the order Perissodactyla and the Rhinocerotidae family consists of five living species, namely the white (*Ceratotherium simum*) and black (*Diceros bicornis*) rhinoceros in Africa and the Indian (*Rhinoceros unicornis*), Javan (*Rhinoceros sondaicus*), and Sumatran (*Dicerorhinus sumatrenis*) rhinoceroses in Asia.[1,2] Perissodactyls are odd-toed ungulates, and obligate herbivores.[3] Based on mitochondrial deoxyribonucleic acid (DNA) data[2] and fossil records, the origin and evolution of African rhinoceros occurred 17 million years (Ma) ago.[3] The divergence of the genus *Diceros* occurred during the late Miocene era in Europe and North Africa and led to the coexistence of the two genera of African rhinoceros (*Ceratotherium* and *Diceros*) in the early Pliocene era about 5 Ma ago.[3] At the end of 2021, it was estimated that 22 137 rhinoceros (6 195 black and 15 942 white) of the 23 432 total African rhinoceros were living on the African continent, with South Africa contributing 33.2% (2 056) black and 81.3% (12 968) white rhinoceroses to the continental population.[4]

The global and unprecedented loss of wildlife species, associated with high rates of extinction, has recently been reported and declared a crisis for humanity.[5] Increased

poaching pressure, illegal wildlife trade, habitat loss and fragmentation due to increasing human population pressure are the main threats responsible for global species loss.[5,6] Concerns about the loss of large and charismatic mammalian species, including the African rhinoceros, have been raised.[5] The abundance and range of occurrence of the two rhinoceros species in sub-Saharan Africa, the southern-central black rhinoceros (*D. bicornis minor*) and the southern white rhinoceros (*C. simum simum*), have drastically been reduced.[7] These black and white rhinoceros are, respectively, listed as critically endangered and near threatened by the International Union for Conservation of Nature (IUCN) *Red List of Threatened Species*.[8,9] At the end of 2021, 1 535 southern-central black and 12 968 southern white rhinoceroses were reported to reside in South Africa.[4] The main factors involved in the decline of these species include the extended duration of illegal harvesting and international trade in black and white rhinoceros horn, worsened by weak law enforcement, combined with habitat fragmentation and loss.[5] Furthermore, a recent report investigating climate change scenarios to predict temperature and precipitation changes in national parks in southern Africa, found that there would be significant negative impacts on the survival probability of black and white rhinoceros should these conditions be reached late in the century.[10] To decrease the probability of extinction of small populations in the wild, strategic conservation measures need to be implemented. Currently implemented rhinoceros conservation strategies include education and awareness campaigns, better equipped and more antipoaching efforts, the use of innovative systems and technologies, and enhancing financial safety nets and livelihoods of local communities.[5] Additional measures include routine dehorning and translocation for population reintroduction, reinforcement, or metapopulation management.[5,11,12]

Translocation of Rhinoceros and Associated Pathophysiological Effects

Translocation is the intentional movement of living organisms, by humans, from one area with release in another.[13] This is an old practice, with the earliest recording of translocation of nondomestic species occurring about 19 000 years ago.[14] Currently, translocation is an effective conservation tool and involves capture often by chemical immobilisation, temporary captivity, transport and release into a new environment.[15,16,17] Exposing animals to various stressors during translocation may result in morbidity and mortality which negatively impact animal welfare.[13,18] In wild animals, these stressors include unfamiliar handling, surroundings, noise, the movement of the transport vehicle, the extended time of standing, and food and water deprivation.[13] The mortality rates associated with rhinoceros translocations in southern Africa are estimated at 5%.[19] High mortality rates in translocated rhinoceros, especially black rhinoceros, have also recently been reported in translocation operations post-release in Kenya and Chad due to stress, specifically nutritional stress.[4,20,21] Acute stress is beneficial in the adaptation process, however, when the physiological stress response system is overwhelmed, chronic stress occurs and persistent or repeated exposure to stressors leads to pathology.[18,22,23] However, due to translocation, there currently exists 10 times more southern white rhinoceros than there would have been if there had been no translocation.[24] Similarly, translocation has been key in increasing black rhinoceros numbers.[24]

Capture myopathy

Capture myopathy (CM) globally accounts for marked morbidity and mortality in translocated wild animals.[25] It is a widely acknowledged physical exertion- or stress-induced muscle degenerative condition that commonly occurs with prolonged or short-distance pursuit, capture, restraint or transportation of wild animals.[25] This condition is associated with a grave prognosis and is often fatal despite intensive, extended and mainly non-specific supportive treatment.[25] Mortality often only occurs after release at a new location and the incidence is therefore underestimated.[13] The primary underlying pathophysiological process is muscle damage (rhabdomyolysis).[25] It is distinguished from other types of muscle cell damage by the fact that both skeletal and cardiac muscles are affected.[13,26] As a result of rhabdomyolysis, myoglobin and creatine kinase (CK) are released from the cytoplasm of damaged muscle fibers into circulation.[25] Additionally, metabolic acidosis and a concurrent increase in body temperature occur during the early stages of CM.[25] The combination of myoglobinuria and renal cast formation, in addition to prolonged splanchnic vasoconstriction secondary to the fight or flight phase of the stress response, results in acute kidney injury.[25] The consequences of myoglobin-induced acute kidney failure are multiple organ failure and death.[25]

Initially, the syndrome is clinically characterised by anxiety, shivering, rapid breathing, torticollis, hyperthermia and pigmenturia from hours to a few days after capture.[25] In protracted cases, animals present with depression, anorexia, constipation, muscular stiffness, severe muscular pain, ataxia, paresis and paralysis.[13] These clinical signs are associated with a poor probability of recovery.[25] However, there is a wide variation in the presentation of CM and four syndromes are described, mainly 1) hyperacute or capture

shock syndrome, 2) acute or ataxic myoglobinuric syndrome, 3) subacute or ruptured muscle syndrome, and 4) chronic debility or delayed peracute syndrome.[25,26]

The quadriceps and gastrocnemius muscles are most commonly affected; however, another distinguishing feature of this syndrome is that cardiac tissue is often severely affected, supporting a possible renaming of this syndrome to capture-induced cardiomyopathy.[25,27,28] Histological evidence of multifocal areas of necrosis in skeletal muscle and variable interstitial fibrosis in cardiac tissue has been demonstrated in wild animals suffering from fatal CM.[25] Capture myopathy in wildlife has been proposed as a model for stress cardiomyopathy in humans, especially peracute CM.[26] The incidence of CM in rhinoceros is unknown, although there appears to be an increased risk in perissodactyls.[26] Investigation into cardiac muscle cell damage biomarkers, specifically cardiac troponin I (cTnI), in translocated rhinoceros, will provide valuable information as to the possible development of capture cardiomyopathy in this species.

There is no treatment currently that ensures recovery from CM and preventative practices that limit the external causes of stress in wild animals are the most successful approach.[25] These preventative practices can be achieved by utilising the correct capture method, adequate planning, the use of tranquilisers and immobilisation drugs, habituation, and cooling of animals and the environment, based on the season, time of day and temperature.[25]

Pathophysiological effects of transport on African rhinoceros

The primary reason for most rhinoceros translocation is to enhance demographic and genetic management of rhinoceros metapopulations.[24] The transportation of rhinoceros is carried out according to the guidelines developed by the Convention of International Trade in Wildlife Endangered Species for the non-air transport of wild animals and plants (CITES 2013) and recommendations for the transport of rhinoceros.[24,29] These guidelines serve as a tool to ensure minimum inputs and standardised procedures are followed, which avoids poorly planned or inappropriate translocations, poor boma management and poor release, with possible adverse effects for rhinoceros and their biological management.[24]

Identified challenges to welfare during transport of rhinoceros include changes in hydration status (haemoconcentration), electrolyte imbalances, acid-base disturbances, a negative energy balance (immobilisation of lipid stores, elevated Ⱦ-hydroxybutyrate (BHB) and non-esterified fatty acids), muscle injury (self-traumatisation, poor muscular tissue perfusion, hypoxic damage to cell walls, skeletal muscle fatigue), protein catabolism, and stress-induced immunomodulation (increased positive acute phase proteins and serum glucocorticoid concentrations) and oxidative stress.[13,15,16] Rhinoceros appear to clinically tolerate prolonged periods of water deprivation but a species difference is suggested based on higher concentrations of serum urea and creatinine, indicative of decreased renal glomerular filtration rate, found in white rhinoceros than in black rhinoceros when experiencing water deprivation.[16,30] The total body water loss may act as a stressor.[16] Rhinoceros's water needs are influenced by transport and also by food intake during transport.[21] Electrolyte imbalances reported

include a transport-induced decrease in serum potassium, magnesium and phosphorus concentrations.[13,21,31] These changes occur due to fasting and a lack of dietary intake of these minerals.[13] Protein catabolism has been demonstrated in black rhinoceros by an increase in blood urea and a decrease in serum creatinine concentrations from before to after transport.[31] Similarly, increased urea concentrations and decreased calcium concentrations have been reported in transported white rhinoceros.[21] Elevated plasma fatty acids due to the mobilisation of lipid stores have been found in transported African rhinoceros, with resultant ketone body formation in the form of BHB.[13,16] Elevated BHB concentrations have been documented in long-distance transported white rhinoceros and represent a negative energy balance.[13,16] Additionally, young animals have a proportionally higher metabolic demand than adult animals. In transported white rhinoceros this was demonstrated by a more pronounced increase in non-esterified fatty acid and BHB concentrations in calves.[16] Oxidative stress is the imbalance between the production of reactive oxygen species (ROS) and the endogenous antioxidant mechanisms that counteract the effects of ROS.[13] Lipid peroxidation is the result of oxidation of unsaturated fatty acids by ROS and an increase of these products has been documented at the end of transport in black rhinoceros.[16] In these animals, the lipid peroxidation was not due to the consumption of plasma antioxidants, as the plasma concentration was not decreased but rather a response of the innate immune response based on the increase in positive acute phase proteins.[16] A major cause of increased disease development after transport is stress-induced immunomodulation.[13] Increased susceptibility to new pathogens after translocation has potentially been illustrated by naïve black rhinoceros translocated to Tsetse fly-infested areas, where 40% of the animals developed trypanosomiasis within a month after transport.[32] Transport-induced

modulation of the immune system has been demonstrated in black and white rhinoceros by demonstration of glucocorticoid-induced redistribution of blood leukocytes.[31,33] Stress is frequently documented in response to the capture and transport of wildlife.[13] Indirect measures of the fight-or-flight response identified capture and the novelty of transport as the main stressors in transported white rhinoceros.[34] In black rhinoceros, increased blood glucocorticoid concentrations, direct markers of a stress response, have been reported after transport.[31]

Skeletal muscle exertion and damage have been demonstrated by elevated serum CK and aspartate aminotransferase (AST) activity and lactate concentration in transported African rhinoceros.[16,21,31] Environmental factors during transport that may contribute to this finding are the limited space to move in and the need to maintain postural balance standing in a transport crate during long journeys.[13] The prolonged standing results in an increased state of muscular contraction with resultant tissue hypoxia resulting in membrane permeability and the release of muscle enzymes into circulation.[13] Knocks and bruising during transport, and not only the length of the journey, contribute to the muscular injury.[21,35] Additionally, black rhinoceros often traumatise themselves during transport and therefore muscle injury may occur.[29] Other factors involved in muscle injury include muscle exertion from being chased during capture (darting), and muscular tremors secondary to certain drugs used in chemical immobilisation, resulting in muscular hypoperfusion due to hypermetabolism.[13,21,36] Repeated intramuscular (IM) injections of tranquilisers or sedatives throughout transport are additional factors that all contribute to skeletal muscle cell damage in transported rhinoceros.[13,21] The biomarkers of muscle damage that increase during transport (CK and AST) are not specific to skeletal

muscle, and cardiac muscle damage will also result in increases in these two enzymes due to cardiac isoforms.[37]

Chemical immobilisation in rhinoceros and adverse cardiovascular effects

The chemical immobilisation of rhinoceros is an essential tool employed in the course of various conservation-related procedures, including translocation, dehorning and treating injured rhinoceros.[17,38] Currently, etorphine forms the basis of most drug combinations, with various tranquilisers and/or sedatives added to etorphine in the dart to try and mitigate some of the adverse effects of chemical immobilisation.[39,40] Etorphine is a semi-synthetic alkaloid derivative of thebaine and is often used in combination with sedative and neuroleptic tranquilisers to immobilise wildlife.[39] Etorphine is a potent opioid with severe side effects, especially in white rhinoceros. These include significant adverse respiratory and cardiovascular changes, muscle rigidity, and tremors.[15,17,39,40] The administration of intravenous butorphanol, as soon as the animal is sufficiently immobilised, does counteract some of these effects.[17,41,42] Butorphanol is a synthetic opioid that reduces the effects of etorphine at the mu-receptors and reported beneficial effects include decreased blood carbon dioxide and lactate concentrations and increased blood oxygenation and pH, and resultant reduction of metabolic acidosis.[43,44] The butyrophenone tranquiliser azaperone is the most commonly used addition for rhinoceros immobilisation to decrease induction times.[45,46] By combining azaperone with etorphine in immobilisation procedures, etorphine-associated hypertension is reduced and it postulated to increase cardiac output.[17,40] Other sedative drugs which may also be used include the benzodiazepine midazolam,[47] and medetomidine, an alpha two (α_2)-agonist.[48] Midazolam is a strong muscle relaxant which potentially combats oxygen

depletion that results from muscle tremors and hypermetabolism secondary to opioid administration.[49,50] When combined with etorphine, instead of azaperone, less severe acidaemia is noted during rhinoceros capture.[15] However, the use of midazolam may exacerbate the respiratory depression brought about by opioids and should be used with caution.[40] The benefits of using medetomidine during rhinoceros immobilisation include strong sedative and analgesic effects, the reduction of anxiety levels and muscle relaxation.[48,51,52] The incorporation of medetomidine to etorphine-based chemical restraint protocols utilised in captive African rhinoceros, often in combination with midazolam or butorphanol, has been shown to maintain clinically acceptable cardiopulmonary physiology.[51]

The reported cardiorespiratory effects of etorphine in rhinoceros include severe hypoxaemia due to hypoventilation and ventilation-perfusion (V/Q) mismatch, hypercapnia, tachycardia, hypertension, and acidaemia secondary to respiratory and metabolic acidosis.[17,41,53-55] It is hypothesised that the cardiovascular response of increased arterial blood pressure and tachycardia associated with the use of etorphine results partially from the hypoxia caused by the drug-induced respiratory compromise.[17] Cardiomyocytes are very sensitive to hypoxia, with tissue ischaemia and concurrent acidosis significantly impairing cardiomyocyte metabolism, resulting in cellular necrosis and apoptosis.[56,57] Furthermore, acute hypoxia results in pro-fibrotic changes in human cardiac fibroblasts that are characterised by epigenetic modifications for example, increased gene and protein expression of collagen 1 and α-smooth muscle actin messenger ribonucleic acid (mRNA).[58,59] Therefore, a single hypoxic incident due to chemical immobilisation may not immediately cause myocardial fibrosis, but it may

initiate cellular and molecular changes that can lead to fibrosis over time, especially if repeated.

Furthermore, when large animals, like rhinoceros, are immobilised there is a risk of rhabdomyolysis due to reduced blood flow and hypoxaemia during recumbency, especially in their limbs.[60,61] The rhabdomyolysis is possibly partly due to compartment syndrome that results in increased interstitial pressure within a closed osteofascial compartment that limits local circulation.[62] Prolonged recumbency due to immobilisation with synthetic opioids as seen when poachers chemically immobilise rhinoceros and then desert the animals without reversing the immobilisation drugs, usually results in extensive rhabdomyolysis and myoglobin-induced kidney injury, which carries a poor prognosis.[60,61] Although not yet investigated, it seems likely that some degree of myocardial injury (either reversible or irreversible) will result from chemical immobilisation in rhinoceros.

Cardiac Troponin I

Physiology

Troponins are proteins that regulate muscle contraction in skeletal and cardiac muscle tissue and are not present in smooth muscle tissue.[63] With actin and myosin, they form part of the thin filaments within the myofibrils and are critical for the calcium-mediated regulation of muscle contraction.[63] The troponin complex consists of three interacting and functionally distinct proteins, namely troponin I, T and C.[63] There are three troponin I

isoforms, two are present in skeletal muscle, while the remaining isoform is present in cardiac tissue.[63] The cardiac isoform is the largest troponin isoform (24,000 kilodalton (kDa)) due to an additional 32 amino acid N-terminal peptide.[63] The rest of the protein has greater than 40% dissimilarity in its amino-acid sequence compared to skeletal muscle TnI.[64] Cardiac troponin I is highly conserved among mammalian species, with a reported nucleotide homology between cTnI of humans, rats, cats, dogs and horses that ranges from 85-95% and amino acid homology from 93-96%.[65-67] The rhinoceros cTnI gene has not specifically been sequenced as such yet, but a basic logical alignment search tool (BLAST)[68] search of the *Ceratotherium simum simum* assembly genome (GenBank assembly [GCA_000283155.1]) using the *Homo sapiens* troponin I cardiac gene sequence (Genbank accession number GU324921.1) revealed a predicted cTnI gene at LOC101390272, with a high homology with the human gene and only three amino acid differences. A similar BLAST search for the black rhinoceros revealed several potential alignments with the *Diceros bicornis minor* whole genome shotgun sequence (Genbank accession PVJY020000135.1). In addition to the tissue isoforms, cTnI can also be phosphorylated, changing the conformation of the protein and modifying its interaction with anti-TnI antibodies.[69] Circulating oxidised, reduced and partially digested post-translationally modified TnI isoforms have also been reported.[69,70]

Biomarkers of cardiomyocyte damage

Cardiac troponins, both cTnI and cardiac troponin T (cTnT), are highly sensitive and specific biomarkers for myocardial damage and are used in human and veterinary medicine to detect cardiac cellular injury.[63] Increased serum troponin concentration occurs secondary to cardiomyocyte damage, which alters the membrane integrity with

the resultant release or leakage of cTnI into circulation.[63] Troponin release occurs secondary to reversible and irreversible cardiomyocyte injury.[63] Blood cardiac troponin concentrations increase within 4 hours after an acute insult and reach peak concentrations at 12-48 hours and 10-16 hours in humans and dogs, respectively.[63] High cTnI blood concentration remains present for seven to 10 days.[63] Sustained increases in cTn for several days after myocardial infarction (MI) are likely due to the ongoing release of cTn from damaged cardiomyocytes.[71] The reported half-life is 2 hours and these proteins are eliminated by renal excretion and the mononuclear phagocytic system.[63,70] In human medicine, cardiac troponins are primarily used for the diagnosis of ischaemic heart disease, for example, acute MI, however, other reported cases of elevated blood troponin levels include for example sepsis, extreme exercise, renal failure and snake envenomation.[69,72] Elevated blood cardiac troponin concentrations in animals have also been reported in various cardiac diseases, some infectious diseases, endocrine disorders, blunt thoracic trauma, sepsis and endurance exercise.[63,70] Cardiac troponin blood concentrations have prognostic value in MI and other non-cardiac diseases in animals, with relation to fatality and duration of intensive care hospitalisation.[63,70,73] Cardiac troponins have not been investigated in rhinoceros species.

Troponin Immunoassays

Blood troponin concentrations are determined with an immunoassay that utilises specific cTn antibodies.[63,70] In contrast to cTnT immunoassays, marketed only by one manufacturer (Roche Diagnostics GmbH, Indianapolis, Indiana, United States of America (US)),[74] a variety of cTnI assays, produced by different manufacturers and with different levels of analytical sensitivity, are available.[63,70] Results obtained from different

cTnI assays are not comparable, as assays are not standardised and different amino acid sequences on the cTnI protein are targeted by assay-specific antibodies.[63,70] In light of this, it is highly recommended to establish laboratory and assay-specific reference intervals (RIs) for the evaluation of blood cTnI concentrations.[63,70] Serum, heparinised plasma or whole blood may be used depending on the assay used.[63] To allow quantitative interpretation of blood cardiac troponin concentrations, high-sensitivity (hs) cardiac troponin assays should be used.[70] The blood cardiac troponin concentration in healthy individuals is usually below the detection limit of conventional cardiac troponin assays.[70,74] To be defined as an hs-cTn assay in human medicine two analytical criteria need to be met, 1) the percentage coefficient of variation (CV) at the 99th percentile upper reference limit (99th URL) should be ≤10%, and 2) measurable concentrations should be attained at a concentration at or above the assay's limit of detection (LoD) for >50% of healthy men and women, individually.[75] Cardiac troponin I and T antibody cross-reactivity of various species with the human assays for blood cardiac troponin levels has been previously demonstrated.[65-67,76] The epitopes that are commonly detected by second and third-generation immunoassays on the cTnI molecule are highly conserved among dogs, cats, horses and humans.[67] However, validation of a candidate assay is recommended before using it for diagnostic purposes in a novel species.[77] Human cTnT assays, mainly hs-cTnT assays, have been validated in horses[78] and investigated in dogs,[79-82] cats,[80,83] cattle,[80] rats,[80,81] rabbits,[80] and, monkeys.[81] Human cTnI assays have been validated in dogs,[84-86] cats,[86] horses,[87] cattle[88] and goats.[89] The iStat point-of-care (POC) cTnI assay has been investigated in dogs[90], cattle[91] and validated in white-tailed deer[92] and shown to detect cTnI in a variety of mammals, excluding rhinoceros, from zoological collections in the United Kingdom.[93] Measurement of cTnI appears to be less useful in birds and useless

in fish due to a smaller ratio of cardiac to skeletal muscle reactivities in these species.[65] Refer to **Table 1**, below, for a summary of the human cTnI immunoassays validated in domestic and non-domestic mammals, laboratory animals and birds.

Table 1: Summary of commercially available cardiac troponin I immunoassays validated for veterinary species.

Validated human cTnI immunoassay	**Species**
Abbott; Architect STAT High Sensitivity Troponin I	Horses[87]
Abbott; Architect STAT Troponin I	Horses[87c]
Beckman Coulter; Access® AccuTnI™ immunoassay	Dogs,[84,94] horses,[87c,74,95] Goats,[89] rats[96,97]
bioMérieux; Vidas Ultra	Goats[89]
Siemens; ADVIA Centaur TnI-Ultra immunoassay	Cattle,[88,91,98b] dogs,[85,86,98] cats,[86] horses,[99] double crested cormorants,[100] rats,[98] mice,[98] marmoset,[98b] pigs,[98b] guinea pigs[98b]
Siemens; Dimensions Vista Troponin I	Horses[87]
Siemens; Stratus® CS Acute Care Troponin I cTnI assay	Dogs,[65,101] sheep,[65b] horses,[65b] pigs,[65b] rabbits,[65b] rats,[65b] mice[65b]
Siemens; IMMULITE 2000 TnI assay	Dogs,[98,102] cats[102]

Singulex; Erenna hs-cTnI assay	Dogs,[103] monkeys,[103] rodents[103]
Abbott; i-STAT cTnI assay*	White-tailed deer,[92] dogs,[90] cattle,[91,104] cats,[105] alpaca,[106] horses,[95] non-domestic mammals[93a]
Dade-Behring; Stratus, cTnI*	Dogs[94]
Quidel; Biosite Triage Meter, cTnI*	Dogs,[94,107] cats[107]
Radiometer; AQT90 FLEX, cTnI*	Horses[108]
Tosoh Bioscience; AIA-360 cTnI assay*	Dogs,[109] cats[109]

*Point-of-care methods.

[a]27 mammalian species: 19 non-human primates (5 prosimians, 8 New World monkeys, 4 Old World monkeys, and 2 ape species), 6 carnivores, 1 rodent and elephant.[93]

[b]No analytical validation, however, the assay demonstrated a high degree of cross-reactivity with the myocardium.[65,98]

[c]Partial analytical validation; demonstrated acceptable linearity.[87]

Cardiac troponin I immunoassays were investigated in this study for several reasons. First, a wide selection of immunoassays is available from various manufacturers. Second, as rhinoceros are treated mainly in field conditions, including POC cTn immunoassays validated in other veterinary species are essential. Most POC cTn immunoassays measure cTnI, specifically those that are locally available for further investigation.[110] Third, cTnT concentration in veterinary species is often below the detection limit of conventional cTnT immunoassays.[74]

Cardiovascular Disease in African Rhinoceros

Captive black rhinoceros suffer from a host of unusual disease syndromes that are associated with increased morbidity and mortality, including haemolytic anaemia, rhabdomyolysis, hepatopathy, ulcerative skin disease, hypophosphataemia and iron overload.[111] Diseases reported in captive southern white rhinoceros include hepatopathy and infertility, gastrointestinal diseases, especially colic and enteritis, and skin diseases.[112,113] However, there is limited information related to cardiovascular disease in captive or free-ranging African rhinoceros. Endocarditis of the right ventricle was found to be the cause of death of an African rhinoceros.[114] Congenital cardiac septal defects have been reported in a neonatal southern-central black rhinoceros.[115] Congestive heart failure and death due to persistent truncus arteriosus, a ventricular septal defect and arterial septal defect, have also been reported in a 6-day-old black rhinoceros.[116] A decrease in the incidence of cardiovascular disease as age increases has also been reported in zoo-kept black rhinoceros, however, no additional details about specific disease entities were provided.[117] Anthracycline cardiotoxicity with resultant fatal congestive heart failure has been reported in a juvenile black rhinoceros after a single low dose of doxorubicin as part of combination chemotherapy for acute lymphoblastic leukaemia.[118] In this case, iron overload resulted in enhanced myocardial susceptibility to the toxic effect of reactive free radicals generated by the iron-catalysed reactions.[118]

Method validation

Analytical method validation is required when the spectrum of use of an existing method is extended, for example, testing on a novel species or specimen.[77,119] This is done to determine the amount of analytical error, contributed by imprecision and systematic error or bias or inaccuracy associated with a particular method and to determine if the amount of error is acceptable based on the intended use of the results.[77] Method validation studies include several procedures and experiments, namely, reportable range or linearity study, short-term replication study (repeatability or within-run), long-term replication study (reproducibility or between-run), method comparison, interference study, recovery study, reference interval determination, detection limit study, quality control (QC) validation, and prozone effect.[77,119] Deciding on which method validation studies are required depends on the budget available, current information on the method available, whether it will be used in a novel species and the clinical setting in which the results will be used.[77] The minimum validation studies to be performed for a method intended to be used in a novel species include reportable range, short- and long-term replication, method comparison, interference, recovery, detection limit, RI determination and QC validation.[77] A summary of each of these experiments is provided here as outlined for veterinary use by the American Society for Veterinary Clinical Pathology (ASVCP).[77]

Analytical quality goals

A quality goal serves as a benchmark of test performance, of which analytical quality goals such as total allowable error (TE_a) or biological variation (BV)-based goals should be decided for each test before initiating validation studies. During a validation study, the total observed error (TE_{obs}) that is inherent to the method or instrument must fall within these preselected goals, and if it exceeds these goals the method or instrument should be rejected. The TE_a includes error due to both bias and imprecision.[77] Generally, a published consensus TE_a is typically used which is based on biological variation, clinical interpretation of results, or state-of-the-art performance. Total allowable error based on biological variation data is often the most stringent quality goal and may be difficult or impossible to achieve for some measurands. When using clinical interpretation of results as a TE_a goal, it is the amount of error that can be tolerated without affecting clinical decision-making. The lower and upper limits of RIs, clinical decision limits or interpretation cut-off values may be used to determine this type of TE_a quality goal. In the state-of-the-art performance approach, the lowest TE_a which can be controlled based on observed instrument or method performance is determined.[120] This can also include information obtained from external quality assurance (QA) or proficiency testing schemes.[121] There are no regulatory body recommendations on TE_a in cTnI for African rhinoceros and also no biological variation data available. The ASVCP guidelines of TE_a for troponin is 70%.[122] The Clinical Laboratory Improvement Amendment ’88 Proficiency Testing Limits, US Federal Register (CLIA) recommendation is a maximum of 20% CV at approximately 50% TE_a.[122]

Linearity study

This study establishes the linearity of the reportable range. A dilution series, using patient serum to maintain the matrix effect,[77] is performed. A minimum of five concentration levels is needed and the measured concentrations are compared to the expected concentrations. The mean value for each specimen is plotted on the y-axis and the expected value is on the x-axis. Visual inspection is performed to detect outliers and assess linearity, and regression analysis is performed to calculate slope and intercept.[77]

Short-term replication study

This experiment provides an estimation of the random error (RE) or imprecision of the method over a short time interval. Pooled patient samples at two different concentrations of analyte should be analysed during a single analytical run. A minimum of twenty replicates for each concentration limit is recommended. The obtained standard deviation (SD) should be $<0.25\ TE_a$.[77]

Long-term replication study

The estimation of the RE or imprecision of the method over a longer time interval, reflecting day-to-day working conditions, is obtained with this experiment. Pooled patient samples at two different concentrations of analyte should be analysed in four to five replicates during a single analytical run for five days. Alternatively, pooled patient samples at two different concentrations of analyte should be analysed once during a single analytical run over 20 days. The between-run SD should be $<0.33\ TE_a$.[77]

Method comparison study

This provides the estimation of bias or systematic error (SE). This study also determines the type of SE, namely proportional or constant, and establishes the degree of bias between the two methods.[77,121] A true "gold standard" reference method or a well-characterised field method may be used as the comparison method. A minimum of 40 independent patient specimens should be tested by both methods and should be conducted over five to 20 days.[77] Statistical analysis of results includes correlation, Bland-Altman difference plots and regression analysis.[77,121] Criteria for acceptable performance depend on the selected TE_a. A Method Evaluation Decision Chart that incorporates TE_a, SE and RE can also be used to determine method acceptability.[77]

Interference study

The SE caused by substances within the specimen being analysed, for example, haemoglobin, lipids or bilirubin, is determined with this experiment. Usually, these errors are constant across the range of patient values and the size of the error is proportional to the concentration of the interfering material. Additional comparisons include that between heparinised plasma and serum and serum collected in plain tubes versus gel tubes. At least five standard solutions of an interferent in pooled patient samples with the analyte of interest are required. The bias is calculated by determining the difference between the interferent-containing sample and the control. The acceptable performance goal is SE measured $<0.5\ TE_a$.[77]

Recovery study

The proportional SE is estimated with this study and is indicative of a possible matrix effect. The magnitude of the proportional SE changes as the concentration of the patient measurand changes. This happens when a specific substance within the sample matrix either reacts with the measurand and, or, the reagent or when there are changes in light transmission or other factors that affect the measurement of results. The proportional SE is calculated as the percentage recovery of a known amount of standard measurand which has been added to a patient specimen. The performance goal depends on the amount of total error that can be accommodated and is either SE measured $<0.5\ TE_a$ or $<\ TE_a$.[77]

Detection limit study

An estimation of the lowest concentration of a measurand that can be measured is provided and this is important for all assays in which a low value or presence or absence of an analyte is of clinical significance. A blank sample without the analyte and samples with very low concentrations of analyte, which usually correspond to the manufacturer's detection limit, are analysed. The quantitative estimations may be reported as limit of blank (LoB), LoD and limit of quantification (LoQ). In the LoQ the estimate of error should not exceed the TE_a.[77]

Quality recommendations for high-sensitivity cardiac troponin I assays

In 2011, the International Federation of Clinical Chemistry (IFCC) Task Force on Clinical Application of Cardiac Biomarkers (TF-CB) was formed to provide evidence-based educational material to assist all biomarker users to better understand important

analytical and clinical aspects of established and novel cardiac biomarkers for clinical and research use.[123] These analytical quality guidelines that apply to hs-cTnI assays, but are not covered by the ASVCP QA guidelines will be briefly mentioned. Firstly, the 99th percentile value is universally endorsed and used as the reference cut-off value to aid in the diagnosis of acute MI in humans. The 99th percentile for hs-cTnI assays should be measured with an analytical imprecision of ≤10% CV.[123] An expert consensus document published two years later proposed that a total absolute error of <3.5 ng/L for hs-cTnI concentrations ≤10 ng/L should be used as performance specifications, based on long-term imprecision and bias obtained with hs-cTnI assays.[75] Secondly, hs-cTnI assay concentrations below the 99th percentile, but above the LoD, may have clinical utility and then LoQ is often used as the lower reporting limit. It is therefore recommended to initially validate the LoB, LoD (outside of the US) or LoQ as applicable per US Food and Drug Administration (FDA) regulations in the US and repeat the validation on an annual basis.[75]

Statistical Quality Control

After the completion of analytical method validation, a QC protocol should be established.[77] Quality control validation or QC planning, involves the selection of candidate quality control rules based on the observed performance of an assay.[124] This allows the laboratory to evaluate the efficacy of error detection by the statistical QC process based on individual analyser performance.[125,126] The components included in the QC validation process are the TE_a quality goal for the assay, observed imprecision (quantified as SD and, or, CV), the observed bias compared to the target mean of the

quality control material (QCM), the desired probability of error detection (P_{ed}) and probability of false rejection (P_{fr}).[124] The goals for P_{ed} and P_{fr} are often set at ≥0.90 and ≤0.05, respectively.[120] However, a P_{ed} ≥0.85 is also acceptable in some circumstances.[127,128] To allow statistical QC to identify deviations from expected stable performance and account for the inherent variability associated with an assay a high P_{ed} is preferred.[120] The sigma metric can be determined if TE_a, CV and bias are available and it provides a unitless measure of assay capability which may help to guide the selection of QC rules. The calculation of sigma metric and use of Westgard Sigma QC Rules is another option available for selecting QC rules.[120]

There are several steps in the QC validation process with the first being the selection of a TE_a quality goal. Secondly, imprecision and bias are quantified by evaluating the performance of QCM. It is recommended to evaluate a minimum of one month of QCM data that includes at least 20 data points, however, three months of QCM data are preferred which allows the inclusion of many factors influencing imprecision and, or, bias in the assay. The evaluation of QCM data is followed by choosing candidate QC rules. Non-normalised or normalised operating process specifications (OPSpec) charts can be used. Performance components, TE_a, P_{ed}, CV and bias can be entered into a computerised program (EZ RULES 3, Westgard QC, Madison, WI, US) and a control rule is automatically selected by the computer. In the rule, *N* indicates the number of QC measures per run, and R indicates the number of runs across which the rules are applied. Manual normalised OPSpecs charts are also available to download from the Westgard website (www.westgard.com).[120] When using these, rules are selected manually by the user.

Recommendations for POC analysers include the use of a simple QC rule, namely the 1_{3s} control rule.[129] A POC analyser qualifies for statistical QC if the 1_{3s} rule has a P_{ed} of ≥0.85 with a concurrent P_{fr} of ≤0.05 for ≥75% of analytes for either one or two levels of QCM.[129] Based on the author's knowledge there is one study available in the literature that demonstrated QC validation based on ASVCP recommendations in a chemistry POC analyser for white rhinoceros.[130]

Reference Intervals

Currently, there exists no gold standard assay for cTnI and until assays are standardised, RIs should be established for each individual assay.[63] Reference intervals are used as an indicator of normal values of a particular measurand or analyte in health. A RI is derived from a group of healthy individuals that is considered representative of the population of interest. A RI should be species- and analyser or method-specific and do not exist for cTnI in African rhinoceros. Based on the IFCC and the Clinical and Laboratory Standards Institute (CLSI) (document C28-A3), the ASVCP has published guidelines for the generation of *de novo* RIs in veterinary species.[131] It is recommended to establish 95% reference limits, with 90% confidence intervals (CI) using non-parametric (NP) methods from at least 120 healthy individuals.[131] The guidelines detail the selection of the reference population, preanalytical and analytical procedures, statistical methods, and the presentation of RIs.

The steps involved in the determination of *de novo* RI study for new analytes, methods, or new populations are as follows:[131] 1) A preliminary investigation of the analyte(s) of interest, which involves investigation into sources of biological variability and interferences affecting the measurement of the measurands in question; 2) The reference population should be defined by inclusion and exclusion criteria. Selection criteria include biological, clinical and geographical factors and exclusion criteria are used to identify animals that should not be included; 3) A questionnaire should be created and completed by the researcher or animal owner to determine whether individuals should be included or not, or belongs to a portioned subgroup; 4) Determine the required or available number of reference individuals; 5) The number of individuals available to provide reference samples should be assessed to allow selecting of the reference individuals that will be included; 6) Collection and processing of samples should be performed in a standardised manner that is consistent with the methods used for testing of animal patients. Preanalytical factors to consider include patient preparation and handling, sample collection, sample handling, time of collection and analyte stability; 7) Samples should be analysed with a quality-controlled and valid method. Details of analytical methods (make and model of the analyser), source of reagents and quality control materials should be recorded; 8) Data should be present in histograms to illustrate data distribution and highlight possible outliers; 9) Outlier analysis should be performed using Dixon's range statistics or Horn's algorithm using Tukey's interquartile fences; 9) Data distribution should be classified as Gaussian (G) or non-Gaussian (NG) by examining the histogram and confirming with a goodness-of-fit test (Anderson-Darling, Kolmogorov-Smirnov, or Shapiro-Wilk); 10) Upper and lower reference limits should be calculated using an appropriate statistical method based on

the number of reference population individuals and data distribution. Confidence intervals around the upper and lower reference limits should be calculated and should not exceed 0.2 times the width of the RI; 11) Determination of whether population partitioning is required based on physiological differences that are expected to result in important clinical differences in RI; and lastly 12) The steps and procedural details should be documented, and this document should be available to users upon request.

When calculating the 99th percentile value used for the diagnosis of acute MI in humans with hs-cTnI assays, the IFCC TF-CB recommends that 300 male and 300 female population reference individuals should be included to allow an appropriately powered analysis.[69,123] Factors that influence the hs-cTnI 99th percentile include the specimen type, sex, age, and assay method. In human medicine, determining the degree of serial changes in cardiac troponin values is the best way to differentiate patients with acute cardiac injury of any kind (including MI) from those that have more chronic elevations mainly related to structural heart disease. Calculation of the reference change values for hs-cTnI assays can be based on BV, with the suggested range of reference change values between 30-85%. The suggested appropriate mean value is approximately 50%. A second option is to use absolute numbers, which are predicated on receiver operating curve analysis, which is more beneficial at higher values where marked percentage changes might not be expected but where absolute changes will be less than BV.[123]

CHAPTER 2: RESEARCH OBJECTIVES

1. To determine the genetic sequence of cTnI of black and white rhinoceros and to compare it to the published cTnI sequences for other species.[a]
2. To evaluate the cross-reactivity of various commercially available POC and reference laboratory cTnI immunoassays for African rhinoceros cTnI.[a]
3. To perform a full analytical method validation of the Siemens Stratus CS Acute Care Troponin I cTnI immunoassay and Siemens Atellica VTLi hs-cTnI immunoassay in both rhinoceros' species.[b]
4. To perform QC validation of the Siemens Stratus CS Acute Care Troponin I cTnI immunoassay and Siemens Atellica VTLi hs-cTnI immunoassay.[b]
5. To establish de novo RIs in both rhinoceros' species for the Siemens Atellica VTLI hs-cTnI immunoassay.[c]
6. To perform RI subclass partitioning in chased and boma-adapted white rhinoceros.[c]
7. To investigate the changes in serum cTnI concentration in translocated African rhinoceros, involving capture by chemical immobilisation and long-distance transport, and in African rhinoceros chemically immobilised using different drug protocols during capture.[d]

[a] Addressed in Chapter 3

[b] Addressed in Chapter 4

[c] Addressed in Chapter 5

[d] Addressed in Chapter 6

CHAPTER 3: GENETIC CHARACTERISATION OF DIAGNOSTIC EPITOPES OF CARDIAC TROPONIN I IN AFRICAN RHINOCEROS

The results presented in this chapter are submitted as a research article.

Manuscript published.

Rautenbach Y, Parsons SD, Loots AK, Goddard A, Meyer LC, Buss PE, Hooijberg EH. Genetic characterization of diagnostic epitopes of cardiac troponin I in African rhinoceros. J Vet Diagn Invest. 2024 Dec 26:10406387241305323.

SUMMARY

African rhinoceros undergo chemical immobilisation and prolonged transport during translocations for conservation purposes and experience several pathophysiological changes, including skeletal muscle injury. Potential concurrent myocardial injury has not been investigated due to a lack of validated immunoassays. This study aimed to use inferred cTnI amino acid sequences of southern white rhinoceros (*C. simum simum*) and southern-central black rhinoceros (*D. bicornis minor*) to assess the potential affinity of several commercial cTnI immunoassays for detecting cTnI in African rhinoceros. This aim was achieved by ribonucleic acid (RNA) extraction from myocardium of deceased rhinoceros followed by primer design, complementary DNA (cDNA) synthesis via reverse transcription polymerase chain reaction (RT-PCR), and Sanger sequencing. The inferred cTnI amino acid sequences were obtained from mRNA transcript sequences. The homology of epitope binding sites recognised by capture and detection antibodies in six human immunoassays was visually evaluated using aligned inferred rhinoceros cTnI amino acid sequences. The percentage identity between white and black rhinoceros cDNA nucleotide sequences was 99%, while inferred amino acid sequences were identical. There were five amino acid differences between humans and rhinoceros in the epitope binding sites of immunoassay antibodies; five assays contained antibodies against epitopes that were not conserved. For one assay the only capture antibody targeted a short heterologous epitope (residue 87-91) and cross-reactivity with rhinoceros cTnI was deemed unlikely. For the five other assays, complete antibody-epitope homology, or the inclusion of multiple detection or capture antibodies, or

targeting of long epitopes indicated that these assays could be suitable for further investigation of cTnI measurement in African rhinoceros.

INTRODUCTION

The southern-central black rhinoceros (*D. bicornis minor*) and the southern white rhinoceros (*C. simum simum*) are, respectively, listed as critically endangered and near threatened by the IUCN Red List of Threatened Species.[16] Poaching, habitat fragmentation and loss, international trade in illegal rhino products, global financial recessions and pandemics resulting in revenue shortfall negatively affecting conservation programs, are all threats to African rhinoceros' conservation.[5,15] Translocation is an important conservation strategy and involves capture using chemical immobilisation, temporary captivity, transport, and release into a new environment.[15-17] Several physiological challenges occur during capture and prolonged transport in particular, including severe hypoxaemia, acidaemia, tachycardia, hypertension, dehydration, electrolyte imbalances, a negative energy balance, muscle injury, protein catabolism, and stress-induced immunomodulation.[13,16] Rhabdomyolysis also occurs due to muscle over-exertion from being chased, the effects of stress and potent opioids used during immobilisation, like etorphine, causing muscle tremors and rigidity resulting in muscular hypoperfusion and hypoxia.[13,36] An increased state of muscular contraction (i.e., standing in a transport crate during long journeys) with resultant tissue hypoxia and repeated IM injections of tranquilisers throughout transport are additional factors that all contribute to muscle damage.[13] The biomarkers of muscle damage that increase during

transport (muscle-specific CK and non-specific AST) are not specific to skeletal muscle or cardiac muscle damage, and hepatocellular injury and haemolysis will also result in increases in these enzymes in the blood.

Furthermore, reported cardiorespiratory effects of chemical immobilisation in rhinoceros include severe hypoxaemia, hypercapnia, tachycardia, hypertension, and respiratory and metabolic acidosis.[17,41,53-55] Cardiomyocytes are very sensitive to hypoxia, with tissue ischaemia and concurrent acidosis significantly impairing cardiomyocyte metabolism, resulting in cellular necrosis and apoptosis.[56,57] To compensate for prolonged increased peripheral resistance secondary to drug-induced vasoconstriction and hypertension, the myocardium enlarges leading to cardiomyocyte hypertrophy and resultant fibrosis due to necrosis and, or, apoptosis.[132] It, therefore, seems likely that some degree of reversible or irreversible myocardial injury will result from chemical immobilisation in rhinoceros, but this has not been investigated.

Troponins are proteins that regulate muscle contraction in skeletal and cardiac muscle tissue.[63] The troponin complex consists of three proteins, namely troponin I, T and C.[63] There are three troponin I isoforms, two are present in skeletal muscle, while the remaining isoform is present only in cardiac tissue.[63] The cardiac isoform is the largest troponin isoform (24 kDa) due to an additional 32 amino acid N-terminal peptide.[63] Cardiac troponin I is highly conserved among mammalian species, with a reported nucleotide homology between cTnI of humans, rats, cats, dogs and horses that ranges from 85-95% and amino acid homology from 93-96%.[65-67] Cardiac troponins are highly

sensitive and specific biomarkers for myocardial damage and are used in human and veterinary medicine for the detection of cardiac cellular injury.[63]

Blood troponin concentrations are determined with immunoassays that utilise specific cTnI antibodies that target varying epitopes on the cTnI molecule.[63,70,133] A variety of cTnI assays, produced by different manufacturers and with different levels of analytical sensitivity, are available.[70,133] Cardiac troponin I human assay antibody cross-reactivity for blood cardiac troponin levels in various species has been previously demonstrated.[65-67,133] The epitopes on the cTnI molecule that are commonly detected by second and third generation immunoassays are highly conserved among dogs, cats, horses, and humans.[67] Even so, validation of a candidate assay is recommended before using it for diagnostic purposes in a novel species.[77] Various human cTnI assays have been validated in domestic and non-domestic animals, however, not in rhinoceros. Information about the epitopes targeted by antibodies contained in cTnI assays is available,[134] and prediction of the degree of antibody binding to these epitopes on the rhinoceros cTnI protein would assist in the screening and selection of assays to be included in full method validation studies.

The study aimed to assess the potential use of several commercially available candidate human cTnI immunoassays for detecting African rhinoceros cTnI. This was done by determining the mRNA transcript sequence of white and black rhinoceros' cTnI and assessing sequence homology. The inferred amino acid sequences were used to evaluate the degree of conservation of the different cTnI epitopes recognised by the capture and detection antibodies used in these immunoassays.

MATERIALS AND METHODS

Animals and sample preparation

Samples of myocardium from the left ventricle were obtained from two horses, two white rhinoceros and one black rhinoceros immediately after death or euthanasia due to causes unrelated to the study. Black rhinoceros skeletal muscle was also obtained, to serve as a negative control. The samples collected from the horses were used for optimisation of the analytical procedures, which included RNA extraction modifications, determination of optimal PCR conditions, and assessment of the preferred combination of the designed primer pairs. For each tissue, duplicate samples of ≤5 mm in any dimension were prepared: one was transferred to a tube containing RNA stabiliser solution (NucleoProtect; Macherey-Nagel), stored at room temperature for 12-24 h, and thereafter at -80°C.

Cardiac troponin I mRNA transcript analysis

Total RNA was extracted using the NucleoSpin RNA Midi Kit (Macherey-Nagel) according to the manufacturer's instructions. The RNA quantity and quality were assessed spectrophotometrically on the Trinean Xpose instrument. Primers targeting the coding sequence of cTnI genes were designed based on the predicted mRNA transcript sequence of the southern white rhinoceros (National Centre for Biotechnology Information (NCBI) reference sequence: XM_004439072.2) and horses (NCBI reference sequence: AY819020.1) using the NCBI Primer-BLAST software (https://www.ncbi.nlm.nih.gov/tools/primer-blast/).[135] The forward primer was 5'-

TCTTGCCCCTTCTCTGCCTC-3'; the reverse primer was 5'-TTCCTCAGGGCCATYCTCAG-3', yielding an amplicon of 708 base pair (bp). Reverse transcription PCR was performed in a Veriti 96-Well Thermal Cycler (Applied Biosystems, Thermo Fisher Scientific) using PrimeScript One Step RT-PCR Kit Ver.2 (Dye Plus) (Takara Bio). Reverse transcription PCR conditions for rhinoceros RNA consisted of an initial reverse transcription for 30 minutes (min) at 50°C, followed by PCR activation for 2 min at 94°C and 30 cycles of denaturation (94°C for 30 seconds (s)), annealing (55°C for 30 s) and extension (72°C for 1 min). Thereafter the samples were kept at 4°C. The PCR conditions for the kit positive control were performed according to the manufacturer's recommendations.

The integrity of the PCR amplicons was visualised by electrophoresis in a 1.5% Tris acetate-ethylenediaminetetraacetic acid (EDTA)-agarose gel stained with ethidium bromide. Amplified PCR products were subsequently submitted to Inqaba Biotec for purification and sequencing. Briefly, PCR products were purified with exonuclease I-shrimp alkaline phosphatase (Exonuclease I and Shrimp Alkaline Phosphatase; New England BioLabs) according to the manufacturer's instructions. The purified products were sequenced on an Applied Biosystems 3730XL Genetic Analyser (Thermo Fisher Scientific) using the BrilliantDye™ Terminator Cycle Sequencing Kit v3.1, BRD3-100/1000 cycle sequencing kit (Nimagen). Generated overlapping sequences were aligned and contigs constructed using Molecular Evolutionary Genetics Analysis (MEGA) v.11 (https://www.megasoftware.net/) software followed by NCBI BLAST analysis.

Commercial human cardiac troponin I immunoassay evaluation

The epitopes recognised by the antibodies in candidate commercially available human cTnI immunoassays, validated in other veterinary species and thus selected for assessment of the degree of identity between various species, are summarised in **Table 2**. Additionally, a novel POC cTnI assay recently validated and released for human medical use was evaluated (Atellica VTLi hs-cTnI, Siemens Healthineers, South Africa).[136] The degree of amino acid conservation of cTnI antibody epitopes was assessed in African rhinoceros, the dog, cat, horse, and rat as follows: the predicted amino acid sequences of the African rhinoceros cDNA nucleotide sequences from the NCBI BLAST analysis were obtained and aligned with the amino acid sequences of the dog (UniProt identifier Q8MKD5), cat (UniProt identifier Q863B6), horse (UniProt identifier Q5PYI0), rat (UniProt identifier P23693), and human UniProt identifier P19429) using the UniProt platform (https://www.uniprot.org/align).[137] Amino acid sequence identity at the epitopes recognised by the capture and detection antibodies utilised in each immunoassay was visually assessed for amino acid differences between the species listed above.

Table 2: Summary of candidate human cardiac troponin I immunoassay evaluated for amino acid sequence homology.

Human cTnI immunoassay	Species validated for	Epitopes recognised by assay antibodies*[134]
Siemens; ADVIA Centaur TnI-Ultra	Bov, Ca, Fe, Eq, Por, rats, mice, marmoset, guinea pigs, double crested cormorants[85,86,88,91,98-100]	C: 41 - 49; 87 - 91; D: 27 - 40
Siemens; Stratus CS Acute Care cTnI	Ca, Eq, Por, sheep, rabbits, rats, mice[65,101]	C: 27 - 32; D: 41 - 56
Siemens; Immulite 2000 XPi TnI	Ca, Fe[98,102]	C: 87 - 91; D: 27 - 40
Beckman Coulter; Access AccuTnI (second generation)	Ca, Eq, goats, rats[74,84,87,89,94-97]	C: 41 - 49; D: 24 - 40
Abbott; iSTAT	Ca, Bov, Fe, Eq, alpaca, white-tailed deer, non-domestic mammals[90-93,95,104-106,138]	C: 41 - 49; 88 - 91; D: 28 - 39; 62 - 78
Siemens; Atellica VTLi hs-cTnI	None	C: 41 - 49; D: 23 - 29; 87 - 91

*Amino acid numbering: Position 1 is the first amino acid after methionine in the human amino acid sequence. Bov = cattle; C = capture antibody; Ca = dogs; cTnI = cardiac troponin I; D = detection antibody; Eq = horses; Fe = cats; hs = high sensitivity; Por = pigs.

RESULTS

Cardiac troponin I mRNA transcript analysis

Extracted RNA yields ranged from 21.2 to 47.0 ng/µL in an elution volume of 500 µL, with optical density ($OD_{260/280}$) values ranging from 2.04 to 2.15. Agarose gel electrophoresis of the cDNA showed amplicons in bp size consistent with cTnI (708 bp) (**Figure 1**).

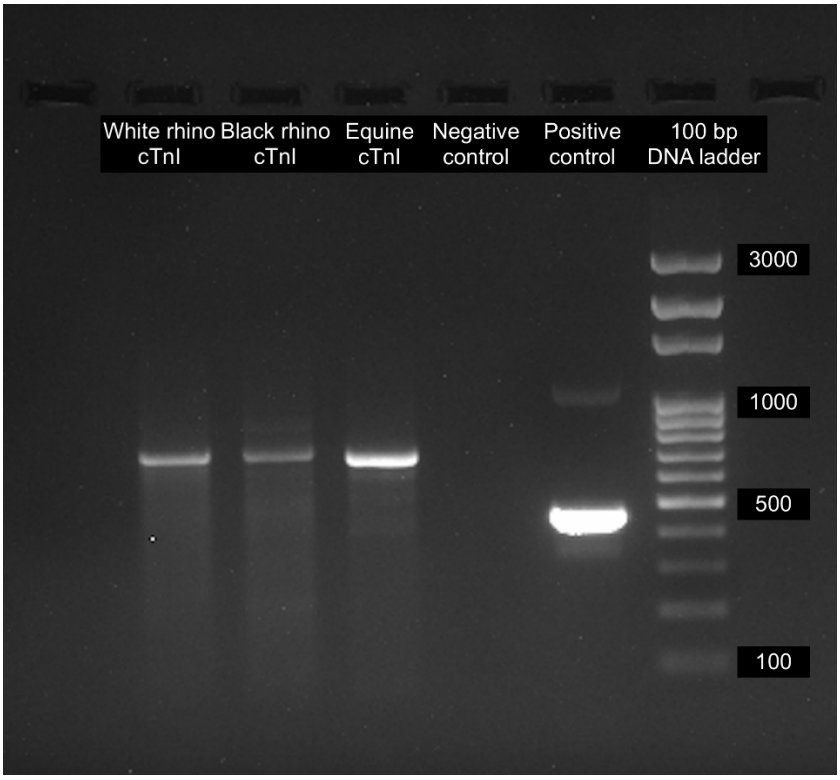

Figure 1: 1.5% agarose gel electrophoresis of cDNA PCR products from myocardium of white and black rhinoceros and horse, amplified using cardiac troponin I (cTnI) specific primers.

A 708 bp amplicon product is present. Skeletal muscle RNA from a southern-central black rhinoceros served as a negative control. Positive control RNA (amplicon, 462 bp) included in the kit (PrimeScript One Step RT-PCR Kit Ver.2 (Dye Plus); Takara Bio). cDNA: complementary deoxyribonucleic acid; RT-PCR: reverse transcription polymerase chain reaction.

Feature annotation of the rhinoceros nucleotide sequences was performed by the NCBI GenBank User Services (https://www.ncbi.nlm.nih.gov/genbank/) and identified regions confirmed by reviewing well-annotated cTnI transcripts of other domestic species.[68] The southern white rhinoceros cTnI cDNA revealed a coding sequence (CDS) that was comprised of 618 nucleotides. The partial nucleotide sequence of the southern-central

black rhinoceros cTnI revealed a partial CDS consisting of 612 nucleotides. These sequences were submitted to the NCBI GenBank (accession OR374027 and OR374028).

When comparing the CDS of the cDNA sequence alignment, the percentage of identity between white and black rhinoceros was 99%. Five single nucleotide variants were identified at the following locations, with the start of numbering from the white rhinoceros CDS: C189G; C192T; C207G; G306T; T444C (**Figure 2**). Comparing the cDNA sequence alignment between white and black rhinoceros versus horses, the percentage identity was 94% and 95%, respectively, and between white and black rhinoceros versus humans, 91%.

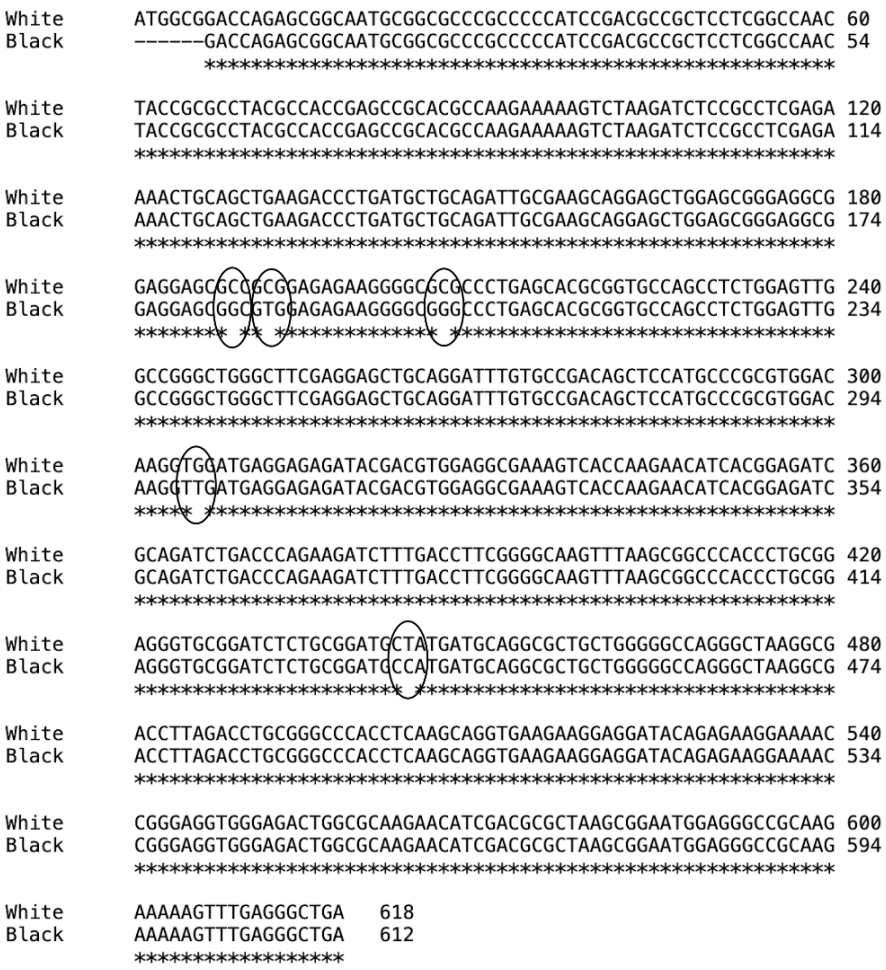

Figure 2: Coding nucleotide sequence alignment of cardiac troponin I (cTnI) mRNA from southern white rhinoceros and southern-central black rhinoceros.

Single nucleotide variants encircled.

Cardiac troponin I protein analysis

Despite the five nucleotide variants, the inferred amino acid sequences of the two rhinoceroses' species were identical (100% identity) with the percentage identity between white rhinoceros and black rhinoceros compared to humans at 94% (**Figure 3**). The percentage identity of the rhinoceros predicted cTnI amino acid sequence compared to the dog, cat, horse, and rat was 95%, 94%, 97%, and 92%, respectively (**Figure 3**).

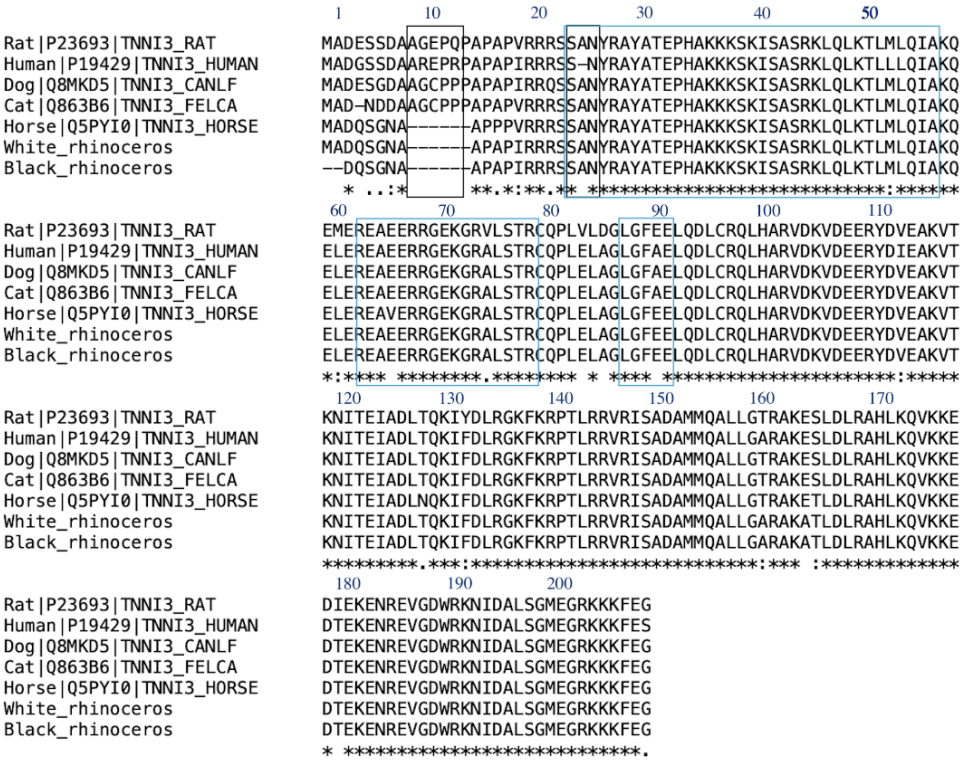

Figure 3: The predicted amino acid sequence of cardiac troponin I (cTnI) from southern white rhinoceros and southern-central black rhinoceros aligned with the cTnI amino acid sequence of human, dog, cat, horse, and rat.

The black boxes highlight gaps in the sequences and the blue boxes highlight regions commonly targeted by cTnI immunoassay capture and detection antibodies. Amino acid numbering is based on the human sequence and is numbered in blue. The cardiac-specific N-terminal region lies from 1-31. "-" = gap; * = sequence conserved; no dots = no sequence conservation; "." and ":" = some conservation, with ":" indicating all pyrimidines or all purines amino acids at position.

Commercial human cardiac troponin I immunoassay evaluation

Amino acid sequence regions described here are highlighted in **Figure 3** and antibody epitopes are presented in **Table 2**. Specific epitopes within the first amino acid sequence region (22-56) were targeted by most antibodies utilised in the immunoassays which

included six detection and five capture antibodies and there was complete amino acid sequence homology between humans and rhinoceros at the epitopes targeted by 4/6 detection antibodies and 1/5 capture antibodies. The entire second amino acid sequence region (62-78) was targeted by a single detection antibody, with complete amino acid sequence homology between humans and rhinoceros. The short third amino acid sequence region (87-91) contained epitopes targeted by three capture antibodies and one detection antibody and sequence homology was absent between humans and rhinoceros.

In terms of specific assays, the same, single amino acid difference was noted in the epitope recognised by the single or one of the pairs of capture antibodies used in the Immulite 2000 XPi TnI, ADVIA Centaur TnI-Ultra and iSTAT cTnI immunoassays in rhinoceros, horses and rats compared to humans, cats and dogs. For the iSTAT assay, a single amino acid difference, at different locations, was also noted in the epitope targeted by one of the pairs of detection antibodies, in the horse and rat. For the Stratus CS cTnI detection antibody, a single amino acid difference was noted in the epitope target in all species compared to humans. Regarding the Atellica vTLI, a single amino acid difference was present at the epitope for one detection antibody in all species compared to humans, and a single amino acid difference was present at the epitope bound by the second detection antibody for the rhinoceros, horse and rat versus humans, cats, and dogs. The epitopes recognised by the capture and detection antibodies in the Beckman Coulter Access AccuTnI immunoassay were conserved across all species evaluated.

DISCUSSION AND CONCLUSION

The complete nucleotide coding sequence of white rhinoceros cTnI cDNA revealed a coding region comprised of 618 nucleotides, with a partial coding sequence of 612 nucleotides found in the black rhinoceros. This is similar to the equine cTnI cDNA coding region of 618 nucleotides but is slightly smaller than the feline and canine coding region of 636 and 633 nucleotides, respectively.[66,67] The southern white rhinoceros and southern-central black rhinoceros are closely related sister-taxa with divergence noted ~5.2 Ma and cessation of ancestral gene flow reported between 3.3 and 4.1 Ma.[3,139] The five nucleotide variants that were identified when comparing the white rhinoceros cDNA sequence to that of the black rhinoceros represent synonymous codon substitution, i.e., the mutations do not alter the encoded amino acid. This is indicative of purifying selection, with synonymous mutations frequently assumed to be neutral concerning fitness.[140]

The inferred amino acid percentage identity between humans, dogs, cats, horses, and rats ranged from 92% - 97%. Our amino acid sequence alignment using the Clustal Omega program in UniProt, showed that white and black rhinoceros cTnI have alanine in the N-terminal region which is also present in canine, feline, equine, and rat cTnI, but missing from human cTnI (**Figure 3**).[141] Interestingly, cTnI of both rhinoceros species lacked the same six amino acids in the N-terminal region as cTnI of horses, a finding not present in all other species studied (**Figure 3**).[67] Considering the evolution of perissodactyls, with the order including the family Equidae, it is not surprising that

identical amino acid deletion was seen in all three of these species.[3] A motif search engine (ScanProsite, https://www.expasy.org/resources/prosite, input UniProt identifier P19429 (TNNI3_HUMAN)) was used to search for functions ascribed to this site on human cTnI. The missing amino acid sequence is an N-myristoylation site (Prosite entry PS00008) common to many eukaryotic proteins. The addition of the saturated fatty acid myristate to glycine (Gly^3 on human cTnI, **Figure 3**) results in acetylation of the N-terminal residue. A similar ProSite search for dog, cat, and rat cTnI, with amino acids present in this region, but with differing sequences (notably, no Gly^3), did not reveal any associated functions. Since all animal species investigated lack the N-myristoylation site contained in human cTnI, the lack of this motif in rhinoceros is probably not of vital importance.

Measurement of blood cTnI concentration is facilitated by immunoassays utilising cTnI-specific antibodies and monoclonal and recombinant antibodies are considered the most suitable options for both capture and detection antibodies (Abs), offering higher specificity.[142] The use of multiple Abs targeting different epitopes, i.e., two or more capture Abs and one or more detection Abs, results in higher assay sensitivity and accuracy.[142] Antibodies specific for epitopes on cTnI located within the central region of the protein should be used since the most stable region of cTnI is between amino acid residues 30 and 110.[143]

The epitopes on the cTnI molecule that are commonly detected by second and third-generation cTnI immunoassays have previously been reported to be highly conserved, with only eight amino acid differences noted among dogs, cats, humans, and horses in the sequence after $Ala^{19/25}$.[67] Our results confirm these findings, with only five amino acid

differences noted in the epitope binding sites of the Abs used in the six candidate immunoassays among both rhinoceros species, dogs, cats, horses, rats and humans.

Despite this high homology, five of the six immunoassays evaluated contain antibodies targeted against the cTnI epitopes that are not conserved between humans and several other species, including rhinoceros. Considering that the Immulite 2000 XPi cTnI immunoassay only utilises a single capture Ab, and the short amino acid sequence of the epitope recognised by this capture Ab (epitope region 87-91; five amino acid length), cross-reactivity with cTnI from species other than the dog and cat (for which the assay has previously been validated), is highly unlikely.[98,102] This supposition is supported by a reported lack of detection of equine cTnI (same amino acid sequence as rhinoceros at epitope region 87-91) by two immunoassays whose capture antibodies target this epitope.[87] One of these two immunoassays was the Immulite 1000 cTnI assay, which uses the same capture antibody as the Immulite 2000 XPi cTnI assay.

A capture antibody used in the ADVIA Centaur cTnI-Ultra also targets the non-conserved 87-91 epitope, but this assay has a second capture Ab (epitope 41-49) and one detection Ab (epitope 27-40) which target epitopes with complete homology. This explains why this assay has been successfully validated in many animal species, including dogs, cats, horses and rats, and the Abs in this assay could be expected to cross-react with African rhinoceros cTnI.[85,86,98,99] However, this assay has now been discontinued and replaced by the ADVIA Centaur XP High-Sensitivity Troponin I assay which has recently been validated in dogs and found to be strongly correlated to the ADVIA Centaur cTnI-Ultra assay.[144] The published epitopes utilised by one of the two capture Abs and single detection Ab in this

new assay[134] are conserved in all the species evaluated in this study, except the rat and it can be assumed that the Abs used in this immunoassay will cross-react with the cTnI molecule in the remaining species.

The iSTAT cTnI assay has been used with success in many species including the dog, cat and many non-domestic mammals (but not rhinoceros), probably because the assay utilises two capture Abs[92,93,138] and the epitopes recognised by one of these Abs are conserved across all the species evaluated in this study. This POC assay therefore shows promise for use in African rhinoceros under field conditions.

The same single amino acid difference (leucine in position 52 replaced by methionine) was present in the epitope recognised by the detection Ab utilised in the Stratus CS Acute care cTnI immunoassay, in all evaluated species, while the epitope recognised by the capture Ab was conserved. Despite the single amino acid difference in the detection Ab epitope, this assay has been validated for use in dogs, horses, rats, mice, sheep, pigs, and rabbits and RIs established for cats.[65,101] The epitope recognised by the Stratus detection Ab consists of 16 amino acids with the amino acid difference located in the last third of the sequence. The degree of homology is thus sufficiently high enough in this relatively long epitope to allow adequate binding of the detection Ab to the captured cTnI antigen. The Stratus CS Acute care cTnI assay is therefore also potentially useful in the evaluation of serum cTnI concentration in rhinoceros.

The epitopes recognised by the capture and detection Abs in the Access AccuTnI immunoassay are conserved across all evaluated species and the assay has been

validated in dogs, horses, goats, and rats.[74,84,87,89,94,96,97] Although not fully validated, the Access AccuTnI has also been used successfully to measure serum cTnI in cats with hypertrophic cardiomyopathy.[145] The Access AccuTnI immunoassay will also potentially be useful in the evaluation of serum cTnI concentration in rhinoceros.

The Atellica VTLi hs-cTnI is a relatively new point-of-care assay that has capture antibodies bound to superparamagnetic nanoparticles.[136] The capture antibodies target only one epitope, which was conserved across all species. Three detection antibodies potentially bind the nanoparticle-capture antibody-cTnI complex; for one of these detection antibodies there was one amino acid difference in all species versus humans at the targeted epitope (23-29) and for the second detection antibody there was one amino acid difference (epitope 87-91) in horses, rhinoceros and rats compared to humans, dogs, and cats. The third detection antibody binds to binary cTnI-cTnC complexes. This new assay has not been investigated in species other than humans, but also holds promise for cTnI measurement in African rhinoceros.

A limitation of this study is that the cTnI mRNA transcript nucleotide sequence of the black rhinoceros was only determined from a single animal. Furthermore, the 5' region of the black rhinoceros coding sequence was incomplete, with six nucleotides missing due to poor signal strength of the sequencing data. Obtaining fresh tissue samples from this critically endangered species is challenging. However, a comparison of our cTnI mRNA transcript sequences to the recently added predicted *D. bicornis minor* cTnI mRNA sequence (NCBI reference sequence: XM_058530778.1) revealed a 100% identity.

By evaluating the homology of rhinoceros cTnI amino acid sequences compared to that of humans and other animal species, and using information about the epitopes targeted by cTnI assay Abs, we could screen available assays before embarking on further investigations. This study identified that five of the six candidate cTnI immunoassays evaluated may be useful for the measurement of blood cTnI concentration in African rhinoceros and could be selected for full analytical method validation studies in the future. The eventual establishment of RIs in healthy populations and assessment of cTnI concentrations using a validated assay in animals at risk for cardiomyocyte injury will enhance the welfare of these animals during conservation-associated activities.

CHAPTER 4: ANALYTICAL VALIDATION OF TWO POINT-OF-CARE CARDIAC TROPONIN I IMMUNOASSAYS IN AFRICAN RHINOCEROS

The results presented in this chapter have been submitted as a research article.

Rautenbach Y., Meyer L.C.R., Goddard, A., Buss P.E., Hooijberg E.H. Analytical validation of two point-of-care cardiac troponin I immunoassays in African rhinoceros. Vet Clin Pathol. Submitted 7 September 2024.

SUMMARY

Muscle damage has been reported in chemically immobilised and transported African rhinoceros during conservation-related activities. The extent of cardiac muscle injury in these rhinoceros is unknown due to a lack of validated cTnI assays. Five automated human cardiac troponin I assays were deemed suitable for analytical validation in African rhinoceros based on cTnI sequencing results. The first objective was to validate two cTnI immunoassay POC analysers in African rhinoceros, and secondly to perform QC validation for the POC analysers. The analytical validation of the Stratus CS Acute Care Troponin I cTnI immunoassay and Atellica VTLi hs-cTnI assay was performed using rhinoceros serum samples and species-specific cardiac muscle lysate. Experiments included precision studies, reportable range, haemoglobin interference studies, recovery studies, and detection limit studies with results assessed against prescribed TE_a performance goals. Commercial QCM data were used to calculate bias and imprecision for QC validation. Imprecision was acceptable and met low cTnI concentration performance goals. The reportable ranges were similar to the manufacturer's specifications (Stratus CS: 0.03-50 ng/ mL; Atellica VTLi: 2 ng/L for lithium-heparin plasma and 4 ng/L for lithium-heparin whole blood to 1250 ng/L). High haemoglobin concentrations in white rhinoceros resulted in a bias in the Stratus CS. A simple 1_{3s} QC rule using two levels of QCM and a TEa of 70% could be used in both analysers, except at very low cTnI concentrations in the Atellica VTLi. In conclusion, both cTnI POC analysers are suitable for use in African rhinoceros and analytical performance goals for low cTnI concentrations in hs-cTnI assays were met.

INTRODUCTION

The two extant species of rhinoceros native to Africa, the black rhinoceros (*D. bicornis minor*) and the white rhinoceros (*C. simum simum*), are endangered, with most animals living in fairly small, isolated populations in conservancies, private game reserves, and intensive protection zones in sub-Saharan Africa.[5,8] National and international rhinoceros conservation strategies include humane horn trimming (dehorning) and translocation of animals to safe areas. Both dehorning and translocation involve capture using chemical immobilisation; translocated animals must also undergo temporary captivity, transport and release into a novel environment.[16] Both chemical immobilisation, which involves the administration of potent opioids like etorphine or thiafentanil, and transport result in pathophysiological changes that impact the cardiovascular and musculoskeletal systems. The pathophysiological changes include severe hypertension, hypoxaemia, tachycardia, electrolyte imbalances and increased activities of serum AST and CK, indicating muscle damage.[16,17] Capture myopathy, a syndrome that involves both skeletal and heart muscle, is well documented during capture and translocation procedures in various wildlife species.[25] Investigation of the extent of cardiac muscle injury during chemical immobilisation and transport and the role of cardiomyocyte injury in capture myopathy in rhinoceros has been hampered by the lack of validated cTnI immunoassays. Cardiac troponins are the biomarkers of choice for the detection of reversible and irreversible cardiomyocyte injury in human and veterinary medicine.[63]

We recently sequenced the cTnI gene of both black and white rhinoceros, and evaluated the homology, compared to humans, of epitopes on the predicted cTnI protein that are targeted by the detection and capture antibodies in six automated human cTnI assays (refer to Chapter 3). Of the five assays that were deemed suitable for further evaluation, we selected two POC methods for further investigation. The Stratus CS Acute Care Troponin I cTnI immunoassay (Siemens Healthineers, Erlangen, Germany) has been used in dogs, horses, sheep, pigs, rabbits, rats and mice.[65,101] The Atellica VTLi hs-cTnI assay (Siemens Healthineers, Erlangen, Germany) has recently been introduced into the human medical environment and has not yet been validated for use in any veterinary species, to our knowledge.

Both assays require analytical validation before use in rhinoceros.[119] Additionally, the implementation of QA and QC strategies is important for POC analysers, which are invaluable in wildlife and often used in field settings.[129,130] Veterinary analytical method validation guidelines and analytical error goals are provided by the ASVCP.[77,122] The IFCC TF-CB additionally mandates analytical performance specifications for hs-cTnI immunoassays.[75] This study aimed to first perform analytical validation of two cTnI immunoassay POC analysers for use in African rhinoceros and secondly, to perform QC validation for the POC analysers.

MATERIALS AND METHODS

Analysers and assay methods

The Stratus CS Acute Care Troponin I method is a two-site sandwich assay with one detection and one capture antibody based on solid phase radial partition immunoassay

technology. Cardiac troponin concentration is reported in ng/mL (µg/L), to two significant figures as per IFCC TF-CB guidelines.[75] The Atellica VTLi utilises a hs-cTnI test which is also a two-site sandwich immunoassay with one detection and three capture antibodies; a key component of this assay is supermagnetic nanoparticles that covalently bind to the capture antibodies.[136] Cardiac troponin concentration is reported in ng/L and as whole numbers without decimal points, as per IFCC TF-CB guidelines.[75] Single-use reagent cartridges, stored at 2-8°C, are used in both analysers. Both analysers used in this study were placed in a veterinary clinical pathology laboratory under recommended operating conditions for analyser and QC validation. Full maintenance, including analysis of manufacturer-recommended QCM (including calibration cartridges in the case of the Stratus CS analyser), was performed on both analysers before the study began. One level of QCM was used in the Stratus (MAS CardioImmune XL, Liquid assayed cardiac marker control Level 2, Lot CXL25042; Thermo Scientific, Fremont, CA, US) and three levels of QCM were used in the Atellica VTLi (Pathonorm, Cardiac Acute Lig L-1, L-2, and L3, Lot 2208906; 2203830; 2208908, respectively, SERO AS, Stasjonsveien, Billingstad, Norway). The QC results performed on the Stratus CS should be reported to three significant figures based on IFCC TF-CB guidelines, however, the analyser only reported two decimal places.[75] In the Atellica VTLi, the QC results were reported with one decimal point as advised by the IFCC TF-CB.[75]

Performance goals

The ASVCP recommended TE_a goal for troponins is 70%.[122] However, this “consensus” goal was based on a single expert opinion and we decided to also compare the analytical performance against a stricter requirement of 30% (or 0.9 ng/mL), as recommended by

the American Association of Bioanalysts (AAB).[146] Based on Westgard and ASVCP guidelines, the requirements for short-term and long-term replication studies are; SD <0.25 TE_a and SD <0.33 TE_a, expressed in the units of the test, respectively.[77,147] The IFCC TF-CB performance specification of total analytical error of <3.5 ng/L for the Atellica hs-cTnI immunoassay at ≤10 ng/L was also applied in the precision experiments.[148] The acceptable performance for the interference and recovery studies was that the estimated proportional SE was <0.5 TE_a, and SE < TE_a, respectively.[77]

Method validation

Samples

Sample materials used were serum and cardiac muscle lysates from black and white rhinoceros. For the preparation of the muscle lysate, myocardium from the left ventricle was obtained from two white rhinoceros and one black rhinoceros immediately after death or euthanasia due to causes unrelated to the study. Multiple muscle blocks, ≤5 mm in any dimension were prepared, blotted on tissue paper to remove excess blood, trimmed of obvious connective tissue and fat, and stored at -80°C in a preservative-free tube. The muscle lysate was prepared as previously described following the manufacturer's instructions (Bio-Plex Cell Lysis Kit; Bio-Rad).[149] Briefly, the frozen muscle was transferred to wet ice and cut into 1 mm x 1 mm pieces (100 – 300 mg, in total), placed in tubes containing steel beads (Bead types type F; Macherey-Nagel) and lysing solution and mechanically homogenised (Precellys 24 homogeniser; Bertin Technologies). The tissue homogenate was centrifuged and the resulting supernatants were stored at -80°C.

The rhinoceros serum samples had been previously collected, as soon as animals were recumbent (inductions times <10 min), for other studies and included healthy animals immobilised for translocation or dehorning. Blood was collected from the auricular vein directly into serum tubes (BD Vacutainer; Becton and Dickinson, Plymouth, UK), stored in a cooler box with ice packs and centrifuged within 24 hours. The serum was aliquoted into cryovials and stored at -80°C. Samples were up to two years old. Samples were excluded if gross haemolysis, lipaemia, or icterus was present. Small amounts of cardiac muscle lysate supernatant were added to aliquots of stored serum to achieve the desired concentrations of cTnI.

Short-term imprecision

A high and low sample pool was created for each analyser and species, using the species-specific cardiac muscle homogenates and serum. Pools were kept at 4°C after being prepared and were used within 8 hours. Twenty measurements were performed on each pool. Both pools were measured in the same analytical run, except in the case of the Atellica VTLI, white rhinoceros, where the pools were measured in two separate analytical runs due to the analyser's results storage capacity being reached, necessitating the download of results to the service software program before analysis could proceed.

Long-term imprecision

A high and low sample pool was created and aliquoted for each analyser and species. The aliquots were stored at -20°C. Four replicate measurements on each pool were

performed daily for five days. Both low- and high-concentration pooled aliquots were measured in the same analytical run.

Reportable range and linearity

A high and low sample pool was created for each species and analyser, using each analyser's manufacturer's reportable range as a guideline. The high pool and low pool were designated as level five and level one, respectively. A dilution series was prepared using the low pool (level one) and the high pool (level five) in ratios of 3:1 (level two), 1:1 (level three) and 1:3 (level four). Levels one to five were only measured in duplicate on the Atellica VTLi, due to cost constraints, and in triplicate on the Stratus CS, in the same analytical run.

Haemoglobin interference

White rhinoceros hemolysate was prepared using EDTA-anticoagulated whole blood samples leftover from research samples unrelated to this project, obtained from healthy white rhinoceros orphans. The plasma was discarded and the red cell pellets were frozen at -20°C. For this experiment, the pellets were thawed to room temperature and mechanically lysed by repeated passage through a small gauge needle attached to a 20 mL plastic syringe. Thereafter the samples were centrifuged at 1520 *g* for eight min and the supernatant was harvested. The haemolysate was added to pooled serum samples of each rhinoceros species. The pooled serum used was free of gross haemolysis and haemolysate was added at three different concentrations, namely 1.0, 2.0 and 3.0 g/L of haemoglobin, as determined spectrophotometrically (ADVIA 2120i, Siemens, Germany) (**Figure 4**). Distilled water was added to the control serum specimens at equivalent

volumes. The pairs of samples were analysed in duplicate. The same samples were measured on both analysers, in the same analytical run for each analyser.

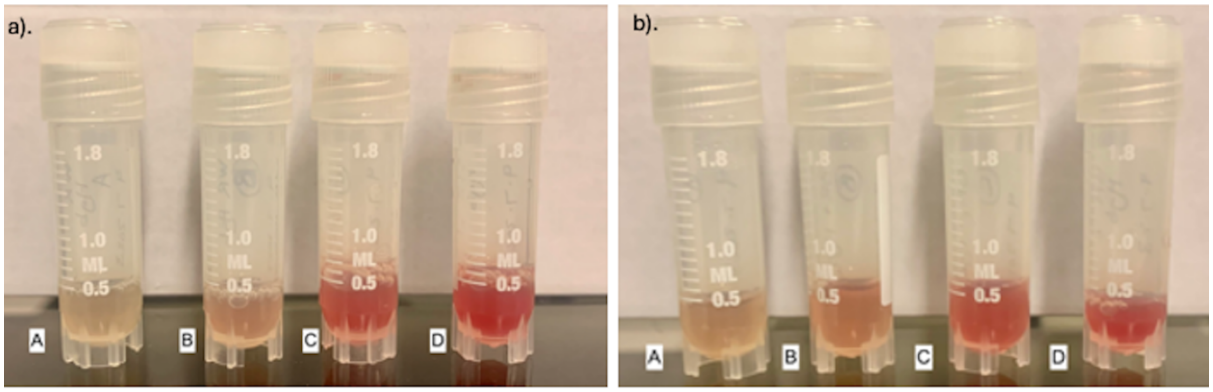

Figure 4: Haemoglobin interference study, different quantities of haemoglobin added to a.) white rhinoceros and b.) black rhinoceros serum samples to reach increased concentrations that are anticipated to occur in patient samples.

A: 0 g/L haemoglobin; B: 1 g/L. A: 0 g/L hemoglobin; B: 1 g/L hemoglobin; C: 2 g/L hemoglobin; D: 3 g/L hemoglobin.

Recovery

Species-specific cardiac muscle homogenate was diluted with pooled serum samples from the same species to create a standard spiking solution for each analyser, with relatively high cTnI concentrations to allow the adding of small amounts to minimise specimen dilution (dilution should be <10%). Serum samples were spiked to four different cTnI concentrations, for each species per analyser. Distilled water was added to the control serum specimens at equivalent volumes. Duplicate measurements of both spiked and control specimens were performed during one analytical run.

Detection limit study

The LoB and LoD were determined. In the LoB experiment, 20 replicates were performed on distilled water in one analytical run on both analysers. In the LoD experiment, species-specific pool serum samples of low concentration (close to the manufacturer-reported LoD of each analyser) were aliquoted and stored at -20°C. Four replicate measurements of each aliquot were performed daily for five days.

Statistical analysis and calculations

The statistical analysis and calculations were performed as previously described and are briefly outlined below.[77,147]

For both imprecision studies SD, mean and CV were calculated. The CV was expressed in percentage and calculated by dividing the SD by the mean multiplied by 100 for each pool.[77]

For the reportable range study, means were calculated from the duplicate or triplicate measurements and plotted against the target values of the dilution series. The resultant graphs were inspected visually for linearity over the range of values, and the slope and intercept were calculated using linear regression analysis.

Calculation of the SE due to haemoglobin interference was performed by determining the mean for the duplicates of the haemoglobin-containing sample and the control and determining the difference between the two. Lastly, the mean difference or bias for all specimens at a given concentration of interferent was calculated.

In the recovery studies the concentration of the measurand added was calculated as follows: $\text{Concentration of standard} \times \left(\frac{\text{mL standard added}}{(\text{mL of standard added} + \text{mL of sample added})}\right)$

The mean of the replicate measurements of all samples was then calculated, followed by the calculation of the difference between the spiked sample and the control. The recovery was then calculated by dividing the difference by the amount added. This was followed by calculating the mean of the recoveries of all the pairs tested and finally calculating the proportional SE as 100% – recovery%.

In the detection limit studies, the LoB and LoD were estimated by the mean value of the blank + (1.65 x SD) of the blank and the low-concentration sample, respectively. The LoQ was estimated as the mean of the blank + (2 x SD) of the low-concentration sample.[77]

The programs and statistical tools used were Microsoft Excel spreadsheets (Microsoft Corp., Redmond, WA, US) and MedCalc version 22.021 (MedCalc Software Ltd, Ostend, Belgium).

Quality control validation

Quality control validation, specifically setting control limits, was performed using the observed analytical performance of both analysers and a TE_a of 30% or 70%. One to three levels of QCM were analysed at the start of each analytical run on both analysers during the method validation experiments. This data was used to calculate the CV (formula described earlier). The target mean values provided by the QCM suppliers of the material used in the Stratus CS and Atellica VTLi were method and analyser-specific. However, as bias was present with initial QCM measurements and because calibration cannot be

performed by an operator for the Atellica VTLi to correct the bias, target mean values for QCM for both analysers for this study were calculated from the initial five to 10 measurements per QCM level. The bias was subsequently calculated as[77]

$$\text{Bias}(\%) = \frac{(\text{mean}_{\text{measured}} - \text{mean}_{\text{target}})}{\text{mean}_{\text{target}}} \times 100$$

The TE_{obs} was expressed in percentage and calculated as[77]

$$\text{TE}_{\text{obs}}(\%) = (2 \times \text{CV}) + |\text{bias}|$$

Analyser performance was also evaluated by calculating the sigma metric (σ).

$$\sigma = \frac{\text{TE}_{\text{a}}\ (\%)}{\text{CV}(\%)}$$

The selection of appropriate control rules was performed by visual analysis of the normalised Westgard OPSpecs Charts after using the Normalised OPSpecs Calculator to determine the various operating points (www.westgard.com).[150,151] A sigma-metric QC design tool was also used to identify candidate rules if none were found using the OPSpecs Charts.[152] The final rule selection was based on the criteria of $n \leq 2$, with a P_{ed} of >85% and a P_{fr} of <5%, and a simple rule was preferred over a multirule.

RESULTS

Method validation

Short-term imprecision

In the Stratus CS the CV ranged from 2.3 – 3.8% and 2.5 – 8.0% in the black and white rhinoceros, respectively. The CV ranged from 5.3 – 5.5% and 8.1 – 10.3% in the black and white rhinoceros, respectively, in the Atellica VTLi. In the Stratus CS all the SDs met both performance goals in the two rhinoceros species, with the SDs measured in the low pools

equal to the AAB performance goal (30% TE_a). In the Atellica VTLi when using AAB cTnI TE_a (30%) the white rhinoceros high pool SD failed. The low pool SDs of the black and white rhinoceros were equal to both the AAB and IFCC TF-CB performance goals (30% and 3.5 ng/L TE_a, respectively). The results are summarised in **Tables 3** and **4**.

Long-term imprecision

In the Stratus CS the CV ranged from 2.8 – 8.3% and 1.9 – 6.3% in the black and white rhinoceros, respectively. The CV ranged from 2.8 – 8.0% and 7.4 – 8.0% in the black and white rhinoceros, respectively, in the Atellica VTLi. All SDs met both performance goals, with the SD measured on the Stratus CS in the white rhinoceros low pool being equal to the AAB performance goal (30% TE_a). The results are summarised in **Tables 3** and **4**.

Table 3: Precision study results for the Siemens Stratus CS in African rhinoceros.

Black rhinoceros	Pool mean (ng/mL)	SD (ng/mL)	CV (%)	Acceptable SD at 70% TE_a*	Acceptable SD at 30% TE_a*	White rhinoceros	Pool mean (ng/mL)	SD (ng/mL)	CV (%)	Acceptable SD at 70% TE_a*	Acceptable SD at 30% TE_a*
Short term imprecision											
Low pool	0.13	0.01	3.8	0.02	0.01	Low pool	0.14	0.01	8.0	0.03	0.01
High pool	11.39	0.26	2.3	1.99	0.86	High pool	8.60	0.22	2.5	1.51	0.65
Long term imprecision											
Low pool	0.16	0.01	8.3	0.04	0.02	Low pool	0.11	0.01	6.3	0.03	0.01
High pool	10.91	0.31	2.8	2.52	1.08	High pool	7.91	0.15	1.9	1.83	0.78

* TE_a in ng/mL calculated as 70% or 30% of pool mean and acceptable SD: <0.25 TE_a for short-term imprecision; <0.33 for long-term imprecision. CV: coefficient of variation; SD: standard deviation; TE_a: total allowable error.

Table 4: Precision study results for the Siemens Atellica VTLi in African rhinoceros.

Black rhinoceros	Pool mean (ng/L)	SD (ng/L)	CV (%)	Acceptable SD (ng/L) at 70% TE_a*	Acceptable SD (ng/L) at 30% TE_a*	Acceptable SD (ng/L) at 3.5 ng/L TE_a#	White rhinoceros	Pool mean (ng/L)	SD (ng/L)	CV (%)	Acceptable SD (ng/L) at 70% TE_a*	Acceptable SD (ng/L) at 30% TE_a*	Acceptable SD (ng/L) at 3.5 ng/L TE_a#
Short term imprecision													
Low pool	11	1	5.3	2	1	1	Low pool	12	1	10.3	2	1	1
High pool	1062	59	5.5	186	80		High pool	988	80	8.1	173	**74**	
Long term imprecision													
Low pool	11	1	8.0	3	1	1	Low pool	12	1	8.0	3	1	1
High pool	1237	35	2.8	286	122		High pool	1186	88	7.4	274	117	

*TE_a in ng/L calculated as 70% or 30% of pool mean and acceptable SD: <0.25 TE_a for short-term imprecision; <0.33 for long-term imprecision. #TE_a of 3.5 ng/L at low cTnI concentrations (≤10 ng/L) as defined by IFCC TF-CB.

cTnI: cardiac troponin I; CV: coefficient of variation; IFCC TF-CB: International Federation of Clinical Chemistry Task Force on Clinical Applications of Bio-Markers; SD: standard deviation; TE_a: total allowable error. Bold text - performance goal exceeded.

Reportable range

Cardiac troponin I showed a linear range under dilution for both species, in both analysers. The analytic range, slope and intercept of the regression lines are shown in **Table 5**. Level one and five values were close to the manufacturer's reportable range for both species in both analysers.

Table 5: Results of the linearity study of cardiac troponin I in African rhinoceros serum obtained by regression analysis.

Analyser	Rhinoceros species	Analytical Range	*r*	Intercept	Slope
Stratus CS (ng/mL)	Black rhinoceros	0.03-40.13	0.99	2.62	0.98
	White rhinoceros	0.05-38.39	1.00	1.10	0.96
Atellica VTLi (ng/L)	Black rhinoceros	3-1250	0.99	-6.45	0.97
	White rhinoceros	3-1250	1.00	-33.18	1.01

r: Correlation coefficient

Haemoglobin interference

Both performance goals were met in all the haemoglobin interference experiments, except white rhinoceros samples analysed on the Stratus CS. The average observed interference was 0.02 ng/mL in white rhinoceros and it was equal to the acceptable allowed SE using the 70% TE_a performance goal but exceeded the 30% TE_a performance

goal. In black rhinoceros, the average observed interference on the Stratus CS was also 0.02 ng/mL. The interference was associated with the samples that had increased concentrations of haemoglobin (>2 g/L). The average observed interference noted in the Atellica VTLi for black and white rhinoceros, respectively, was -2 ng/L and 1 ng/L. In white rhinoceros, the observed interference in the Atellica VTLi was equal to the acceptable allowed SE using the 30% TE_a performance goal. Results are summarised in **Table 6.**

Table 6: Haemoglobin interference results for the Siemens Stratus CS and Atellica VTLi in African rhinoceros.

	Mean of paired sample measurements	**Average interference/ SE**	**Acceptable SE at 70% TE_a ***	**Acceptable SE at 30% TE_a ***
Siemens Stratus CS (ng/mL)				
Black rhinoceros	0.25	0.02	0.09	0.04
White rhinoceros	0.05	0.02	0.02	**0.01**
Siemens Atellica VTLi (ng/L)				
Black rhinoceros	36	-2	13	5
White rhinoceros	6	1	2	1

*TE_a in ng/mL (Stratus CS) or ng/L (Atellica VTLi) calculated as 70% or 30% of measurements mean and acceptable SE: <0.5 TE_a. SE: systematic error; TE_a: total allowable error. Bold text - performance goal exceeded.

Recovery

In the Stratus CS the average recovery in black and white rhinoceros samples was 110% and 102%, respectively. The proportional SE was -10% and -2% in the black and white rhinoceros, respectively. Both the performance goals (TEa of 30% or 70%) were met. In the Atellica VTLi the average recovery in black and white rhinoceros samples was 85%

and 58%, respectively. The proportional SE was 15% and 42% in the black and white rhinoceros, respectively. When using the ASVCP cTnI TE_a, the performance goals were met; however, only the black rhinoceros samples met the performance goal when assessed against the AAB cTnI TE_a.

Detection limit study

For both black and white rhinoceros, the LoB and LoD on the Stratus CS were <0.03 ng/mL and 0.04 ng/mL, respectively. The manufacturer reported analytical sensitivity is <0.03 ng/mL. The LOQ for both species on the Stratus CS was 0.05 ng/mL.

The LoB for both rhinoceros species was 0.06 ng/L on the Atellica VTLi, which is lower than reported by the manufacturer (0.55 ng/L). The LoD on this analyser was 0.88 ng/L for black rhinoceros and 0.81 ng/L for white rhinoceros. This is lower than the manufacturer-reported LoD (1.2 – 1.6 ng/L). The LoQ was 1.05 ng/L and 0.97 ng/L in the black and white rhinoceros, respectively.

QC validation

The results are summarised in **Table 7**, with suitable candidate QC rules as advised for POC analysers highlighted in blue.[129,130]

For the Stratus CS, the target mean calculated using 10 measurements was the same as the method-specific target mean provided by the manufacturer and therefore the manufacturer target mean was used for bias calculations. For the Stratus CS, 42 QCM data points were available in a six-month period for level two of the commercial QCM.

The assay was suitable for statistical QC using the $1_{2.5s}$ n = 2 rule at the 30% TE_a performance goal, however, it was associated with a P_{ed} of 80% and a P_{fr} of 3%; no rules were available for a higher P_{ed}. When using the 70% TE_a performance goal, three suitable QC rule candidates were available with a P_{ed} of ≥90 % and a P_{fr} of 0-3% using two levels of QCM.

For the Atellica VTLi, 18, 14 and 23 QCM data points were available for levels one to three of the commercial QCM, respectively, in an eight-month period. Target means were calculated using eight, five and 10 measurement data points, respectively, for QCM levels one, two and three. These values differed from those provided by the manufacturer and the calculated measurement means were used for bias calculations. The remaining data points for each QCM level were used for QC performance calculations. When using the 30% TE_a performance goal, candidate QC rules with a P_{ed} of ≥90 % and a P_{fr} of 0-3% using two levels of QCM were not available for any of the QCM levels. This was also true when using the 70% TE_a performance goal for QCM level one, but three suitable QC rule candidates were available with a P_{ed} of ≥90 % and a P_{fr} of 0-3% using two levels of QCM for QCM levels two and three.

Table 7: Results of the quality control (QC) validation from observed analytical performance using commercial QC material.

	Stratus CS: MAS CardioImmune XL, Level 2 (ng/mL)	**Atellica VTLi:** Pathonorm Cardiac Acute Lig L-1 (ng/L)	Pathonorm Cardiac Acute Lig L-2 (ng/L)	Pathonorm Cardiac Acute Lig L-3 (ng/L)
Target mean	0.62*	16	33	277
Observed mean	0.59	16	32	278
SD	0.04	3	2	25
CV (%)	7.2	17.7	7.1	9.0
Bias (%)	-5.5	3.9	-1.1	0.5
TE_{obs} (%)	19.8	39.3	15.4	18.4
Sigma metric (σ) at 30% TE_a	4.2	1.7	4.2	3.3
Sigma metric (σ) at 70% TE_a	9.8	3.9	9.8	7.8

QC rule candidates at 30% TE_a, n = 2	$1_{2.5s}$ P_{ed}: 80% P_f: 3%	1_{2s} P_{ed}: 5% P_f: 3%	1_{2s} P_{ed}: 90% P_f: 9% $1_{2.5s}$ P_{ed}: 80% P_f: 3%	$1_{2.5s}$ P_{ed}: 40% P_f: 3%
QC rule candidates at 70% TE_a, n = 2	1_{3s} P_{ed}: >90% P_f: 0% $1_{3.5s}$ P_{ed}: 90% P_f: 0% $1_{2.5s}$ P_{ed}: 90% P_f: 3%	$1_{2.5s}$ P_{ed}: 75% P_f: 3%	1_{3s} P_{ed}: >90% P_f: 0% $1_{3.5s}$ P_{ed}: 90% P_f: 0% $1_{2.5s}$ P_{ed}: 90% P_f: 3%	1_{3s} P_{ed}: >90% P_f: 0% $1_{3.5s}$ P_{ed}: 90% P_f: 0% $1_{2.5s}$ P_{ed}: 90% P_f: 3%

*Used the QCM manufacturer-provided target mean. The remaining target means were calculated from analyser-specific measurements. The rules in blue met the simple rule requirements, with a P_{ed} >85% and a probability of false rejection <5% using two levels of quality control material.

CV: coefficient of variation; n: number of quality control level measurements performed; P_{ed}: probability of error detection; P_{fr}: probability of false rejection; SD: standard deviation; TE_a: total allowable error; TE_{obs}: total observed error; σ: sigma metric; QC: quality control; QCM: quality control material.

DISCUSSION AND CONCLUSION

Both POC analysers fulfilled most of the method validation requirements and can be used for black and white rhinoceros. Furthermore, candidate QC rules suitable to control the level of error as suggested by the ASVCP, were identified based on observed analytical performance. These rules can be used to assess the performance of the analysers once validated with prospective QC data points, which is the penultimate step before implementation.[120]

Deciding on whether an instrument or method is suitable for its intended use in veterinary testing is based on predetermined quality goals, namely the TE_a, and the strictest requirement should be used.[77] The consensus ASVCP TE_a for cardiac troponin is 70% which is based on clinical interpretation of results.[122,146] A consensus TE_a can also be based on BV which is the most stringent quality goal or state-of-the-art performance where analytical performance is used to determine the lowest TE_a that can be controlled.[153] A more stringent TE_a of 30%, recommended by the AAB was also used in this study, as the ASVCP guideline was derived from a single expert opinion based on cardiac troponin results in dogs and cats and because of the lack of published data on clinical and biological variation of cardiac troponin results in African rhinoceros. The AAB recommendation is based on the "final rule" mandated by the Centers for Medicare & Medicaid Services according to the Clinical Laboratory Improvement Amendments of Proficiency Testing Regulations Related to Analytes and Acceptable Performance, published in the US Federal Register in July 2022 and revised on the 8th of July 2024, which

will be implemented in January 2025.[154] Furthermore, additional analytical performance goals are required by the IFCC TF-CB for hs-cTnI assays as cardiac troponin testing is the standard of practice for the diagnosis of acute myocardial infarction, early rule-out, risk stratification and outcome assessment in patients with acute coronary syndrome.[155] Among other things, these recommendations focus on QC utilisation, validation of the lower reportable analytical limits, correct reporting of units in measurable concentration for patients and QCM, and imprecision goals at the 99th URLs.[75] All the method validation experiments performed for both assay methods met the ASVCP TE_a performance goal. The AAB TE_a performance goal was not met in the white rhinoceros short-term replication experiment on the Atellica VTLi, but the imprecision at concentrations near 10 ng/L was acceptable for the Atellica VTLi based on the IFCC TF-CB hs-cTnI assay guidelines. The AAB TE_a performance goal was not met for the white rhinoceros haemoglobin interference experiment on the Stratus CS, indicating that haemolysed samples from white rhinoceros are not suitable for cTnI measurement with the Stratus CS. The error present with the white rhinoceros recovery experiment on the Atellica VTLi also exceeded the AAB TE_a. However, based on all results, both analysers are suitable for the preliminary measurement of cTnI in both rhinoceros species despite not fulfilling all criteria for the 30% performance goal.

Although the Stratus CS has not been validated for use in veterinary species, it has been used to generate feline and canine cTnI RIs and assess cardiac muscle reactivity in pigs, sheep, rabbits, rats and mice.[65,101] There are therefore no veterinary-specific repeatability results available for comparison, but the reported within-run CV in human validation studies using commercial QCM is low (<3%).[156] In our study, only the imprecision for the

white rhinoceros low pool was higher than reported for low pool and QCM concentrations by the manufacturer (2.7 – 4.3%; only repeatability data available). The Atellica VTLi has not been used or validated for use in veterinary medicine and repeatability results can only be compared to results obtained from human validation studies and the manufacturer's findings. The reported CV% ranged from 4.1 – 8.0% and 3.4 – 9.3%, in a recent validation study and the manufacturer results, respectively.[136] These CV% results are comparable to the CV obtained in the white rhinoceros high pool which failed the 30% TE_a performance goal. The experiment was performed by one operator under standardised conditions and procedures and therefore the CV obtained at a high cTnI concentration in white rhinoceros serum most likely reflects the analyser's inherent analytical performance capability. However, the CV was <10%, fulfilling the recommended high precision for a hs-cTnI method.[69,157]

In human medicine, very low cTnI concentrations, only measurable by hs-cTnI assays, are used for medical decision-making, necessitating the evaluation of analytical performance for hs-cTnI assays at low cTnI concentrations (≤10 ng/L), as significant bias has previously been identified in hs-cTn assays.[148] Therefore, the IFCC TF-CB recommends that long-term analytical performance at low cTnI concentrations be monitored using a performance goal of SD 3.5 ng/L.[148] Furthermore, the 99th URLs are used as diagnostic cut-off values in the diagnosis of acute coronary syndrome in humans[158] and it is recommended that hs-cTnI assays should have a CV of ≤10% at the 99th URLs, which allows confident reporting of hs-cTnI values and serial changes in cardiac troponin results over time.[155] According to the Atellica VTLi manufacturer information and a recent study,[136] this could be achieved for the assay based on their

validations. Similarly, it was confirmed in our study, and the performance goal was achieved with CVs of <10% obtained for the low sample pools (11 ng/L), which approximates the concentration of the 99th URLs determined for black and white rhinoceros, 9 ng/L and 6 ng/L, respectively (refer to Chapter 5).

Haemoglobin, up to a concentration of 10 g/L, in the Stratus CS has been shown by the manufacturer to not result in a significant level of interference and has been confirmed in validation studies.[156] The lack of significant interference was also true for all the black rhinoceros samples in the haemoglobin interference experiment, but not for the white rhinoceros samples containing ≥2 g/L haemoglobin. A possible explanation for this discrepancy between the two rhinoceros species is that the troponin concentration in the white rhinoceros experiment was very low and near the detection limit of 0.04 ng/mL (0.05 ng/mL vs 0.25 ng/mL in black rhinoceros, **Table 6**) and the SE may not be evident at higher cTnI concentration in the Stratus CS. Similar to human validation studies, haemoglobin did not significantly affect the Atellica VTLi results in rhinoceros, supporting manufacturer claims.[136]

The proportional SE from the recovery experiment in white rhinoceros samples analysed on the Atellica VTLi exceeded the 30% total error goal, but performed within acceptable limits when compared to the ASVCP guidelines. Conversely, the Atellica VTLi recovery in the black rhinoceros samples met both performance goals. The same reagent cartridge lot was used in the black and white rhinoceros experiment, therefore, it is plausible that a species-specific matrix effect was the cause of the bias present in white rhinoceros. As this analyser has not been used in publications concerning other animal species it is

difficult to determine the source of bias. Although not investigated in this study due to costs and time constraints, assessing whether similar results are noted in plasma or whole blood samples will be useful.

When validating an immunoassay, detection limits experiments are recommended especially when a low value may be of clinical significance.[77] With the advent of hs-cTn assays and the use of the 99th URLs for clinical decision-making, clinicians are incorporating both detectable and nondetectable hs-cTn concentrations into their clinical decision-making process.[75] Emerging clinical evidence suggests that "undetectable levels", concentrations less than either the LoB or LoD, can be used to safely rule out acute MI using a single cardiac troponin value with high clinical sensitivity.[155] It is recommended that the LoD should be defined as the lower analytical reportable limit and be communicated to clinicians, especially if the LoQ is being utilised for the lower reporting limit.[75] To ensure that detectable hs-cTn concentrations can be consistently quantified over time due to reported drift over time at the 99th percentile medical decision limit of hs-cTn assays, it is advised that clinical laboratories should validate LoB, LoD (outside of the US) and LoQ (as per US FDA regulations) at a minimum on an annual basis or more frequently as deemed necessary.[75] Even though high values of cTnI rather than low concentrations are considered significant in veterinary patients, detection limit studies also guide decisions on whether an assay will be useful in a particular species, i.e., is the concentration of the analyte of interest quantifiable in health by the assay. Serum cTnI in healthy dogs and cats as measured on the Stratus CS has been reported to often be below the detection limit of the assay (0.03 ng/mL).[101] It is reasonable to extrapolate this to rhinoceros based on the LoD at 0.04 ng/mL determined

in this assay. The hs-cTnI assay utilised by the Atellica VTLi is likely to be more useful for serum cTnI quantification, especially when establishing reference intervals in healthy animals or for monitoring changes at low concentration levels.

The ASVCP QA guidelines recommend weekly measurement of QCM for unit devices in POC analysers.[129] Quality control limits established during validation instead of manufacturer target ranges should be used in assessing method performance. A simple QC rule, such as $1_{2.5s}$ or 1_{3s} with a P_{ed} of ≥85% and P_{fr} of ≤5%, using ≤2 levels of QCM is preferred in veterinary medicine POC analysers.[129] This guideline contrasts with the IFCC TF-CB's recommendation of daily measurement of three QCM concentrations for hs-cTn assays and two for contemporary cTn assays, which is impractical for POC analysers in veterinary practices.[75] Using ASVCP cTnI TE_a, appropriate QC rules could be achieved for both analysers, except for the Atellica VTLi when using the low QCM level, where the P_{ed} was below the 85% requirement. In the Atellica VTLi, this was made achievable in part by decreasing the bias associated with utilising the provided method-specific QCM manufacturer target means and instead using target means determined from initial QCM measurements in our laboratory. The calculation of mean was done since calibration, which is used to reduce bias in many reference laboratory instruments, could not be conducted on the Atellica VTLi.[77,119,122] A master calibration curve is created for each lot of cartridges and included in the cartridge's radio frequency identification tag, defining the correlation between signal change differences and analyte concentration.[120,159] Calibration could be done on the Stratus CS during each reagent lot change, and the computed measured target mean was comparable to the target mean provided by QCM manufacturers. Because the Atellica VTLi analyser's target means were calculated

internally, the evaluation of QCM results was initially focused on precision. It was also limited by the low number of QCM data points available, which were further reduced due to the calculation of method-specific target means. To calculate bias and CV, a minimum of 20 QCM data points are required.[77]

When using a total error goal of 30%, neither analyser qualified for the use of a simple candidate QC rule with the desired P_{ed} and $P_{fr.}$ The lack of a valid QC rule was primarily due to the high CVs obtained. The CVs for the lower concentration QCM target means were comparable to the CVs observed in the low pool precision studies, except for the Atellica VTLi's low-level QCM, which exceeded the highest low pool CV obtained (10.3% in the low pool vs 17.7% for level one QCM). This is also the only QCM level with a σ <3.0, indicating inadequate analytical performance and the need for additional statistical and non-statistical QC.[120] Currently, there are no published guidelines for the concentration of QCM that should be used to monitor the performance of cTnI assays, as well as the permissible imprecision and bias at concentrations equal to or less than the 99th percentile.[75] However, monitoring analytical performance at low concentrations is critical, as reported quantitative shifts may result in analytical variations within the low concentration ranges that exceed the stated degree of concentration change utilised in clinical research for serial cTnI measurements.[75] High-sensitivity-cTnI POC analysers should adhere to the proposed performance specification of <35% of the TE at low hs-cTnI concentrations.[75] To achieve this, the "reverse approach"[129] can be used to determine the minimum total error that can be managed with a simple QC rule.[120] However, manual OPSpecs Charts are not sufficient for this purpose, and a statistical computerised software program like EZ Rules 3 (EZ RULES 3,

Westgard QC, Madison, WI, US) is necessary.[120] When the CVs from both analysers (excluding the Atellica VTLi, level one) were entered into a "reverse approach" calculation using the Westgard sigma-metric design tool, the total error that could be controlled with a simple QC rule ($1_{2.5s}$, P_{ed} of 90% and P_{fr} of 3%, n = 2) was 33-42%. This computed minimum total error that can be controlled may subsequently be used as a consensus TE_a goal for cTnI in veterinary laboratory medicine. The next step is to select a final simple rule, calculate control limits at the specified QCM levels, and utilise statistical QC to monitor analytical performance on both analysers over time.

Based on its small size, Atellica VTLi lends itself to potential use in field conditions. However, operating specifications advise ambient temperatures of 5-27°C and single-use reagent cartridges should be stored between 2-8°C, which may be difficult to maintain during field operations. Furthermore, the battery life allows the analysis of up to 60 tests, and the data of 100 tests can be stored on the analyser before it needs to be downloaded to the service software program using a local area or wireless network. Considering these specifications, the efficiency and practicality of use in field conditions should be assessed.

This study has certain limitations that should be considered. Firstly, the validation of the assays was conducted using biobank-stored species-specific serum pools, however, the assays have not been validated for the use of serum. The Stratus CS assay requires lithium or sodium heparin anticoagulated whole blood, while the Atellica VTLi assay has been validated for use with lithium-heparinised whole blood or plasma.[136,160] Ideally, the effect of serum should have been compared to lithium heparinised plasma samples

using an interference study, but only serum and not plasma samples were available. The contribution of a matrix effect due to the use of serum, or species-specific differences could be a reason for performance goals not being met in the recovery experiment for the white rhinoceros.

Troponin I exists in various subforms, and it is found as slow-twitch and fast-twitch skeletal (skTn) or cardiac (cTn) isoforms. The sequence homology between skTnI and cTnI is approximately 40%.[161] The analytical specificity of the investigated immunoassays, i.e., cross-reactivity of the immunoassay with skTnI, was not evaluated in this study. Nevertheless, a veterinary study found cross-reactivity with skTnI when assessing cardiac muscle reactivity to Stratus CS antibodies.[65] However, this assay was highly selective for myocardium, with a reactivity level over 1 000 times greater than that of skeletal muscle.[65] Furthermore, it is unlikely that significant cross-reactivity will occur, given the low reported degrees of cross-reactivity for skTnI in the Atellica VTLi and Stratus CS cTnI assays, which are less than 0.01% and 0.04%, respectively. Although cross-reactivity is expected to be minimal in these assays, it is essential to consider it when interpreting serum cTnI levels in rhinoceros that have experienced substantial skeletal muscle injury.

In conclusion, two POC cTnI immunoassays have been successfully validated for use in African rhinoceros. The Atellica VTLi has potential for use in the field due to its small size and low detection limits. Ideally, field performance evaluation should be performed to assess the effect of ambient outside temperatures and ease of use. Although a simple QC rule has been validated for both analysers based on ASVCP guidelines on total error,

further investigation into the lowest total error that can be controlled, based on the analytical performance of each analyser, is needed.

CHAPTER 5: GENERATION OF REFERENCE INTERVALS FOR CARDIAC TROPONIN I IN AFRICAN RHINOCEROS USING A HIGH-SENSITIVITY POINT-OF-CARE IMMUNOASSAY

The results presented in this chapter will be submitted as a research article.

Manuscript is prepared:

Rautenbach Y., Meyer L.C.R., Goddard, A., Buss P.E., Hooijberg E.H. Generation of reference intervals for cardiac troponin I in African rhinoceros using a high-sensitivity point-of-care immunoassay.

SUMMARY

Chemical immobilisation and transport are essential for managing and conserving African rhinoceros and have several documented adverse effects, including on the cardiovascular system. The presence and nature of cardiomyocyte injury in African rhinoceros have not been investigated. A hs-cTnI assay has recently been validated for use in both black and white rhinoceros, but RIs are lacking. The main purpose of the study was to establish RIs for African rhinoceros using the hs-cTnI assay. A secondary aim was to assess if subset partitioning could be applied in animals that were chased during immobilisation versus those that were not. Biobanked serum samples of apparently healthy free-living immobilised black (*n* = 62) and white (*n* = 87) rhinoceros from the Kruger National Park, South Africa were used. Serum cTnI concentration was measured on the Atellica VTLi hs-cTnI immunoassay. The RIs were calculated according to the ASVCP recommendations. The RIs for serum cTnI were 1.47-8.17 ng/L and 2.22-5.84 ng/L in black and white rhinoceros, respectively. The 99th URLs were 9 ng/L and 6 ng/L in black and white rhinoceros, respectively. Subclass partitioning was possible for white rhinoceros based on whether they were immobilised in a boma or chased by a helicopter before being immobilised. In conclusion, the generation of species-specific hs-cTnI RIs on a POC analyser in black and white rhinoceros allows for the identification of potential cardiomyocyte damage in these species.

INTRODUCTION

Global conservation strategies are currently being upscaled due to recent reports of unprecedented loss of wild species associated with high extinction rates.[5] Specifically, the loss of large mammalian species is accelerated by increased poaching pressure, wildlife trade and increasing human population pressure leading to habit loss and fragmentation.[5] Approximately 89% of the remaining African rhinoceros populations, which encompass the northern white (*C. simum cottoni*), southern white (*C. simum simum*), and southern-central black (*D. bicornis minor*) rhinoceros, are located in southern Africa.[162] The IUCN has classified two of the rhinoceros species in sub-Saharan Africa (the range extending from Kenya in the north to South Africa), namely the southern-central black and southern white rhinoceros, as critically endangered and near threatened, respectively.[5] In addition to population loss due to illegal harvesting of, and trade in, rhinoceros horn, recent studies have shown that climate change and associated temperature changes are likely to have significant impacts on habitat suitability for southern African rhinoceros.[10]

National and international rhinoceros conservation plans often involve translocation for population reintroduction or reinforcement, or metapopulation management.[16] Translocation involves capture, temporary captivity, transport, and release into a novel environment.[16] Dehorning, or horn trimming, is another commonly performed conservation intervention that serves as an antipoaching tool and is occasionally performed at capture when translocating the animals.[163] Rhinoceros are large and

dangerous animals, and chemical immobilisation to capture and restrain individuals is essential for most veterinary and conservation-related procedures.[17] Helicopter capture is used to capture free-living animals in the wild and involves chasing the animal for variable distances before darting it with potent immobilising drugs.[17] Alternatively, lower doses of these drugs are used during ground-darting of rhinoceros that are confined in a holding-facility or enclosure (boma) after being previously chased during the initial immobilisation. Animals may be kept in bomas or camps in rehabilitation facilities or if undergoing veterinary treatment, but may also be confined for a specified period after helicopter capture for adaptive purposes.[16,164] Boma adaptation in translocated rhinoceros, which varies from weeks to months, allows transition to new diets, and restricted living space, and allows quarantine for disease screening and treatment of injured animals.[164]

Reported welfare challenges in the capture and transport of black and white rhinoceros include dehydration, mobilisation of energy reserves, stress-induced immunomodulation, oxidative stress, and muscular damage.[16] Severe hypoxaemia and acidaemia due to respiratory compromise and metabolic acidosis are commonly associated with chemical immobilisation and capture, particularly when using potent opioids, such as etorphine, combined with exertion due to prolonged chase distances or time.[17,164] These drugs can lead to cardiac arrhythmias, and muscle and nervous system dysfunction due to acidosis and hypoxaemia, which may result from muscle rigidity or tremors, tachycardia, and decreased tissue perfusion.[164]

Cardiac troponins, which are intracellular proteins that regulate the contractile mechanism in cardiomyocytes, serve as highly specific and sensitive indicators of myocardial damage.[63,70] From a clinical perspective, troponins' most significant advantage lies in their exceptional negative predictive value for both cardiac and non-cardiac conditions. In general, low troponin concentrations correlate with improved chances of survival, whilst increased concentrations indicate individuals at higher mortality risk.[70] Blood troponin concentration serves as a quantitative measure of the extent of myocardial injury; however, it provides no information regarding the underlying cause of the injury or the mechanism of troponin release.[70] In a canine model of acute MI, cardiac troponin serum concentration begins to rise 4-12 hours after the infarction, with peak concentrations reached within 10-16 hours.[63]

Information related to the cTnI nucleotide sequence in black and white rhinoceros and validation of two POC cTnI immunoassays facilitates the investigation of potential cardiomyocyte damage in African rhinoceros (refer to Chapters 3 and 4). The primary aim of this study was to generate serum cTnI RIs from a population of apparently healthy free-living black and white rhinoceros using the recently validated Atellica VTLi hs-cTnI assay. A secondary aim was to evaluate whether RI subclass partitioning could be applied to animals being chased before immobilisation and blood sampling compared to boma-adapted animals that were immobilised without chasing.

MATERIALS AND METHODS

Study population

The black rhinoceros (*D. bicornis minor*) reference individuals originated from the population of apparently healthy free-living rhinoceros living within the Kruger National Park (23°49'60"S, 31°30'0"E) in the north-eastern part of South Africa. Blood samples were collected from June 2021 to April 2023 from animals immobilised for dehorning, except for one animal that was immobilised for relocation.

The white rhinoceros (C. *simum simum*) reference individuals were subcategorised either as free-living animals chased by a helicopter during immobilisation or boma-adapted animals. Animals originated from the population of free-living or boma-kept rhinoceros living within the Kruger National Park, except for 21 boma-adapted animals that were in a rhinoceros sanctuary close to the national park. Blood samples were collected from animals immobilised for translocation, dehorning or other management purposes from July 2018 to April 2023 (chased) and October 2020 to August 2023 (boma-adapted).

Healthy calves, subadults and adults of both sexes were included. Age was determined from body size and horn development. Calves are individuals still with dam and <2.5 years, subadults are 2.5-7 years and adults are animals >7 years.[165]

Animals were determined to be healthy based on a physical examination carried out during immobilisation at the time of blood collection. If there were repeat samples from the same animal, only the most recent sample was used, and any others were excluded.

Sample collection and storage

Rhinoceros were immobilised according to the South African National Parks Standard Operating Procedure for the Capture, Transport and Maintenance in Holding Facilities of Wildlife. A combination of etorphine hydrochloride (9.8 mg/mL, Captivon®; Wildlife Pharmaceuticals, White River, South Africa) or thiafentanil (9.8 mg/mL, Thianil®, Wildlife Pharmaceuticals, White River, South Africa), and azaperone tartrate (40 mg/mL, Stresnil®; Janssen Pharmaceutical Ltd., Halfway House, South Africa) or midazolam hydrochloride (50 mg/mL, Dazonil®, Wildlife Pharmaceuticals, White River, South Africa) was used.

The dose in adult male and female black rhinoceros was 4.0-4.5 mg thiafentanil and 60 mg azaperone. The dose in adult male and female white rhinoceros was 3.5-4.5 mg of etorphine and 20-40 mg azaperone or midazolam. The etorphine dose was based on the rhinoceros age and estimated body weight. Butorphanol tartrate (50 mg/mL, Butonil®, Wildlife Pharmaceuticals, White River, South Africa) was routinely administrated intravenously (IV) to white rhinoceros immediately after recumbency as a partial opioid antagonist at a dose of 10-20 mg for every 1 mg of etorphine.

In the chased group, rhinoceros were located and darted from a helicopter, while boma animals were darted from the ground. The immobilisation drug combination was

administrated IM using a 3 mL plastic dart plus a 60 mm uncollared needle fired from a compressed air rifle (DAN-INJECT®; International S.A., Skukuza, South Africa).

Blood was collected from an auricular or medial radial vein directly into a serum vacutainer tube (Greiner Bio-One, Lasec S.A., Pty Ltd., Cape Town, South Africa or BD Vacutainer, Becton and Dickinson, Oxford, UK) within 15 min of a rhinoceros becoming recumbent. Samples were transported on ice packs in a cooler box until they could be processed in a laboratory, within 3-24 hours of collection. Samples were centrifuged at 1300 *g* for 10 min and the serum was decanted into a cryotube (Greiner Bio-One, Lasec S.A., Pty Ltd., Cape Town, South Africa). Samples were frozen at -80°C.

Samples were subsequently transported frozen on dry ice to the clinical pathology laboratory at the Onderstepoort Veterinary Academic Hospital and again stored at -80°C. Samples were between three and 65 months old at the time of analysis and most samples had undergone a single freeze-thaw cycle. Samples were excluded if gross haemolysis, lipaemia, or icterus was present. Each sample was thawed, mixed, and analysed on the same day, and all RI data were collected over eight non-consecutive days.

Analytical methods

Serum cTnI concentration was measured on the Atellica VTLi hs-cTnI assay, using the POC Atellica VTLi Immunoassay analyser (Siemens Healthineers, Midrand, South Africa), previously validated in African rhinoceros (refer to Chapter 4). The Atellica VTLi hs-cTnI assay test is a two-site immunoassay, with monoclonal cTnI detection antibodies bound

to superparamagnetic particles.[136] One level of quality control material (Pathonorm Cardiac Acute Liq, Sero) was run at the start of each analytical run.

Statistical analysis

Generation of 95% RIs was performed in line with the ASVCP guidelines and recent guidelines for small sample sizes.[131,166,167] Additionally, the 99th URLs were also determined. The results of the 99th URLs were reported as whole numbers according to the IFCC TF-CB.[123] Calculations were performed using MedCalc for Windows, version 22.021 (MedCalc Software, Ostend, Belgium) and *Reference Value Advisor* version 2.1.[168] Descriptive statistics were compiled, data distribution in the form of histograms was examined visually, and normality and symmetry were additionally assessed using the Shapiro-Wilk test (level of significance P <0.2)[166] and McWilliams runs test (level of significance P <0.05). Outliers were identified using Tukey's and Dixon methods. Because true determination of health in these animals could not easily be performed, a strict approach to outlier exclusion was followed. To calculate the 95% RIs, when samples comprised 60-100 reference individuals, the parametric method (P) was used if the data had a G distribution, and the NP in all other instances. If the sample size consisted of <60 reference individuals the NP was used regardless of the data distribution.[166,167] A non-parametric bootstrap method was used to calculate the 90% CI of the limits. To calculate the 99th URLs and CIs the P was used for both species. According to recent international guidelines related to MI in humans, an increase in cTnI over the 99th URLs is considered clinically relevant.[158] The ratio of the width of the CI to the width of the RIs was also calculated.[131]

Partitioning of the rhinoceros reference populations was statistically evaluated following Lahti *et al*. recommendations for G distributions, which support partitioning into subclasses if >4.1% or <0.9% of a subclass falls outside the upper or lower limits of the entire population RI, and discourages partitioning if the proportion of the subclass which falls outside the combined RI is between 1.8% and 3.2%.[169] An independent samples t-test was used to further investigate whether significant statistical differences were present between subgroups.

The effect of sample age (in months) on the black and white rhinoceros' serum cTnI concentration was evaluated using linear regression analysis. A *P*-value of ≤0.05 was considered significant.

RESULTS

Study population

Seventy black rhinoceros were enrolled of which only one animal was boma-adapted. Repeat samples for four individuals and two invalid test results were excluded, as were two suspected outliers. The outliers were both males, a calf and subadult, and the serum cTnI concentration was 0 ng/L and 17 ng/L, respectively. The final reference population consisted of 62 individuals and included 16 adult males, 22 adult females, 11 subadult males, seven subadult females, and three male and three female calves.

A hundred and eight white rhinoceros, 52 chased and 56 boma-adapted, were enrolled. Two repeats, two invalid samples, and 17 suspected outliers were removed based on outlier testing and visual inspection of the data distribution. Eleven of the white rhinoceros outliers were boma-adapted, and six chased. There were 12 subadult males, three subadult females, and one female calf. The range of serum cTnI concentration was 2 to 101 ng/L (average concentration: 14 ng/L). The reference population consisted of 87 individuals (44 chased and 43 boma-adapted) and included 14 adult males, 10 adult females, 31 subadult males, 10 subadult females, 12 male and 10 female calves.

Reference interval and subclass partitioning

Descriptive statistics and the final 95% RIs for serum cTnI obtained using P are presented in **Table 8**. The RIs of serum cTnI determined in the black and white rhinoceros reference populations were 1.47 to 8.17 ng/L and 2.22 to 5.84 ng/L, respectively. The frequency histograms of the black and white rhinoceros reference population are illustrated in **Figure 5**. The data had a G distribution (P = 0.594 and P = 0.213 in black and white rhinoceros, respectively). The 99th URL was 9 ng/L and 6 ng/L, for black and white rhinoceros, respectively (**Table 8**). The ratio of the width of the CI to the width of the 95% and 99% RIs was <20% (**Table 8**).

Considering the Lahti *et al.* partitioning statistical recommendations,[169] only the white rhinoceros reference population could be partitioned into a chased and boma-adapted (non-chased) group. The NP was used to determine the RIs and descriptive statistics and the final RIs reported in **Table 8**. The ratio of the width of the CI to the width of the non-chased white rhinoceros upper limit RI was >20% (**Table 8**). There were no significant

differences between the two subgroups, based on the independent samples t-test (*P* = 0.571).

Table 8: Reference intervals for serum cTnI in apparently healthy African rhinoceros, measured using the Atellica VTLi high-sensitivity cTnI assay on the Atellica VTLi immunoassay analyser (Siemens Healthineers, Midrand, South Africa).

cTnI	Units	n	Mean	SD	Median	Min	Max	*P*-value	Distribution	Method	LRL of RI	URL of RI	CI 90% of LRL	CI 90% of URL
95% Reference intervals														
Black rhinoceros	ng/L	62	4.82	1.66	4.80	1.50	9.00	0.594	G	P	1.47	8.17	0.92 – 2.04	7.57 – 8.75
White rhinoceros	ng/L	87	4.03	0.91	3.90	2.00	6.30	0.213	G	P	2.22	5.84	1.97 – 2.48	5.57 – 6.11
Non-chased white rhinoceros	ng/L	43	3.87	0.86	3.80	2.00	6.00	0.690	G	NP	2.03	5.99*	2.00 – 2.54	5.16 – 6.00

Chased white rhinoceros	ng/L	44	4.19	0.94	4.25	2.40	6.30	0.408	G	NP	2.41	6.29	2.40 – 2.76	5.85 – 6.30
99% Reference intervals														
Black rhinoceros	ng/L	62	4.82	1.66	4.80	1.50	9.00	0.594	G	P	1	9	0 – 1	9 – 10
White rhinoceros	ng/L	87	4.03	0.91	3.90	2.00	6.30	0.213	G	P	2	6	1 – 2	6 – 7

The Shapiro-Wilk test was used for evaluating the distribution of data (*P* <0.2). CI: confidence interval; cTnI: cardiac troponin I; G: gaussian; LRL: lower reference limit; n: number of included reference individuals; NG: non-Gaussian; NP: non-parametric method; P: parametric method; RI: reference interval; SD: standard deviation; URL: upper reference limit.

*The CI to RI ratio exceeded 20%.

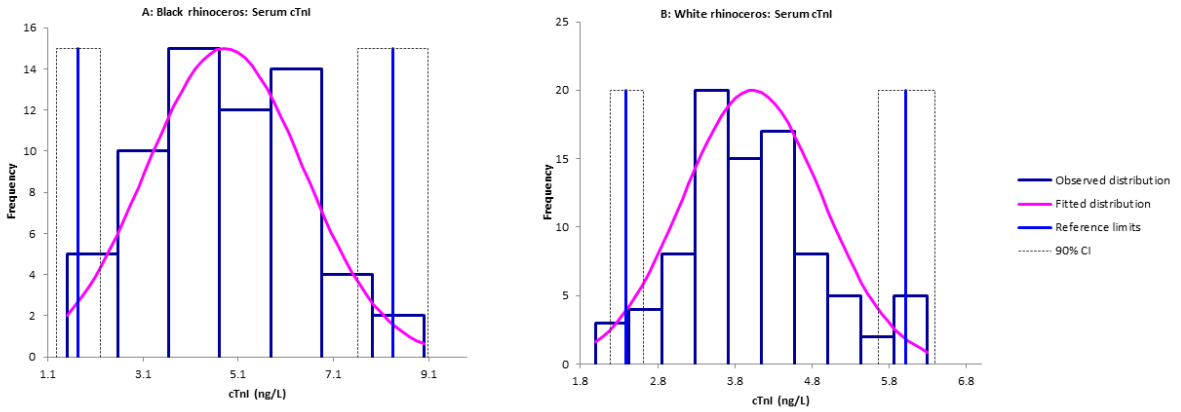

Figure 5: Histograms depicting the frequency distribution for serum cardiac troponin I (cTnI) concentration in the (a) black rhinoceros reference population (n = 62) and (b) white rhinoceros reference population (n = 87).

Effect of sample age

The black rhinoceros sample age ranged from seven to 35 months and in white rhinoceros the sample age ranged from three to 65 months. There was no significant effect of storage time on cTnI; the coefficient of determination (r^2) between the sample age and serum cTnI in black and white rhinoceros was 0.011 (*P* = 0.424) and 0.014 (*P* = 0.273) respectively and the regression plots are presented in **Figure 6**.

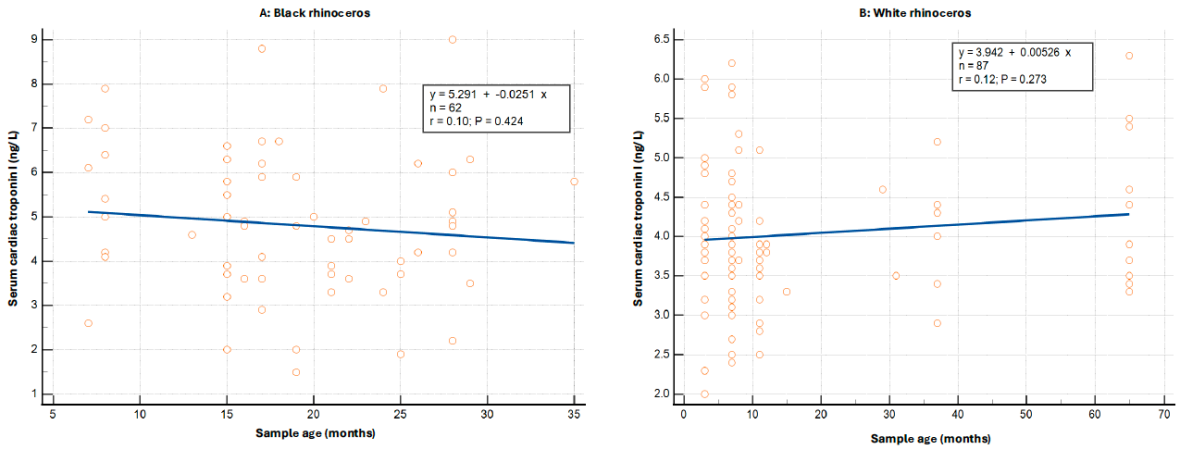

Figure 6: Regression analysis between the sample age (months) and the serum cardiac troponin I concentration in (a) black and (b) white rhinoceros.

DISCUSSION AND CONCLUSION

According to the authors' knowledge, this is the first report of cTnI RIs in African rhinoceros generated in compliance with the ASVCP guidelines, with a slightly wider RI established for the black rhinoceros compared to the white rhinoceros. For the generation of RIs, the inclusion of a minimum of 120 reference individuals is advised, however, this could not be achieved in this study and is a limitation commonly encountered in wildlife species especially when endangered or threatened. Considering the effect of small sample sizes on the accuracy of statistical methods used for generating RIs, the latest guidelines on RI estimation in small sample sizes were followed.[166,167] Based on these recommendations the Shapiro-Wilk method was used to assess the data distribution, with significance set at P >0.2. Furthermore, with small sample sizes NP were used in this study, regardless of the data distribution.

Partitioning into subclasses is based on physiological differences that are expected to result in clinically important differences in RIs.[131] In light of reported increases in serum cTnI in chased blesbok (*Damaliscus pygargus phillipsi*) sampled immediately after immobilisation and 40 min later,[133] and capture myopathy identified as a potential problem in captured and translocated rhinoceros,[170] partitioning was based on animals that were chased during chemical immobilisation, compared to those that were confined in bomas and darted from the ground. At least 40 individuals should be included within each subclass, therefore partitioning could only be applied in the white rhinoceros.[131] Studies have shown that increased concentrations of cTnI can be observed in humans following various forms of physical activity. This includes not only extended periods of intense exercise, such as marathon running, but also shorter and intermittent bouts of activity, like half-hour runs or playing basketball.[171] Similarly, elevated cTn concentrations following prolonged physical exertion lasting approximately 16-18 hours and 3-14 hours in sled dogs and horses, respectively, have been reported.[63] Elevated concentrations of cTnI immediately after an hour of show jumping in horses have also been documented.[172] Based on these findings, similar to humans, both heavy and light exercise may cause elevated troponin concentrations. It is believed that after physical exercise slight hypoxia induces the leakage of cytosolic cTn, resulting in low absolute levels within hours and rapid normalisation within 24-38 hours. These findings correspond to cytosolic release and not cellular necrosis and possibly reflect a physiological as opposed to a pathological process.[171,172] Notably, although the reference limits were comparable and the cTnI concentrations did not differ significantly between white rhinoceros subgroups, the upper reference limit (URL) and the median cTnI concentration were elevated in the chased group, relative to the non-chased group. Given the elevated cTnI previously

reported in chased blesbok shortly after capture,[133] it is a reasonable hypothesis that higher serum cTnI serum concentrations would be present in chased rhinoceros (the duration of the chase is typically only 5-10 min before immobilisation). However, significantly elevated cTnI was not noted in the chased group, which can be explained by cTnI kinetics, with elevations noted only 4-12 hours after acute cardiac injury in humans, dogs, and horses.[63] In the case of cytosolic troponin release, i.e., not associated with cellular necrosis, the cTnI values return to baseline values within 24-48 hours.[171] When compared to the chased group, the fairly similar maximum serum cTnI concentrations noted in the boma-kept group could also be due to stress. In humans, myocardial ischaemia during either mental stress (mental stress-induced myocardial ischaemia, MSIMI) or conventional pharmacologic or exercise-induced stress, such as exercise or pharmacological stress testing, is associated with higher resting levels of cTnI.[173] The underlying pathophysiological mechanism causing MSIMI is still unclear, but abnormal vasomotion, depression, platelet reactivity, vitamin D deficiency, inflammation and metabolic risk factors are associated with it.[173] Physiological stress with no habituation has been demonstrated in white rhinoceros confined for extended periods.[174] Based on these results it may be postulated that the slightly higher URL cTnI concentrations in some boma-adapted animals may be due to the stress associated with being confined in holding pens or bomas.

Population-based RIs have been reported to be less useful for interpreting cTnI values due to high individual variation in dogs and humans.[22,23] The utility of population-based reference intervals for cTnI in rhinoceros is unknown due to the lack of data for BV of cTnI measured with hs-cTnI assays in general, and for cTnI in most species. Therefore,

measuring serial changes in values in individual animals, especially those that show cTnI increases at low concentrations measured with high sensitivity assays, is likely to be important for results interpretation. Based on recent international guidelines, including the 2012 Third Universal Definition of Myocardial Infarction edited by a joint task force, increased serum cTnI concentrations above the 99th URL are considered clinically relevant in humans, if this cut-off is measured with an immunoassay with an imprecision of ≤10%.[158] The 99th URL values are strongly dependent on the criteria used to define the reference population, the analytical performance of cTn methods and the statistical methods used for the reference limit calculations.[158] The precision of the Atellica VTLi hs-cTnI immunoassay in both species of rhinoceros meets the analytical quality requirement (2.8 – 7.9% and 7.4 – 10.2%, in black and white rhinoceros, respectively) (refer to Chapter 4), however, the reference population size is underpowered for the calculation of both 95% and 99% RIs.[131,155] Therefore, the diagnostic utility of these 99% URL specific cut-off values in African rhinoceros might be limited and overlap performance should be prospectively evaluated in well-defined healthy and diseased rhinoceros.

Several limitations are associated with the generation of RIs in this study. The effect of capture-induced stress and immobilisation drugs on serum cTnI concentrations, the difficulty in minimising preanalytical errors and ensuring standard sampling protocols as well as the classification of apparent health based on a restricted clinical examination in an immobilised animal, are all limitations previously reported in African rhinoceros RI generation.[175] In addition, the ratio of the width of the CI to the RI of the upper reference limit was >20% in the non-chased white rhinoceros group, indicative that more reference

individuals should be added to this group to decrease the uncertainty associated with these RI limits.[131] Data on age and sex were available for both rhinoceros species, however, subclass partitioning based on these variables was not possible due to the inability to meet the requisite minimum number of individuals per subclass. The IFCC TF-CB recommends sex-specific 99th URLs because women typically have lower URLs than men.[75,176] Although specific URLs according to age or ethnicity are not currently recommended, previous human population-based studies have found that hs-cTnI concentrations increase with age.[75,176] Furthermore, age is associated with cTnI concentration in dogs.[177] Lastly, the age of the serum samples was variable and ranged from three to 65 months, with a large proportion of these having undergone a single freeze-thaw cycle. There are no reports on the stability of cTnI in rhinoceros, but stability has been reported for two to four weeks at -80°C in New World camelids and bovines, with no significant effect after three freeze-thaw cycles.[88,106] In humans, stability of hs-cTnI has been demonstrated for up to one year at -80°C.[178] The age of the samples included in this study may be a limiting factor, however, no statistical association was found between the sample age and serum cTnI concentration. Furthermore, obtaining a large number of fresh blood samples from healthy threatened megaherbivore species such as rhinoceros is challenging.

In conclusion, this study established 95% and 99% RIs for cTnI in a population of apparently healthy free-living black and white rhinoceros using a hs-cTnI POC analyser. The generated RIs allow further investigation into the welfare of these animals in the field during veterinary procedures or translocation. Furthermore, these RIs will allow investigation into cardiovascular disease in captive and free-living rhinoceros, for which

there is currently limited information available. These generated RIs should be utilised as a baseline for chemically immobilised healthy African rhinoceros, darted by helicopter, after chase, or from the ground within a boma.

CHAPTER 6: CHANGES IN SERUM CARDIAC TROPONIN I CONCENTRATION DURING IMMOBILISATION AND TRANSLOCATION OF AFRICAN RHINOCEROS

The results presented in this chapter will be submitted as a research article.

Manuscript in preparation:

Rautenbach Y., Meyer L.C.R., Goddard, A., Buss P.E., Pohlin F., Hooijberg E.H. Serum cardiac troponin I concentrations in translocated African rhinoceros.

SUMMARY

Chemical immobilisation and long-distance transportation of black (*D. bicornis minor*) and white rhinoceros (*C. simum simum*) are essential components of conservation strategies aimed at restoring populations, preventing biodiversity loss, and improving the conservation status of a species. However, several stressors have been identified in transported rhinoceros that impact animal welfare during translocation; therefore, this study aimed to determine whether animals show evidence of cardiac injury as a result of translocation procedures. The first objective of this study was to measure the serum cTnI concentration in rhinoceros immediately after immobilisation (capture), at various time points during long-distance transport and at the point of release. The second objective was to evaluate serum cTnI levels using four different drug-immobilisation protocols. The final objective was to identify any associations between serum cTnI concentrations and biomarkers of muscular damage, indicators of negative energy balance, and markers of hypoxaemia and acidaemia during transport. Serum cTnI concentrations were measured in stored serum samples collected during four long-distance translocation studies in black and white rhinoceros, and one chemical immobilisation study in white rhinoceros. Analysis was performed using the validated Siemens Atellica VTLi hs-cTnI immunoassay. Changes in cTnI concentrations over time were evaluated with a paired t-test, Wilcoxon test, or repeated measures ANOVA/Friedman's test. The Wilcoxon test was used to compare cTnI differences between the groups. Correlation analysis between cTnI concentrations, biochemical analytes, and blood gas measurands was performed using the Spearman's rank order. A *P*-value <0.05 was considered significant. Significantly increased cTnI concentrations were noted during transportation and release compared

to capture in all rhinoceros cohorts. As expected, the degree of cTnI increase was more significant in cohorts chased and darted by helicopters. No significant differences in cTnI concentrations were found among the four immobilisation protocols. Significant associations were observed between cTnI and AST, BHB, CK, lactate, pH, and partial pressure of oxygen (PaO_2). Hypoxaemia and acidaemia were correlated with increased cTnI concentration, providing evidence of hypoxic damage to cardiomyocytes. Concurrent skeletal and cardiac muscle damage was observed in transported black and white rhinoceros, indicating that these animals most likely suffered from capture myopathy.

INTRODUCTION

Translocation of wildlife species is a commonly used tool for the conservation of threatened and endangered animals, with a focus on restoring and enhancing populations.[179] This involves the deliberate capture and movement of animals from one site for release in another.[21] Translocation plays an integral part in national and international conservation plans for the African rhinoceros.[5] The two rhinoceros species in sub-Saharan Africa, the black and white rhinoceros (*D. bicornis minor* and *C. simum simum*), are critically endangered and near threatened,[8,9] respectively, and their abundance and range of occurrence have decreased dramatically.[5] Despite concerns about animal welfare during wildlife translocation, little research has been undertaken into measuring physiological variables to quantify physiological indicators relevant to animal welfare.[13] Capture and transport-related mortalities due to acute stress have been reported in 6.5% of black rhinoceros translocation from South Africa to Namibia.[24]

Furthermore, in recent translocations, >50% to 100% of the rhinoceros translocated from South Africa to Chad and Kenya, respectively, died within months of introduction to their new environment.[4,20] The main pathophysiological changes identified in long-distance rhinoceros translocation are haemoconcentration due to dehydration, electrolyte imbalances, a negative energy balance characterised by increased BHB concentrations, muscle damage, protein catabolism, stress-induced immunomodulation and oxidative stress.[16,31]

When transported, animals often have to maintain postural balance and have limited movement space.[180] The prolonged increased muscular tone and stress response potentially lead to poor tissue perfusion, with resultant hypoxic damage to cell walls causing membrane permeability, resulting in muscle enzyme release into the bloodstream.[35,181] Increases in the activities of muscle enzymes, such as AST, CK, and lactate dehydrogenase, have been documented during and after transport in black and white rhinoceros.[16,31] Capture myopathy is a syndrome that affects both skeletal and cardiac muscles due to muscular over-exertion and stress. It is thought to affect most captured and transported mammals, although the proportion that is affected to the point of displaying clinical signs is unknown.[26] The prevalence of capture myopathy associated with wild mammal translocation is likely underestimated because of the lack of thorough necropsies, unobserved post-release deaths, and difficulty in diagnosing CM as it is a continuum of effects and misattribution of impairment.[25,26] Capture myopathy has not specifically been documented in rhinoceros, however, it is believed to occur frequently in perissodactyls, which include horses, tapirs, and rhinoceroses.[26] It is therefore important to monitor biomarkers of skeletal and cardiac muscle cell damage when

translocating rhinoceros and to undertake post-release monitoring in these animals. In veterinary medicine, serum AST and CK enzymatic activities are commonly used biomarkers for skeletal muscle injury, and cardiac troponins are sensitive and specific for myocardial damage.[63,182]

Translocation can be extremely stressful for rhinoceros; therefore, stress should be considered a predictable factor in translocation which may affect the outcome.[22] Capture and transport are often considered the most stressful components of translocation as they mainly induce severe acute stress responses.[13,16] However, significant stress also occurs after animals are released into unfamiliar environments as the change from their natural environment to the destination environment is a large stress factor for rhinoceros, inducing both acute and chronic stress responses.[174,183] There are two approaches to the translocation of rhinoceros: (1) they can either be captured, loaded immediately into transport crates and transported directly to their destination ("field-to-field"), or (2) they can first undergo a post-capture adaptation period of at least 6 weeks in bomas before transportation to their final destination ("boma-to-field").[24,184] If the journey is going to be longer than 8-10 hours, it is preferable to first boma-train animals.[184] Despite the potential effect of confinement-specific stressors,[185] the confinement phase of translocation is a very important part of the adaptation period (boma-training) during which rhinoceros are introduced to water containers and cultivated fodder.[174] It is also advised that a rhinoceros should be kept in a boma after the journey for a few days before release. This approach reduces release-stress and allows for adaptation to a new environment.[24,184]

Chemical immobilisation is often performed in rhinoceros for conservation-related procedures, such as translocation and dehorning, as well as veterinary treatment and sample collection.[49] Potent opioids are used as the basis in the majority of drug combinations, with etorphine being the most commonly used. Rhinoceros, especially white rhinoceros, are sensitive to the effects of opioids and common side effects include respiratory compromise, hypertension, and tachycardia.[17,39,40] Etorphine-induced respiratory compromise is worsened by heavy intra-abdominal organs of rhinoceros during recumbency, with resultant inadequate ventilation leading to marked hypoxaemia and hypercapnia.[40,53] The butyrophenone tranquiliser azaperone is commonly used together with etorphine as an opioid synergist.[49] A key benefit of azaperone is that it reduces the hypertension caused by etorphine[17] and also potentially improves cardiac output.[40] However, etorphine and azaperone drug combinations have a greater negative effect on ventilatory function than etorphine alone[186] and increase limb rigidity and muscle tremors.[187] Midazolam, an imidazobenzodiazepine, has also been used as an opioid synergist in rhinoceros as it is believed to improve muscle relaxation and sedation; although it may amplify respiratory depression caused by etorphine.[40,47,50] Medetomidine, and other α_2-adrenoreceptor agonists, have also been used as opioid synergists to immobilise rhinoceros for various procedures and have been associated with improved muscle relaxation and analgesia; although they may cause V/Q mismatching and decrease tissue perfusion.[51] Butorphanol tartrate, a synthetic opioid, is often included in rhinoceros immobilisation protocols as it has a mixed partial agonist-antagonist effect.[40] The beneficial effects of butorphanol include improved respiration, lower incidence of metabolic acidosis and hypoxaemia, and reduced muscle tremors, with it being administered IV as soon as the animal is immobile and in a recumbent position as a

standard practice.[44,188] Despite the beneficial effects of incorporating opioid synergist drugs into etorphine-based immobilisation protocols and adding butorphanol, animals may still suffer from hypoventilation, hypoxaemia, hypercapnia, acidosis and other metabolic derangements.

Considering the availability of a recently validated POC hs-cTnI immunoassay in rhinoceros, the Siemens Atellica VTLi (refer to Chapter 4), the first objective of this study was to determine whether black and white rhinoceros that were translocated over long distances suffered from myocardial damage by comparing the serum cTnI concentrations at various time points during the journey. The second objective was to investigate the changes in serum cTnI concentrations in a cohort of white rhinoceros immobilised with four different immobilisation drug combinations over eight weeks. The third objective was to evaluate whether there were any associations between markers of muscle damage (AST and CK), indicators of negative energy balance (BHB), blood gas measurands (pH and PaO_2), lactate concentration and serum cTnI concentrations at any point of the translocation.

MATERIALS AND METHODS

Study population and samples

Stored serum collected from African rhinoceros in previous and ongoing research studies at the Faculty of Veterinary Science (FVS), University of Pretoria, was analysed for serum cTnI. Information related to the study population and sample collection for each FVS

study is summarised below (study groups 1 – 5). Retrospective biochemistry and blood gas results that were included in the statistical analysis, if available, included serum AST and CK activity, BHB and lactate concentrations, blood pH, and PaO_2.

Study group 1: Investigation of the pathophysiological changes in black rhinoceros transported for up to 23 hours (600 km).[16]

This study was approved by the University of Pretoria Animal Research and Ethics Committee (REC), project V067-17, and the samples were collected during October 2017. This study investigated changes in serum biochemistry, acute phase proteins, and oxidative stress biomarkers in 14 boma-adapted adult black rhinoceros at capture and after transport. The animals were sedated by darting within the boma, allowing conscious loading into a crate. The dart contained a low dose of etorphine (Captivon®, 9.8 mg/mL, Wildlife Pharmaceuticals, Karino, South Africa) combined with 60 mg azaperone (Azaperone tartrate, 50 mg/mL, Wildlife Pharmaceuticals) delivered remotely using 1.5 mL plastic darts (DAN-INJECT®, International S.A., Skukuza, South Africa) with 60 mm uncollared needles propelled by compressed air. Once the animal was in the crate, blood samples were collected from the auricular vein (16 ± 7 min to crate individual animals). The sedative effects of etorphine were partially reversed after crating with intravenous administration of 1.2 mg diprenorphine (Activon®, 12 mg/mL, Wildlife Pharmaceuticals) and 10 mg butorphanol (Butorphanol tartrate, 50 mg/mL, Wildlife Pharmaceuticals). A tranquiliser, zuclopenthixol acetate (Clopixol-Acuphase®, 50 mg/mL, H. Lundbeck Pty. Ltd., Randburg, South Africa), was administered IM via hand injection (150-220 mg/animal) immediately after loading into the crates. During transport, the vehicles were stopped at 2-4 hour intervals to allow for additional IM administration of azaperone and

midazolam (Dazonil®, 50 mg/mL, Wildlife Pharmaceuticals) if animals were restless. Eight animals received at least one top-up dose of 40-100 mg of azaperone, of which two animals required one to three additional doses of azaperone and midazolam (15-30 mg). At release, the animals were immobilised with etorphine (3.5-4 mg/animal) and azaperone (40 mg/animal) via a pole syringe or hand injection into the nuchal hump while standing in the transport crate. They were released from their crates and manually restrained with ropes before becoming recumbent. Subsequently, the blood samples collected at release were from the radial or auricular veins. To reverse immobilisation, naltrexone hydrochloride (Trexonil®, 50 mg/mL, Wildlife Pharmaceuticals) was administered IV at 20 times the etorphine dose in milligrams.

Study group 2: Investigation of the pathophysiological changes in white rhinoceros transported for up to 34 hours (1 300 km).[16]

This study was approved by the University of Pretoria REC, project V067-17, and the samples were collected during September 2017. Changes in serum biochemistry measurands, acute phase proteins, and oxidative stress biomarkers were investigated in 40 semi-captive white rhinoceros, consisting of adults and juveniles, at capture and after transport. Animals were captured by darting from a helicopter using 2.0 mL darts (Pneu-dart, Inc.®, Williamsport, Pennsylvania, US) with 63.5 mm barbed needles. Chemical immobilisation consisted of etorphine (3-5 mg/adult or 0.1-0.5 mg/juvenile) combined with azaperone (20-40 mg/adult or 0-10 mg/juvenile), and 5 000 IU hyaluronidase (Hyalase®, Jyron Laboratories, Johannesburg, South Africa) in adults. The flying time per animal ranged from 5-12 min. Blood samples were collected from the auricular vein immediately after the animals became recumbent (induction time was 5 min).

Butorphanol (2-5 times the etorphine dose in milligrams) was administrated IV to recumbent animals that displayed marked muscular tremors. To facilitate loading, partial reversal of the immobilisation effects of etorphine was obtained by IV administration of diprenorphine (0.2-0.8 mg/adult or 0-0.1 mg/juvenile; M5050®, 12 mg/mL, Novartis, Midrand, South Africa). Once loaded in the crate, each adult animal received another 2.5-15 mg of IV diprenorphine to reverse etorphine's effects further. The tranquiliser, zuclopenthixol acetate, was administrated IM via hand injection (150-220 mg/adult or 10-50 mg/juvenile) immediately after loading into the crates. During transport, all rhinoceros received at least one top-up dose of azaperone (80-120 mg/adult or 10-80 mg/juvenile), combined with 2.5-15 mg midazolam in the juveniles. During transport, 16 adults and six juveniles required up to three additional top-up doses of azaperone alone or in combination with midazolam (10-20 mg/adult). At release, the adults were immobilised again with etorphine (3.5-6 mg/animal) and azaperone (20-40 mg/animal) via pole syringe or hand injection into the nuchal hump while standing in the transport crate. Etorphine (0.5-2.5 mg/animal) and midazolam (5 mg/animal) were administered IM to juveniles using a pole syringe. They were released from their crates and manually restrained with ropes before becoming recumbent. Subsequently, these "release" blood samples were collected from the radial or auricular veins. To reverse immobilisation, naltrexone hydrochloride (Trexonil®, 50 mg/mL, Wildlife Pharmaceuticals) was administered IV at 20 times the etorphine dose in milligrams.

Study group 3: *Investigation of the pathophysiological changes in white rhinoceros transported for up to 6 hours (280 km).*[15,33]

This study was approved by the University of Pretoria REC, project V067-17, and the samples were collected during July 2018. A total of 23 free-living male white rhinoceros were captured, four animals at a time (three animals during one translocation), and transported, with six resultant translocation events over three successive weeks. Changes in haematological and immunological variables, serum biochemistry measurands, and venous acid-base levels were investigated in this study. Animals were located by direct observation and then captured by IM darting from a helicopter, using 3.0 mL plastic darts with a 60 mm uncollared needle (DAN-INJECT®, International S.A., Skukuza, South Africa). No chasing time was recorded, but the animals ran approximately 905 ± 500 meters. Two chemical immobilisation protocols were administrated alternately during capture: (1) etorphine combined with azaperone (n = 11 animals, group A) and (2) etorphine and midazolam (n = 12, group M). The etorphine doses (aiming to administer 2 μg/kg) were based on standardised estimated categories, animal size, and body condition (1 250-1 500 kg = 3 mg; 1 500-1 750 kg = 3.5 mg; 1 750-2 000 kg = 4 mg). Azaperone or midazolam was administrated at 5 times the etorphine dose (mg). Once immobilised, blood was collected from the radial vein (time of sample capture sample = TC). The induction time was 8 ± 3 min. Some animals were dehorned for conservation-related reasons before being loaded into crates. After sample collection and auricular vein catheter placement for subsequent sample collection, butorphanol (5 mg per mg etorphine) was administered IV to partially reverse the immobilisation effect of etorphine. Once crated, IV diprenorphine (3 mg per mg etorphine) was administered to further antagonise the residual immobilisation effect of etorphine. Once all four

rhinoceros were captured and loaded into transport crates, transportation started and a blood sample was collected (start of transport sample, T0) using the auricular catheter. The time lag from TC to T0 between individuals was approximately 153 ± 79 min. Intramuscular re-administration of midazolam or azaperone (standard dose of 25 times the etorphine dose in mg), via hand injection into the nuchal hump, was at the start (T0), and at 2 hours (T2) and 4 hours (T4) of transport. Blood samples were collected at the T2 and T4. The destination was reached after 6 hours of transport, and a final blood sample was collected (6-hour transport sample, T6) from the auricular catheter before it was removed. At release, naltrexone hydrochloride (20 mg/mg etorphine) was administered IV to completely reverse the residual effects of etorphine.

Study group 4: Investigation of the acute stress response and cardiorespiratory effects in white rhinoceros immobilised with four different drug protocols.[49]

This study was approved by the University of Pretoria REC (projects REC 011-21, REC 057-21 and samples were collected from June to August 2021. Eight adult male boma-adapted white rhinoceros were immobilised either with etorphine or a combination of etorphine and azaperone (Stressnil®,40 mg/mL, Janssen Pharmaceutical Halfway House) or midazolam, or medetomidine (50 mg/mL, Wildlife Pharmaceuticals, Nelspruit, South Africa) using a randomised crossover design. The immobilisation protocols were as follow: (1) Treatment EB – etorphine (2.0 μg/kg) IM, then butorphanol (20 μg/kg) intravenous 15 min after positioning into lateral recumbency; (2) Treatment EAzaB - etorphine (2.0 μg/kg) and azaperone (10 μg/kg) IM, then butorphanol (20 μg/kg) IV 15 min after positioning into lateral recumbency; (3) Treatment EMedB - etorphine (2.0 μg/kg) and medetomidine (5 μg/kg) IM, then butorphanol (20 μg/kg) IV 15 min after positioning

into lateral recumbency; (4) Treatment EMidB - etorphine (2.0 μg/kg) and midazolam (10 μg/kg) IM, then butorphanol (20 μg/kg) IV 15 min after positioning into lateral recumbency. The animals were darted within the precincts of the bomas, and each animal was immobilised at least 4 times, with a two-week wash-out period between the immobilisation protocols. Dart placement sites included the hump or rump muscles, and immobilisation drugs were administered using 3 mL DAN-INJECT® darts with a 2 × 60 mm uncollared needle fired by a carbon dioxide-powered dart gun. Immobilisation doses were calculated based on the estimated body mass of the animals. Induction ranged from 10-15 min in the majority of animals. Blood samples were collected from the auricular vein at immobilisation (T0) and twice at 20 min intervals from the radial vein (T20 and T40). Immobilisation was reversed using IV naltrexone hydrochloride (20 times the etorphine dose). Atipamezole hydrochloride (Antisedan®, 5 mg/mL, Zoetis South Africa Pty. Ltd., Bute Lane, Sandton, South Africa) was administered IV to reverse the effect of medetomidine.

Study group 5: Effect of fluid administration on transported white rhinoceros

This study was approved by the University of Pretoria REC (project REC 043-20) and samples were collected during August 2023. This simulated transportation used 24 boma-adapted or semi-captive sub-adult and adult white rhinoceros divided equally into a control group and two treatment groups. Rhinoceros in the treatment groups were given either IV Ringer's lactate solution or potable luke-warm water per rectum. Intravenous fluids were administered continuously via a portable fluid administration system from the time of crating until release.[189] In the animals that received rectal fluid, a slow bolus was administered into the rectum using a bucket and funnel connected to an endogastric

tube and was repeated at 4-hour intervals. Animals were placed in transport crates after capture, but the crates remained stationary during the 24 hours of the study to simulate transport. For capture, rhinoceros were immobilised using etorphine and azaperone. Standard drug doses for white rhinoceros (as described above) were used and delivered IM using 3.0 mL DAN-INJECT® plastic darts with a 60 mm uncollared needle projected by a compressed air dart rifle. Intravenous butorphanol was administered once the animals were recumbent. Top-up doses of IM azaperone were given every 2 hours. Etorphine was administered to all animals before release (0.5-2 mg) to facilitate blood sampling, after which the animals received naltrexone hydrochloride (15 mg for subadults and 50 mg for adults). Samples were collected from the radial vein at capture (8-30 min after darting) and from an auricular vein at 12 hours post-capture (T12) and 24 hours post-capture/time of release (T24).

Sample analysis

For clinical biochemistry analysis, blood was directly collected into serum tubes (BD Vacutainer®, Becton and Dickson, Oxford, UK), stored in a cooler box with ice packs, and centrifuged within one to 24 hours. The serum was aliquoted and stored at -80°C until analysis. The samples from study groups 2 and 5 were first stored at -20 °C for one month (study group 2) or one to 10 days (study group 5) before being stored at -80 °C. Blood collected for blood gas analysis was directly collected into lithium-heparinised tubes (BD Vacutainer®, Becton and Dickson, Oxford, UK) or heparinised syringes and analysed immediately. The majority of stored samples from study groups 1-3 had undergone one previous freeze-thaw cycle, while the samples from female rhinoceros in study groups 1 and 2 had undergone two previous freeze-thaw cycles.

Cardiac troponin I

Serum samples were thawed on a sample roller for 10–20 min at room temperature, and cTnI was measured using the validated Atellica VTLi hs-cTnI immunoassay (Siemens Healthineers, Erlangen, Germany) (refer to Chapter 4). The analyser was maintained according to the manufacturer's guidelines and assay performance was monitored by running one level of QCM at the start of each analytical run. The analyses were performed by the primary investigator. Results were reported as whole numbers with no decimals, as advised by the IFCC TF-CB.[75]

Clinical biochemistry analytes

Serum clinical biochemistry analysis for AST, BHB, CK, and lactate (study groups 1-2) were performed using a Cobas Integra 400 Plus automated wet biochemistry analyser (Roche Diagnostics Ltd., Rotkreuz, Switzerland)[175] and commercially available kits (Roche Diagnostics Ltd. for AST, CK and lactate; Randox Laboratories, Crumlin, Antrim, UK for BHB), using spectrophotometry. Aspartate aminotransferase serum activity was measured using the kinetic pyridoxal-5'-phosphate IFCC standardised method. Serum BHB levels were measured using a kinetic enzymatic assay, with D-hydroxybutyrate converted to acetoacetate. Serum CK activity was measured using a kinetic enzymatic method with creatine and adenosine diphosphate. Serum lactate concentration was determined using an enzymatic colorimetric method with L-lactate and oxygen (O_2). The Cobas Integra 400 Plus analyser was maintained and calibrated according to the manufacturer's instructions and internal laboratory standard operating protocols. The

commercial QCM was run daily at the start of the analytical run. Trained veterinary laboratory technologists performed the analyses.

Blood gas measurands

The blood pH, PaO_2 and lactate (study groups 3-5) were measured on a POC blood gas analyser with pre-calibrated test cards (EPOC® Portable analyser system and EPOC® BGEM test cards, Siemens Healthineers, Erlangen, Germany). The pH was measured via potentiometry using a pH-selective electrode. The PaO_2 was measured by amperometry using a membrane covered O_2 sensing cathode electrode. The O_2 reduction current is proportional to the dissolved O_2 concentration. Lactate was also measured by amperometry using β-D-lactate converted to hydrogen peroxide. Analyser maintenance and sample analyses were performed according to the manufacturer's guidelines by the investigators of the original studies.

Data analysis

First, the results from each time point in each study were examined using descriptive statistics, and data distribution was determined using the Shapiro-Wilk test. As the samples taken at different time points in each study were from the same individual, differences in cTnI concentrations between time points in each study were evaluated using a paired t-test or Wilcoxon test (two time points) or repeated measures ANOVA or Friedman's test (> two time points), depending on data distribution. In study group 3, cTnI levels in the dehorned versus non-dehorned and midazolam versus azaperone groups were assessed using the independent samples t-test or the Mann-Whitney test, depending on sample distribution. For study group 4, where rhinoceros were immobilised

4 times over eight weeks, additional analysis included the comparison of T0 and T40 values over the four immobilisation events using repeated measures ANOVA or Friedman's test, depending on data distribution. To assess the cumulative effect of repeated immobilisation on the cTnI concentration in rhinoceros, the results obtained in chronological order of immobilisation events were compared using repeated measures ANOVA or Friedman's test, depending on data distribution. Post-hoc testing was performed using the Bonferroni multiple comparison test (for repeated-measures ANOVA) or a Conover test (Friedman test). A *P*-value of <0.05 was considered significant for all these tests. Data is presented as median or mean (interquartile range (IQR) or SD). Correlation analysis between cardiac troponin I concentration and AST, CK, lactate, pH, and PaO_2 levels was performed using Spearman's rank-order or Pearson *r* correlation, depending on the data distribution. Statistical analysis was performed using MedCalc Statistical software for Windows, version 22.016 (MedCalc Software Ltd, Ostend, Belgium). Post-hoc sample size power calculations were also performed using the serum cTnI results of each study group and α set at 0.05. The ClinCalc.com Post-hoc Power Calculator (https://clincalc.com/stats/power.aspx) was used.

RESULTS

Study group 1: Investigation of the pathophysiological changes in black rhinoceros transported for up to 23 hours (600 km)

Fourteen adult black rhinoceros were initially included in this study, and blood samples collected at capture and release were used for analysis. Paired serum cTnI results were

only available for 12 animals; one animal was excluded due to insufficient sample volume to repeat the cTnI measurement, and another was excluded as invalid test results were obtained. The 12 animals included consisted of seven females and five males. Retrospective data on AST and CK serum activity and lactate and BHB concentrations at both collection points were available for 11/12 animals, with the capture data in one animal unavailable due to marked haemolysis (**Table 9**).

Table 9: Median or mean (IQR or SD) concentrations or activities of blood clinical chemistry analytes of translocated black rhinoceros at capture and release.

	Capture	**Release**
Aspartate aminotransferase (U/L)	69* (60-84)	148* (98-367)
β-hydroxybutyrate (mmol/L)	0.19 (0.03)	0.20* (0.16-0.34)
Cardiac troponin I (ng/L)	5 (2)	8* (5-22)
Creatine kinase (U/L)	412* (266-718)	4 440* (2 575-16 883)
Lactate (mmol/L)	1.76* (1.53-2.16)	8.13 (3.45)

*Median values. IQR: interquartile range; SD: standard deviation

The serum cTnI concentration was significantly increased at release compared to that at capture ($P = 0.039$) (**Figure 7**).

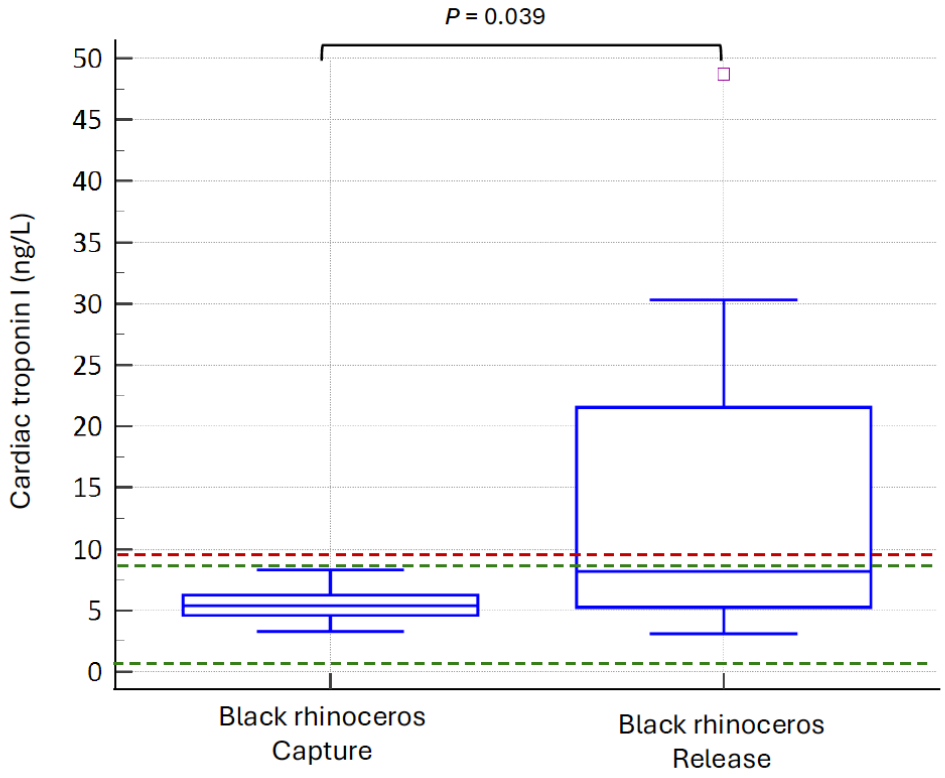

Figure 7: Comparison of serum cardiac troponin I concentration at capture and release in translocated black rhinoceros.

Box-and-whisker plot: The central box encompasses the 25th-75th percentiles. The middle line represents the median. The outside horizontal lines indicate the minimum and maximum excluding far-out values. Open square: Outside values (value higher than the upper inner fence but not higher than the upper outer fence). Red stippled line: 99th percentile upper reference limit (9 ng/L); Green stippled lines: 95% reference limits (1.47 – 8.17 ng/L) (refer to Chapter 5).

The correlation results of serum cTnI with AST, BHB, CK, and lactate are summarised in **Tables 10** and **11** below. Statistically significant and moderate positive correlations were observed between capture AST, CK, and lactate activity/concentrations and release serum cTnI concentration.

Table 10: Correlations between capture or release serum cTnI and corresponding capture or release AST, BHB, CK, and lactate in translocated black rhinoceros.

	Capture cTnI	Release cTnI
AST	Rho: 0.041; *P* = 0.905	Rho: 0.424; *P* = 0.170
BHB	Rho: 0.369; *P* = 0.265	Rho: 0.285; *P* = 0.369
CK	Rho: 0.374; *P* = 0.258	Rho: 0.382; *P* = 0.221
Lactate	Rho: 0.355; *P* = 0.284	Rho: -0.406; *P* = 0.191

AST: aspartate aminotransferase; BHB: β-hydroxybutyrate; CK: creatine kinase; cTnI: cardiac troponin I; Rho: Spearman's rank correlation coefficient.

Table 11: Correlations of release serum cTnI with capture AST, BHB, CK, and lactate in translocated black rhinoceros.

	Release cTnI
Capture AST	Rho: 0.711; *P* = 0.014*
Capture BHB	Rho: -0.115; *P* = 0.736
Capture CK	Rho: 0.620; *P* = 0.042*
Capture lactate	Rho: 0.724; *P* = 0.012*

AST: aspartate aminotransferase; BHB: β-hydroxybutyrate; CK: creatine kinase; cTnI: cardiac troponin I; Rho: Spearman's rank correlation coefficient. *Statistically significant correlations.

The post-hoc power based on the sample size of 12 rhinoceros was 56.5%.

Study group 2: Investigation of the pathophysiological changes in white rhinoceros transported for up to 34 hours (1 300 km)

Forty white rhinoceros were initially included in this study, and blood samples collected at capture and release were used for analysis. Paired serum cTnI results were only available for 35 animals, and five animals were excluded due to insufficient sample volume to measure cTnI concentrations at release. The 35 animals included 15 adults (12 females and three males) and 20 juveniles (12 females and five males). Sex information for the three juvenile animals was unavailable. Retrospective data on AST and CK serum activity and lactate and BHB concentrations at both collection points were available for 34/35 animals, with the release data in one animal unavailable due to marked haemolysis (**Table 12**).

Table 12: Median or mean (IQR or SD) concentrations or activities of blood clinical biochemistry analytes of long-distance translocated white rhinoceros at capture and release.

	Capture	Release
Aspartate aminotransferase (U/L)	65 (13)	223* (116-315)
β-hydroxybutyrate (mmol/L)	0.22 (0.08)	0.32 (0.12)
Cardiac troponin I (ng/L)	5* (4-6)	15* (8-30)
Creatine kinase (U/L)	190* (151-265)	9 057* (1 843-11 852)
Lactate (mmol/L)	16.63 (7.58)	5.76 (2.85)

*Median values. IQR: interquartile range; SD: standard deviation

The serum cTnI concentration was significantly increased at release compared to that at capture ($P < 0.001$) (**Figure 8**).

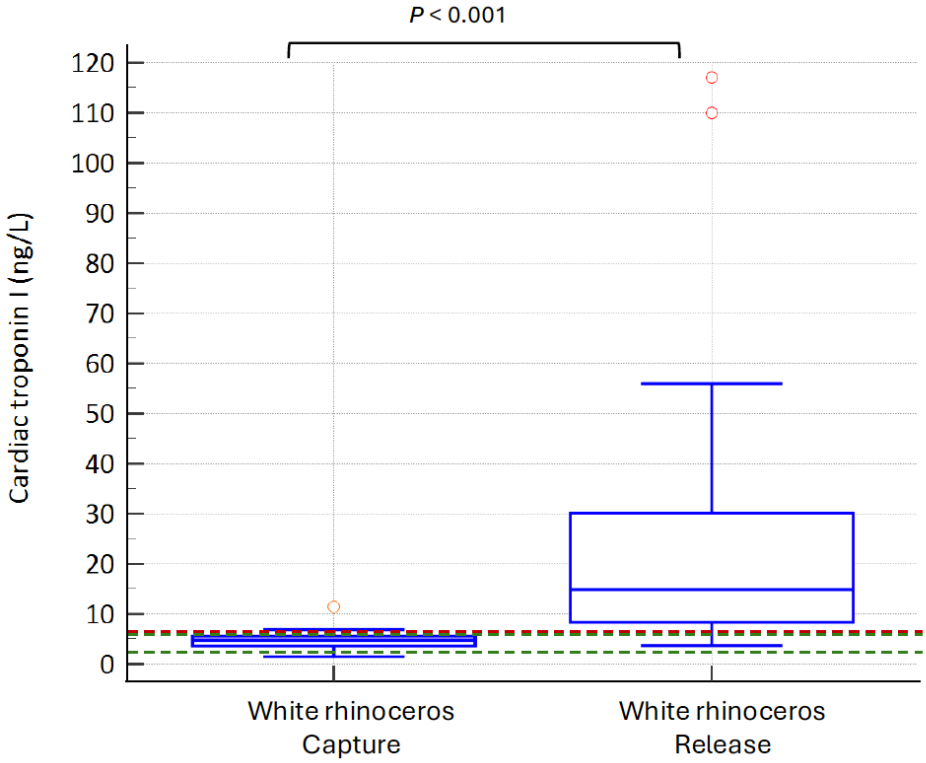

Figure 8: Comparison of serum cardiac troponin I concentration at capture and release in translocated white rhinoceros.

Box-and-whisker plot: Central box represents values from the 25 to 75 percentile. The middle line represents the median. The horizontal lines extend from the minimum to the maximum value. Open circles: Outside values (value higher than the upper inner fence but not higher than the upper outer fence).

Red stippled line: 99th percentile upper reference limit (6 ng/L); Green stippled lines: 95% reference limits (2.41 – 6.29 ng/L) (refer to Chapter 5).

The correlation results of serum cTnI with AST, BHB, CK, and lactate are summarised in **Tables 13** and **14** below. Significant and mild-to-moderate positive correlations existed between release AST and release cTnI levels, and between capture CK and capture cTnI

levels. No significant correlations were found between capture AST, BHB, CK, and lactate levels and release cTnI concentration.

Table 13: Correlations between capture or release serum cTnI and corresponding capture or release AST, BHB, CK, and lactate in long-distance translocated white rhinoceros.

	Capture cTnI	**Release cTnI**
AST	Rho: -0.189; *P* = 0.277	Rho: 0.355; *P* = 0.040*
BHB	Rho: -0.207; *P* = 0.233	Rho: -0.086; *P* = 0.634
CK	Rho: 0.555; *P* = 0.001*	Rho: 0.276; *P* = 0.114
Lactate	Rho: -0.144; *P* = 0.410	Rho: 0.149; *P* = 0.399

AST: aspartate aminotransferase; BHB: β-hydroxybutyrate; CK: creatine kinase; cTnI: cardiac troponin I; Rho: Spearman's rank correlation coefficient. *Statistically significant correlations.

Table 14: Correlations of release serum cTnI with capture AST, BHB, CK, and lactate in long-distance translocated white rhinoceros.

	Release cTnI
Capture AST	Rho: -0.082; *P* = 0.638
Capture BHB	Rho: -0.077; *P* = 0.657
Capture CK	Rho: 0.159; *P* = 0.362
Capture lactate	Rho: 0.168; *P* = 0.335

AST: aspartate aminotransferase; BHB: β-hydroxybutyrate; CK: creatine kinase; cTnI: cardiac troponin I; Rho: Spearman's rank correlation coefficient. *Statistically significant correlations.

The post-hoc power based on the sample size of 35 rhinoceros was 99.5%.

Study group 3: Investigation of the pathophysiological changes in white rhinoceros transported for up to 6 hours (280 km)

Twenty-three white rhinoceros were initially included in this study, and blood samples collected at TC, T0, T2, and T6 were used for analysis. Paired serum cTnI results were only available for 22 animals, and one animal was excluded due to invalid test results at all time points. In one animal, an insufficient volume of serum was left to measure cTnI concentration at T6; however, results were available for all other time points and the results were retained. The 22 animals included 13 adults and nine subadult males. Ten of these animals were dehorned during immobilisation before loading and transport. Eleven animals received midazolam and azaperone, respectively. Retrospective data on AST, CK, and BHB serum activity and concentrations were available at TC, T0, and T6; however, lactate concentration and pH data were available at all collection points investigated in this study (TC, T0, T2, and T6). Data were available for 21/22 animals, with the AST, CK, and BHB T6 data for one animal unavailable due to insufficient sample volume (**Table 15**).

Table 15: Median or mean (IQR or SD) concentrations or activities of blood clinical chemistry analytes of white rhinoceros transported for 280 km at capture, start of transport, 2 hours after the start of transport and at release.

	Capture (TC)	Start of transport (T0)	Two hours after start of transport (T2)	Release (T6)
Aspartate aminotransferase (U/L)	54 (12)	55 (12)	No data available	71* (59-71)
β-hydroxybutyrate (mmol/L)	0.17* (0.15-0.18)	0.24* (0.21-0.39)	No data available	0.46* (0.17-0.63)
Cardiac troponin I (ng/L)	4* (4-6)	104* (41-240)	190* (122-290)	179* (104-353)
Creatine kinase (U/L)	199* (181-223)	524* (296-738)	No data available	1 311* (1 038-2 204)
Lactate (mmol/L)	10.70 (4.71)	2.61* (1.66-3.18)	1.73* (1.56-2.84)	2.24* (1.75-3.10)
pH	7.15 (0.11)	7.43* (4.47-7.49)	7.45 (0.04)	7.48 (0.06)

*Median values; IQR: interquartile range; km: kilometre; SD: standard deviation.

Significant differences in the cTnI concentration were found between all the time points evaluated ($P < 0.001$) (**Figure 9**).

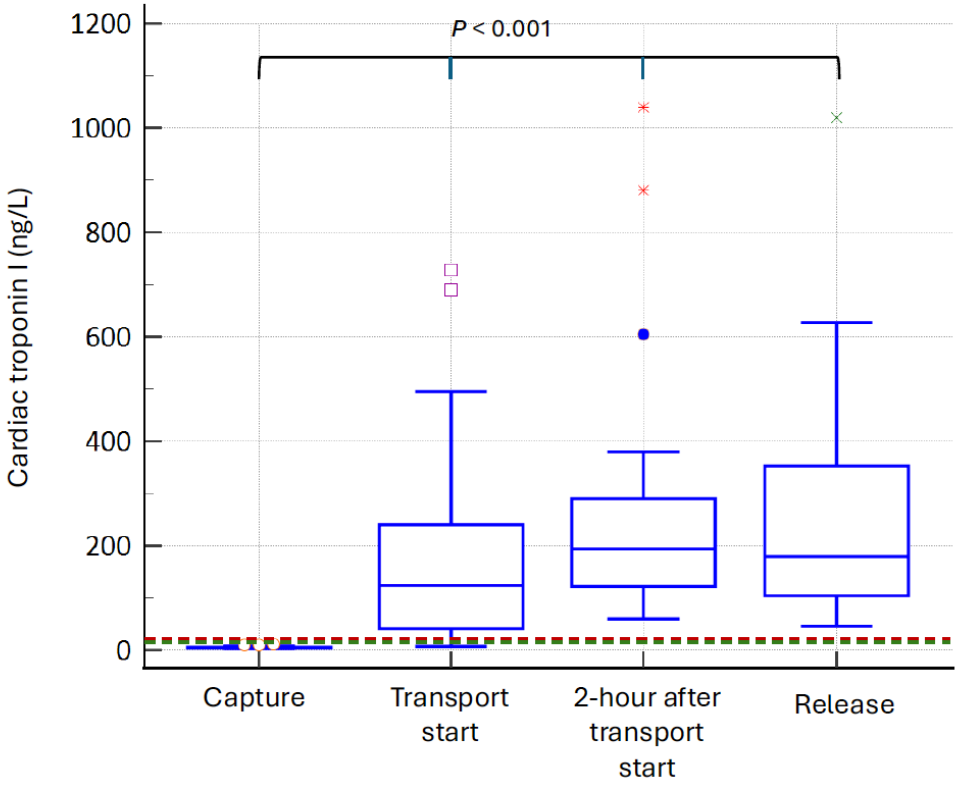

Figure 9: Comparison of serum cardiac troponin I concentration at capture, start of transport, 2-hours after start of transport and release in white rhinoceros transported for 280 km.

Box-and-whisker plot: The central box encompasses the 25th-75th percentiles. The middle line represents the median. The outside horizontal lines indicate the minimum and maximum excluding far-out values. Open squares and blue dot: Outside values (value higher than the upper inner fence but not higher than the upper outer fence). Stars and cross: Far-out values (value higher than the upper outer fence).

Red stippled line: 99th percentile upper reference limit (6 ng/L); Green stippled line: 95% upper reference limit (6.29 ng/L), the lower limit (2.41 ng/L) not displayed (refer to Chapter 5).

When grouped according to whether they were dehorned during immobilisation, the serum cTnI concentration was significantly higher in animals that were dehorned than in those that were not at TC, T0, and T2. However, no difference in serum cardiac troponin concentration was observed at release (T6). The results are summarised in **Table 16**.

The correlation results of serum cTnI with AST, BHB, CK, lactate, and pH are summarised in **Tables 17** and **18**. Significant and moderate positive correlations were observed between the start of transport CK activity and BHB concentration compared to the serum cTnI concentration at the same time point. The release serum activities of AST and CK compared to the release serum cTnI concentration showed significant moderate positive correlations. No significant correlations were found between lactate and pH compared to serum cTnI concentration at any of the investigated time points. Additionally, AST activity at the capture and start of transport showed significant moderate positive correlations with the released serum cTnI concentration.

Table 16: Comparison of serum cTnI concentration (ng/L) in white rhinoceros that were dehorned or not and animals that received midazolam or azaperone at immobilisation at all the time points investigated during the 280 km translocation.

	White rhinoceros dehorned	**White rhinoceros Not dehorned**	**White rhinoceros receiving Azaperone**	**White rhinoceros receiving Midazolam**
At capture				
Number of animals	10	12	11	11
Median or mean cTnI	7	4	4*	4*
IQR or SD	3	1	4-6	3-6
P - value	**0.017**		0.430	
Start of transport				
Number of animals	10	12	11	11
Median or mean cTnI	332	34*	128*	103
IQR or SD	230	12-75	53-436	104
P - value	**<0.001**		0.133	
Two hours after the start of transport				

Number of animals	10	12	11	11
Median or mean cTnI	266*	141	194*	189
IQR or SD	211-605	90	120-539	102
P - value	**0.002**		0.652	
Release				
Number of animals	10	11	11	10
Median or mean cTnI	167*	211	155*	221
IQR or SD	142-443	155	93-441	123
P - value	0.324		0.805	

*Median values. cTnI: cardiac troponin; IQR: interquartile range; km: kilometre; SD: standard deviation. Bold text indicates a *P*-value <0.05.

Table 17: Correlations between capture, start of transport,2 hours after the start of transport or release serum cardiac troponin I and corresponding values at the same timepoints of AST, BHB, CK, lactate and pH in white rhinoceros transported for 280 km.

	Capture cTnI	Start of transport	Two hours after transport start	Release cTnI
AST	Rho: -0.252; *P* = 0.257	Rho: 0.300; *P* = 0.174	No data available	Rho: 0.665; *P* = 0.001*
BHB	Rho: 0.255; *P* = 0.253	Rho: 0.586; *P* = 0.004*	No data available	Rho: 0.054; *P* = 0.813
CK	Rho: 0.017; *P* = 0.940	Rho: 0.658; *P* <0.001*	No data available	Rho: 0.478; *P* = 0.028*
Lactate	Rho: 0.092; *P* = 0.683	Rho: 0.146; *P* = 0.516	Rho: 0.312; *P* = 0.158	Rho: -0.028; *P* = 0.904
pH	Rho: -0.220; *P* = 0.322	Rho: -0.141; *P* = 0.531	Rho: 0.321; *P* = 0.145	Rho: 0.184; *P* = 0.425

AST: aspartate aminotransferase; BHB: β-hydroxybutyrate; CK: creatine kinase; cTnI: cardiac troponin I; km: kilometre; Rho: Spearman's rank correlation coefficient.

*Statistically significant correlations.

Table 18: Correlations of release serum cTnI with capture or start of transport AST, BHB, CK, lactate and pH in white rhinoceros transported for 280 km.

	Release cTnI
Capture AST	Rho: 0.575; *P* = 0.006*
Start of transport AST	Rho: 0.687; *P* = 0.001*
Capture BHB	Rho: -0.026; *P* = 0.911
Start of transport BHB	Rho: -0.183; *P* = 0.426
Capture CK	Rho: -0.123; *P* = 0.5960
Start of transport CK	Rho: 0.222; *P* = 0.333
Capture lactate	Rho: 0.404; *P* = 0.069
Start of transport lactate	Rho: 0.170; *P* = 0.463
Capture pH	Rho: -0.414; *P* = 0.062
Start of transport pH	Rho: -0.273; *P* = 0.231

AST: aspartate aminotransferase; BHB: β-hydroxybutyrate; CK: creatine kinase; cTnI: cardiac troponin I; Rho: Spearman's rank correlation coefficient. *Statistically significant correlations.

The post-hoc power based on a sample size of 22 rhinoceros was 99.5%; however, it was 67.2% and 8.3% for the dehorned versus non-dehorned groups and azaperone versus midazolam groups, respectively.

Study group 4: Investigation of the acute stress response and cardiorespiratory effects in white rhinoceros immobilised with four different drug protocols.

Eight subadult male white rhinoceros were included in this study, and blood samples collected at capture and 40 min after capture (T40) were used for analysis. Paired serum cTnI results were available for all animals. Retrospective data on the lactate concentration, pH, and PaO_2 were available at both collection points.

In each immobilisation protocol, no significant differences were noted between the serum cTnI concentrations obtained at capture and T40. The results are summarised in **Table 19**.

Table 19: Comparison of capture and 40-minute post-capture serum cardiac troponin concentrations in eight subadult white rhinoceros in four different immobilisation protocols.

Immobilisation protocol	Mean or median (IQR or SD) cTnI at capture (ng/L)	Mean or median (IQR or SD) cTnI at 40-minutes post-capture (ng/L)	*P*-value
Etorphine & azaperone (EAzaB)	6 (1)	6 (2)	0. 934
Etorphine (EB)	6* (4-6)	6 (2)	0.735
Etorphine & midazolam (EMidB)	5 (2)	6 (2)	0.735
Etorphine & medetomidine (EMedB)	6 (2)	5 (2)	0.076

*Median values. cTnI: cardiac troponin I; IQR: interquartile range; SD: standard deviation.

There were no significant differences between the capture cTnI concentrations among the four different immobilisation protocols ($P = 0.432$) (**Figure 10**). Similarly, no significant differences were noted in the T40 serum cardiac troponin concentration when the four immobilisation protocols were compared ($P = 0.445$) (**Figure 11**).

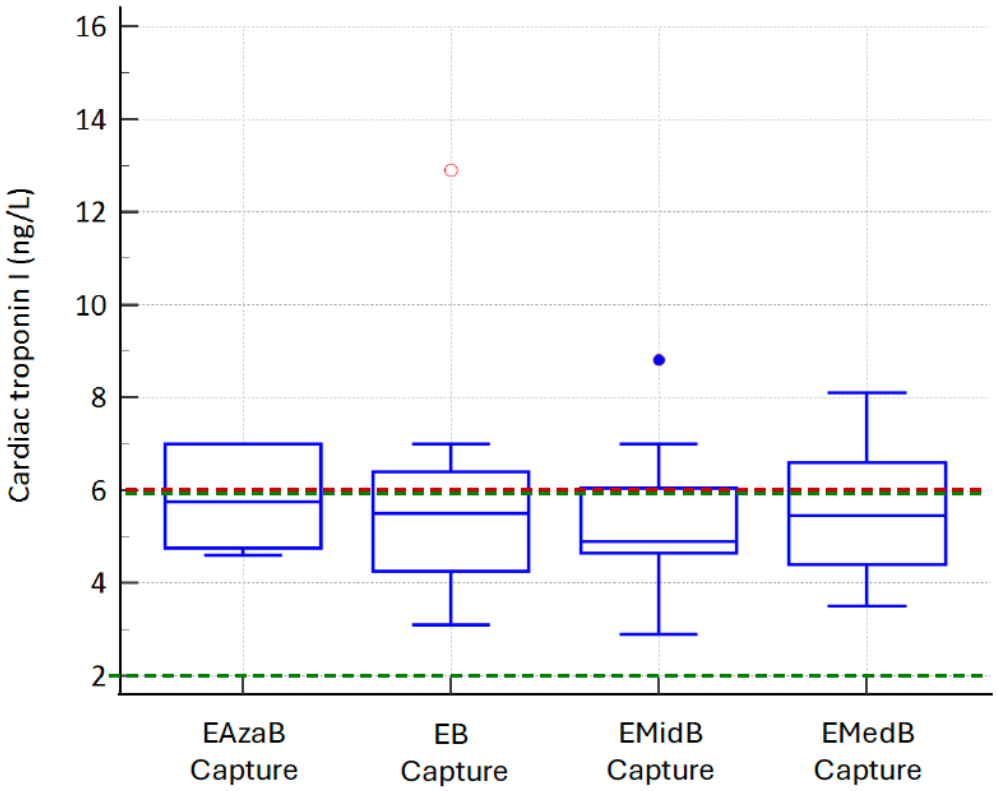

Figure 10: Comparison of serum cardiac troponin I concentration at capture in four different immobilisation protocols used in white rhinoceros.

Box-and-whisker plot: The central box encompasses the 25th-75th percentiles. The middle line represents the median. The outside horizontal lines indicate the minimum and maximum excluding far-out values. Blue dot: Outside value (value higher than the upper inner fence but not higher than the upper outer fence). Open circle: Far-out value (value higher than the upper outer fence).

Red stippled line: 99th percentile upper reference limit (6 ng/L); Green stippled lines: 95% reference limits (2.03 – 5.99 ng/L) (refer to Chapter 5).

EAzaB: Etorphine, azaperone, and butorphanol; EB: Etorphine and butorphanol; EMidB: Etorphine, midazolam, and butorphanol; EMedB: Etorphine, medetomidine, and butorphanol.

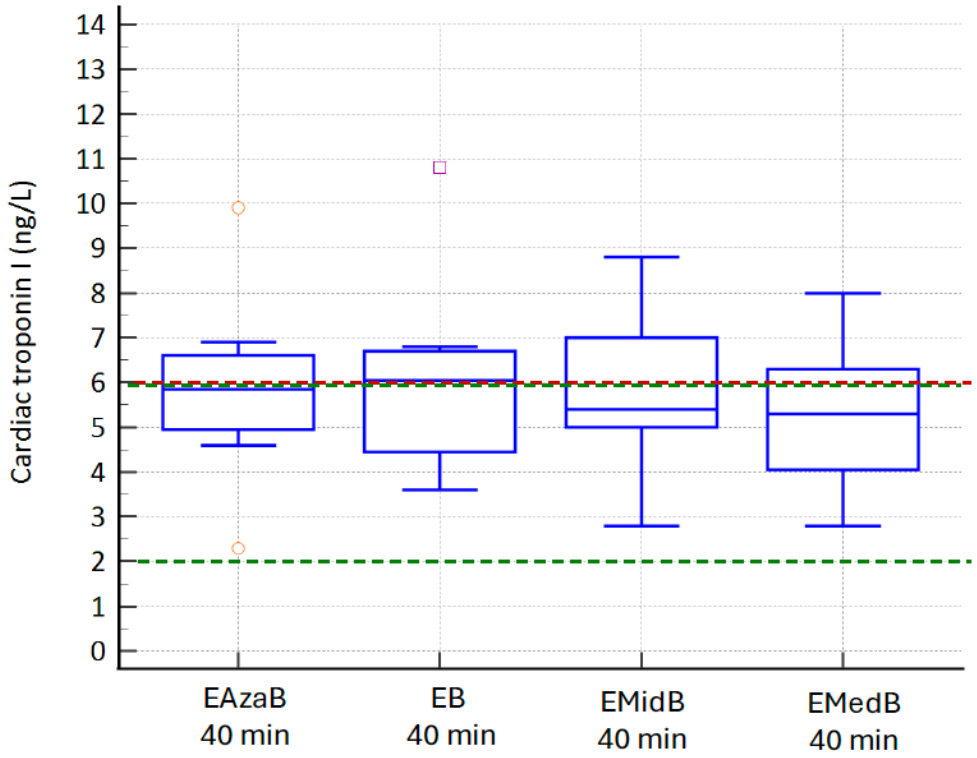

Figure 11: Comparison of serum cardiac troponin I concentration at 40 minutes after capture in four different immobilisation protocols used in white rhinoceros.

Box-and-whisker plot: The central box encompasses the 25th-75th percentiles. The middle line represents the median. The outside horizontal lines indicate the minimum and maximum excluding far-out values. Open circles and open square: Outside values (value higher than the upper inner fence but not higher than the upper outer fence).

Red stippled line: 99th percentile upper reference limit (6 ng/L); Green stippled lines: 95% reference limits (2.03 – 5.99 ng/L) (refer to Chapter 5).

EAzaB: Etorphine, azaperone, and butorphanol; EB: Etorphine and butorphanol; EMidB: Etorphine, midazolam, and butorphanol; EMedB: Etorphine, medetomidine, and butorphanol. Min: minutes.

A significant and moderate positive correlation was observed between capture lactate concentration and capture serum cTnI concentration in the EAzaB immobilisation protocol. And, in the same immobilisation protocol, a significant and moderate negative

correlation was observed between capture pH and capture serum cTnI concentration. The T40 serum cTnI concentration was significantly moderately and negatively correlated with PaO_2 in the EB immobilisation protocol. The results are summarised in **Table 20**.

Table 20: Correlations of serum cardiac troponin I with lactate and PaO_2 at capture and 40 minutes later, in four different immobilisation protocols used in white rhinoceros.

	Capture cTnI	40 minute cTnI
Etorphine & azaperone (EAzaB)		
Lactate	Rho: 0.773; *P* = 0.024*	Rho: 0.036; *P* = 0.933
PaO_2	Rho: -0.393; *P* = 0.336	Rho: -0.204; *P* = 0.629
pH	Rho: -0.884; *P* = 0.004*	Rho: -0.110; *P* = 0.795
Etorphine (EB)		
Lactate	Rho: -0.143; *P* = 0.787	Rho: -0.120; *P* = 0.778
PaO_2	Rho: 0.024; *P* = 0.955	Rho: -0.762; *P* = 0.028*
pH	Rho: 0.539; *P* = 0.168	Rho: 0.663; *P* = 0.073
Etorphine & midazolam (EMidB)		
Lactate	Rho: -0.119; *P* = 0.779	Rho: -0.073; *P* = 0.863
PaO_2	Rho: -0.024; *P* = 0.955	Rho: 0.122; *P* = 0.774
pH	Rho: -0.287; *P* = 0.490	Rho: -0.368; *P* = 0.370
Etorphine & medetomidine (EMedB)		
Lactate	Rho: 0.357; *P* = 0.385	Rho: -0.455; *P* = 0.257
PaO_2	Rho: 0.060; *P* = 0.888	Rho: 0.024; *P* = 0.955
pH	Rho: 0.289; *P* = 0.487	Rho: 0.473; *P* = 0.237

cTnI: cardiac troponin I; PaO_2: Partial pressure of oxygen; Rho: Spearman's rank correlation coefficient.

*Statistically significant correlations.

When organising the immobilisation protocol administration in chronological order for each animal and comparing the serum cTnI concentrations between the protocols administered first to last, the serum cTnI concentration was significantly higher at the first immobilisation compared to the second, third, and last immobilisation (P = 0.020). The results are summarised in **Table 21**. The first administered immobilisation protocol in 3/8 animals was EAzaB, EB in 2/8 animals and the remaining 3/8 animals received protocol EMedB. None of the animals received protocol EMidB at the first immobilisation.

Table 21: Serum cardiac troponin I concentration (ng/L) comparison when the immobilisation protocol was chronologically administered in eight subadult white rhinoceros.

	Median cTnI	IQR
First immobilisation	7*	6-8
Second immobilisation	6	5-7
Third immobilisation	5	5-7
Fourth immobilisation	5	5-5

cTnI: cardiac troponin I. IQR: Interquartile range *The serum cTnI was significantly different in the first immobilisation compared to 2nd, 3rd and 4th immobilisation.

The post-hoc power based on a sample size of 8 rhinoceros, in each of the four immobilisation protocols, was ≤7%;

Study group 5: Effect of fluid administration on transported white rhinoceros

Twenty-four white rhinoceros were initially included in this study, and blood samples at capture, T12, and T24 were used for the analysis. Paired serum cTnI results were available for 23 animals, and one animal was excluded because of invalid test results at all time points. The 23 animals included 11 adults (eight males and three females) and 12 subadults (nine males and three females). Retrospective data on AST and CK serum activity and BHB and lactate concentrations were available at all collection points investigated (**Table 22**).

Table 22: Median or mean (IQR or SD) concentrations or activities of blood clinical chemistry analytes of translocated white rhinoceros at capture, 12 hours after the start of transport and at release.

	Capture	12 hours after start of transport (T12)	Release (T24)
Aspartate aminotransferase (U/L)	80 (14)	110* (84-139)	155* (112-227)
β-hydroxybutyrate (mmol/L)	0.18* (0.16-0.19)	0.39 (0.10)	0.32* (0.25-0.48)
Cardiac troponin I (ng/L)	4 (1)	16* (11-106)	13* (8-40)
Creatine kinase (U/L)	217 (52)	1 628* (1 013-3 415)	3 805* (2 249-5 856)
Lactate (mmol/L)	2.58* (1.81-3.83)	1.56* (1.33-2.16)	1.39* (1.23-1.66)

*Median values. IQR: interquartile range; SD: standard deviation

The serum cTnI concentration was significantly different between all the different time points investigated ($P < 0.001$) (**Figure 12**).

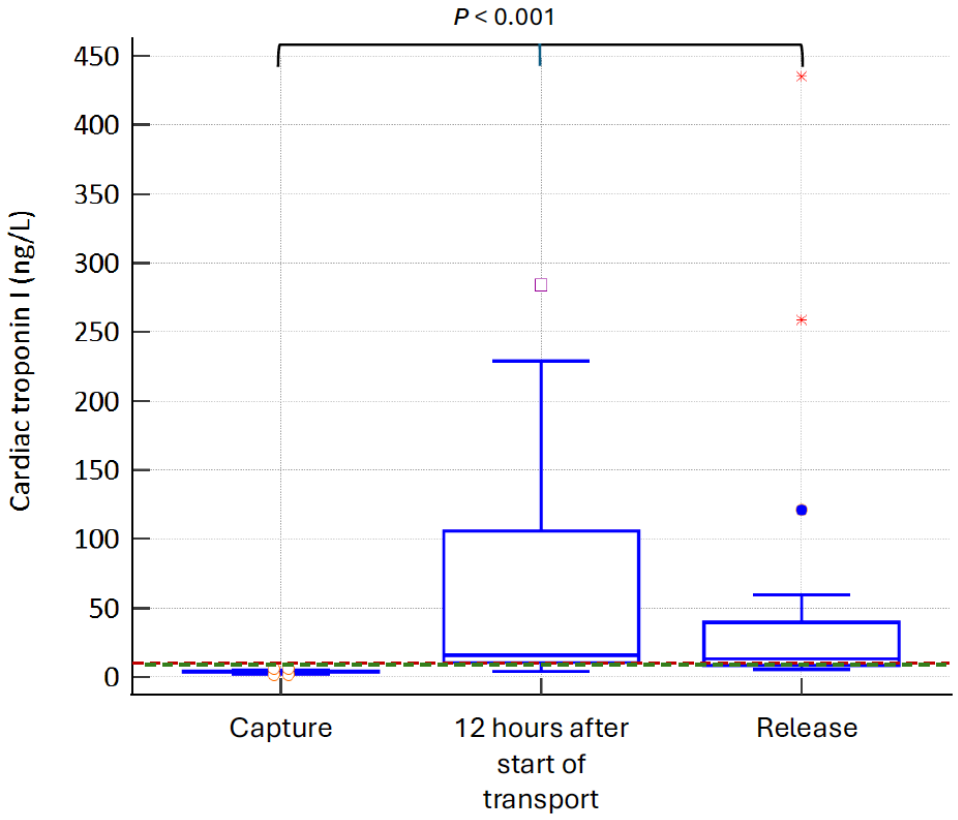

Figure 12: Comparison of serum cardiac troponin I concentration at capture, 12-hours after the start of transport and release in translocated white rhinoceros.

Box-and-whisker plot: The central box encompasses the 25th-75th percentiles. The middle line represents the median. The outside horizontal lines indicate the minimum and maximum excluding far-out values. Open squares and blue dot: Outside values (value higher than the upper inner fence but not higher than the upper outer fence). Stars: Far out values (value higher than the upper outer fence).

Red stippled line: 99th percentile upper reference limit (6 ng/L); Green stippled line: 95% upper reference limit (5.99 ng/L), the lower limit (2.03 ng/L) not displayed (refer to Chapter 5).

There were no significant correlations between AST serum activity and lactate concentration compared with serum cTnI concentration at any of the investigated time points. However, moderate and significant positive correlations were found between CK activity at T12 and serum cTnI concentration at T12 and release. In addition, moderate

and significant negative correlations were found when the capture BHB concentration was compared with the serum cTnI concentration at T12 and release. The results are summarised in **Table 23**.

Table 23: Serum cTnI correlations with AST, BHB, CK and lactate at capture, 12-hours after the start of transport and release in translocated white rhinoceros.

	Capture cTnI	T12 cTnI	Release cTnI
AST capture	Rho: -0.061 *P* = 0.783	Rho: -0.245 *P* = 0.260	Rho: -0.158 *P* = 0.473
AST T12		Rho: 0.246 *P* = 0.257	Rho: 0.310 *P* = 0.150
AST release			Rho: 0.275 *P* = 0.204
BHB capture	Rho: -0.132 *P* = 0.537	Rho: -0.528 *P* = 0.010*	Rho: -0.471 *P* = 0.024*
BHB T12		Rho: 0.092 *P* = 0.678	Rho: 0.093 *P* = 0.673
BHB release			Rho: 0.228 *P* = 0.296
CK capture	Rho: -0.092 *P* = 0.676	Rho: -0.029 *P* = 0.897	Rho: -0.092 *P* = 0.677

CK T12		Rho: 0.516 *P* = 0.012*	Rho: 0.487 *P* = 0.018*
CK release			Rho: 0.290 *P* = 0.179
Lactate capture	Rho: -0.064 *P* = 0.772	Rho: 0.341 *P* = 0.111	Rho: 0.198 *P* = 0.366
Lactate T12		Rho: 0.190 *P* = 0.385	Rho: 0.234 *P* = 0.283
Lactate release			Rho: -0.395 *P* = 0.069

AST: aspartate aminotransferase; BHB: β-hydroxybutyrate; CK: creatine kinase; cTnI: cardiac troponin I; Rho: Spearman's rank correlation coefficient. T12: 12-hours after the start of transport. *Statistically significant correlations.

The post-hoc power based on a sample size of 23 rhinoceros was 64.8%;

DISCUSSION AND CONCLUSION

Serum cTnI concentrations in translocated African rhinoceros were significantly increased during transport and at the time of release compared with the serum concentrations at capture. The degree of serum cTnI increase was more marked in animals that were chased, darted and immobilised from a helicopter than in animals that were darted from the ground and sedated while confined to a boma. Serum cTnI

concentrations in these animals also correlated with other serum biomarkers of muscle injury.

Myocardial injury was evident in all groups of translocated rhinoceros. In the boma-adapted transported black rhinoceros cohort, although the cTnI concentration was increased at the time of release, the median cTnI concentration (8 ng/L) was at the upper end of the black rhinoceros serum cTnI reference interval (RI: 1.47-8.17 ng/L, refer to Chapter 5) and below the 99th URL (9 ng/L, refer to Chapter 5). However, in five animals, serum cTnI concentration at the time of release exceeded these limits, peaking at 49 ng/L, while their capture concentrations were below the 99th URL. In the three white rhinoceros transport studies, the median cTnI concentration exceeded the 99th URL at release in all groups. In the study with a six-hour transport of 22 male white rhinoceros over 280 km, the median cTnI at capture was within reference limits, with only a few animals exceeding RI limits (chased RI: 2.41-6.29 ng/L, refer to Chapter 5). However, compared with the 99th URL (6 ng/L, refer to Chapter 5), the median cTnI at the start of transport was 17 times higher and 30-32 times higher at subsequent collection points. In the 24-hour translocation study of boma-adapted white rhinoceros, median serum cTnI concentrations significantly increased at 12 and 24 hours post-capture, being approximately 2-2.5 times higher than the white rhinoceros 99th URL. Significantly increased cTnI concentrations were recorded in the semi-captive white rhinoceroses transported 1 300 km. Baseline cTnI concentrations at capture were within reference limits and below the 99th URL, while the median release cTnI concentration was approximately 2.5 times above the 99th URL (15 ng/L). Conversely, no significant differences in serum cTnI concentrations were observed between immobilisation and 40

min later in the drug protocol comparison study. The median serum cTnI concentrations at each time point for all drug protocols were below or at the 99th URL for white rhinoceroses.

The human European Society of Cardiology and the American College of Cardiology recommend diagnosing spontaneous MI when at least one cTnI concentration exceeds the 99th percentile of a normal population, provided baseline levels are below this threshold.[72] Based on this criteria, the serum cTnI findings indicate acute myocardial injury in all cohorts of translocated rhinoceros. Intracellularly, cTnI exists in two pools, most structurally bound within the thin filaments of the cardiac muscle tissue contractile apparatus, and a small proportion as free cytosolic cTnI.[63] The early rise of cTnI in the blood after acute cardiac injury is due to the release of cytosolic cTnI, while sustained increases are due to the slower release of structurally bound troponin representing cell necrosis with contractile apparatus destruction.[63,190] As a leakage marker, the early release of cytosolic cTnI into the circulation is due to cardiomyocyte damage and membrane integrity loss.[63,171,191] This mechanism alone cannot account for the high cTnI levels observed in rhinoceroses in this study, given that the free cytosolic cTnI constitutes only a small fraction (2-4% in humans).[63,191] It is more likely that the increased cTnI concentrations result from the release of the structurally bound cTnI pool. Whether cTn release occurs with reversible or irreversible cardiac injury is debatable. A delayed but continuous release of troponin at high concentrations suggests irreversible cardiomyocyte injury (due to degradation of myofilaments with the release of structurally bound troponin), as shown in a swine model of ischaemic heart disease and patients with unstable angina.[192,193] In these studies, an early and transient release of cTnI at lower

concentrations is hypothesised to be associated with cTn leakage from the free pool through reversibly damaged cardiomyocyte membranes.[190,192,193] Some human studies of prolonged strenuous exercise, which reported transient cTnI increases, also support the idea that the release is from the cytosolic pool.[194,195] However, these findings are in contrast to the significant troponin release in marathon runners with myocardial scarring.[196] Furthermore, a biphasic cTn release pattern has been demonstrated in humans with acute MI, with peaks documented 14 hours and three to five days after the insult.[197] Unfortunately, serum cTnI concentrations cannot differentiate between the two intracellular cTnI pools.[198] Increased cTnI concentrations in these rhinoceroses could not be further investigated for transience (indicating reversible injury) or persistence (irreversible injury) as no follow-up samples were collected post-release. Additionally, myocardial histological examination is required to distinguish between reversible and irreversible damage.

No significant difference was found in the serum cTnI concentration at initial immobilisation and 40 min later in the eight subadult white rhinoceros. In humans and dogs, cTnI concentrations begin to rise 4-12 hours after acute MI.[199,200] It is plausible that no change might have been detected in these white rhinoceros because of the short time interval between initial immobilisation and the 40 min sample collection, with insufficient time to detect cTnI release from damaged cardiomyocytes. However, in humans with acute MI, a single admission measurement of a hs-cTnI test level above the 99th URL has a sensitivity and specificity of >90% for the diagnosis of acute MI, regardless of the time of chest pain onset.[201] Furthermore, a single hs-cTnI test concentration above the 99th URL, performed within 3 hours of the onset of chest pain, is highly predictive that

serum cTnI concentration will increase further by 30% within 6 hours.[201] In both the EAzaB and EB immobilisation protocols, although not different, the median serum cTnI concentration at both time points in each protocol was at the 99th URL, 6 ng/L, established in white rhinoceros (refer to Chapter 5). Additionally, this cut-off value was exceeded by individual animals in each immobilisation protocol at both evaluated time points. Based on the findings in humans with acute MI, it can be hypothesised that the serum cTnI concentration in these individual white rhinoceros may also be increased if it was measured serially within 3-6 hours of the immobilisation event.[201]

The degree of serum cTnI increase was more marked in white rhinoceros that were chased and darted from a helicopter compared to boma-adapted transported white rhinoceros darted from the ground while confined in a boma. This finding was particularly evident in the 280 km transportation group of free-living white rhinoceros within Kruger National Park (study group 3). These cohorts received fairly similar etorphine-based immobilisation drug protocols to the boma-captured transport simulation group (study group 5), and therefore cardiomyocyte damage due to hypoxaemia and hypercapnia, associated with etorphine-based protocols was not the only underlying mechanism of troponin release involved.[40,53] During capture, the total distance run by animals in study group 3 before and after darting ranged from 200 meters to 3.5 kilometre (km).[15,33] Troponins increase in humans after short-term and intermittent exercise, like basketball or running.[171] In humans, the pattern of troponin concentrations post-exercise corresponds to the release from the cytosolic compartment of cardiomyocytes.[171] The increased membrane permeability that allows troponin leakage might be caused by the production of ROS, alterations in calcium, pH, glucose or fat metabolism or changes in

communication between integrins and cytoskeleton of stretched cardiomyocytes.[171,202] Furthermore, troponin release as part of the general inflammatory process associated with exercise may also contribute to increased troponin concentrations after exercise.[171] The previously reported acidaemia, acute phase response, decreased unsaturated fatty acids and increased lipid peroxidation products in the chased white rhinoceros group most likely contributed to increased cardiomyocyte membrane permeability and cardiomyocyte damage.[15,33]

Interestingly, a similar degree of serum cTnI increase was noted in the transported boma-adapted white rhinoceros (study group 5) compared with the group of free-living white rhinoceros that were transported for 36 hours (1 300 km) after being immobilised by darting from a helicopter following a chase (study group 2). This may be partly explained by the reduced intensity of the chase in study group 2, with the reported flying time ranging on average between 5-12 min per animal (the distances run not reported).[16] Furthermore, the behavioural responses shown by adult white rhinoceros in response to natural enemies, mainly humans, are varied, and there is also great individual variation in such behaviour which might be learned in juveniles from observing adults.[203] These helicopter-darted animals in study group 2 originated from a private 340-hectare game farm and were semi-captive consisting of adults and juveniles. Therefore, the fight-and-flight response in these animals might have been less intense than those of the free-living rhinoceros bulls originating from Kruger National Park (study group 3) and more similar to that of the boma-adapted animals (study group 5). The white rhinoceros in study group 2 consisted predominantly of females, while the white rhinoceros in study group 3 were all bulls. Reports on sex-associated differences in the degree of troponin release post-

exercise in humans are contradictory.[171] It is unknown whether this contributed to the difference in serum cTnI concentrations in these two cohorts of chased rhinoceros. The difference between the timing and number of blood samples evaluated in these two studies most likely also played a role, as cTnI concentration normalisation occurs 24-28 hours post-exercise in human athletes,[171] which may explain the lower degree of serum cTnI concentrations at release (~34 hours) in study group 2 versus the six-hour release time in study group 3.

The median cTnI concentration of the boma-adapted black rhinoceros population was below the species-specific 99th URL (refer to Chapter 5), whereas in boma-adapted white rhinoceros, the median serum cTnI concentration was 2-2.5 times higher than that of the white rhinoceros 99th URL (refer to Chapter 5). The immobilisation drugs used in these two studies (study groups 1 and 5, respectively) were similar, and the differences noted in the degree of cTnI increase between the boma-adapted species of rhinoceros might be related to different drug doses and their different responses to the drugs used. This possible drug effect is supported by the findings that black rhinoceros immobilised with etorphine and azaperone are not as hypermetabolic as white rhinoceros immobilised with etorphine.[204] Furthermore, during capture, the black rhinoceros were sedated by darting within the boma using a low-sedative dose of etorphine that allowed conscious loading into the transport crates.[13] These animals were thus not recumbent at capture and did not experience high levels of muscular (metabolic) activity before initial immobilisation, or severe respiratory depression potentially reducing the degree of muscular injury.

Ten animals in study group 3 were dehorned for conservation purposes unrelated to the original study. The median serum cTnI concentration in this group was significantly higher than that in the non-dehorned animals at all evaluated time points, except for release. Studies of the hormonal, physiological, and behavioural effects of dehorning in black and white rhinoceros have reported no adverse reproductive, physiological, or behavioural effects,[12,205,206] and no adverse pathophysiological effects directly associated with the dehorning procedure have been reported. The initial immobilisation period required for dehorning before loading into a crate was the same as that required for non-dehorned animals. In this study, the animals were transported four at a time and captured individually. The animals captured first waited in transport crates until all four were caught, resulting in capture-to-transport times varying between 1-4.5 hours.[15,33] Dehorned animals were captured first, so their T0, T2, and T6 blood samples were taken at later time intervals post-capture than those of non-dehorned animals. The significant increases in serum cTnI concentrations in dehorned compared to non-dehorned rhinoceros during initial sampling are likely due to troponin release kinetics, with longer sampling intervals allowing sufficient time for cTnI release into the circulation. Other potential causes for the increased serum cTnI concentration in the dehorned group should be further investigated, but these findings are likely related to capture and immobilisation logistics rather than the dehorning procedure itself.

Additionally, in study group 3, white rhinoceros were captured either with etorphine-azaperone or etorphine-midazolam. The serum cTnI concentrations at all time points investigated were not different between the two immobilisation groups. This finding is interesting, since an etorphine-midazolam combination, compared to an etorphine-

azaperone combination, is expected to cause less muscle rigidity and tremors, respiratory depression, and respiratory and metabolic acidosis in immobilised rhinoceros.[53,207] These differences occur because midazolam enhances the effect of the neurotransmitter gamma-aminobutyric acid (GABA) at the $GABA_A$ receptor which causes better skeletal muscle relaxation, anxiolysis, and sedation during immobilisation.[15,50,208] Lower concentrations of serum cTnI would therefore have been expected in the midazolam group of rhinoceros. These findings suggest that the type of opioid synergist drug (azaperone vs. midazolam) combined with etorphine did not change the degree of cardiomyocyte damage during immobilisation of free-living white rhinoceros.

In the study that investigated four different immobilisation drug protocols in white rhinoceros (study group 4), a moderate positive correlation between the capture lactate and cTnI concentration was noted in the EAzaB immobilisation protocol. The capture pH also revealed a moderately negative association with the capture cTnI concentration. This finding was most likely due to the muscle tremors, hypoxaemia, and acidaemia that are reported in white rhinoceros when this protocol is used, despite the addition of butorphanol in all the immobilisation protocols used in this study.[49,209] The addition of butorphanol and oxygen insufflation has been shown to decrease the intensity of the muscle tremors caused by catecholamine released by the immobilisation drugs.[209] In the etorphine-only immobilisation protocol (EB), there was a moderate negative correlation between the PaO_2 and serum cTnI concentration, 40 min after immobilisation. White rhinoceros are extremely sensitive to the effects of opioids, with hypoxaemia and hypercapnia, due to respiratory depression, inadequate gas exchange in the lungs, and increased oxygen consumption due to hypermetabolism and muscle tremors, commonly

reported side effects when immobilised with etorphine.[41] Importantly, cardiomyocytes are sensitive to the effects of hypoxia, as demonstrated with cTnI released into the bloodstream within 30 min of reperfusion and continued to increase for 180 min in a porcine ischaemia-reperfusion model.[210,211] These correlation results suggest that hypoxaemia, and acidaemia caused by the etorphine immobilisation protocols resulted in a degree of cTnI release, and thus possible cardiomyocyte damage.

Interestingly, when the four immobilisation events in study group 4, in each animal, were arranged sequentially, the median serum cTnI concentration was significantly higher during the first immobilisation event, compared to all the other immobilisations. Increased serum cTnI levels have been reported in a significant number of human trauma intensive care unit patients (both medical and surgical or trauma), related to the degree of physiological stress and illness and not due to mechanical chest injury.[212] Despite allowing the rhinoceros used in this study to acclimatise for six weeks in a boma at initial capture and administration of long-acting tranquilisers,[49] animals might have experienced more physiological stress prior to the first immobilisation event, resulting in higher serum cTnI levels, with gradual habituation to the activities related to the immobilisation events taking place over the weeks that followed. However, rhinoceros have variable individual responses to capture and confinement, and despite evidence of behavioural habituation, no physiological habituation has been demonstrated in a study that assessed the stress response in confined white rhinoceros.[174]

Serum cTnI concentrations in the transported rhinoceros showed moderate positive correlations with other serum muscle injury biomarkers at various time points. In study

group 2, involving 1 300 km transport of white rhinoceros, CK activity was moderately positively associated with serum cTnI concentration at the time of capture. However, the median capture CK activity fell within the RIs for white rhinoceros using the same method.[175] Similarly, the median cTnI concentration at capture was within the method-specific RIs for chased white rhinoceros (refer to Chapter 5).[175] Moderate positive correlations were also observed between CK activity and serum cTnI concentration at the start of transport, and between AST and CK activities and serum cTnI concentration at the time of release in the white rhinoceros transported for 280 km (study group 3). Median serum cTnI concentration was significantly increased, 17 times the URL at the start of transport and 30-32 times at release. Corresponding CK increases were 1.2 times and 3 times above the URL, respectively, while AST activity was at the upper end of the AST RI.[175] In boma-adapted transported white rhinoceros (study group 5), a moderate positive correlation was found between CK activity and serum cTnI concentration midway through transport. The median serum cTnI concentration was 2-2.5 times the URL, and CK activity was 4 times the URL.[175] The mild to moderate positive correlations between serum AST and CK activities and cTnI concentration indicate concurrent skeletal and myocardial muscle damage in these captured and transported rhinoceroses. The increased muscle marker activities may partly result from exertion during capture in study groups 2 and 3. Additionally, rhinoceroses likely experience fatigue and muscle contraction in transportation crates, leading to poor tissue perfusion, particularly if they stand throughout the journey. Repeated IM administration of tranquilisers may have caused further skeletal muscle damage.[213] Transport in crates and vehicle motion, known to cause muscle exertion in transported red deer,[35,214] likely contributed to skeletal muscle damage in rhinoceroses in study groups 2 and 3. These findings suggest the presence of

CM, characterised by damage to both the skeletal and cardiac muscles due to exertion and stress.[13]

In the boma-adapted black rhinoceros transport study (study group 1), capture AST and CK activity, and in the 280 km white rhinoceros transport study (study group 3), only capture AST activity was moderately and positively correlated with serum cTnI concentration at release. In the boma-adapted white rhinoceros transport study (study group 5), CK activity halfway during transport was moderately and positively correlated with serum cTnI concentration at release. These findings suggest that animals with evidence of muscular damage at capture or during transport, based on increased serum AST and CK activities, also suffered from myocardial injury. However, an increased concentration of cTnI was not detected at the time of the first sampling, and the cytosolic release of cTnI into circulation was only evident during and at the release or end of transport due to troponin kinetics. The enzymatic kinetics of AST, CK, and serum cTnI are unknown in African rhinoceros and can only be extrapolated from what is known in other species. Serum cTnI concentrations only begin to increase 4-12 hours after acute MI in humans and peak at 12-48 hours,[63] while in a dog model of acute MI, similar release times are observed, with peak concentrations reached within 10-16 hours.[199] As a cytosolic enzyme, CK is released rapidly from injured muscle, and in horses CK activities peak at 6-12 hours after an insult, while AST peaks at 24-36 hours after an insult.[215] Furthermore, because the half-life of CK is short (~2 hours) in most species, increased serum activities are indicative of recent muscular injury.[37,215] Based on the kinetics of these muscle biomarkers, increased serum activities will likely be noted earlier than increased serum cTnI concentrations, which explains the positive correlations of these muscular injury

biomarkers with serum cTnI concentrations several hours later at the end of transport. In addition, cardiac isoenzyme forms of CK (especially CK-3 of the MB isoenzyme) and AST originating from cardiomyocytes are also released into the blood and are included in the total AST and CK serum activities measured,[37] and therefore, the likely contribution of cytosolic leakage of AST and CK from cardiomyocytes at capture and release cannot be excluded.

In contrast to the other included studies, no associations were found between serum AST activity and serum cTnI concentrations in the boma-adapted white rhinoceros transport study (study group 5). In a recent study that investigated the effects of feeding and transport duration on the welfare of transported white rhinoceros, greater increases in muscle enzymes (AST and CK) were indicative of greater fatigue in non-fed rhinoceros over longer times.[21] As some animals in this study received fluids, this intervention may have reduced the occurrence of energy deficits, dehydration, muscle damage, and potential cardiomyocyte damage typically experienced by rhinoceros during long-duration transportation.

A positive correlation between lactate concentrations at capture and cTnI concentrations at the time of release was found in boma-adapted transported black rhinoceros (study group 1). The serum lactate concentrations were found to be increased from capture to after transport in these animals.[16] High lactate concentrations are indicative of muscular hypoxia and hypoperfusion as lactate production is increased in these conditions due to anaerobic energy production.[216] The muscles of rhinoceros are in a contracted state during chemical immobilisation, leading to vessel compression and resultant poor tissue

perfusion, which explains the increased lactate concentrations.[16] Furthermore, etorphine can cause increased sympathetic tone and a rise in cellular metabolism with increased oxygen consumption.[41] Despite only being sedated with etorphine, these results suggest poor tissue perfusion at capture could have resulted in cardiomyocyte damage, as evidenced by the increased serum cTnI concentration at the time of release. Increased cardiac troponin levels have been demonstrated in humans with abnormal tissue-level perfusion,[217] supporting the hypothesis that myocardial hypoperfusion might have contributed to the increased cTnI levels found in these animals. The lack of significant correlations between serum lactate concentration and serum cTnI concentrations in the three white rhinoceros transport studies (study groups 2, 3, and 5) may indicate that myocardial hypoperfusion and hypoxia were not the only underlying mechanisms that contributed to the increased serum cTnI concentrations noted in these animals.

Oxidative stress has been documented in white rhinoceroses transported for 280 km over 6 hours.[33] There was a moderate positive correlation between capture BHB and cTnI concentration at the start of transport (study group 3), likely due to increased membrane permeability from altered fat metabolism releasing cytosolic cTnI, as previously discussed.[171] Increased cTnI concentrations in cattle with ketosis are attributed to lipotoxicity and oxidative stress, leading to greater membrane permeability and cell death.[202] Similarly, fatty acid incorporation and micelle formation in myocardial plasma membranes destabilise and disrupt cell membranes, causing troponin release.[202] Focal degenerative changes and increased cTnI concentrations have also been observed in sheep with pregnancy toxaemia.[202] In contrast, in study group 5, BHB concentration at

capture was negatively correlated with serum cTnI concentration midway through transport and release. Experimental models of ischaemic heart disease have shown conflicting roles for BHB. Ischaemic heart disease, caused by nutrient and oxygen shortages, paradoxically leads to further irreversible myocardial cell death upon blood flow restoration, known as lethal myocardial reperfusion injury, and is responsible for up to 50% of the final infarct size in animal studies.[218,219] In a mouse ischaemia and reperfusion injury model, D-β-hydroxybutyrate infusion demonstrated a cardioprotective effect by reducing infarct size and cTnI concentration, attenuating myocardial apoptosis, and preserving cardiac function.[219] This effect is due to enhanced autophagic flux, reduced mitochondrial ROS formation, increased adenosine triphosphate production, reduced mitochondrial swelling, and partial restoration of mitochondrial membrane potential.[219] Further investigation into the energy metabolism of transported white rhinoceros and its role as a potential stressor and involvement in the myocardium is required.

This study has a few limitations. First, the measurement of serum cTnI concentration was performed on stored samples, and the samples used were collected between December 2017 and August 2023, with the oldest samples stored for approximately six years at the time of troponin analysis, and approximately half had undergone a single freeze-thaw cycle. A small proportion of samples underwent two freeze-thaw cycles. In humans, cTnI stability has been demonstrated for up to one year at -80 °C, and no significant changes were found after three freeze-thaw cycles in bovines and New World camelids.[220] However, there was no statistical evidence that sample age affected the cTnI results in the samples used (up to 5.5 years old) to determine cTnI reference intervals in rhinoceros

(refer to Chapter 5). In addition, in certain samples, the serum was separated from the red cell pellet approximately 24 hours after collection, resulting in haemolysis that may have increased the serum AST activities. But, the degree of haemolysis reported for the majority of samples was negligible to mild, and consequently, the impact should not have significantly affected the results. Haemolysis up to an H index of 25 (approximate haemoglobin concentration of 0.25 g/L or 16 µmol/L) does not result in significant interference in the Cobas AST method. However, the contribution of increased serum AST activities due to haemolysis cannot be excluded. Furthermore, different test methodologies have been used to determine serum lactate concentrations in rhinoceros transport studies, with the degree of bias between the methods unknown. However, the lactate results were only used for correlation analysis; therefore, the trend of change and not the absolute values were used to interpret these results. The sample sizes of the different study groups were small and underpowered for statistical analysis in three of the study groups, particularly in study group 4. The probability of false negatives, namely, the non-detection of significant differences in serum cTnI concentration when evaluating the four distinct chemical immobilisation protocols, warrants consideration in the interpretation of the results. Based on the findings reported in this chapter, further investigations with larger sample sizes are necessary to reevaluate if potential cardiomyocyte injury is associated with the use of specific chemical immobilisation protocols in rhinoceros. Finally, the serum cTnI results were interpreted and analysed based on retrospective study data, and information might have been incorrectly transcribed, incomplete or missing.

In conclusion, significantly increased concentrations of cTnI were demonstrated in both black and white rhinoceros transported over long distances when capture cTnI concentrations were compared to concentrations measured at various time points during the journey and at release. Increased serum cTnI concentration was found in chased helicopter-darted animals, as well as in boma-adapted animals, although the degree of cTnI increase was more significant in some groups of chased rhinoceros. Increased cTnI concentrations were associated with increased serum activities of AST and CK, indicative of capture myopathy based on concurrent cardiac and skeletal muscle injury. However, whether cardiomyocyte damage was reversible or irreversible could not be determined in this study. Furthermore, adverse physiological aspects typically associated with the drugs used in chemical immobilisation protocols have also been found to be associated with cTnI concentrations. The findings of this study demonstrate the need to monitor biomarkers of skeletal and cardiac muscle cell damage after capture and during the transportation of rhinoceros over long distances and to implement post-release monitoring in these animals. This biomarker monitoring will aid in the identification of animals possibly suffering from capture myopathy, even if asymptomatic, with corrective measures being implemented to improve the welfare aspects of rhinoceros translocations.

CHAPTER 7: DISCUSSION AND CONCLUDING REMARKS

The research presented in this thesis included an investigation into a cardiac biomarker, specifically cTnI, in African rhinoceros (*C. simum simum* and *D. bicornis minor*). This study focused specifically on the validation of cTnI immunoassays in both species, the generation of RIs, and the investigation of changes in serum cTnI during the translocation and immobilisation of rhinoceroses, using the validated assays.

The genetic characterisation of cTnI in the southern-central black and southern white rhinoceros revealed a 99% identity between the two species' cDNA nucleotide sequences and identical inferred amino acid sequences. When comparing the CDS cTnI cDNA nucleotide sequence alignment between black and white rhinoceros and humans, the percentage identity was 91%. The degree of identity between the inferred amino acid sequences of the two rhinoceros species versus humans was 94%, while ranging from 92-97% when compared to those of dogs, cats, horses, and rats. These findings are consistent with the reported degree of homology between cTnI in humans, rats, dogs, and horses and support the high conservation of cTnI among mammalian species.[65-67] The mRNA nucleotide sequences of both rhinoceros species were submitted to the NCBI GenBank (accession OR374027 and OR374028) and available for use by other scientists (refer to Chapter 3).

Six candidate commercially available human cTnI immunoassays were evaluated for amino acid sequence homology based on the visual inspection of epitopes targeted by assay detection and capture antibodies in both rhinoceros species utilising the aligned inferred rhinoceros cTnI amino acid sequences. The following five immunoassays were found to be suitable for further analytical validation of cTnI measurement in African rhinoceros: 1) Siemens ADVIA Centaur TnI-Ultra, 2) Siemens Stratus CS Acute Care cTnI, 3) Beckman Coulter Access AccuTnI (2nd generation), 4) Abbott iSTAT cTnI, and 5) Siemens Atellica VTLi hs-cTnI. The Siemens Immulite 2000 XPi cTnI assay is unlikely to detect rhinoceros cTnI, as this immunoassay utilises a single capture Ab which recognises a five-amino acid epitope region, and cross-reactivity with cTnI other than that of the dog and cat is highly unlikely (refer to Chapter 3).[87,98,102]

Based on the information obtained from the genetic characterisation of the cTnI gene in both species two POC cTnI immunoassays, the Siemens CS Acute Care Troponin I conventional cTnI immunoassay and the Siemens Atellica VTLi hs-cTnI immunoassay, were successfully validated for use. The analytical method validation was performed according to ASVCP guidelines, and these immunoassays fulfilled most of the method validation requirements and can be used preliminary for the measurement of serum cTnI concentration in black and white rhinoceros. All method validation experiments performed for both assay methods met the ASVCP TE_a of 70%. However, the more stringent AAB TE_a of 30% was exceeded only in the white rhinoceros short-term replication experiment (Atellica VTLi), haemoglobin interference study (Stratus CS), and recovery experiment (Atellica VTLi) (refer to Chapter 4). To our knowledge, this is the first report of cTnI immunoassays validated for use in African rhinoceros. These

immunoassays will most likely be able to measure cTnI in other subspecies of African rhinoceros based on the high percentage of identity found in the cTnI mRNA nucleotide sequence between the southern-central black and southern white rhinoceros. However, because of the early evolutionary divergence of these species from Indian and Asian rhinoceros species, the epitopes targeted by the Abs used in these two immunoassays might not be conserved, but, synonymous mutations cannot be excluded in which case then assays will be able to detect the cTnI. Further investigation into the genetic characteristics of cTnI in these species is needed.

In addition, of clinical importance was that the IFCC TF-CB recommendation of a CV of ≤10% at the 99th URLs, generated using the Atellica VTLi hs-cTnI immunoassay, was achieved as well as the analytical performance goal of SD 3.5 ng/L at low cTnI concentration (≤10 ng/L).[148,155] The requirement of a low CV% is important as changes at low serum cTnI concentrations are used for clinical decision-making and poor analytical performance should not be attributed to pathophysiological causes of changes in serum cTnI measurements in patients (refer to Chapter 4). To the best of our knowledge, this is the first veterinary study that incorporated hs-cTnI performance goals, as outlined by the IFCC TF-CB, in the analytical validation of hs-troponin immunoassays and highlights important analytical considerations that have a clinically significant impact on medical decision-making by clinicians.

To monitor analytical performance, the ASVCP QA guidelines recommend weekly measurement of QCM for unit devices in POC analysers.[129] In veterinary medicine a simple QC rule, for example, $1_{2.5s}$ or 1_{3s} with a P_{ed} of ≥85% and P_{fr} of ≤5%, using ≤2 levels

of QCM is preferred for POC analysers.[129] Using the 70% TE_a performance goal, a simple 1_{3s} QC rule using two levels of QCM with acceptable P_{ed} and P_{fr} when using the 70% TE_a performance goal could be used in both analysers, except at very low cTnI concentrations (16 ng/L) in the Atellica VTLi. At the moment there are no established guidelines regarding the appropriate concentration of QCM for evaluating cTnI immunoassays and there is a lack of defined standards for permissible imprecision and bias at concentrations equal to or less than the 99th URL.[75] Interestingly, the total error that could be controlled using a simple QC rule, utilising a "reverse approach" based on the CVs from the Stratus CS and Atellica VTli, ranged from 33-42% (refer to Chapter 4). This information may be useful when the current ASVCP cTnI TE_a of 70% is revisited and a more stringent performance goal is considered.

Reference intervals for serum cTnI concentration in rhinoceros can be used to assess whether cardiomyocyte injury occurred or not. Using the Atellica VTLi and adhering to ASVCP guidelines, 95% and 99% cTnI RIs were generated for both rhinoceros species in apparently healthy free-living populations that were chemically immobilised. The 95% RIs of serum cTnI in black and white rhinoceros reference populations were 1.47-8.17 ng/L and 2.22-5.84 ng/L. The 99th URL was 9 ng/L and 6 ng/L for black and white rhinoceros, respectively. The imprecision of the Atellica VTLi at the generated 99th URLs met the imprecision goal of ≤10%.[158] Subclass partitioning of the white rhinoceros reference population generated RIs of 2.03-5.99 ng/L and 2.41-6.29 ng/L in non-chased and chased cohorts during immobilisation, respectively. These subclass RIs were not statistically different (refer to Chapter 5). It should be noted that the reference population size was underpowered for the calculation of both 95% and 99% RIs. Generating RIs for

wildlife, particularly for endangered species, is often challenging. Moreover, determining the health status of these animals is difficult.[221] When the sample size of the reference population is underpowered for statistical analysis, alternative approaches such as multicentre or subject-based RIs may be utilised (refer to Chapter 8).[131] However, for the latter to be considered, BV data of cTnI in rhinoceros is necessary, which is currently unavailable. The best practice is to use species-specific and analyser-specific RIs when evaluating patient results, however, these RIs may be considered guidelines when evaluating serum cTnI concentrations in other rhinoceros species, i.e., elevated or not. However, it is advised to perform reference interval transference[131] at the very least when evaluating cTnI concentrations in other species of rhinoceros.

Measurement of serum cTnI concentration, using the Atellica VTLi, in black and white rhinoceros captured and transported over long distances demonstrated significantly increased cTnI concentrations, exceeding the species-specific 99th URLs, during transportation and release compared to capture, which is indicative of acute myocardial injury. Serum cTnI concentrations cannot differentiate between the two intracellular cTnI pools,[198] and whether these findings represented reversible or irreversible cardiomyocyte injury could not be established in this study either (refer to Chapter 6).

Transported white rhinoceros chased and darted from a helicopter exhibited a more marked increase in serum cTnI concentrations compared to those that were boma-adapted and darted from the ground while confined in a boma. In humans, increased cTnI concentrations have been reported after short-term and intermittent exercise, such as running,[171] and in one cohort of rhinoceros the total distance run by animals before and

after darting ranged from 200 meters to 3.5 km. The acidaemia, acute phase response, decreased unsaturated fatty acids, and increased lipid peroxidation products in the chased rhinoceros likely contributed to increased cardiomyocyte membrane permeability causing cardiomyocyte damage, and subsequent cTnI release (refer to Chapter 6).[15,33]

The serum cTnI concentration of the transported animals also correlated positively with serum CK and AST activities which serve as other biomarkers of muscle injury at various time points, with increased serum concentrations and activities documented during transportation. These findings are highly suggestive of CM, which is characterised by damage to both the skeletal and cardiac muscles due to exertion and stress (refer to Chapter 6).[13] Although this syndrome has been suspected to occur during rhinoceros translocation projects, it has not previously been formally reported in scientific literature.

Investigation of the serum cTnI concentration in transported rhinoceros captured either with etorphine-azaperone or etorphine-midazolam showed no significant differences at any of the time points investigated between the immobilisation groups. The lack of difference suggests that the type of opioid synergist drug combined with etorphine did not change the degree of cardiomyocyte damage during the immobilisation of free-living rhinoceros. Furthermore, in the group of boma-adapted white rhinoceros that were immobilised with four different immobilisation drug protocols, hypoxaemia, and acidaemia caused by immobilisation with the etorphine-only protocol resulted in cardiomyocyte injury. However, no increase in serum cTnI was documented in any of the animals when comparing the serum cTnI concentrations between the protocols

administrated first to last, that is, no evidence of cumulative myocardial injury over 8 weeks (refer to Chapter 6). A significant finding was that the boma-adapted white rhinoceros in the simulated transport study also developed increased serum cTnI concentrations. These animals were not chased before darting and were only standing in crates during the mock translocation, indicating that capture by chemical immobilisation alone could play a role in cardiomyocyte injury.

The main aim of this thesis was to validate commercially available cTnI immunoassays and subsequently investigate the potential cellular myocardial injury in translocated African rhinoceros. The findings of this study will aid in the conservation of these species, and their implementation will potentially contribute to improved welfare during the translocation of these animals. Veterinarians can use these validated immunoassays to measure the serum cTnI concentrations in African rhinoceros. The Atellica VTLi is a small (analyser length 25 centimeters (cm), height 5.2 cm and width 8.5 m), portable, easy-to-use POC analyser utilising an hs-cTnI assay with low detection limits, which lends itself to its potential use in the field by wildlife veterinarians (refer to Chapter 8). Approximately 60 tests can be analysed on a fully charged battery, and the analyser memory can store up to 100 test results before it needs to be downloaded to the service software program using a local area network or wireless network (installed on a computer, laptop preferred for mobility). New QC lots can be registered manually on the analyser or using service software. A simple QC rule was validated for both analysers that can be implemented and used by general practitioners to ensure the accurate analytical performance of the analyser and reliable results. Lastly, regardless of the journey duration, a “boma-to-field” approach to rhinoceros translocation should be implemented where possible, as

opposed to “field-to-field”, to reduce chasing and immobilisation before transport (refer to Chapter 8).

CHAPTER 8: FUTURE RESEARCH DIRECTIONS

The findings of this thesis, particularly the research on method validation and assessment of serum cTnI changes in long-distance translocated rhinoceros, provide a foundation for various future investigations. Potential areas for further research are briefly discussed below.

Evaluation of the use of the Siemens Atellica VTLi analyser under anticipated field conditions. The stated operating temperature is 5-27°C, however, these ambient temperatures will be exceeded in the field. Furthermore, validation of the measurement of cTnI in whole blood, lithium-heparinised whole blood, or plasma of African rhinoceros will also be advantageous.

The generated 95% and 99% RIs were underpowered, and the use of subject-based RIs is an alternative. Investigation of BV in African rhinoceros of cTnI, will enable this. The significance of changes in cTnI in serial samples, especially at low concentrations, can then be determined.

To evaluate the clinical significance (reversible versus irreversible cardiomyocyte injury) of increased serum cTnI concentrations in translocated rhinoceros, prospective measurements of serum cTnI concentration should be performed during long-distance transportation and for a specified period after transport before hard release of rhinoceros. The various timepoints used to evaluate the serum cTnI concentrations in

these studies can also be used to better define the cTnI kinetics in rhinoceros. Furthermore, if any unfortunate mortality is experienced during translocation, histopathology should be performed to evaluate whether myocardial ischaemia or fibrosis (especially in animals exposed to repeated translocations) is correlated with serum cTnI concentrations. Furthermore, investigation of serum cTnI concentrations during the post-translocation phase will also identify animals potentially suffering from CM and provide additional information related to capture cardiomyopathy in these species.

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APPENDICES

CONGRESS PRESENTATIONS AND POSTERS RELATED TO THIS THESIS

1. Rautenbach Y, Parsons SDC, Goddard A, Meyer LCR, Buss PE, Hooijberg EH. Sequencing of the southern white rhinoceros (*Ceratotherium simum simum*) cardiac troponin I gene and analytical validation of a point-of-care cardiac troponin I immunoassay. Joint European Congress of Veterinary Pathology and Clinical Pathology (ESVP/ECVCP/ESVCP/ECVCP), Lisbon, Portugal, poster, 31 August – 2 September 2023.

2. Rautenbach Y, Parsons SDC, Goddard A, Meyer LCR, Buss PE, Hooijberg EH. Sequencing of the southern white rhinoceros (*Ceratotherium simum simum*) cardiac troponin I gene and analytical validation of a point-of-care cardiac troponin I immunoassay. University of Pretoria, Faculty of Veterinary Science, Faculty Day, Pretoria, South Africa, research presentation (Award: Best Postgraduate Oral presenter), 21 September 2023.

3. Rautenbach Y, Goddard A, Meyer LCR, Buss PE, Pohlin F, Hooijberg EH. Cardiac troponin I in African rhinoceros: Immunoassay validation, reference interval determination and evaluation of serum concentration changes during

immobilisation and transport. 39th World Veterinary Association Congress, Cape Town, South Africa, research presentation, 16 April 2024.

4. Rautenbach Y, Meyer LCR, Goddard A, Buss PE, Pohlin F, Hooijberg EH. High-sensitivity cardiac troponin I in African rhinoceros: Point-of-care immunoassay validation, reference interval determination and evaluation of serum concentration changes during immobilisation and transport. University of Pretoria, Faculty of Veterinary Science, Faculty Day, Pretoria, South Africa, research presentation (Award: Runner-up Postgraduate Oral presenter), 7 August 2024.

5. Rautenbach Y, Meyer LCR, Goddard A, Buss PE, Pohlin F, Hooijberg EH. High-sensitivity cardiac troponin I in African rhinoceros: Point-of-care immunoassay validation, reference interval determination and evaluation of serum concentration changes during immobilisation and transport. European Congress of Veterinary Clinical Pathology (ESVCP/ECVCP), Budapest, Hungary, research presentation, 28 – 31 August 2024.

RESEARCH AND ANIMAL ETHICS CERTIFICATES

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Veterinary Science
Research Ethics Committee

20 February 2024

LETTER OF APPROVAL

Ethics Reference No	**REC205-21**
Protocol Title	**Investigation of cardiac troponin I in the southern-central black (Diceros bicornis minor) and southern white (Ceratotherium simum simum) rhinoceros: Gene sequencing and assay validation**
Principal Investigator	**Dr Y Rautenbach**
Supervisors	**Prof EH Hooijberg**

Dear Dr Y Rautenbach,

We are pleased to inform you that your submission conforms to the requirements of the Faculty of Veterinary Sciences Research Ethics committee.

Please note the following about your ethics approval:

1. Please use your reference number (REC205-21) on any documents or correspondence with the Research Ethics Committee regarding your research.
2. Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.
3. Please note that ethical approval is granted for the duration of the research as stipulated in the original application (for Post graduate studies e.g. Honours studies: 1 year, Masters studies: two years, and PhD studies: three years) and should be extended when the approval period lapses.
4. The digital archiving of data is a requirement of the University of Pretoria. The data should be accessible in the event of an enquiry or further analysis of the data.

Ethics approval is subject to the following:

1. 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.
2. Note: All FVS animal research applications for ethical clearance will be automatically rerouted to the Animal Ethics committee (AEC) once the applications meet the requirements for FVS ethical clearance. As such, all FVS REC applications for ethical clearance related to human health research will be automatically rerouted to the Health Sciences Research Ethics Committee, and all FVS applications involving a questionnaire will be automatically rerouted to the Humanities Research Ethics Committee. Also take note that, should the study involve questionnaires aimed at UP staff or students, permission must also be obtained from the relevant Dean and the UP Survey Committee. Research may not proceed until all approvals are granted.

We wish you the best with your research.

Yours sincerely

PROF M. OOSTHUIZEN
Chairperson: Research Ethics Committee

100 YEARS OF VETERINARY EDUCATION

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Faculty of Veterinary Science
Fakulteit Veeartsenykunde
Lefapha la Disaense tša Bongakadiruiwa

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Veterinary Science
Research Ethics Committee

29 July 2024

LETTER OF APPROVAL

Ethics Reference No	**REC057-23**
Protocol Title	**Serum cardiac troponin I in Southern-central black rhinoceros (Diceros bicornis minor) and the Southern white rhinoceros (Ceratotherium simum simum): generation of reference intervals and evaluation of cardiac troponin I in immobilised, transported and sick rhinoceros**
Principal Investigator	**Dr Y Rautenbach**
Supervisors	**Prof A Goddard** **Prof LCR Meyer** **Prof EH Hooijberg**

Dear Dr Y Rautenbach,

We are pleased to inform you that your submission conforms to the requirements of the Faculty of Veterinary Sciences Research Ethics committee.

Please note the following about your ethics approval:

1. Please use your reference number (REC057-23) on any documents or correspondence with the Research Ethics Committee regarding your research.
2. Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.
3. Please note that ethical approval is granted for the duration of the research as stipulated in the original application (for Post graduate studies e.g. Honours studies: 1 year, Masters studies: two years, and PhD studies: three years) and should be extended when the approval period lapses.
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We wish you the best with your research.

Yours sincerely

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Chairperson: Research Ethics Committee

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UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Veterinary Science
Animal Ethics Committee

12 April 2022

Approval Certificate
New Application

AEC Reference No.:	REC205-21
Title:	Investigation of cardiac troponin I in the southern-central black (Diceros bicornis minor) and southern white (Ceratotherium simum simum) rhinoceros: Gene sequencing and assay validation
Researcher:	Dr Y Rautenbach
Student's Supervisor:	Prof EH Hooijberg

Dear Dr Y Rautenbach,

The **New Application** as supported by documents received between 2022-02-14 and 2022-03-28 for your research, was approved by the Animal Ethics Committee on its quorate meeting of 2022-03-28.

Please note the following about your ethics approval:

1. The use of species is approved:

Species	Number
Horses	2
Samples	**Number**
Ceratotherium simum simum - Serum (Stored- Historic/Retrospective)	12
Ceratotherium simum simum - Skeletal muscle (Stored- Historic/Retrospective)	3
Ceratotherium simum simum - Ventricular myocardium (Stored- Historic/Retrospective)	3
Diceros bicornis minor - Serum (Stored- Historic/Retrospective)	12
Diceros bicornis minor - Skeletal muscle (Stored- Historic/Retrospective)	3
Diceros bicornis minor - Ventricular myocardium (Stored- Historic/Retrospective)	3
Equus ferus caballus - Ventricular myocardium (Samples from live animals)	2

2. Ethics Approval is valid for 1 year and needs to be renewed annually by 2023-04-12.
3. Please remember to use your protocol number (REC205-21) 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.
5. **All incidents** must be reported by the PI by email to Ms Marleze Rheeder (AEC Coordinator) within 3 days, and must be subsequently submitted electronically on the application system within 14 days.
6. The committee also requests that you record major procedures undertaken during your study for own-archiving, using any available digital recording system that captures in adequate quality, as it may be required if the committee needs to evaluate a complaint. However, if the committee has monitored the procedure previously or if it is generally can be considered routine, such recording will not be required.

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Fax +27 12 529 8321
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Fakulteit Veeartsenykunde
Lefapha la Diseanse tša Bongakadiruiwa

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 A Tordiffe
DEPUTY CHAIRMAN: UP-Animal Ethics Committee

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Veterinary Science
Animal Ethics Committee

3 August 2023

Approval Certificate
New Application

AEC Reference No.: REC057-23
Title: Serum cardiac troponin I in Southern-central black rhinoceros (Diceros bicornis minor) and the Southern white rhinoceros (Ceratotherium simum simum): generation of reference intervals and evaluation of cardiac troponin I in immobilised, transported and sick rhinoceros

Researcher: Dr Y Rautenbach
Student's Supervisor: Prof EH Hooijberg

Dear Dr Y Rautenbach,

The **New Application** as supported by documents received between 2023-06-09 and 2023-07-31 for your research, was approved by the Animal Ethics Committee on its quorate meeting of 2023-07-31.

Please note the following about your ethics approval:

1. The use of species is approved:

Species	Number
Samples	Number
Rhino - Serum from 10 White, 2 black rhino Clinical cases Stored- Historic/Retrospective	12 (CP Biobank)
Rhino Serum from 14 Black rhino; 2 collection time point Stored- Historic/Retrospective	28 (V067-17)
Rhino Serum from 23 White rhino, 5 collection time point Stored- Historic/Retrospective	115 (V067-17)
Rhino Serum from 30 White rhino, 3 collections points Stored- Historic/Retrospective	90 (REC043-20)
Rhino Serum from 32 White rhino, 2 collection time point Stored- Historic/Retrospective	64 (V067-17)
Rhino Serum from 50 White, 8 black rhino Clinical cases Stored- Historic/Retrospective	58 (VWS Biobank)
Rhino Serum from 70 White& 70 Black rhino (RI) Stored- Historic/Retrospective	140 (VWS Biobank)
Rhino Serum from 8 White rhino, 2 col. points, 4 x/ anim Stored- Historic/Retrospective	64 (REC057-21)

2. Ethics Approval is valid for 1 year and needs to be renewed annually by 2024-08-03.
3. Please remember to use your protocol number (REC057-23) 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.
5. **All incidents** must be reported by the PI by email to Ms Marleze Rheeder (AEC Coordinator) within 3 days, and must be subsequently submitted electronically on the application system within 14 days.

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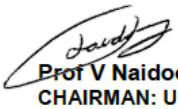
We wish you the best with your research.

Yours sincerely



Prof V Naidoo
CHAIRMAN: UP-Animal Ethics Committee