PHYSIOLOGICAL RESPONSES TO CAPTURE AND TRANSPORT IN SOUTHERN WHITE RHINOCEROS (*CERATOTHERIUM SIMUM SIMUM)* AND SOUTHERN-CENTRAL BLACK RHINOCEROS *(DICEROS BICORNIS MINOR)*

By

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Submitted in fulfilment of the requirements for the degree

Doctor of Philosophy (PhD)

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October 2019

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DEDICATION

"We have a responsibility toward the other life-forms of our planet whose continued existence is threatened by the thoughtless behaviour of our own human species. . . . Environmental responsibility – for if there is no God, then, obviously, it is up to us to put things right."

Jane Goodall, pioneer woman scientist, conservationist, and peacemaker

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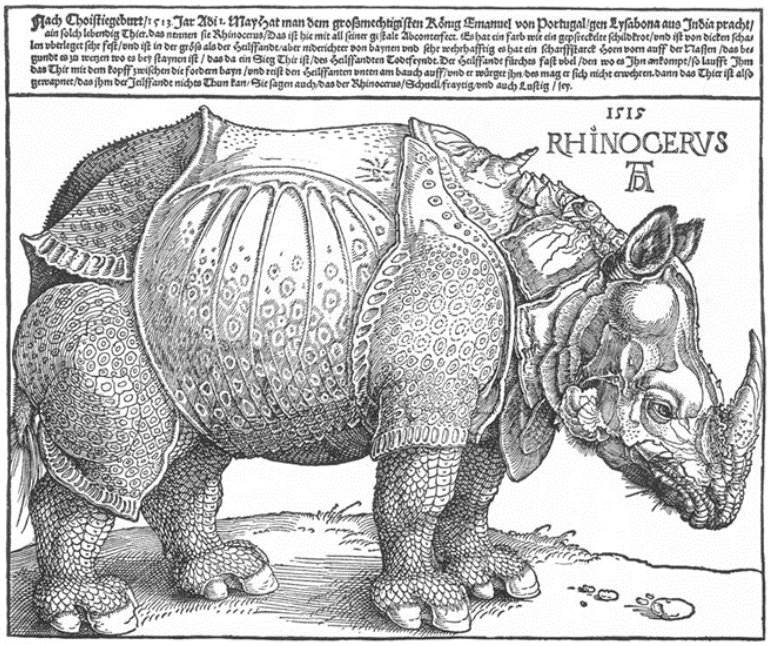
This thesis is dedicated to all fellow women conservationists who dare to discover and to protect.

"Run, rhino, run"

KOBEN, female artist, punk, animal lover and friend

PREFACE

THE TALE OF THE MOST INFAMOUS RHINOCEROS TRANSLOCATION IN HISTORY



Albrecht Dürer 1515, *Rhinoceron*, National Gallery of Art, Washington

On May 20th 1515, the Portuguese ship *Nostra Senara de Ajuda* anchored in the harbour of Lisbon, Portugal, returning from the Far East. It had very valuable cargo on board: the first living one-horned Indian rhinoceros to reach Europe since the ancient Roman times. In early 1514, *Afonso de Albuquerque*, governor of Portuguese India, sent ambassadors to *Sultan Muzafar II*, ruler of Gujarat, to negotiate the use of the island of Diu. The Sultan responded with gifts in return – one of which was the live rhinoceros. *Albuquerque* decided to forward the gift, known by its Gujarati name of *Ganda*, to *King Manuel I* of Portugal. The rhinoceros begun its journey from Goa, India, in early January 1515. The animal was accompanied by its Indian care-keeper, named *Ocem*, who fed it with vast quantities of rice throughout the sea journey of 120 days. The ship, captained by *Francisco Pereira Coutinho*, sailed across the Indian Ocean, around the Cape of Good Hope and north through the Atlantic with only three stops in port - Mozambique, St Helena and the Azores.

The rhinoceros arrived in a Europe that was obsessed with the rediscovery of its own past (Renaissance period in European history). The arrival of a living example, described by the ancient Roman author Pliny in his writings, provided evidence of

the reliability of texts from the classical antiquity. Thus, the rhinoceros was examined by scholars, and letters describing the "fantastic creature" were sent to correspondents throughout Europe. One sketch reached Albrecht Dürer in Nuremberg who illustrated the rhinoceros in his famous woodcut *Rhinoceron (1515)*. After arranging a fight with a young elephant from his collection, *King Manuel I* decided to give the rhinoceros as a gift to the *Medici Pope Leo X*. Wearing a collar of green velvet, the rhinoceros embarked in December 1515 for the voyage from Lisbon to Rome. On January 24th 1516, the animal disembarked briefly on an island off Marseilles to be beheld by *King Francis I* of France. After resuming its journey, the Portuguese ship sank in a sudden storm on the coast of Liguria, Italy. The rhinoceros, chained and shackled to the deck of the vessel, was unable to swim and drowned.

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ACKNOWLEDGEMENTS

This study was funded by the International Rhino Foundation (IRF), the Wildlife Group of the South African Veterinary Association (SAVA), the National Research Foundation (NRF) and research funds from the Department of Paraclinical Sciences of the Faculty of Veterinary Science, University of Pretoria.

I would like to thank South African National Parks, Rhinos Without Borders (Great Plains Conservation and & Beyond), the World Wildlife Fund and Ezemvelo KZN Wildlife for allowing access to rhinoceros transports, and Wildlife Pharmaceuticals for sponsoring the midazolam. Many thanks to DEFFink and the students of the University of Veterinary Medicine Vienna for raising awareness and additional funds for the project - how epic!

I am grateful to the University of Pretoria for the financial support granted to complete this PhD and to the AAZV Murray Fowler International Conference Scholarship Program and Cayman Chemical for supporting my active participation at the AZWV/AAZV/Leibniz-IZW conference 2018 and the International Society of Wildlife Endocrinology conference 2019.

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

Prof Leith CR Meyer, my supervisor, for the initiation of the rhino translocation project and providing me with the opportunity to complete a PhD at the University of Pretoria. Thank you for believing in my ideas, your trust and ongoing support!

Prof Emma H. Hooijberg, my co-supervisor, for sharing her knowledge in clinical pathology, her time invested in, and her motivation for, the project, and her many positive words. Thank you so much for your friendship!

Dr Peter Buss, my other co-supervisor, for his commitment to the project and his guidance and patience during some slightly chaotic data collection periods. Being able to call you my mentor, I must have done something right!

Drs Markus Hofmeyr and Dave Cooper, my field-supervisors, for sharing their experience and passion for rhinos.

Drs Mmadi Reuben, Francois Vilioen, Dee Blackhurst, and Nikolaus Huber, my co-workers, for your enthusiasm, time and help with the project.

My colleagues, students, and friends for helping with sample collection, analysis and logistics during various phases of my fieldwork: Sara & Sven Bourquin, Keagan Boustead, Caron Botes, Louis Bredenkamp, Grant Brokensha, Kyle Burger, Dylan Carulei, Damian & Les Charlisle, Kelly Cunningham, Hayden Cuthill, Keri-Lee Dobbie, Johannes Eksteen & Thomas Lengate (Kroonstad Dierehospitaal), Maria Fabregas, Jacques Flamand, Carolin Frei, Justin Grace, Guy Hausler, Harry Hensberg, Rob Jackson, Patrick Kahler, Henna Kaarakainen, Julian Keles, Travis Kersten, Ivan Kotze, Laurenz Krüger, Rowan Leeming, Johan Malan, Tebogo Manamela, Uldri van der Merwe, Penelope Miya, Thembeka Mtetwa, Janine Meuffels, Carien Muller, Mary Nasr, Comfort Nkgowe, Charlotte Nkuna, Margaret Nolan, Jacques O'Dell, Brett Pearson, Kelvin Pieterse, Jenni Pohlin, Kelly Du Preez, Christine Prechtl, Elisabeth Raich, Yolandi Rautenbach, Leana Rossouw, Ursina Rusch, Calvin Smith, Camilla & Lucy Smyth, Grant Tracy, Judi Uys, Malcom White, Marci Witczak, Madelyn deWet, Stefanie Wetzels and Gareth Zeiler.

My family and friends for always caring about me (and my pets).
Without your help none of this would have been possible!

My dad for his emotional and financial support and Radek for his unconditional love and the many hours of helping me with statistical analyses.

Miezi & Mali for their company during the writing process….meouw!

LIST OF ABBREVIATIONS

AG	Anion gap
ALP	Alkaline phosphatase
ANS	Autonomic nervous system
APRs	Acute phase reactants
AST	Aspartate aminotransferase
Atot	Total non-volatile weak acids
AUC	Area under the curve
AUCC	Animal Use and Care Committee
BANDS%	Percentage immature neutrophils
BE	Base excess
BHB	Beta hydroxybutyrate
BRREP	Black rhino range expansion project
iCa^{++}	Ionised calcium
CD	Conjugated dienes
CITES	Convention on International Trade in Endangered Species of Wild Fauna and Flora
CK	Creatine kinase
Cl^-	Chloride
CTAD	Sodium-citrate
EDTA	Ethylenediaminetetraacetic acid
EOS%	Percentage eosinophils
EPOC	Enterprise point-of-care
FGM	Faecal glucocorticoid metabolites
GABA	Gamma-aminobutyric-acid
GGT	Gamma-glutamyl transferase
GLDH	Glutamate dehydrogenase

HCO_3^-	Bicarbonate
HGB	Haemoglobin
HPA	Hypothalamic-pituitary-adrenal
HPLC	High performance liquid chromatography
HR	Heart rate
HRV	Heart rate variability
IATA	International Air Transport Association
IUCN	International Union for Conservation of Nature
K^+	Potassium
KNP	Kruger National Park
LCC	Leukocyte coping capacity
cLCC	Corrected LCC (per neutrophil)
LH	Luteinising hormone
LYM%	Percentage lymphocytes
LYM	Absolute lymphocyte count
MCV	Mean cell volume
MCH	Mean cell haemoglobin
MCHC	Mean cell haemoglobin concentration
MDA	Malondialdehyde
Mg^{++}	Magnesium
MON%	Percentage monocytes
MPV	Mean platelet volume
Na^+	Sodium
NEFA	Non-esterified fatty acid
NEU	Absolute neutrophil count
N:L ratio	Relative proportion of neutrophils to lymphocytes
ORAC	Oxygen radical absorbance capacity
PBR	Peripheral benzodiazepine receptors

PCT	Plateletcrit
PCV	Packed cell volume
PCW	Platelet distribution width
Pi	Inorganic phosphate
PBS	Phosphate-buffered saline
PLT	Platelet blood count
PMA	Phorbol 12-myristate 13-acetate
$PvCO_2$	Partial pressure of venous carbon dioxide
RBC	Red blood cell count
RDW	Red blood cell distribution width
RLU	Relative light units
ROS	Reactive oxygen species
SAA	Serum amyloid A
SANParks	South African National Parks
SD	Standard deviation
SEG%	Percentage mature neutrophils
SIDm	Measured strong ion difference
SIG	Strong ion gap
TBARS	Thiobarbituric acid reactive substances
T3	Triiodothyronine
T4	Thyroxine
TSH	Thyroid stimulating hormone
TSP	Total serum protein
WBC	White blood cell count
WWF	World Wildlife Fund

LIST OF DEFINITIONS

Translocation: the human mediated movement of living organisms from one area, with release in another (1).

Animal welfare: the state of an animal, including its physiological and psychological state, as regards its ability to cope with its environment (2).

Stress: constellation of events,

consisting of a

stressor: an unexpected stimulus,

that precipitates a

stress perception: reaction in the brain,

which activates the

stress response: physiological systems in response (3)**.**

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SUMMARY

Physiological responses to capture and transport in southern white rhinoceros (*Ceratotherium simum simum*) and southern-central black rhinoceros (*Diceros bicornis minor*)

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The southern-central black rhinoceros is currently listed as "critically endangered" and the southern white rhinoceros as "near threatened" by the International Union for Conservation of Nature Red List of Threatened Species. Translocation is an important tool for rhinoceros conservation and is widely used to reinforce declining populations or to restore extirpated populations. Capture and transport are part of translocation and expose the rhinoceros to a variety of stressors that ultimately can lead to morbidity and mortality.

The broad objectives of this thesis were therefore to: (1) establish a better understanding of the physiological responses to capture and road-transport in African rhinoceros, (2) to identify challenges associated with transport that should be addressed in order to improve animal welfare, and (3) to investigate whether the use of midazolam, instead of azaperone, would be able to mitigate some of these challenges.

The third chapter of this thesis investigated physiological responses to capture and long road transport in black and white rhinoceros in a "real-world" setting. Paired venous blood samples were collected from an auricular vein at capture and after transport in 14 boma-adapted black, and 32 semi-captive white rhinoceros and were evaluated for changes in: (1) clinical chemistry analytes; (2) acute phase reactants and (3) oxidative stress biomarkers. The Wilcoxon rank sum test was used to compare changes in measured analytes from capture and after transport.

The fourth and fifth chapter explored some of these changes in more detail and investigated if there were differences between rhinoceros sedated with midazolam compared to azaperone. Twenty-three wild white rhinoceros bulls were captured with a combination of etorphine plus either azaperone or midazolam. Azaperone or midazolam, respectively, were re-administered intramuscularly at capture (TC), start of transport (T0), and at two (T2) four (T4) and six (T6) hours of transport. Acid-base status was evaluated at these time points. Adrenaline and cortisol concentrations, as well as haematological (erythron, thrombon, leukon) and immunological (leukocyte coping capacity, acute phase reactants and oxidative stress biomarkers) changes, were measured at TC, T0 and T6. Changes in measured variables over time and between groups were compared using general mixed effects models.

Black and white rhinoceros transported over a long time experienced total body water loss, mobilisation of energy reserves, muscular damage, and stress-induced immunomodulation.

White rhinoceros bulls experienced respiratory acidosis combined with a lactic- and non-volatile weak acid acidosis during capture, followed by a mild metabolic- and strong ion alkalosis during transport. Increases in plasma adrenaline and serum cortisol concentrations indicated that rhinoceros mounted a stress response to capture and transport. The stress response was associated with characteristic haematological and immunological changes including stress-haemoconcentration, a progressive increase in neutrophil to lymphocyte ratio, the mounting of an acute phase reaction and oxidative stress. The acidaemia and associated alterations in acid-base indices were significantly less pronounced in midazolam-sedated compared to azaperone-sedated rhinoceros. Plasma adrenaline and serum cortisol concentrations did not differ between the groups. Midazolam, however, appeared to greater influence immunological responses to stress than azaperone.

Based on these results, we identified the following challenges to animal welfare during rhinoceros capture and transport: (1) life-threatening acid-base abnormalities associated with the unique challenges of rhinoceros capture; (2) fear and the mounting of a stress response to capture and the novelty of transport; (3) stress-induced immunomodulation; and (4) skeletal muscle fatigue, energy imbalance and dehydration that likely become more relevant with transport time.

Midazolam proved to be able to partly mitigate the first challenge and may therefore be a safer alternative to azaperone when combined with etorphine for the capture of wild white rhinoceros. Further research looking at behavioural benefits of using midazolam over azaperone, and possible consequences of its immunological effects, are required to demonstrate the value of midazolam administrations during transport. To optimise rhinoceros translocation, additional measures that aim to mitigate challenges to animal welfare during capture and transport, such as administering fluids during long journeys, need to be included in the planning of future translocations. The effectiveness of these measures in mitigating these challenges need to be systematically investigated.

CHAPTER 1: BACKGROUND

1.1 TRANSLOCATING THE BLACK AND WHITE RHINOCEROS

1.1.1. Translocation: what & why

Translocation is the human-mediated (intentional) movement of living organisms from one area, with release in another (1). Recent archaeological research suggests that translocations of non-domesticated species began as long ago as the late Pleistocene (4). The earliest known instance involved the introduction of the grey cuscus (*Phalanger orientalis*), a small marsupial weighing about 2 kg, to New Ireland about 19,000 years ago (4,5). From then on, animals have been translocated around the world for food, companionship, and by accident (5). There are many reasons to translocate animals. Seddon et al. (2012) provide a framework for considering all motivations for wild animal translocations (6). First, this framework distinguishes translocations that relate to the conservation of the species from those for which conservation is not the primary aim. These translocations include the non-lethal management of problem animals, commercially and recreationally motivated translocations (e.g. for hunting), the introduction of exotic natural enemies to control exotic pest species, translocations for aesthetic or religious reasons, the capture and release of rehabilitated wildlife, and illegal liberations of captive animals by animal rights activists (6). Translocations with the primary objective of improving the status of the targeted population, species or ecosystem, and not only provide a benefit to the translocated individual, are conservation translocations (1). In the face of increasing species extinction rates and global biodiversity loss, conservation translocation represent a powerful tool for species conservation (7). Conservation translocations within a species' indigenous range are classified as population restoration and comprise reinforcement and reintroduction (1,6). Reinforcement involves the release of individuals into an existing population of conspecifics in order to enhance population viability and reduce the

risks of genetic or demographic collapse due to stochastic effects. Translocations for reinforcement are used to overcome barriers to natural dispersal from other wild populations, to enable rapid population growth, or to allow for genetic exchange and avoid inbreeding depression (1,6). Reintroduction is the release of individuals into an area that was once part of the species' range but from which it has disappeared (1). Conservation translocations outside of a species' indigenous range are classified as conservation introduction and comprise assisted colonisation and ecological replacement (1,8). Assisted colonisation is the translocation of a species to favourable habitat beyond their native range to protect them from current or future threats (1,8). Ecological replacement refers to the release of a species outside its historic range to fill an ecological niche left vacant by the extinction of a native species (6,8). The term "translocation" as used in this thesis refers to conservation translocation, specifically population restoration *(*reinforcement*:* black rhinoceros in chapter 3 and white rhinoceros in chapter 4 and 5; reintroduction*:* white rhinoceros in chapter 3*)*. Routine wildlife translocations typically consist of: capture, transport and release (9). Following capture and, or, prior to release, translocation procedures frequently entail a period of temporary captivity to allow for veterinary examinations and for adaptation (10). This thesis focuses on the "capture" and "transport" of black and white rhinoceros translocated within southern Africa.

1.1.2. The need for translocating the black and white rhinoceros

Rhinoceritidae (rhinoceros) are iconic mega-herbivores belonging to the order Perissodactyla, the odd-toed ungulates. Other members of the order include Equidae (horses, assess, zebra) and Tapiridae (tapirs)(11). Rhinocerotidae comprise five living rhinoceros species, which are all threatened with extinction (12): the greater one-horned Indian (*Rhinoceros unicornis*), lesser one-horned Javan (*Rhinoceros sondaicus*) and two-horned Sumatran (*Dicerorhinus sumatrensis*) rhinoceros in southern Asia, and the black (*Diceros bicornis*) and white (*Ceratotherium simum*) rhinoceros in sub-Saharan Africa (12). There are three living subspecies of black rhinoceros occupying different

areas of Africa (13): the *critically endangered* eastern (*D. bicronis spp. michaeli*), *vulnerable* south-western (*D. bicronis spp. bicornis*) and *critically endangered* southern-central (*D. bicronis spp. minor*) black rhinoceros. The western black rhinoceros (*D. bicronis spp. longipes*) once ranged through the savannah zones of central-west Africa but it is now considered to have gone extinct (14). There are two subspecies of white rhinoceros, the *critically endangered* northern (*C. simum spp. cottoni*) and the *near threatened* southern (*C. simum spp. simum*) white rhinoceros (15). Since March 2018, there are only two known northern white rhinoceros left, both of which are female (16,17). The term "black rhinoceros" as used in this thesis refers to the southern-central black rhinoceros, the term "white rhinoceros" as used in this thesis refers to the southern white rhinoceros.

The name rhinoceros originates from the Greek *rhinokerōs* and translates to nose (*rhis*) and horn (*keras*)(18). The horn does not only give the rhinoceros its name, it is also the reason more than 5,000 rhinoceros have been killed in South Africa alone over the past five years (12). Rhinoceros species have faced many different threats over the past decades. Habitat loss and fragmentation as well as climate change (droughts) are important factors, but illegal hunting (poaching) for the illegal international rhinoceros horn trade currently represents a main threat (12,19). Increased demand for rhinoceros horn together with an increased involvement of organised international criminal syndicates in rhinoceros poaching have caused a dramatic increase in this threat over the last decade (13,15,19). Throughout Africa, rhinoceros poaching rates increased from 62 rhinoceros poached per year in 2007 to 1,349 in 2015 and 1,124 in 2017 (12). This increase in rhinoceros poaching has slowed black rhinoceros population growth and induced a decline in white rhinoceros numbers since 2012 (12). Most of the world's wild rhinoceros are conserved within South Africa (19,20). By the end of 2017, this country hosted 75 % of Africa's (37 % black and 86 % white) rhinoceros comprising 2,046 black (80 % of which were the southern-central subspecies) and 15,625 white rhinoceros (12). The largest white rhinoceros population

is found within the Kruger National Park (KNP) and has also been severely affected by poaching since 2007. Poaching rates in the KNP from 2014 - 2017 are estimated to have averaged 10 – 12 % of the population per year (20).

To ensure long-term survival of viable and valued African rhinoceros populations in the wild, the African Rhino Conservation Plan was recently developed at a continental level (21). Additionally, most rhinoceros range states implemented national rhinoceros conservation plans (12). The Biodiversity Management Plan for the Black Rhinoceros in South Africa (2011 – 2020) (22) and the Biodiversity Management Plan for the White Rhinoceros in South Africa (2015 – 2020) (23) address five key areas for rhinoceros conservation, namely: security; community empowerment; biological management; responsive legislative provisions that are effectively implemented and enforced; and demand management (12). Moreover, the World Wildlife Fund (WWF) Black Rhino Range Expansion Project (BRREP) contributes to the expansion of black rhinoceros numbers and range within the country (chapter 3) (12,16). Rhinoceros (conservation) translocation plays a central role in the "biological management" of these conservation plans (12). As a matter of fact, translocation has been identified as key to successful rhinoceros conservation (24).

1.1.3. Rhinoceros translocation history & current technique

Historically, rhinoceros have been translocated for commercial, recreational and aesthetic reasons. From the first century BC to the third century AD, skilled Roman animal handlers translocated a number of rhinoceros to be exhibited in arenas in Alexandria and Rome. The majority of these animals were likely black rhinoceros originating from regions around the Red Sea coast or the hinterland of present Eritrea and Ethiopia, where these animals were extant until the last century (25). In Asia, (Asian) rhinoceros were frequently translocated as gifts by the rulers and citizens of the different Chinese provinces and neighbouring countries such as Malaysia and Indonesia (25).

From the third to the sixteenth centuries AD, no rhinoceros was seen in Europe and from the 16th to the 18th century, only eight (Indian) rhinoceros survived their translocation from India to be displayed in Europe (26). In an era dependent upon sail and horse power, the logistics of moving a rhinoceros were tremendous and time consuming and many rhinoceros died during their voyage (26).

The first rhinoceros arriving in Europe after Roman times was the Indian rhinoceros *Ganda* that reached the harbour of Lisbon on May 20th 1515 and was eternalised on a woodcut by the German artist Albrecht Dürer (25,26) (see preface). The first black rhinoceros is said to have arrived in Antwerp, Belgium, in 1858. During the 19th century, only a dozen black rhinoceros were imported from Africa to Europe and half a dozen to the United States. These dates are remarkably recent and likely reflect the difficulty of capturing and handling a black rhinoceros due to their large size, inaccessible habitat and wild temper (25,26). Since 1930, there has been a steady increase in black rhinoceros translocations from Africa (mainly *D. bicornis spp. michaeli* from Kenya and Tanzania; and *D. bicornis spp. minor* from Zimbabwe and South Africa) to various parts of the world (25,27).

The first pair of (northern) white rhinoceros arrived in Antwerp as late as in April 1950 (25). In the early nineteen-hundreds, due to uncontrolled hunting, the white rhinoceros was on the verge of extinction with only a few animals left in Zululand (KwaZulu-Natal), South Africa (15,25). The rapid grow of this population together with the development of chemical immobilisation techniques resulted in a series of conservation translocations of white rhinoceros back into their former range (28). In 1961, the Natal Parks Board started to translocate white rhinoceros from Umfolozi and Hluhluwe national parks to other national parks and game reserves in South Africa as well as to privately owned properties (25). Initially, rhinoceros had been caught by net or with ropes from a chase vehicle (28,29), which often resulted in mortality. The first attempts of chemical capture were made with the dissociative anaesthetic phencyclidine and the curariform muscle relaxant gallamine triethiodide, but were associated with a high mortality rate (29). The first chemical capture with opioids

included the use of Themalon (diethylthiambutene hydrochloride) and Sernyl (l-(l-PhenylcycIohexyl) piperidine monohydrochloride)(28).

In 1963, M99 (etorphine) was made available for rhinoceros capture (28,30), the potent opioid that is still being used today (31,32). Once these new chemical capture techniques had been implemented, the Natal Parks Board authorised export of white rhinoceros to zoological gardens all over the world and to former ranges within the African continent (25,28).

Over the last 50 years, these capture techniques have not changed substantially (28), although they have been refined. Today, rhinoceros are most commonly captured with a combination of etorphine and azaperone (24,31,32). White rhinoceros appear to be more sensitive to the effects of the potent opioid compared to black rhinoceros and exhibit muscle tremors, limb paddling, hypoxaemia, hypercapnia, and hypertension (32–34). Oxygen supplementation by nasal insufflation is therefore advised (35). Post-induction intravenous administration of butorphanol has been shown to improve arterial oxygenation by reducing oxygen consumption in association with muscle tremors (36). Butorphanol and diprenorphine are both opioid agonist-antagonists and are used to partially reverse etorphine-immobilisation enabling the rhinoceros to stand up and walk into the transport crate (24,31,37). Full antagonism of the etorphine is achieved by administering naltrexone (24,31). During transport, rhinoceros are most commonly tranquilised with azaperone (duration of effect 2 – 6 hours) (24,31). Long acting neuroleptics such as zuclopenthixol acetate (duration of effect 3 days) or perphenazine enanthate (only takes effect after 12 hours but lasts 7 to 10 days) are administered to calm the animals during long transports, temporary captivity, and release (31,32).

Despite these improvements of translocation techniques over the years, rhinoceros frequently traumatise themselves during transport or become sick and die after they have been released (29,38). Black rhinoceros commonly experience knocking off of horns, fracture of nasal bones, bruising and swelling of the lips, muscle damage and heat stress (29). White rhinoceros tend to develop post-capture anorexia, diarrhoea

and enterocolitis due to *Clostridium* and, or, *Salmonella* infections (38). While the morbidity rates are not exactly known, the current mortality rate for rhinoceros translocations in South Africa and Namibia is estimated to be 5% (38). Only last year (2018), eleven out of eleven black rhinoceros died following a translocation within Kenya (39) and four out of six black rhinoceros following translocation from South Africa to Chad (40). According to the inquiry team, the cause of all the deaths in the Kenya translocation was "multiple stress syndrome intensified by salt poisoning and complicated by the following conditions: dehydration, starvation, proliferation of opportunistic bacteria in upper respiratory tract (*Pasteurella* species), gastric ulcers and gastritis" (39).

These incidences show that there is still room for improvement of current rhinoceros translocation techniques. Understanding the physiological responses to capture and transport and their effect on animal welfare in rhinoceros is therefore important.

The effects of capture have been extensively studied in the rhinoceros over the past few years (33–35,41) and are summarised in chapter 1.2. The effects of transport have not yet been investigated in the white rhinoceros. Chapter 1.3 reviews the effects of transport on animal welfare in mammalian species, including the black rhinoceros.

1.2. THE PHYSIOLOGICAL EFFECTS OF CAPTURE IN THE RHINOCEROS

1.2.1. Fight or flight response

In Africa, wild rhinoceros are typically captured by remote darting from a helicopter (24). The environmental disturbance by the helicopter (stressor) causes a stress response in the rhinoceros (10). The stress response is one of nature's fundamental survival mechanisms (3). It is a cascade of events, mediated by an integrated network of neuroanatomical structures and peripheral organs, that produce the behavioural and physiological responses necessary to enable immediate survival needs (42). Although many factors are involved, the major mediators of the stress response are: (1) the catecholamines that are released by the adrenal medulla (and sympathetic nerves) following activation of the sympathetic nervous system (or withdrawal of the parasympathetic nervous system tone) (43), and (2) the glucocorticoids that are released following activation of the hypothalamic-pituitary-adrenal (HPA) axis (Fig. 1) (3,44). The catecholamines (e.g. noradrenaline, adrenaline) initiate an immediate fight-or-flight response increasing arousal, elevating heart rate (HR) and blood pressure, and mobilising energy sources to the central nervous system and somatic muscle (45). Like an antelope being chased by a lion, the rhinoceros will start running away from the helicopter.

The fight or flight response is then followed by the HPA axis response, which takes slightly longer to effect. Neural input to the hypothalamus induces secretion of the corticotrophin-releasing hormone, which in turn stimulates adrenocorticotropic hormone release from the anterior pituitary gland, which promotes release of glucocorticoids (e.g. cortisol) from the adrenal cortex (9). The major metabolic effect of the increased glucocorticoid secretion is to further mobilise energy (46), which allows the rhinoceros to continue running.

These high levels of muscular activity associated with excitement and pursuit result in an elevated oxygen consumption, lactate build-up and an increase in body temperature (44,47). Increasing lactate concentrations lead to increasing hydrogen ion

generation and a drop in blood pH with subsequent metabolic (lactic) acidosis (34). Some animals may develop capture myopathy, a syndrome that is characterised by depression, muscular stiffness, severe muscle pain, ataxia, paresis and paralysis, metabolic acidosis, and death (48). Capture myopathy affects skeletal and cardiac muscles in response to muscular exertion and stress and likely occurs in the majority of all captured mammals, with an unknown proportion of those being affected to the point of showing clinical signs (48).

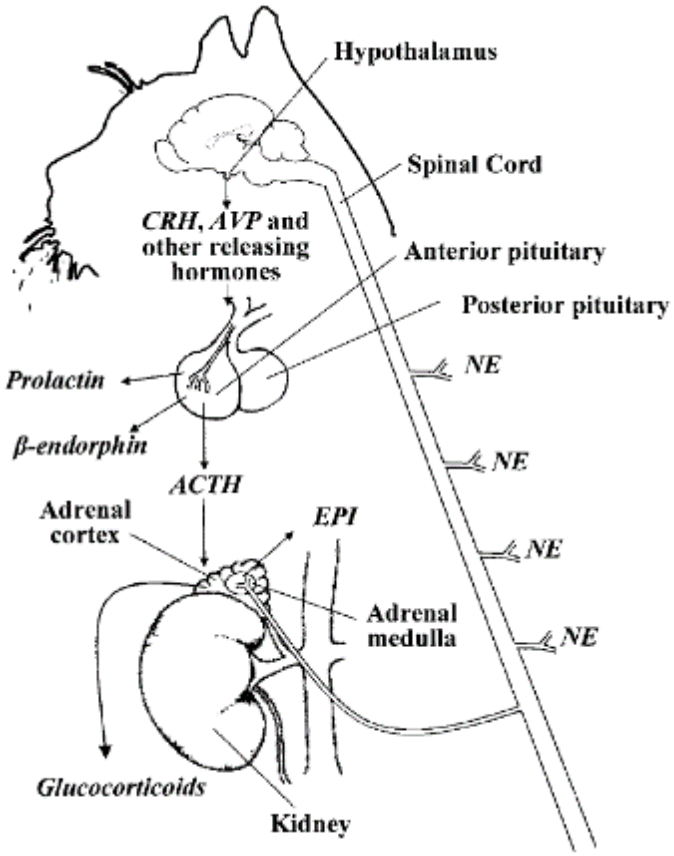

Figure 1: The hypothalamic–pituitary–adrenal axis and the sympathetic nervous system release hormones in response to a stressor (taken from Figure 1. Reeder DM, Kramer KM. Stress in free-ranging mammals: integrating physiology, ecology, and natural history. J Mammal. 2005; 86(2):225–35. License number 4692921357305).

Abbreviations: NE norepinephrine (noradrenaline), EPI epinephrine (adrenaline), CRH corticotrophin-releasing-hormone, AVP arginine vasopressin, ACTH adrenocorticotropic-releasing-hormone.

1.2.2. Etorphine chemical immobilisation

Over the past 60 years, etorphine has become the standard opioid for the chemical capture of black and white rhinoceros (29,31,32). Etorphine is a full μ, κ and δ opioid receptor agonist and is 5,000 – 10,000 times more potent as an analgesic than morphine (49). It is well-known to cause respiratory neuronal depression in the rhinoceros (50) leading to hypoxaemia, hypercapnia and acidaemia (33,34). These respiratory depressant effects are enhanced by an increase in upper airway resistance and a decrease in chest and abdominal wall compliance (31,50). Oxygenation and ventilation are closely related to, and affected by, acid-base status and changes in electrolyte concentrations. Changes in body temperature, respiratory pattern, and metabolic demands can alter this balance, resulting in neurological or myocardial dysfunction, multi-organ failure, or capture myopathy (44). It is therefore not surprising that mortalities have been associated with etorphine chemical immobilisation in rhinoceros (24,51,52).

Recent research aimed at improving rhinoceros immobilisation suggests that hypoxaemia and hypercapnia are associated with an increase in alveolar-arterial oxygen gradient and an elevation in oxygen consumption and carbon dioxide production (36). Rhinoceros immobilised with etorphine are in a hypermetabolic state associated with tremors, tachycardia, an increase in cardiac output, systemic arterial hypertension and pulmonary hypertension (36,53,54). These effects could be the consequence of an upregulated sympathetic nervous system activity directly caused by the etorphine, or indirectly mediated by the cardiopulmonary effects of the etorphine, or both (54).

Regardless of the causes of these effects, during translocation, rhinoceros likely endure profound physiological effects caused by capture, before transport starts. The duration of these effects and their importance during translocation remain to be investigated.

1.3. A REVIEW ON THE EFFECTS OF TRANSPORT ON ANIMAL WELFARE IN WILD MAMMALIAN SPECIES

This sub-chapter has been submitted as a research paper for publication and is currently under review by the Journal of Zoo and Wildlife Medicine:

Pohlin F, Hooijberg EH, Meyer LCR. A review on the effects of transport on animal welfare in wild mammalian species.

ABSTRACT

Wild mammals are translocated for a variety of reasons including population restoration, conservation introductions, commercial or recreational purposes, or due to religious beliefs. Transport represents a crucial part of the translocation process, exposing animals to stressors such as physical confinement, motion, vibrations, unfamiliar smells, noise, micro-climatic changes and temporary deprivation of food and water. Studies that determine the impact of these stressors on wild mammal welfare during transport are few and often show variable results that are difficult to interpret. Here, we discuss behavioural, physiological, hormonal, haematological, biochemical, and immunological responses that have been used to assess animal welfare of transported wild mammals and factors associated with these responses.

1.3.1. Introduction

For as long as people have been moving from one place to another, animals have been moved with them. The International Union for Conservation of Nature (IUCN) defines the human-mediated movement of living organisms from one area with release in another, as translocation (1). The earliest recorded translocation occurred about 20,000 years ago, when the northern common cuscus (*Phalange orientalism*) was taken to New Ireland as an important source of food (4,5,55). Nowadays, translocation

represents an effective tool for conservation and is increasingly used to reinforce declining populations or to re-establish extirpated animal populations (1,8). According to the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) Trade Database, more than 200,000 living individual mammals have been exported by CITES partners over the past five years (27). There are many motivations to translocate wild mammals and not all of them relate to the conservation of the species being moved (e.g. commercial, recreational, or religious motivations) (6). In fact, from a commercial point of view, the global demand for wildlife and wildlife products is estimated to be worth billions of US dollars each year (56,57).

Despite their wide use and importance, many translocations result in failure, for reasons such as not adequately adapting to, or breeding in, the release area, predation, or contracting illnesses that lead to death (9,10,58). Although the direct causes for these mortalities are often related to external factors, such as novel pathogens, the vulnerability to these factors is exacerbated by the adverse effects of translocation-induced stress (9).

Stress was first described by Hans Selye in 1936, who defined it as a non-specific response of the body to any demand for change (59). Later, this concept was refined by introducing the terms stressor and stress response. A stressor is a stimulus that threatens homeostasis (balance of bodily functions), whereas the stress response is the reaction of the organism to a stressor to regain homeostasis (60).

The two most commonly studied physiological systems that initiate a stress response are the autonomic nervous system (ANS) and the hypothalamic-pituitary-adrenal (HPA) axis (42). The response of the ANS to a stressor is almost instantaneous and results in an increased release of the catecholamine neurohormones noradrenaline (from the adrenal medulla and post ganglionic sympathetic adrenergic nerves) and adrenaline (from the adrenal medulla). Stimulation of the HPA axis results in a simultaneous, but generally slower and more sustained, release of glucocorticoid steroid hormones from the adrenal cortex (45,61). Behaviourally, the response to stress

may consist of a fight or flight reaction or, when the threat is ill-defined and no alternative behaviour to end the threat is clear, an increased state of vigilance that is enhanced by fear (62).

Transport is an integral part of the translocation process. From an animal's perspective, the process of being transported involves several stressors including isolation, confinement in a novel environment, motion, vibrations, foreign sounds and smells, changes in temperature and humidity, inadequate ventilation and temporary deprivation of food and water (63–65). Animals react to these challenges through several interactive mechanisms involving behavioural, physiological, hormonal, haematological, biochemical, and immunological responses (66). Numerous studies in laboratory- and domestic animals have measured these responses in order to assess the effect of transport on animal welfare (64,66–69). Animal welfare refers to the state of an animal, including its physiological and psychological state, and its ability to cope with its environment (2,66,70,71). It is a continuum that ranges from poor to excellent, and includes both the extent of failure to cope and the ease or difficulty in coping with a physiological or psychological stressor (72,73).

In wildlife species, the effects of transport on an animal's welfare have been poorly studied and mortality records are commonly the only records available (56). For example, eight out of 84 bontebok (*Damaliscus pygargus*) died during a two and a half hour translocation in South Africa in 1957 (74), and 35 out of 50 sea otters (*Enhydra lutris*) died during translocation from Alaska to Washington State, USA, between 1955-1958 (75). The mortality rate of red colobus monkeys (*Procolobus kirkii*) transported within Zanzibar was 13.3% in 1978 (76) and for African elephants (*Loxodonta africana*) transported within southern Africa in 2003, was up to 10% (77,78). Between 1989 and 2006, 8.5% of black rhinoceros (*Diceros bicornis*) died during translocations in southern Africa (24). The use of tranquilising drugs, to reduce capture stress, has led to a decrease in mortality rates over the years (28). However, animals still die during, or after, transport, which raises serious concerns for animal welfare (10,79,80). To reduce mortality rates and improve animal welfare during

transport, it is necessary to understand how stress and other physiological responses to transport in wild animals impact their welfare, and to develop translocation strategies to mitigate negative stress responses.

This review describes the results from recent research on wild animal transport, with special reference to the indices that have been measured to assess stress and animal welfare in transported mammalian species, and the factors associated with these responses.

1.3.2. Behavioural responses to transport

Fear is a major psychological stressor in animals during transport (65). Fear elicits HPA axis activity and can be measured by the degree of behavioural aversion to a fearful stimulus (2,81). Behavioural observations can be made without handling the animal and therefore represent a valuable tool for assessing wild animal welfare (65,66). Transported animals may, for example, remain motionless, move away, attempt to escape, jump, kick, vocalise, or lie down (65,69,81). Some species start exploring the compartment in which they are placed in to find a suitable place to sit or lie down, whereas others do not settle and may resist confinement within the transport container or fight with conspecifics (66). A prime example is the black rhinoceros, who often will traumatise themselves during transport and commonly experience fracture of the nasal bones, avulsed horns, and bruising or swelling of the lips (29). In zoo-housed tigers (*Panthera tigris*), pacing, a pinned back ear orientation, and an increased respiratory rate have been attributed to transport stress. Previous experience did affect the tigers' stress responses as naïve tigers performed these repertoires with a greater intensity than tigers that had been transported before (82). A similar habituating-effect of temporary confinement has been described in zoo-housed bongo (*Tragelaphus eurycerus*) (83), nyala (*Tragelaphus angasi*) (84) and white-, black-, Indian- and Sumatran rhinoceros (85,86). This habituating-effect highlights the importance of exposing zoo animals to transport-elements by crate training, and positive reinforcement prior to translocation. In wild mammals, habituation to

repeated transports has only been investigated in red deer (*Cervus elaphus*), which appeared to habituate poorly (87). Whilst animals in captivity become acclimated to people and a variety of different handling events, wild species do not have the opportunity to do so, and may therefore have a more pronounced fear response (65). Further studies exploring behavioural effects of the transport experience in captive and wild individuals of the same species are warranted to better compare habituating-effects.

1.3.3. Physiological responses to transport

Changes in body weight and heart rate (HR) are traditional measurements used to assess animal welfare (88). Farm animals subjected to transport commonly exhibit a decline in body weight due to dehydration and exertion (68,89). Similarly, chimpanzees (*Pan troglodytes*) (90) and wild-caught owl monkeys (*Aotus nancymai*) (91) transported for over 20 hours, experienced a loss of body weight, from before to after transport. In contrast, captive-reared guanacos that were transported for only two hours did not lose any weight (92). It is known that the degree of body weight loss during transport is strongly correlated with transport distance (69). In domestic animals, additional factors such as: (1) species (ruminant vs monogastric); (2) age, physiological state and temperament of the animal; and (3) thermal conditions, were found to also influence body weight and hydration status during transport (68). These factors need to be considered in present and future studies investigating the effect of transport length on wild animal body weight dynamics.

Changes in HR represent an indirect measure of ANS activity and are commonly used to assess short-term effects of a stress response. Because HR positively correlates with oxygen consumption, changes in HR are often used as indicator for metabolic rate and energy expenditure (93). As HR increases, so too does metabolic heat production leading to an elevation in body temperature, which can be detrimental and cause mortality (94). Hot and humid environmental conditions contribute to hyperthermia and are important factors to be taken into consideration when transporting wild

animals (1, 29). In wild ungulate species, HR and body temperature were found to be elevated during initial transport. Both variables progressively decreased with transport time indicating a habituating-effect and that exercise and the novelty associated with loading and journey commencement are particularly strong stressors (92,95–97). Red deer transported at a high density had higher HRs and temperatures than those transported at lower density (96). Loading density is a critical factor to take into consideration when assessing the potential risks to animal welfare during transport (69). Several wild mammal herd species, such as deer, impalas, and zebras, are commonly mass captured and transported collectively as a single entity (98). At high loading densities animals may increasingly suffer from heat stress and may not be able to lie down and rest during transport (68,69). Furthermore, separating family groups (e.g. zebra), or males and females (e.g. bighorn sheep), from each other, might represent another important factor in reducing transport stress (1).

These factors need to be explored further across many mammalian species.

Heart rate variability (HRV) is progressively emerging as a suitable indicator of stress- and welfare status in domestic and wild animals (99). Measurement of HRV investigates the functioning of the ANS, especially the balance between the sympathetic- and parasympathetic nervous system tone (99). Schmidt et al. (2010) non-invasively measured HRV in horses (*Equus caballus*) during road transport by using a mobile recording system (f810i, POLAR, Kempele, Finland) attached to the animals' thorax with an elastic girth. They identified a stress response to transport by measuring a decrease in HRV, along with an increase in HR and salivary cortisol concentrations (100). In wild mammals, it is difficult to investigate the effect of transport on HRV by using this method, as baseline measurements will be influenced by the animal's stress response to capture. However, the non-invasive measurement of HRV has been adapted and applied in southern chamois (*Rupicapra pyrenaica*) (97) and roe deer (*Capreolus capreolus*) (95) to investigate differences in the effects of anxiolytic drugs during transport. Although no significant differences were found between the treatment groups, HRV represents a valuable tool for future research in

transported wild animals. By using implantable cardiac monitors, some of the technical issues associated with the non-invasive measurement of HRV could be avoided – an option worth exploring (101,102).

1.3.4. Hormonal responses to transport

The most frequently examined hormones in mammals responding to transport-induced stress include, but are not limited to, the mediating hormones of the HPA axis (e.g. adrenocorticotropic hormone, glucocorticoids), catecholamine neurohormones (e.g. noradrenaline, adrenaline), thyroid hormones (e.g. total and free triiodothyronine (T3), thyroxine (T4), thyroid stimulating hormone (TSH)), and reproductive hormones (e.g. luteinising hormone (LH), progesterone, testosterone) (103).

Currently, in free ranging and captive wildlife research glucocorticoid steroid hormones, such as cortisol or corticosterone, are primarily used to measure a stress response (104–106). There are a number of sources from which glucocorticoids and their metabolites can be measured, such as faeces, urine, saliva, blood, and hair (104,105,107). Faecal glucocorticoid metabolites (FGM) for example, represent an integrated measure of a stress response and have commonly been investigated in wildlife-transport studies due to the non-invasiveness of the method (104–106,108). Increased FGM after- compared to before transport, were measured in African elephants (109,110), African and Indian rhinoceros (86,111,112), fallow deer (*Dama dama*) (113), Garnett's bushbabies (*Otolemur garnettii*) (114), Grevy's zebras (*Equus grevyi*) (115), Przewalski's horses (*Equus ferus przewalskii*) (116) and the woylie (*Bettongia penicillata*) (117). Additional stressors, such as capture and release into a novel environment may have influenced the magnitude and duration of FGM excretion in these studies (104–106). To account for these confounders, Dembiec et al. (2004) simulated a short transport in five zoo-housed tigers (82). All tigers voluntarily entered the transport crate within five minutes of having access to it and, after 30 minutes of driving, each tiger was released back into their familiar enclosure. In line

with the other studies, they found an increase in FGM three to six days after transport, demonstrating that transport on its own is a strong stressor (82).

More instantaneous measures of a stress response are blood (serum or plasma) catecholamine and glucocorticoid concentrations (104,105). Champagne et al. (2012) detected elevated plasma adrenaline concentrations in yearling northern elephant seals towards the end of an over-night physical restraint in a transport cage (118), and Martucci et al. (1992) documented higher catecholamine concentrations in net-gun captured and helicopter-transported desert bighorn sheep (*Ovis Canadensis*) compared to reference intervals from domestic animals (119) indicating a sympathetic nervous system response. Increased blood glucocorticoid concentrations after transport, compared to at capture, have been documented across a range of species including artic foxes (*Vulpes lagopus*) (120), dromedary camels (*Camelus dromedarius*) (121), guanacos (*lama guanicoe*) (92), northern elephant seals (*Mirounga angustirostris*) (118), red deer (96), reindeer (*Rangifer tarandus*) (122) and a variety of African mammals (123,124) indicating HPA axis activation. An activation of the HPA axis has been suggested to change the composition of gut microbiota, which in turn could persistently elevate serum glucocorticoid concentrations (125). Transport-induced changes in gut microbial composition and diversity may thus impact animal welfare and may be worth investigating in transported wild- and domestic animal species.

Similarly, the impact of an altered hypothalamus-pituitary-thyroid axis on animal welfare is not yet completely understood, but is currently being studied in different fields of research (103). Glucocorticoids may affect thyroid function, often by suppressing baseline serum T3 and T4 concentrations and reducing TSH secretion (126). However, in habituated dromedary camels and farm animals, transport has shown to cause an increase in thyroid hormones with considerable variability in preferential hormone-release across species (103,121). These increases have been attributed to higher metabolic requirements associated with a stress response to transport (103). The effect of transport on thyroid function remains to be investigated in wild mammals.

Thyroid function is known to have significant effects on reproductive hormone metabolism, fertility, and pregnancy patterns in domestic animal species (103). Wild animals that have been captured, transported, and released into a novel environment commonly experience a decreased reproductive capacity (9,10,127,128). Interestingly, an increase in serum testosterone concentrations has been measured immediately after transport in Arctic fox females that travelled for over eight hours (120), and a peak in faecal androgen and progesterone metabolites has been measured in African rhinoceros for 4-16 days after capture and transport, prior to decreasing below pre-capture levels (112). This short-term increase of reproductive hormones in response to a stressor has been suggested to play a role in reducing emotional stimulation and fear during situations the animal perceives as life-threatening (120,129). Changes in gonadotropin concentrations in response to transport may largely depend on the animal's phase of the oestrous cycle (130,131). In the domestic horse transported during the luteinising phase of the oestrous cycle, plasma LH concentrations increased initially, followed by a gradual decrease, whereas in horses transported during the luteal phase of the oestrous cycle, plasma LH concentrations remained low (131). Additionally, transport may affect birth-sex-ratio bias depending on the animal's stage of gestation (132). Even though the exact mechanisms remain to be investigated, translocation-induced birth-sex-ratio bias has been observed between early (86% male births) and mid (38% male births) gestation in female rhinoceros due to failure of female embryo development (132). Depending on the species, it may therefore be crucial to consider the phase of the oestrous cycle, or gestation stage, when investigating reproductive hormonal responses to transport in females.

1.3.5. Haematological and biochemical responses to transport

In domestic animals, the effect of transport on animal welfare has often been evaluated by measuring blood haematological and biochemical alterations (66,69). During a stress response, in dogs and likely other mammals, circulating catecholamine neurohormones will stimulate alpha-1-adrenergic-receptors of the splenic capsule and

parenchyma causing splenic contraction and release of erythrocytes into the peripheral circulation (133). Chemically and physically immobilised mammals may have increased packed cell volume (PCV), red blood cell count (RBC), and haemoglobin concentration (HGB) in comparison to reference intervals owing to this effect (134–138). It is important to note, that comparing once-off measurements of haematological changes, associated with transport, to population-based reference intervals is generally of limited value, and trends of chosen variables over time should be monitored within one individual (139). Monitoring these trends has been done in wild ungulate species and PCV, RBC and HGB were found to gradually decrease from capture throughout transport, supporting the assumption that capture might be associated with catecholamine-induced splenic contraction (95,97,124,140,141). Animals that have been sedated with acepromazine experienced lower PCV, RBC and HGB during capture and transport compared to control animals, likely due to the drug's alpha-1-adrenergic-blocking effect inducing relaxation of the spleen and sequestration of erythrocytes (95,97).

In domestic animals, total serum protein (TSP), albumin, and PCV are known to increase with the duration of transport if animals suffer from water deprivation (64,89,142–144). Higher TSP and albumin concentrations after- compared to before transport have been demonstrated in chimpanzees indicating a loss of total body water (90). Changes in the water content of the body fluid compartments are best reflected by changes in serum sodium concentrations (145). Increases in serum sodium concentrations after transport have been documented in several wild mammal species, and most likely indicate a relative water loss in the extracellular fluid compartment (87,90,95).

Other common findings in transported wild animals are increases in the muscle enzymes creatine kinase (CK), aspartate transaminase (AST), and lactate dehydrogenase, indicating intense exercise and muscle cell damage (90,95,97,122,124,141). Transport can take a physical toll on animals and trauma is not uncommon. During the journey, animals often have limited space to move and may

have to maintain postural balance. This lack of movement and increased muscle tone can potentially lead to poor muscular tissue perfusion, increased muscle cell permeability, and the release of muscle enzymes into the blood stream (68). Additionally, increased sympathetic nerve activity contributes to restricted muscle blood flow through peripheral vasoconstriction (146).

In order to generate energy, lactate production typically increases when metabolic demand exceeds aerobic metabolism capacity as seen with intense exercise (147). Accordingly, elevated serum lactate concentrations have been recorded in transported wild mammals (87,95,97,121). Rehbinder (1990) reported degenerative lesions in both skeletal and myocardial muscles in reindeer transported over 71 km to a slaughterhouse (148). Another study in transported reindeer found that intramuscular lactate concentrations increased concurrently with the depletion of glycogen and triglycerides, reflecting anaerobic metabolism and the utilisation of carbohydrates and fatty acids to fuel the muscle's energy demands (149). The breakdown of glycogen in response to increased glucocorticoid and catecholamine concentrations leads to an elevation in plasma glucose and has been documented in transported black rhinoceros (124), chimpanzees (90) and dromedary camels (121). Interestingly, plasma glucose concentrations remained stable in transported guanacos (92) and red deer (87), whereas they decreased in transported wild small ruminants (95,97,141). Hypoglycaemia has been associated with prolonged transport, fasting, and exhaustion in domestic animals (68). When glucose reserves are depleted, fatty acids are mobilised from the adipose tissue and the gluconeogenic and ketogenic pathways are activated. Mobilisation of lipid stores results in elevated plasma fatty acid concentrations, which has been reported in transported dromedary camels (121). As the vast majority of plasma fatty acids are bound to albumin, ketone bodies, such as β-hydroxybutyrate (BHB), are generated. Thus, elevated BHB concentrations after transport have been associated with a negative energy balance in domestic animals (150–152). Saeb et al. (2009) investigated changes in serum BHB concentrations in dromedary camels transported over five hours. Even though serum non-esterified

fatty acid (NEFA) concentrations were increased after transport, serum triglyceride, cholesterol, and BHB concentrations did not change significantly. However, transport increased serum urea nitrogen concentrations in these camels indicating fluid shifts and protein catabolism in response to the deprivation of water and food (121).

Similar changes in serum electrolyte and metabolite concentrations have been reported in transported domestic animals and were found to correlate with journey duration (69,89,144,153). Although animal transport of long duration has the potential to negatively compromise animal welfare compared to a shorter journey, it is important to emphasise that it is not just transport duration, but the associated negative aspects, such as fear, extreme ambient conditions, and the lack of food, water and rest, which also have a major impact on animal welfare (69,144). It is thus crucial to investigate the influence of these factors on wild animal welfare during transport and explore possible measures to mitigate the effects of these factors. López-Olvera et al. (2006) and Montané et al. (2002) examined the effects of the tranquilising drug acepromazine on haematological and serum biochemical measures in transported southern chamois and roe deer. All animals were captured by means of mesh drive-nets and received either intramuscular acepromazine (treated group) or saline (control group) before being transported for 1.5 (southern chamois) or 9 (roe deer) hours. Blood samples were taken at capture (southern chamois) and before- and after transport (southern chamois and roe deer). In both species, the administration of acepromazine resulted in reduced serum biochemical alterations indicating the drug's capability of mitigating adverse effects of transport on animal welfare (95,97). Similarly, northern elephant seals that received anaesthetic agents (tiletamine, zolazapam, ketamine, and diazepam) during capture and transport did not exhibit a cortisol stress response in contrast to animals that were only physically restrained (118).

These studies demonstrate that animal welfare during transport can be improved by the administration of tranquilising or sedative drugs. Future research can focus on comparing the ability of different anxiolytic drugs, solitary or in combination, to most

effectively mitigate the adverse effects of transport-induced stress in a given species. However, even simple measures such as the consistent provision of water and food, or fluid and metabolic supplements, scheduled rest intervals during transport, and avoiding extreme ambient conditions, should vastly improve wild animal welfare during transport and need to be explored in more detail in controlled studies.

1.3.6. Immunological responses to transport

Measurement of leukocyte profiles is becoming more popular with wildlife researchers, particularly because they are directly related to changes in glucocorticoid concentrations (154,155). Transport was found to increase white blood cell count and neutrophil numbers and decrease lymphocyte numbers in several wild mammal species (90,95,97,134,141,148). These changes reflect glucocorticoid-induced transmigration of lymphocytes from the circulation into tissues, and the influx of neutrophils from the bone marrow into the blood (154). Shift of neutrophils from the marginating to the circulating blood pool further contributes to the glucocorticoid-induced neutrophilia (156). As neutrophils and lymphocytes counts are inversely affected, the relative proportion of neutrophils to lymphocytes (N:L ratio) is frequently used as measure of a stress response (92,155,157–159). Similar to studies conducted in domestic animals, significant increases in N:L ratio were measured in transported European badgers (*Meles meles*) (159), captive-reared guanacos (92), and cynomolgus monkeys (*Macaca fascicularis*) (157). In response to external stimuli leukocytes are activated and release inflammatory mediators (e.g. cytokines) that potentially damage healthy tissue (160,161). Montes et al. (2004) described an increase in circulating activated neutrophils in transported European badgers (159), whereas Schapiro et al (2012) found a decreased natural killer cell activity along with increased interferon γ cytokine levels in transported chimpanzees (90). In healthy individuals, leukocytes (particularly neutrophils) release reactive oxygen species (ROS) that destroy bacteria and other pathogens (162). The so-called Leukocyte Coping Capacity (LCC) measures ROS production in real time and is substantially decreased in stressed

individuals (163–165). Whether LCC can be used as an indicator of animal welfare during transport remains to be investigated.

As a potential application to assess animal welfare, the measurement of an acute phase response has recently been proposed (166). The acute phase response represents a complex systemic reaction of the innate immune system to non-specific stimuli characterised by systemic, metabolic and physiological alterations including the release of acute phase proteins and the generation of ROS (166,167). Acute phase proteins are glycoproteins synthesised mainly by hepatocytes upon stimulation by pro-inflammatory cytokines and released into the bloodstream (168). Haptoglobin, serum amyloid A, and fibrinogen were found to increase dramatically in transported water buffalo calves (*Bubalus bubalis*) when compared with control calves (169), but did not change significantly in dromedary camels during and after transport (170). However, both of these species had an increase in plasma malondialdehyde (MDA) concentrations, indicating lipid peroxidation and the generation of ROS (169,170). Cellular defence against oxidative damage from these substances is through an effective and complex antioxidant system (171). In dromedary camels, the activity of the antioxidant catalase gradually increased with transport distance indicating defence against the oxidative damage (172). In contrast, in water buffalo calves, the antioxidants nitric oxide, superoxide dismutase and glutathione significantly decreased during transport (169). This decrease in plasma antioxidants along with the increase of plasma oxidants (such as MDA) is indicative of oxidative stress which could lead to tissue damage and increased susceptibility to pathogens (171,173).

Stress-induced immunosuppression and oxidative stress have been identified as major factors for the development of disease after transport (174–176). Therefore, transported individuals may exhibit recrudescence of latent and normally innocuous pathogens, increased shedding of pathogens, and an increased vulnerability to new pathogens (174,175). For instance, naïve black rhinoceros translocated to tsetse-infested areas were found to develop life-threatening trypanosomiasis despite their ability to ecologically thrive in areas with tsetse (177). This outcome is concerning

because transport may exacerbate the impact of disease on species vulnerable to extinction and even trigger the spread of emerging infectious diseases (174). It is therefore crucial to start assessing the risk of disease development after transport in wild animals. In domestic animals, simple interventions that mitigate oxidative stress, such as the administration of the ascorbic acid or vitamin E, has demonstrated decreased incidence of certain diseases (171,176). These interventions and their effects on transport-triggered diseases need to be investigated in wild animals.

1.3.7. Future directions

Transport of wild mammals is associated with stress- and other physiological responses that adversely impact animal welfare. Where animals are under human care, such as during transport, it is the human's responsibility to monitor the animals' welfare and mitigate welfare-impacts if unacceptable (56,79,178). In order to provide a logical and comprehensive framework for good animal welfare, the Farm Animal Welfare Council of the United Kingdom established the concept of the five freedoms, defining ideal states for an animal's physical and mental condition (179). To a certain degree, most of these aspects appear to be solvable for many wild animal species subjected to transport: (1) freedom from hunger and thirst- by providing access to food and water or supplements; (2) freedom from discomfort- by providing transport containers appropriate for the species, number of animals and ambient conditions; (3) freedom from pain, injury, or disease- by careful loading and driving, and rapid diagnosis and treatment; (4) freedom to express normal behaviour- by allowing the animal to rest during long journeys; and (5) freedom from fear and distress- by reducing stressors and administering stress-reducing drugs (73,179). Based on the International Air Transport Association (IATA) Live Animals Regulations, CITES has developed practical guidelines for the non-air transport of live wild animals and plants, addressing these points (180,181). The guidelines for example, emphasise the importance of providing water and food at appropriate intervals during transport, and appropriate bedding to guarantee animal comfort. If an animal injures itself

during transport, it should receive appropriate veterinary treatment or, if necessary, undergo emergency euthanasia. Depending on the species and length of transport, animals should be transported together and offered rest periods at appropriate intervals. The use of sedatives is only recommended when necessary to ensure positive animal welfare (181). However, these guidelines are mainly adapted from domestic animal transport regulations and based on experience rather than scientific assessment. So, what is "appropriate" for wild species? More species-specific research on transport-factors, such as the deprivation of food and water, ambient conditions, container design, bedding substrate, loading density, travel time, frequency and duration of rest intervals, and their interaction, is needed. Many wildlife practitioners administer ancillary drugs, such as anti-inflammatories, or metabolic supplements, to animals undergoing transport. In some instances (e.g. dehydration), some of these drugs may have detrimental physiological effects to the animals and further investigations of their effect on animal welfare during transport are warranted. With respect to the different tranquilising or sedative drugs used for their anxiolytic properties, their effects on the long-term consequences of capture and transport need to be investigated, especially after animals are released. In fact, the effectiveness of any intervention used to improve welfare should not only be measured during transport, but also assessed post-release and as an indicator of translocation success. Science-based data will be critical for strengthening current guidelines and developing species-specific interventions and recommendations for optimal transport.

1.3.8. Conclusion

The understanding of how transport affects animal welfare in wild animals is still embryonic and needs to be investigated further. Wild animal welfare during transport can be assessed by measuring a range of behavioural, physiological, hormonal, haematological, biochemical, and immunological changes. We recommend collecting information on a diverse set of measures and monitoring the trends of chosen

variables over time, comparing serial samples with each other. Although, in this review, we focused on the effects of transport on animal welfare in mammalian species, other taxa, such as birds, reptiles, and fish should receive equal, if not greater attention as they are often traded and transported in large numbers, and may be more conservation status sensitive. Factors influencing animal welfare during transport have been identified in domestic animals and are likely to be of greater relevance in the transport of wild species. Fisher et al. (2009) describe three main challenges to animal welfare during transport: (1) stress and fear due to capture, loading and novelty of transport; (2) hydration, energy, and fatigue challenges that increase with transport duration; (3) risks to the thermal comfort and physical integrity of the animals (68). It is crucial to critically explore these, and other, challenges in transported wild animals and investigate the efficacy of interventions that aim to mitigate these challenges. Reducing the impact of these challenges on the physiology and behaviour of transported animals will improve wild animal welfare during transport and reduce the incidence of transport-related problems, which in turn will decrease the likelihood of translocation failure.

The identification of challenges to animal welfare associated with capture and transport in black and white rhinoceros represent a major aim of this thesis and are explored in chapter 3 to 5.

1.4. SEDATION TO IMPROVE RHINOCEROS WELFARE DURING CAPTURE AND TRANSPORT

1.4.1. The current use of azaperone in rhinoceros capture and transport

Beyond its physiological effects, transport likely represents a psychological stressor to the animal and is associated with fear (182). Fear has been identified as a main factor compromising animal welfare (179). A number of studies on a diverse range of species have demonstrated the benefits of using drugs that reduce fear and anxiety during transport (10). In the rhinoceros, the use of these drugs has been shown to reduce the risk of injury, aggression and excitement and facilitate introductions (29,183). The choice of drug is based on the desired duration of action and expected outcome (183). Azaperone is the most commonly used (short-acting) tranquiliser for rhinoceros transport (29,31,183). This butyrophenone provides sedation of 4 to 6 hours with an onset of effect within 15 to 20 minutes after intramuscular injection (31). Total doses of 80 – 200 mg per adult rhinoceros are usually administered (32). The behavioural calming effects of the azaperone are mediated primarily by blockade of dopamine receptors in the basal ganglia and limbic system of the brain (184). At therapeutic doses, butyrophenones inhibit conditioned avoidance behaviour and decrease spontaneous motor activity. At higher doses, or when administered intravenously, extrapyramidal symptoms, such as “dog-sitting”, “star-gazing”, ataxia or paradoxical excitement can occur (184,185). Butyrophenones also bind to autonomic (adrenergic and muscarinic), serotonin and histamine receptors (184). Blockade of alpha-1-receptors in peripheral arterioles causes peripheral vasodilation and reduces mean arterial blood pressure (186). Therefore, because rhinoceros immobilised with etorphine experience severe tachycardia and hypertension (33,54), azaperone is typically combined with the etorphine for the capture of rhinoceros (31) to reduce etorphine-induced hypertension (41). However, rhinoceros still develop muscular rigidity, tremors, respiratory impairment and respiratory and lactic acidosis with this combination (34,51,187).

1.4.2. How midazolam could improve rhinoceros capture and transport

Midazolam is a benzodiazepine that enhances the effects of the neurotransmitter gamma-aminobutyric acid (GABA) at the $GABA_A$ receptor in the central nervous system, primarily at the level of the limbic system and the reticular formation (188,189). The resulting increase in chloride conductance and hyperpolarisation of postsynaptic cell membranes produces the powerful skeletal muscle relaxant, anticonvulsant, anxiolytic, amnestic, hypnotic and sedative effects of the drug (188). Recently, co-administration of midazolam with etorphine has been advocated for rhinoceros capture (32,183,190). Improved muscle relaxation by this drug might reduce muscular rigidity and thus, oxygen consumption, anaerobic metabolism and lactic acidosis (190). In captive and wild rhinoceros, midazolam (5 - 50 µg/kg) has occasionally been used to calm the animals during transport (32,183). This benzodiazepine was found to provide sedation of 4 to 6 hours to the rhinoceros with an onset of effect within 5 to 10 minutes after intramuscular injection (183).

An advantage of administering midazolam instead of azaperone for the capture and transport of rhinoceros could be that benzodiazepines are relatively free from undesirable cardiovascular side-effects and can be antagonised (e.g. using flumazenil) (188). Midazolam might have also greater fear-reducing and anxiolytic effects than azaperone. Benzodiazepines have been shown to decrease stress-induced hyperthermia in rodents (191), which is a common test to determine the anxiolytic properties of a drug (192). Midazolam reversed the behavioural deficits of rats following prolonged stress (193) and had a dose-dependent cytoprotective effect on corticosterone-induced astrocyte damage (194). Moreover, benzodiazepines reversed stress-induced immunosuppression in adult male Wistar rats and Swiss albino mice (195).

Recent research suggests that benzodiazepines also bind to peripheral benzodiazepine receptors (PBR), or translocator proteins (18 kDa), which are widely expressed throughout the body (196). Peripheral benzodiazepine receptor densities

are particularly high in steroidogenic tissues, such as the adrenal gland (196,197). Midazolam might therefore be able to directly modulate the stress response.

The benefits of using midazolam, instead of azaperone, for rhinoceros capture and transport remain to be investigated and are explored in chapter 4 and 5 of this thesis.

CHAPTER 2: RESEARCH OBJECTIVES

1. To establish a more comprehensive understanding of the physiological responses to capture and transport in black and white rhinoceros. [a-c]
2. To identify challenges to animal welfare associated with capture and transport in black and white rhinoceros. [a-c]
3. To evaluate physiological responses to capture and long road transport in black and white rhinoceros in a "real-world" setting. [a]
4. To investigate biochemical, including electrolyte and acid-base, responses to capture and transport in black and white rhinoceros. [a,b]
5. To determine whether electrolyte and acid-base responses can be modified by using midazolam instead of azaperone during the capture and transport of white rhinoceros. [b]
6. To examine the stress responses to capture and transport in black- and white rhinoceros. [a,c]
7. To investigate haematological responses to capture and transport in white rhinoceros. [c]
8. To determine whether haematological responses can be modified by using midazolam instead of azaperone during the capture and transport of white rhinoceros. [c]
9. To investigate immunological responses to capture and transport in black and white rhinoceros. [a,c]
10. To determine whether immunological responses can be modified by using midazolam instead of azaperone during the capture and transport of white rhinoceros. [c]

[a] Addressed in Chapter 3

[b] Addressed in Chapter 4

[c] Addressed in Chapter 5

CHAPTER 3: CHALLENGES TO ANIMAL WELFARE ASSOCIATED WITH CAPTURE AND LONG ROAD TRANSPORT IN BOMA-ADAPTED BLACK (*DICEROS BICORNIS MINOR*) AND SEMI-CAPTIVE WHITE (*CERATOTHERIUM SIMUM SIMUM*) RHINOCEROS

This chapter has been published as a research paper with the Journal of Wildlife Diseases ("online first"):

Pohlin F, Hofmeyr M, Hooijberg EH, Blackhurst D, Reuben M, Cooper D, Meyer LCR. Challenges to animal welfare associated with capture and long road transport in boma-adapted black (*Diceros bicornis minor*) and semi-captive white (*Ceratotherium simum simum*) rhinoceros. J Wildlife Dis 2020; 56(2): 000-000 (in press).
Doi: 10.7589/2019-02-045

This research paper has been adjusted to this thesis format.
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ABSTRACT

Translocation is an important conservation tool used to reinforce declining populations or to restore extirpated populations. Capture and transport are part of translocation and expose animals to a variety of stressors that ultimately can lead to morbidity and even mortality. We aimed to establish a better understanding of the physiological responses to capture and transport in black (*Diceros bicornis*) and white (*Ceratotherium simum*) rhinoceros in southern Africa. Fourteen adult black rhinoceros were transported by vehicle 600 km, and 32 white rhinoceros, 24 adults and 8 juveniles, were also road-transported 1300 km. The black rhinoceros had been wild-caught and boma-adapted over 6 weeks prior to the translocation and were only sedated to allow for loading into the transport crates. The white rhinoceros originated from a game-farm and were chemically immobilised from a helicopter and then loaded. Paired blood samples were collected from animals at loading (capture) and after transport and evaluated for changes in: (1) clinical chemistry analytes; (2) acute phase reactants and (3) oxidative stress biomarkers. The Wilcoxon rank sum test was used to compare changes in measured analytes from capture and after transport.

All rhinoceros survived capture and transport. Similar to transported domestic animals, rhinoceros experienced total body water loss, mobilisation of energy reserves, and muscular damage. Alterations in acute phase reactants suggest that animals mounted a stress response. Oxidative stress, by means of an increase in plasma oxidants, was observed in black rhinoceros. Based on these results, we identified the following challenges to animal welfare during transport: (1) hydration status, (2) energy balance, (3) skeletal muscle fatigue, and (4) stress-induced immunomodulation. Measures that aim to mitigate these challenges, such as administering fluids during long journeys, need to be included in the planning of future translocations and systematically investigated for their effectiveness.

3.1. INTRODUCTION

The southern-central black rhinoceros (*Diceros bicornis minor*) is listed as critically endangered, and the southern white rhinoceros (*Ceratotherium simum simum*) as near threatened, by the International Union for Conservation of Nature (IUCN) Red List of Threatened Species (13,15). The main reasons for these assessments are the continued and increased poaching threat and increasing illegal demand for rhinoceros horn associated with the increased involvement of organised international criminal syndicates in rhinoceros poaching (198). Translocation for population reintroduction or reinforcement represents an essential tool for the management of these species and is an integral part of national and international rhinoceros conservation plans (21,23). Translocation involves capture, temporary captivity, transport, and release into a novel environment, exposing the animals to a variety of stressors, such as prolonged periods of water and food deprivation that can lead to morbidity or mortality (9,10). Rhinoceros frequently traumatise themselves during transport (29) or become sick and die after they have been released (24,38,39). The current mortality rate for rhinoceros translocations in South Africa and Namibia is estimated to be 5% (38). The prevalence of morbidity is likely underestimated (38).

The morbidity and mortality associated with rhinoceros translocation raises serious concerns for animal welfare (10,79,80). The physiological responses to translocation must be understood to reduce adverse events.

Handling and transport of farm animals influences the activity of certain enzymes and hormones, mobilisation of energy and protein metabolism, concentration of acute phase reactants (APRs), and the balance between oxidants and antioxidants (67,173,199,200). However, these effects have been poorly studied in rhinoceros. Increases in creatine kinase (CK) and aspartate transaminase (AST), changes in serum electrolyte concentrations, and a marked rise in blood cortisol and glucose concentrations occur during capture and transport of black rhinoceros (124), but similar investigations have not been carried out in white rhinoceros. Furthermore, the

effects of capture and transport on APRs and oxidative balance have not been evaluated in either black or white rhinoceros. APRs are a group of positive and negative immunomodulatory proteins and other analytes whose serum concentrations increase or decrease in response to stressors (166,168). Concurrent stress-induced alterations in plasma oxidants and antioxidants might result in an imbalance in favour of oxidants which may induce oxidative stress, causing cellular damage and increased susceptibility to disease associated with translocation (140,171,201).

Our aim was to better understand the physiological responses to capture and transport by evaluating clinical chemistry analytes, APRs, and oxidative stress biomarkers during actual translocation operations of black and white rhinoceros, and to identify challenges associated with transport that should be addressed in order to improve animal welfare.

3.2. MATERIALS AND METHODS

Fourteen boma-adapted black rhinoceros were transported just over 600 km from KwaZulu-Natal to the north of South Africa and 32 white rhinoceros, originating from a game-farm, were transported just over 1300 km from the Free State in South Africa to Botswana. Trucks and International Air Transport Association (IATA) approved rhinoceros crates were used in both translocations, which followed the practical guidelines for transport of live wild animals (181) and rhinoceros (24,29). The translocations were planned and took place independently of this study and the University of Pretoria Animal Ethics Committee (V067-17) approved opportunistic sample collection from these animals.

3.2.1. Rhinoceros capture

Black rhinoceros. All black rhinoceros were adults (seven females and seven males) captured from the wild and confined, for adaptation purposes, in bomas for 6 weeks prior to translocation. Temporary confinement facilitated veterinary examinations,

disease screening and quarantine, and allowed for a quicker capture process on the day of transport.

The translocation took place overnight in October 2017 in order to avoid extreme ambient temperatures. The rhinoceros were sedated, via darting, within the boma with a combination of 0.6 – 0.8 mg etorphine (Captivon®, 9.8 mg/ml, Wildlife Pharmaceuticals, Karino, South Africa) and 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. Using a low sedative-dose of etorphine is a common capture method in boma-adapted rhinoceros, which allowed for conscious loading of the animals without the need for full immobilisation. Once in the crate, a blood sample was collected from an auricular vein. To partially reverse some of the sedative effects of etorphine, 1.2 mg diprenorphine (Activon®, 12 mg/ml, Wildlife Pharmaceuticals) and 10 mg butorphanol (Butorphanol tartrate, 50 mg/ml, Wildlife Pharmaceuticals) were administered intravenously. Additionally, 1 g carprofen (Rimadyl®, 50 mg/ml, Zoetis, South Africa, Sandton), a non-steroidal anti-inflammatory drug, was given intramuscularly to all animals.

White rhinoceros. The white rhinoceros comprised 24 adults (18 females, six males) and eight juveniles (five females, three males) and originated from a 340 ha private game farm where they received additional water and supplementary food prior to the translocation. Animals were captured in the early morning, in September 2017 by darting from a helicopter with a combination of etorphine (3 – 5 mg/adult or 0.1 – 1.5 mg/juvenile), azaperone (20 – 40 mg/adult or 0 – 10 mg/juvenile) and 5000 IU hyaluronidase (adult only; Hyalase®, Kyron Laboratories, South Africa, Johannesburg) delivered remotely using 2.0 ml darts (Pneu-dart, Inc®., Williamsport, USA, Pennsylvania) with 63.5 mm barbed needles.

The animals became recumbent within 5 minutes of darting and a blood sample was collected immediately from an auricular vein. If an animal tremored severely (n = 10),

butorphanol, at 2 – 5 times the etorphine dose in mg, was administered intravenously to mitigate the hypoxaemia associated with the muscle tremors (53). Within 10 minutes of darting, diprenorphine (0.2 – 0.8 mg/adult or 0 – 0.1 mg/juvenile; M5050®, 12 mg/ml, Novartis, South Africa, Midrand) was administered intravenously to partially reverse the etorphine's immobilising effects, which facilitated loading. Each rhinoceros, including the juveniles, was loaded into its own transport crate where adult animals received another 2.5 – 15 mg of intravenous diprenorphine to complete the etorphine's reversal. Additionally, either 5 g vitamin C (n = 4; ascorbic acid, 500 mg/5ml, Fresenius Kabi, South Africa, Bloemfontein) or 500 mg vitamin E and 50 mg selenium (n = 4; vitamin E acetate 17 mg/ml, sodium selenite 1.67 mg/ml, Kyron Laboratories) were given intramuscularly to some adult rhinoceros to support the animals' antioxidant defences (171).

3.2.2. Rhinoceros transport

The tranquiliser zuclopenthixol acetate (Clopixol-Acuphase®, 50 mg/ml, H. Lundbeck Pty. Ltd., South Africa, Randburg) was administered intramuscularly via hand-injection (150 – 220 mg/black rhinoceros or 100–250/adult white rhinoceros or 10 – 50 mg/juvenile white rhinoceros) just after loading into the crates. Transport commenced once all rhinoceros had been captured and loaded, which took 3.25 ± 0.75, mean ± standard deviation (SD), hours in both translocations.

During transport, the vehicles stopped at 2 – 4 hour intervals to allow for additional intramuscular administration of azaperone and midazolam (Dazonil®, 50 mg/ml, Wildlife Pharmaceuticals) to restless animals. Eight black rhinoceros received at least one top-up dose of 40 – 100 mg azaperone, of which two animals required additional one to three top-up doses of azaperone and midazolam (15 – 30 mg). All white rhinoceros received at least one top-up dose of azaperone (80 – 120 mg/adult or 10 – 80 mg/juvenile), which was combined with 2.5 – 15 mg midazolam in the juveniles. Sixteen adult- and six juvenile white rhinoceros required up to three additional top-up doses of azaperone, alone, or in combination with midazolam (10 –20 mg/adult)

during transport. Water was not provided to the animals, as past experience has shown that rhinoceros don't drink during transport and affixed water containers are known to cause injury (181). At the heat of the day, however, white rhinoceros were doused with water during stops and small amounts of alfalfa hay were offered to some of the animals.

At the release site, all adult rhinoceros were re-immobilised with etorphine (3.5 – 4 mg/black rhinoceros or 3.5 – 6 mg/white rhinoceros) and azaperone (40 mg/black rhinoceros or 20 – 40 mg/white rhinoceros) via pole syringe or hand-injection into the nuchal hump while standing in the transport-crate. Juvenile white rhinoceros received 0.5 – 2.5 mg etorphine and 5 mg midazolam intramuscularly via pole-syringe. Before animals became immobile they were released from their crates and manually restrained with ropes until they became recumbent. Subsequently, blood samples were collected from the immobilised animals from cephalic or auricular veins. Naltrexone (Trexonil®, 50 mg/ml, Wildlife Pharmaceuticals), at 20 times the etorphine dose in mg, was administered intravenously to reverse the immobilisation and release the rhinoceros into the private game reserve (black rhinoceros) or national park (white rhinoceros).

3.2.3. Blood sample analysis

Blood from rhinoceros was collected directly into serum and potassium oxalate/ sodium fluoride tubes (BD Vacutainer; Becton and Dickinson, Plymouth, UK), stored in a cooler box with ice packs, and centrifuged within 24 hours. Serum and plasma were aliquoted and stored at -80 °C until analysis. Samples from the white rhinoceros were stored at -20 °C for 1 month prior to being stored at -80 °C.

Samples were analysed in the clinical pathology laboratory of the Onderstepoort Veterinary Academic Hospital (Pretoria, South Africa) and the University of Cape Town Division of Chemical Pathology laboratory (Cape Town, South Africa).

Clinical chemistry analytes. Serum clinical chemistry analysis was done using a Cobas Integra 400 Plus automated biochemistry analyser (Roche Diagnostics Ltd., Rotkreuz,

Switzerland) using commercially available kits. Measured analytes included: total serum protein (TSP), albumin, globulin, sodium, chloride, urea, creatinine, potassium, magnesium, phosphorus, total calcium, total bilirubin, cholesterol, triglycerides, alkaline phosphatase (ALP), γ- glutamyl transferase (GGT), glutamate dehydrogenase (GLDH), AST, CK. Plasma glucose and lactate concentrations were measured by spectrophotometric methods with the same analyser. Serum beta hydroxybutyrate (BHB) and non-esterified fatty acid (NEFA) concentrations were measured by kinetic enzymatic and colorimetric methods, respectively, using BHB and NEFA kits (Randox Laboratories, Crumlin, Antrim, UK). Serum cortisol concentration was assessed by a chemiluminescent immunoassay using the Immulite/Immulite 1000 Cortisol® following manufacturer's instructions (Siemens Healthcare, Erlangen, Germany).

Acute phase reactants. Serum haptoglobin was determined by the haemoglobin-binding method using a commercial kit (PHASE Haptoglobin Assay, Tridelta Development Limited, Kildare, Ireland) with the Cobas Integra 400 Plus analyser. Concentrations of serum amyloid A (SAA) were determined by a solid-phase sandwich enzyme-linked immunoassay using a commercial kit (PHASE SAA Assay, Tridelta Development Limited), previously validated for use in black and white rhinoceros (202,203). Serum iron was measured with the Cobas Integra 400 Plus biochemistry analyser.

Oxidative stress biomarkers. The lipid peroxidation products, conjugated dienes (CD) and thiobarbituric acid reactive substances (TBARS), were measured by spectrophotometric methods (204). The antioxidant capacity of plasma was assessed by the oxygen radical absorbance capacity (ORAC) method (205,206). These methods are described in more detail in chapter 5.

3.2.4. Statistical analysis

Statistical tests were performed using R 3.3.1 for Windows (The R Foundation, Vienna, Austria) (243). Descriptive tables and scatter plots were generated. Mean ± SD were calculated for each analyte and presented for descriptive purposes. Due to the small

sample size, nonparametric analyses were used to compare concentrations of measured analytes between capture and release by using the Wilcoxon rank sum test with data divided by species and age. A p value < 0.05 was considered significant.

3.3. RESULTS

All rhinoceros survived capture and transport. The mean ± SD overall time animals spent in the transport crates was 19.7 ± 2.3 (range 16.3 to 23.0) hours for black rhinoceros and 34.3 ± 3.2 (range 30.5 to 40.3) hours for white rhinoceros. Environmental temperatures ranged from 7.9 °C and 13.7 °C during the night to 28.2 °C and 40.3 °C during the day in the black and white rhinoceros translocations, respectively.

Clinical chemistry analytes. Capture and transport influenced many clinical chemistry analyte concentrations. Means ± SD for measured analytes at capture and after transport and the corresponding p values for the Wilcoxon rank sum test are shown in Table 1 for the different species and age groups. Briefly, albumin (Fig. 2a) concentrations increased in black rhinoceros (p = 0.004). TSP and sodium (Fig. 2b) concentrations increased from capture to after transport in black (p = 0.004 and p < 0.001, respectively) and adult white rhinoceros (p = 0.043 and p < 0.001, respectively). Chloride concentrations increased and potassium concentrations decreased in all animals (p = 0.020 and p < 0.001 in black, p < 0.001 and p = 0.011 in adult white, p = 0.010 and p = 0.007 in juvenile white rhinoceros, respectively). Magnesium and phosphorus concentrations decreased in adult rhinoceros (p = 0.037 and p < 0.001 in black, p < 0.001 and p < 0.001 in white rhinoceros, respectively). Total calcium concentrations increased in black (p = 0.022), but decreased in juvenile white rhinoceros (p < 0.001). Only in white rhinoceros did creatinine (Fig. 2c) and urea concentrations increase (p < 0.001 and p < 0.001 in adults, p = 0.027 and p = 0.002 in juveniles, respectively). Total bilirubin concentrations rose in all animals (p = 0.014 in black, p < 0.001 in adult white, p = 0.001 in juvenile white rhinoceros). Non-esterified fatty acids (Fig. 3a) and BHB concentrations (Fig. 3c) increased in adult- (p < 0.001 and p = 0.044, respectively) and

juvenile (p = 0.001 and 0.004, respectively) white rhinoceros. Cholesterol declined in black (p = 0.044) and adult white rhinoceros (p = 0.011), whereas triglyceride concentrations (Fig. 3b) increased in black (p = 0.001), but decreased in adult white rhinoceros (p = 0.022). Capture and transport did not appear to change the liver enzymes ALP and GGT ($p > 0.05$); however, GLDH activities were lower following transport in white rhinoceros (p = 0.016). Serum CK (Fig. 4a) and AST (Fig. 4b) were markedly elevated after transport compared to capture in all adult ($p < 0.001$, both analytes) and juvenile ($p < 0.010$ and p = 0.003, respectively) rhinoceros. Lactate (Fig. 4c) concentrations were higher after transport in black ($p < 0.001$), but lower in white ($p < 0.001$) rhinoceros. No changes in cortisol (Fig. 5a) and glucose concentrations were detected in any rhinoceros group ($p > 0.05$).

Acute phase reactants. Mean ± SD of APR concentrations at capture and after transport and the corresponding p-values for the Wilcoxon rank sum test are shown in Table 2 for the different species and age groups. There were no changes in haptoglobin concentrations from capture to after transport in the groups ($p > 0.05$). Serum amyloid A (Fig. 5b) concentrations in white rhinoceros capture samples were below the detection range (< 7.0 mg/l), but increased to detectable concentrations in adult white rhinoceros after transport (p = 0.013) and were also higher in black rhinoceros after transport (p = 0.002). Iron (Fig. 5c) decreased from capture to after transport in all animals (p = 0.006 in black, p = 0.006 in adult white, p = 0.005 in juvenile white rhinoceros).

Oxidative stress biomarkers. Mean ± SD of oxidative stress biomarker concentrations at capture and after transport and the corresponding p-values for the Wilcoxon rank sum test are shown in Table 2 for the different species- and age groups. Namely, CD concentrations were higher after transport compared to capture in black rhinoceros only (p = 0.001). No significant changes in TBARS and ORAC were detected ($p < 0.05$).

Table 1. Mean ± SD concentrations or activities of blood clinical chemistry analytes of black- (*Diceros bicornis minor*) and adult and juvenile white rhinoceros (*Ceratotherium simum simum*) at capture and after transport. A significant difference (shown in bold) between capture and after-transport samples represented the effects of transportation.

Clinical chemistry analyte	Units	Mean ± SD concentrations								
		Black rhinoceros (*n* = 14)			Adult white rhinoceros (*n* = 24)			Juvenile white rhinoceros (*n* = 8)		
		At capture	After transport	*p*	At capture	After transport	*p*	At capture	After transport	*p*
Albumin	g/L	32 (1)	34 (2)	**0.004**	31 (3)	32 (2)	0.085	31 (2)	32 (2)	0.798
Alkaline phosphatase)	U/L	82 (41)	92 (28)	0.239	82 (29)	81 (26)	0.959	175 (34)	141 (22)	0.093
Aspartate transaminase	U/L	78 (32)	206 (130)	**<0.001**	62 (13)	313 (313)	**<0.001**	64 (13)	375 (384)	**0.002**
Beta hydroxybutyrate	mmol/L	0.20 (0.04)	0.23 (0.10)	0.942	0.24 (0.07)	0.30 (0.11)	**0.044**	0.16 (0.06)	0.39 (0.15)	**0.004**
Chloride	mmol/L	94 (2)	96 (3)	**0.020**	89 (2)	95 (2)	**<0.001**	90 (1)	94 (3)	**0.010**
Cholesterol	mmol/L	1.34 (0.23)	1.09 (0.35)	**0.044**	2.13 (1.42)	1.83 (0.57)	**0.011**	4.54 (1.59)	3.98 (1.80)	0.442
Cortisol	nmol/L	49 (25)	44 (16)	0.627	76 (25)	78 (26)	0.853	52 (16)	49 (33)	0.798
Creatine kinase	U/L	499 (300)	7919 (7514)	**<0.001**	199 (71)	9097 (6554)	**<0.001**	3234 (64)	6062 (5453)	**<0.001**
Creatinine	μmol/L	93 (15)	89 (14)	0.382	141 (21)	177 (45)	**<0.001**	127 (19)	153 (20)	**0.027**
Globulin	g/L	52 (5)	55 (4)	0.159	55 (4)	56 (4)	0.212	43 (7)	43 (7)	1.000
Glucose	mmol/L	4.97 (0.69)	5.49 (0.49)	0.080	7.56 (1.39)	8.15 (1.06)	0.490	7.68 (1.26)	7.58 (0.77)	0.959
Glutamate dehydrogenase	U/L	3 (1)	3 (1)	0.225	4 (2)	3 (1)	**0.016**	3 (2)	2 (1)	0.082
γ glutamyl transferase	U/L	14 (3)	17 (4)	0.055	15 (5)	14 (3)	0.773	10 (4)	9 (3)	0.599
Lactate	mmol/L	2.44 (1.77)	7.91 (3.23)	**<0.001**	17.03 (6.58)	6.77 (2.74)	**<0.001**	19.79 (7.58)	2.95 (0.81)	**<0.001**

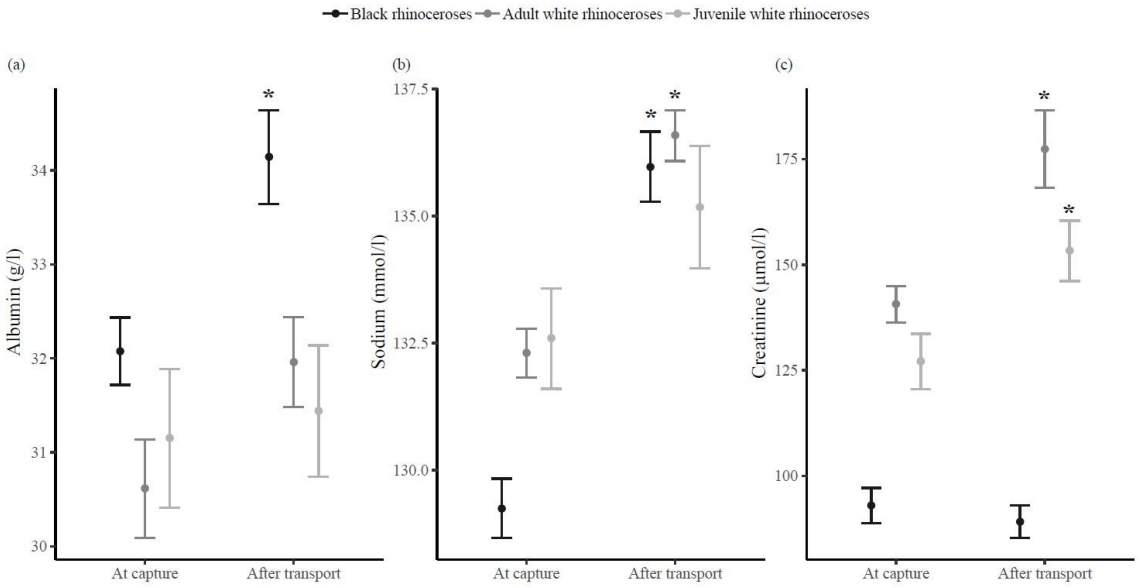
Table 1 continued.

Clinical chemistry analyte	Units	Mean ± SD concentrations								
		Black rhinoceros (*n* = 14)			Adult white rhinoceros (*n* = 24)			Juvenile white rhinoceros (*n* = 8)		
		At capture	After transport	*p*	At capture	After transport	*p*	At capture	After transport	*p*
Magnesium	mmol/L	0.89 (0.12)	0.8 (0.08)	**0.037**	1.12 (0.11)	0.80 (0.07)	**<0.001**	1.14 (0.14)	1.02 (0.17)	0.092
Non-esterified fatty acids	mmol/L	0.39 (0.31)	0.47 (0.28)	0.481	0.10 (0.10)	0.42 (0.23)	**<0.001**	0.06 (0.03)	0.75 (0.43)	**0.001**
Potassium	mmol/L	6.8 (1.4)	5.4 (0.4)	**<0.001**	4.9 (0.6)	4.6 (1.4)	**0.011**	4.72 (0.55)	3.81 (0.61)	**0.007**
Phosphorus	mmol/L	1.56 (0.26)	1.03 (0.30)	**<0.001**	1.48 (0.23)	0.67 (0.37)	**<0.001**	2.02 (0.24)	1.87 (0.38)	0.234
Sodium	mmol/L	129 (2)	136 (3)	**<0.001**	132 (2)	137 (2)	**<0.001**	133 (3)	135 (3)	0.195
Total calcium	mmol/L	2.99 (0.08)	3.07 (0.10)	**0.022**	2.95 (0.18)	2.85 (0.22)	0.061	3.13 (0.13)	2.81 (0.11)	**<0.001**
Total bilirubin	µmol/L	3.2 (1.2)	4.7 (1.8)	**0.014**	2.0 (0.4)	4.5 (1.8)	**<0.001**	2.1 (0.5)	6.7 (2.6)	**0.001**
Total serum protein	g/L	84 (5)	90 (4)	**0.004**	85 (5)	88 (5)	**0.043**	74 (6)	75 (5)	0.959
Triglycerides	mmol/L	0.29 (0.12)	0.60 (0.33)	**0.001**	0.51 (0.14)	0.40 (0.13)	**0.022**	0.60 (0.12)	1.16 (0.68)	0.066
Urea	mmol/L	5.0 (0.8)	5.7 (0.8)	0.058	3.6 (0.6)	4.9 (1.2)	**<0.001**	2.5 (0.9)	4.6 (1.2)	**0.002**

Table 2. Mean ± SD concentrations or activities of acute phase reactant and oxidative stress biomarkers of black- (*Diceros bicornis minor*) and adult and juvenile white rhinoceros (*Ceratotherium simum simum*) at capture and after transport. Significant differences (shown in bold) between capture and after-transport samples represented the effects of transportation.

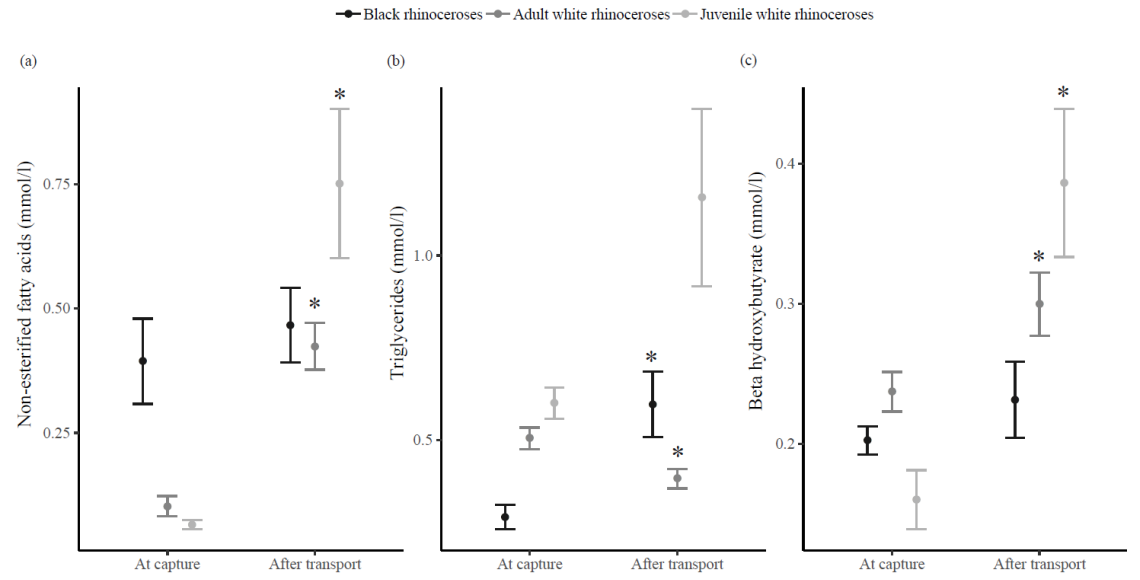
	Units	Mean ± SD concentrations								
		Black rhinoceros (*n* = 14)			Adult white rhinoceros (*n* = 24)			Juvenile white rhinoceros (*n* = 8)		
		At capture	after transport	*p*-value	At capture	After transport	*p*-value	At capture	After transport	*p*-value
Acute phase reactant										
Haptoglobin	g/L	4.0 (1.0)	3.5 (1.9)	0.209	2.2 (0.8)	2.3 (1.0)	0.676	1.6 (0.6)	1.3 (1.0)	0.336
Serum amyloid A	mg/L	24 (53)	104 (82)	**0.002**	< 7 (0)	10 (7)	**0.013**	< 7 (0)	14 (17)	0.310
Iron	µmol/L	25.8 (4.3)	19.5 (6.7)	**0.006**	23.6 (4.2)	19.7 (5.3)	**0.006**	26.3 (3.3)	16.4 (5.4)	**0.005**
Oxidative stress biomarker [1]										
ORAC	µmol/L trolox equivalents	764 (272)	974 (389)	0.183	781 (255)	660 (194)	0.113	1136 (493)	919 (432)	0.505
CD	µmol/L	41.5 (8.0)	53.7 (7.5)	**0.001**	56.0 (10.1)	53.4 (8.6)	0.726	58.0 (10.2)	53.4 (10.4)	0.958
TBARS	µmol/L MDA equivalents	0.39 (0.15)	0.29 (0.07)	0.135	0.32 (0.14)	0.34 (0.18)	0.570	0.24 (0.08)	0.26 (0.22)	0.527

[1] Oxidative stress biomarker: oxygen radical absorbance capacity (ORAC), conjugated dienes (CD) and thiobarbituric acid reactive substances (TBARS)



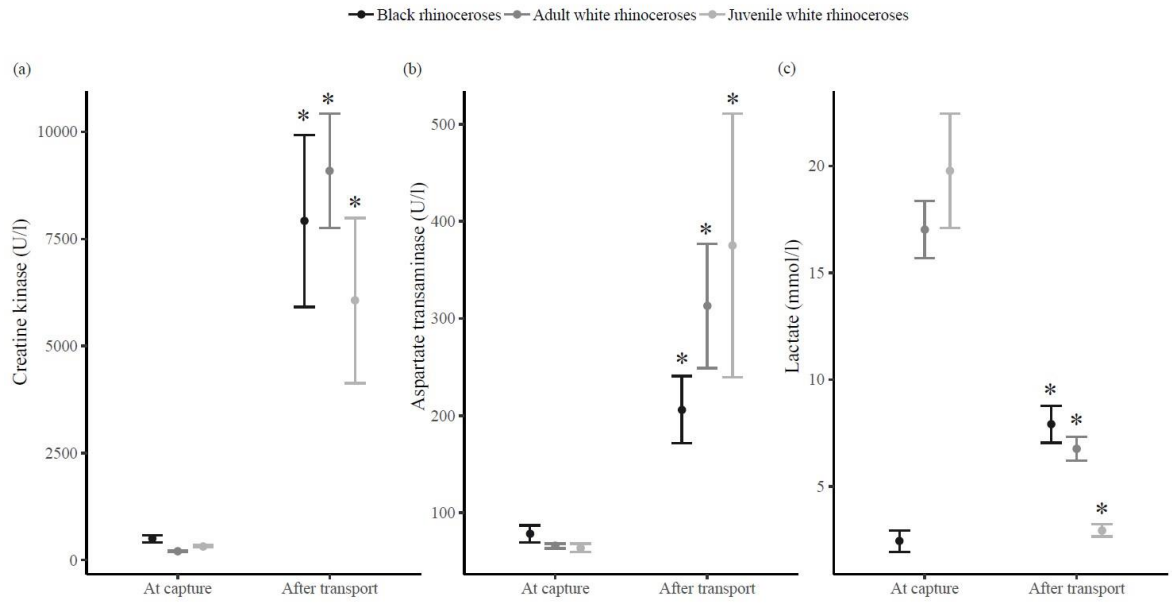
* Significantly different ($p < 0.05$) from "At capture" to "After transport"

Figure 2: Mean ± SD concentrations of (a) albumin, (b) sodium, and (c) creatinine in black and adult and juvenile white rhinoceros at capture and after transport indicating challenges in hydration status (see 3.4.1.).



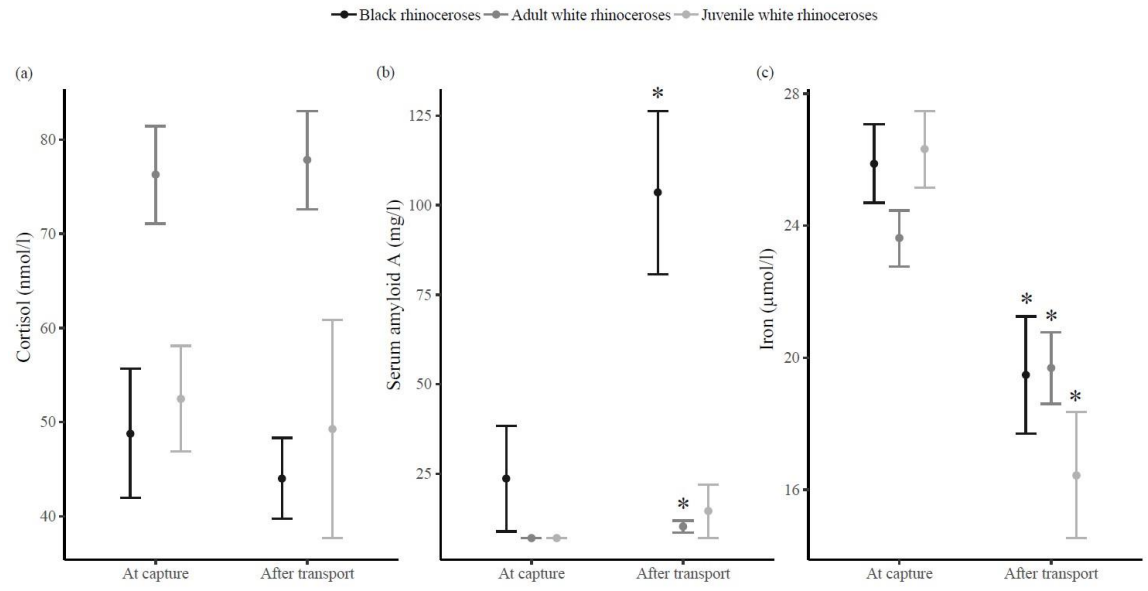
* Significantly different ($p < 0.05$) from "At capture" to "After transport"

Figure 3: Mean ± SD concentrations of (a) non-esterified fatty acids, (b) triglycerides and (c) beta hydroxybutyrate in black and adult and juvenile white rhinoceros at capture and after transport indicating challenges in energy balance (see 3.4.2.).



* Significantly different ($p < 0.05$) from "At capture" to "After transport"

Figure 4: Mean ± SD concentrations of (a) creatine kinase, (b) aspartate transaminase and (c) lactate in black and adult and juvenile white rhinoceros at capture and after transport indicating skeletal muscle fatigue (see 3.4.3.).



* Significantly different ($p < 0.05$) from "At capture" to "After transport"

Figure 5: Mean ± SD concentrations of (a) cortisol, (b) serum amyloid A and (c) iron in black and adult and juvenile white rhinoceros at capture and after transport indicating stress-induced immunomodulation (see 3.4.4.).

3.4. DISCUSSION

Capture and transport of black and white rhinoceros induced changes in serum electrolyte, enzyme and metabolite concentrations, APRs, and, in the black rhinoceros, plasma oxidants. Based on these changes, we identified the following challenges to animal welfare during transport: (1) hydration status, (2) energy balance, (3) skeletal muscle fatigue, and (4) stress-induced immunomodulation. Most clinical chemistry analytes remained within published reference intervals for black and white rhinoceros measured at capture (207–209). However, because these reference intervals were established using different laboratory methods, and the value in comparing once-off measurements to population-based reference intervals can be limited (139), the changes of variables within individuals was used to identify these challenges.

3.4.1. Hydration status

The increase in TSP and albumin concentrations from capture to after transport point to a decrease in plasma volume, most likely because rhinoceros did not drink (210). The parallel increase in the concentrations of sodium and chloride also indicates a relative body water loss and dehydration. Despite this increase, both analytes remained within normal limits for the species (207) suggesting that rhinoceros are fairly tolerant to the effects of water deprivation. Depending on the season and location, rhinoceros sometimes only drink every second day (29,211).

However, the white rhinoceros responded to the water deprivation with an additional increase in serum urea and creatinine concentrations, indicating a decreased renal glomerular filtration rate (212). Similar changes in serum electrolytes, urea and creatinine concentrations have been correlated with journey-duration and ambient temperatures in transported domestic animals (69,89). The white rhinoceros were transported over a longer time-period than the black rhinoceros and were exposed to higher ambient temperatures, therefore likely experienced a greater degree of dehydration. Even though the animals appeared to clinically cope with the prolonged time of water deprivation, total body water loss could pose as an additional stressor.

Rhinoceros, especially those caught from the wild, seldom drink water during transport (M Hofmeyr, pers. comm.), therefore future actions should include research on effective methods of fluid administration and planning and scheduling appropriate administration of fluids during long transports.

3.4.2. Energy balance

Transportation induced an increase in total bilirubin concentrations and decrease in serum potassium concentrations in all animals. Magnesium and phosphorus concentrations decreased in the adults, and calcium concentrations in the juveniles. These changes are known to occur after a period of fasting (210,213) and a lack of dietary intake of these electrolytes (213,214). Food deprivation leads to protein catabolism and mobilisation of lipid stores from the adipose tissue for energy (215). Mobilisation of lipid stores results in elevated plasma NEFA concentrations, which was observed in the white rhinoceros in our study and reported in transported domestic animals (121,216,217). Typically, only a part of NEFA is utilised as energy source and the remainder is converted into triglycerides or metabolised to ketone bodies such as BHB (215). Accordingly, we found an increase in triglyceride concentrations in transported black rhinoceros and an increase in BHB concentrations in transported white rhinoceros indicating a negative energy balance. Cholesterol concentrations, however, decreased in adult rhinoceros possibly in response to a release of inflammatory cytokines and acute phase reaction (218). The observed negative energy balance during the long transports was likely a stressor and should be mitigated. Whether feeding can correct this imbalance, especially in animals unaccustomed to eating unnatural food, or if metabolic supplements or parenteral feeding could be used successfully requires further investigation. This investigation should focus on nutritional planning (i.e. type and amount of food) and consider the gastrointestinal side-effects of the tranquilising drugs.

3.4.3. Skeletal muscle fatigue

Animals likely become tired if they remain standing during a long journey. Plasma indicators of muscle exertion (AST and CK) increased from capture to after transport in all rhinoceros. Additionally, calcium concentrations increased in the black rhinoceros, most likely due to increased muscular activity (219). Elevated muscle enzymes have occurred in a variety of transported animals (95,124,141,220). When an animal is immobilised by physical or chemical restraint, or standing in a transport crate, its muscles are in an increased state of contraction. Consequent compression of vessels may lead to poor muscular perfusion resulting in tissue hypoxia and ultimately muscle cell damage (48). Repetitive intramuscular administration of the tranquilising drugs throughout transport may have also caused additional muscle cell injury and release of CK into the blood stream (221). Serum lactate concentrations increased from capture to after transport in the black rhinoceros but decreased in the white rhinoceros. Lactate is produced in skeletal muscle and other tissues as a direct result of increased metabolic rate and glycolytic carbon flow. Following intense exercise, muscular hypoperfusion, or hypoxia, lactate production will increase, creating energy anaerobically and allowing for the continuation of exercise (147). Normal resting lactate concentrations in rhinoceros are not available in the literature; however, mean values for ground-immobilised white rhinoceros (4.6 mmol/l (222)) indicate that the lactate concentrations in our helicopter-captured white rhinoceros were profoundly elevated. This finding is not unexpected, as the helicopter-captured rhinoceros likely experienced higher muscular activity prior to immobilisation compared to the ground-immobilised rhinoceros in Cole et al.'s (2017) study (222), or to our boma-confined black rhinoceros. Additionally, the white rhinoceros in our study were fully immobilised and likely endured greater hypoxia (33,34) than did the black rhinoceros, which were only sedated.

These results suggest that differences in capture techniques had substantial implications for animal welfare during transport. Temporary confinement of

rhinoceros in bomas allowed for a smoother capture before transportation, without the need for full immobilisation, thereby mitigating hyperlactataemia and other capture associated pathophysiology, at the beginning of the transport. However, temporary captivity itself may adversely affect animal welfare (38) and its value, as a component of the translocation process, remains to be investigated in rhinoceros.

3.4.4. Stress-induced immunomodulation

Elevations in plasma cortisol and glucose concentrations have been documented in transported domestic (64,68,89) and wild animal species (92,121), including black rhinoceros (124), indicating a stress-response to transport. Interestingly, we did not find any significant changes in serum cortisol and glucose concentrations in our rhinoceros. Previous studies in farm animals have shown that blood cortisol concentrations peak within the first three hours of transport and then return to baseline concentrations within nine hours (220,223). If the timing of cortisol release is similar in rhinoceros, we may have missed sampling at peak plasma concentrations. Alternatively, it could be that cortisol concentrations were elevated at both sample points or that, due to the sedative drugs, rhinoceros were not stressed. In order to identify a stress response during long transports, future studies need to collect serial blood samples at shorter time intervals or include other analytes that indicate a stress response and whose concentrations change more slowly. Both of these suggestions have been implemented in chapter 5 of this thesis.

There appears to be a link between stress responses and an increase in APR concentrations and oxidative stress biomarkers during transport (169,224,225). By inducing pro-inflammatory cytokines in immunity-related cells, the activation of the HPA axis promotes the initiation of an acute phase response (226). In white rhinoceros, haptoglobin and SAA are positive APRs, which increase during an acute phase response, and albumin and iron are negative APRs, which decrease during an acute phase response (203). Serum amyloid A has also been shown to be a positive acute phase protein in black and white rhinoceros (202). We observed an increase in

SAA concentrations and a decrease in iron concentrations from capture to after transport indicating immunomodulation in response to stress. An acute phase response is often accompanied by alterations in plasma oxidants and antioxidants that may lead to an excessive production of reactive oxygen species (ROS), resulting in oxidative stress (166). Oxidative stress has been implicated in numerous disease processes so that oxidative parameters have been proposed as biomarkers to identify animals at risk of disease (171). Of all the oxidative stress biomarkers that we measured, transport elevated the concentration of CD only in the black rhinoceros, indicating an increased production of free radicals in these animals (67). Unlike the white rhinoceros, we gave the black rhinoceros a non-steroidal anti-inflammatory drug, which might have contributed to the oxidative damage (227). However, the administration of the potent antioxidants vitamin C or E to some of the white rhinoceros could have prevented an increase in lipid peroxidation products (171); therefore, the role of oxidative stress in translocation requires further research.

Our primary aim was to determine the physiological responses to capture and transport of African rhinoceros. Because the translocations took place for conservation purposes, independently of this study, it was not possible to standardise interventions or change how the animals were managed. Therefore, a number of confounding variables, like the varying use of sedatives and other drugs, immobilisation techniques, or the different times spent in the transport crate could have influenced the results in some animals. Interspecies and interage group differences were only noted within and between time points, but were not statistically compared as this was beyond the scope of this study. However, these comparisons should be directly investigated in the future. Nevertheless, we believe this study highlighted some important effects of capture and transport, which likely influenced the welfare of translocated rhinoceros in a real-world setting. These effects likely resulted from challenges such as the lack of water, food and rest, and stress-induced immunomodulation, which could be minimised by implementing simple measures

such as providing water and food or administering fluids or metabolic supplements. However, some measures, such as feeding or providing water, might be difficult to implement, or might cause side-effects such as colic, and therefore need to be systematically investigated. Practical guidelines developed for the non-air transport of live wild animals (181) and for rhinoceros (24,29) mention measures that are currently undertaken to guarantee animal welfare during transport. These measures mainly focus on management considerations around boma housing and transport, and transport crate design. For rhinoceros, currently there are no recommended limits for transport-duration, or water and food deprivation times, importantly these should be established in future studies.

3.5. CONCLUSION

This multidisciplinary assessment indicates that capture and transport of long duration compromises animal welfare of black and white rhinoceros. It is important to emphasise that it is not just the stress induced by transport itself, but the associated challenges, such as the lack of water, food and rest, and stress-induced immunomodulation, which have major implications on animal welfare. Interventions that aim to mitigate these challenges need to be included in the planning of translocation operations and systematically investigated for their effectiveness.

CHAPTER 4: ELECTROLYTE AND ACID-BASE RESPONSES TO CAPTURE AND TRANSPORT IN WILD SOUTHERN WHITE RHINOCEROS BULLS (*CERATOTHERIUM SIMUM SIMUM*) SEDATED WITH EITHER AZAPERONE OR MIDAZOLAM

This chapter has been submitted as a research paper for publication and is currently under review by the Journal of Veterinary Anaesthesia and Analgesia:

Pohlin F, Buss P, Hooijberg EH, Meyer LCR. Midazolam alters acid-base status less than azaperone during the capture and transport of wild southern white rhinoceros (*Ceratotherium simum simum*).

ABSTRACT

The aim of this study was to describe acid-base changes in wild white rhinoceros bulls during capture and transport using the Henderson-Hasselbalch and the Stewart's approach; and to compare these changes between rhinoceros sedated with midazolam (group M) to azaperone (group A).

Twenty-three sub-adult white rhinoceros bulls were captured with a combination of etorphine (3 – 4 mg, intramuscularly) plus either azaperone (n = 11 group A) or midazolam (n =12 group M), at five times the etorphine dose, mg. Azaperone or midazolam, respectively, were re-administered intramuscularly at 25 times the etorphine dose, in mg, at capture (TC), start of transport (T0), and at two (T2) four (T4) and six (T6) hours of transport. Venous blood samples were also collected at these times. Changes in measured and calculated acid-base variables were compared over time and between groups using a general linear mixed model.

At capture, blood pH was 7.109 ± 0.099 and 7.196 ± 0.111 for group A and group M, respectively. Blood pH increased from TC to T0 (7.441 ± 0.035 and 7.430 ± 0.057, p = 0.001), but did not change significantly from T0 to T6. Rhinoceros from group M had significantly higher pH, base excess and strong ion gap, and lower anion gap and lactate at TC than rhinoceros from group A.

Results indicate that rhinoceros experienced respiratory acidosis combined with a lactic- and non-volatile weak acid acidosis during capture, followed by a mild metabolic- and strong ion alkalosis during transport. Rhinoceros captured with etorphine-midazolam suffered less from lactic acidosis than rhinoceros captured with etorphine-azaperone. Therefore, midazolam may be a safer alternative to azaperone when combined with etorphine for the capture of wild white rhinoceros.

4.1. INTRODUCTION

Capture and transport are essential procedures used in the management and conservation of the southern white rhinoceros (23). Capture, through immobilisation with etorphine-based drug combinations, is known to cause severe respiratory and lactic acidosis (34,187,228). However, the consequences of these acid-base changes and the acid-base status of rhinoceros during transport are unknown. Acidaemia, in particular, is characterised by an increase in hydrogen ions and can have life-threatening consequences such as reduced enzyme function, electrolyte disturbances, depression of cardiac muscle contractility or altered oxygen delivery to the tissues (229). Therefore, in order to reduce the risks associated with these procedures, it is important to identify changes in acid-base status and determine the underlying mechanisms causing these changes.

Two different methods are commonly used to describe alterations in acid-base status: (1) the traditional Henderson-Hasselbalch bicarbonate based approach and (2) Stewart's quantitative strong ion difference based approach (230). To date, in white rhinoceros, only the traditional approach has been applied (187,228,231,232). The traditional Henderson-Hasselbalch approach is based on the assumption that the ratio of carbonic acid and bicarbonate determine hydrogen ion concentration and thus pH (233,234). The limitation of this approach is that accuracy can only be assumed if plasma protein and electrolyte concentrations are within normal limits (235). Wild white rhinoceros captured with etorphine experience severe physiological disturbances resulting from the effects of the potent opioid combined with a fight or flight response (36,53,54). Therefore, protein or electrolyte concentrations are unlikely to be within normal limits during the capture of these animals. The Stewart's quantitative strong ion difference-based approach includes these variables and explains how alterations in plasma protein and phosphate concentrations, as well as changes in the concentration of strong ions, such as sodium and chloride, affect pH

(235). Thus, this quantitative approach may be better suited to comprehensively assess acid-base disturbances during capture and transport.

In order to reduce some of the physiological disturbances resulting from the effects of the etorphine (i.e. hypertension), this potent opioid is routinely combined with the butyrophenone azaperone (31,32). However, rhinoceros still develop muscular rigidity, tremors, respiratory impairment and respiratory and lactic acidosis with this combination (34,51,187). Co-administration of midazolam, instead of azaperone, with the etorphine has been advocated to mitigate some of these disturbances in rhinoceros (190). Midazolam is a benzodiazepine that enhances the effects of the neurotransmitter GABA at the GABAA receptor resulting in skeletal muscle relaxation, anxiolysis and sedation (236). Combining the etorphine with midazolam could therefore reduce muscular rigidity and the magnitude of a fight or flight response, thereby reducing oxygen consumption, anaerobic metabolism and possibly lactic acidosis (190). Moreover, relaxation of thoracic muscles could reduce respiratory impairment and thus respiratory acidosis.

The aim of this study was to 1) investigate changes in acid-base status during the capture and transport of white rhinoceros bulls using the traditional Henderson-Hasselbalch and the Stewart's quantitative strong ion difference based approach, and 2) to determine whether these changes can be reduced by using midazolam instead of azaperone for the capture and transport of wild white rhinoceros. We hypothesised that 1) blood pH would be lowest during the capture and changes in protein and electrolyte concentrations would play an important role in the acid-base status during capture and transport; and 2) that midazolam use results in less changes in the rhinoceros' acid-base status than azaperone.

4.2. MATERIALS AND METHODS

Twenty-three sub-adult wild white rhinoceros bulls were transported 280 km within the KNP (24859944.5099S, 31835911.1799E; altitude 317 m), South Africa, for management purposes unrelated to the study. Three to four animals were captured

and transported at a time, resulting in six translocation events taking place over a three week period in July 2017 (southern hemisphere wintertime). All procedures were performed according to the Standard Operating Procedure for the Capture and Transportation of Wildlife protocol approved by South African National Parks (SANParks) Animal Use and Care Committee (AUCC). The University of Pretoria Animal Ethics and Research Committee (protocol V067-17) and the SANParks AUCC (protocol 009/17) approved the use of these rhinoceros for this study.

4.2.1. Capture

Rhinoceros were located by direct observation and drugs were delivered remotely by darting (Dan-Inject®; International S.A., Skukuza, South Africa) from a helicopter into the gluteal muscle using 3.0 ml plastic darts with a 60 mm uncollared needle. Rhinoceros were captured with two different immobilisation protocols in a randomly alternated fashion: (1) etorphine (etorphine hydrochloride 9.8 mg/ml, Captivon®; Wildlife Pharmaceuticals, Karino, South Africa) combined with azaperone (group A, n = 11; azaperone tartrate 50 mg/ml, Wildlife Pharmaceuticals) or (2) etorphine combined with midazolam (group M, n = 12; midazolam hydrochloride 50 mg/ml, Dazonil®; Wildlife Pharmaceuticals). Etorphine doses were based on standardised estimated weight categories: 1250 – 1500 kg = 3 mg; 1500 – 1750 = 3.5 mg; 1750 – 2000 = 4 mg. Azaperone or midazolam were administered at five times the etorphine dose in mg. The time from successful darting to recumbency was recorded as the induction time. Once immobilised, rhinoceros were approached quietly, positioned in lateral recumbency and blindfolded to reduce stimuli during handling. A blood sample was collected immediately from the cephalic vein (TC) as this venipuncture site was easily accessible in all rhinoceros and blood collection was quick. At the same time, the auricular skin was aseptically prepared and a 16 gauge 20 cm over-the-wire intravenous catheter (Arrow®, PA 19605 USA) was inserted into an auricular vein and sutured to the skin to allow for serial blood sample collection during transport. Heart rate, respiratory rate, and body temperature were monitored throughout the 30

minute procedure and oxygen was delivered at a constant rate of 10 l/min by nasal insufflation (after the TC blood sample was collected). Once the catheter was in place, butorphanol (5 mg for every mg of etorphine; butorphanol tartrate 50 mg/ml, Wildlife Pharmaceuticals) was administered intravenously to partially antagonise the μ-opioid receptor effects of the etorphine (232) and allow for loading of the rhinoceros into the transport crate. Once in the crate, an intravenous bolus of diprenorphine (3 mg for every mg of etorphine; diprenorphine hydrochloride 12 mg/ml Activon®; Wildlife Pharmaceuticals) was administered to further antagonise the immobilising, but not the sedative, effects of the etorphine (37). Additional sedation was achieved by re-administering azaperone (group A) or midazolam (group M) intramuscularly. Because there are no pharmacokinetic studies investigating the optimal dose of azaperone or midazolam in rhinoceros, these drugs were administered at 25 times the etorphine dose, in mg, for practical reasons.

4.2.2. Transport

The animals were transported on trucks in International Air Transport Association (IATA) approved rhinoceros crates, following the practical guidelines for transport of live wild animals (181) and rhinoceros (24,29). Transport started once all four rhinoceros (three rhinoceros on one translocation) had been captured and loaded into the transport crates, which took 162 ± 84 (mean ± SD) minutes. Azaperone (group A) or midazolam (group M) was re-administered intramuscularly at a standard dose of 25 times the etorphine dose, mg, at the start of transport (T0) and at two (T2) and four (T4) hours of transport. This time interval was chosen for logistical reasons, as during drug-administration stops, the behaviour of the rhinoceros was monitored and a blood sample was also collected. The destination was reached after six hours of transport. Final blood samples were collected (T6) and the auricular catheters removed. Naltrexone (80 mg; naltrexone hydrochloride 50 mg/ml, Trexonil®; Wildlife Pharmaceuticals) was administered intravenously to completely antagonise the

residual sedative effects of etorphine so that the rhinoceros could be released back into the national park.

4.2.3. Sample collection and analysis

Blood samples were collected from a cephalic vein at capture (TC), and from the auricular intravenous catheter (Fig. 6) at the start of transport (T0), and at two (T2), four (T4) and six (T6) hours of transport. Blood sample collection always preceded azaperone or midazolam re-administration.

Blood was collected directly into lithium-heparinised tubes (BD Vacutainer; Becton and Dickinson, Plymouth, UK) and analysed immediately using the portable pre-calibrated Enterprise point-of-care (EPOC) blood gas analyser with recalibrated test cards (EPOC® Portable analyser system + EPOC® BGEM test cards, Kyron Laboratories). The device measured pH, partial pressure of venous carbon dioxide ($PvCO_2$), concentrations of sodium (Na^+), potassium (K^+), ionised calcium (iCa^{++}), chloride (Cl^-), glucose, and lactate; and calculated bicarbonate (HCO_3^-), base excess (BE), and anion gap (AG) using the traditional Henderson-Hasselbalch equation (233,234). Blood collected into serum tubes (BD Vacutainer; Becton and Dickinson) was stored in a cooler box with ice packs, centrifuged within 24 hours of sample collection and stored at -80°C for one month until analysed in the clinical pathology laboratory of the Onderstepoort Veterinary Academic Hospital. Concentrations of selected clinical chemistry analytes were measured using a Cobas Integra 400 Plus automated biochemistry analyser (Roche Diagnostics Ltd., Rotkreuz, Switzerland) and commercially available kits. Magnesium (Mg^{++}), inorganic phosphate (Pi), albumin and globulin (calculated as total protein minus albumin) were measured at all time points. Urea, creatinine, beta hydroxybutyrate (BHB), creatine kinase (CK) and aspartate aminotransferase (AST) were measured at TC, T0 and T6.

Figure 6: The auricular intravenous catheter that was used for blood sample collection during transport in white rhinoceros bulls.

4.2.4. Calculated variables

Quantitative analysis of acid-base status was assessed using the Stewart's quantitative approach to acid-base chemistry (237) simplified by Constable (1997) (238). Measured strong ion difference (SIDm), total non-volatile weak acids (Atot) and strong ion gap (SIG) were estimated using the following formulas, derived for horses (238):

SIDm (mmol/l) = $(Na^+ + K^+ + iCa^{++} + Mg^{++}) - (Cl^- + lactate)$

Atot (mmol/l) = 0.225 * albumin (g/l) + 0.14 * globulin (g/l) + 1.8 * Pi (mmol/l)

SIG (mmol/l) = Atot / (1 + 10 pKa - pH) - AG

The value used for pKa of plasma was 6.65 as experimentally determined for horses, a domesticated member of the order Perissodactyla related to the rhinoceros (238). Finally, plasma osmolality was calculated as (239):

Osmolality (mOsm/kg) = 2 * (Na^+ + K^+) + glucose (mmol/l) + urea (mmol/l)

Interpretation of acid-base status was performed using the (1) traditional Henderson-Hasselbalch bicarbonate based approach and the (2) Stewart's quantitative strong ion difference based approach. Due to a lack of reported acid-base reference intervals from non-immobilised white rhinoceros, we used reference intervals from horses to interpret our findings (240–242) (Table 3).

4.2.5. Statistical analysis

All statistical analyses were performed with the software R version 3.3.1 (243). Data were assessed for normality by calculating descriptive statistics and plotting of histograms. Mean ± standard deviation (SD) were calculated for each analyte per sample point and group and interval plots were generated for descriptive purposes. Changes over time and between groups for variables of interest were compared using a general linear mixed model (fixed factors: time and group; random factors: rhinoceros; interactions: time and time x group). Start of transport (T0) and group A were used as reference category to (1) better differentiate the effects of capture (TC to T0) from the effects of transport (T0 to T6) and because (2) azaperone is the drug that is currently most commonly added to etorphine for rhinoceros capture and transport. Induction times (period between darting and immobilisation) were compared between groups using a Mann-Whitney-Test. A p value < 0.050 was considered significant.

Table 3. Reference intervals used to interpret our findings. Due to a lack of reported venous acid-base reference values from white rhinoceros, we used reference intervals from horses, the domestic species most closely related to the rhinoceros (240–242). Reference intervals for white rhinoceros were used if available (209,222).

(unit)	Equine reference interval	Rhinoceros reference interval
pH	7.31 – 7.45	
$PvCO_2$ (mm Hg)	36.3 – 54.0	
HCO_3^- (mmol/l)	24– 30	
BE (mmol/l)	-6 – 6	
AG (mmol/l)	7 – 15	
SIDm (mmol/l)	38 – 44	
Atot (mmol/l)	14 – 15.6	
SIG (mmol/l)	-2 – +6	
Osmolality (mOsm/kg)	270 – 300	
Lactate (mmol/l)	< 2	4.6 *
Na+ (mmol/l)	133 – 141	
K^+ (mmol/l)	3.0 – 4.6	
Cl- (mmol/l)	90 – 100	
iCa^{++} (mmol/l)	1.34 – 1.72	
Mg++ (mmol/l)	0.6 – 1.0	
Pi (mmol/l)		0.73 – 1.88
Albumin (g/l)		18–32
Globulin (g/l)		51–87
Glucose (mmol/l)		2.3 – 12.1
Urea (mmol/l)		1.3–6.7
Creatinine (µmol/l)		90–195
CK (U/l)		95–435
AST (U/l)		11–76

Abbreviations: pH (pH), partial pressure of carbon dioxide ($PvCO_2$), bicarbonate (HCO_3^-), base excess (BE), anion gap (AG), measured strong ion difference (SIDm), non-volatile weak acids (Atot), strong ion gap (SIG), sodium (Na+), potassium (K+), chloride (Cl-), ionised calcium (iCa++), magnesium (Mg++), inorganic phosphorus, creatine kinase (CK) and aspartate aminotransferase (AST).

* Cole et al. (2017) measured white rhinoceros lactate concentrations ranging from 0.9 to 14.3, mean 4.6, mmol/l with the Cobas Integra 400 Plus automated biochemistry analyser (Roche Diagnostics Ltd., Rotkreuz, Switzerland).

4.3. RESULTS

All rhinoceros survived capture and transport. Ambient temperatures ranged from 16.9 ± 1.2 (mean ± SD) °C to 27.7 ± 4.3 °C during the translocations. Induction time did not differ between the azaperone and midazolam group ($p = 0.717$) and was 07:37 ± 2:57 minutes and 07:55 ± 2:54 minutes, respectively.

Variables used in the interpretation of acid-base status are presented in Table 4, showing that the largest changes in blood acid-base status occurred from TC to T0. Measured clinical chemistry analyte concentrations used to calculate dependent variables, or aid in the interpretation of results, are presented in Table 5.

In both groups of animals the blood pH was low at TC, increased from TC to T0 ($p < 0.001$) and did not change significantly thereafter (Fig. 7a). Partial pressure of venous carbon dioxide (Fig. 7b), lactate (Fig. 7f), AG and Atot (Fig. 7d) decreased from TC to T0 ($p < 0.001$ all variables) and HCO_3^- (Fig. 7c), BE, SIDm (Fig. 7e), and SIG increased from TC to T0 ($p < 0.001$ all variables). No statistically significant differences in these variables were found from T0 to T6. A decrease from TC to T0 occurred in the electrolytes K^+, iCa^{++}, Cl^-, Mg^{++} and iP ($p < 0.001$), glucose concentration ($p = 0.005$), osmolality ($p < 0.001$), and albumin and globulins ($p < 0.001$). Whilst most of these variables did not change significantly thereafter, K^+ and Mg^{++} progressively decreased from T0 to T6 ($p < 0.001$) and albumin increased moderately from T0 to T4 ($p = 0.001$). A significant increase from T0 to T6 was observed in serum urea ($p < 0.001$), CK ($p < 0.001$), AST ($p < 0.001$) and BHB ($p = 0.020$). Creatinine concentrations did not change significantly throughout capture and transport.

Rhinoceros captured with etorphine-midazolam had higher pH ($p = 0.012$), BE ($p = 0.027$) and SIG ($p = 0.002$), and lower AG ($p = 0.003$), lactate ($p = 0.002$), Na^+ ($p = 0.006$), K^+ ($p = 0.002$) and osmolality ($p = 0.011$) at TC than rhinoceros captured with etorphine-azaperone. At T6, midazolam-sedated rhinoceros had slightly higher albumin concentrations than azaperone-sedated rhinoceros ($p = 0.025$).

Table 4. Mean ± SD for venous pH, partial pressure of carbon dioxide ($PvCO_2$), bicarbonate (HCO_3^-), base excess (BE), anion gap (AG), measured strong ion difference (SIDm), non-volatile weak acids (Atot), strong ion gap (SIG) and plasma osmolality in white rhinoceros captured and transported with either azaperone (group A) or midazolam (group M) as a sedative. Time points: capture (TC), start of transport (T0), two (T2), four (T4) and six (T6) hours of transport.

Variable (unit)	Group	Time points				
		TC	T0	T2	T4	T6
pH	A	7.109 ± 0.099 *	7.441 ± 0.035	7.443 ± 0.04	7.479 ± 0.055	7.474 ± 0.068
	M	7.196 ± 0.111 *†	7.430 ± 0.057	7.463 ± 0.037	7.469 ± 0.046	7.474 ± 0.056
Traditional (Henderson-Hasselbalch) blood acid-base variables						
$PvCO_2$	A	73.3 ± 9.9 *	49.6 ± 0.1	51.2 ± 6.6	46.1 ±7.1	45.9 ± 8.9
(mm Hg)	M	65.4 ± 10.3 *	48.7 ± 7.4	47.6 ± 6.6	46.6 ± 6.9	46.7 ± 8.0
HCO_3^-	A	23.7 ± 5.3 *	33.9 ± 2.0	34.8 ± 2.1	33.9 ± 1.7	33.1 ± 2.2
(mmol/l)	M	25.9 ± 5.8 *	32.5 ± 4.6	33.8 ± 3.0	33.6 ± 2.6	33.9 ± 3.0
BE	A	-5.8 ± 6.7 *	9.7 ± 1.8	10.7 ± 2.3	10.4 ± 1.2	9.5 ± 2.0
(mmol/l)	M	-2.2 ± 7.3 *†	8.3 ± 5.1	10.0 ± 2.9	9.9 ± 2.4	11.3 ± 4.5
AG	A	21 ± 5 *	12 ± 1	11 ± 2	12 ± 2	13 ± 2
(mmol/l)	M	17 ± 5 *†	13 ± 4	12 ± 2	13 ±2	13 ± 2
Quantitative (Stewart's) blood acid-base variables						
SIDm	A	35.2 ± 5.4 *	46.0 ± 1.7	46.1 ± 1.5	46.1 ± 1.0	45.9 ± 2.4
(mmol/l)	M	36.7 ± 5.8 *	44.4 ± 4.5	45.7 ± 2.7	45.5 ± 2.6	46.7 ± 2.2
Atot	A	17.5 ± 0.6 *	15.5 ± 1.3	15.9 ± 1.3	15.7 ± 1.3	15.4 ± 1.3
(mmol/l)	M	17.2 ± 0.7 *	14.9 ± 0.5	15.3 ± 0.7	15.1 ± 0.5	15.1 ± 0.5
SIG	A	-8.0 ± 5.4 *	1.1 ± 1.9	2.7 ± 1.7	1.3 ± 2.2	0.4 ± 1.7
(mmol/l)	M	-3.6 ± 5.8 *†	-0.5 ± 3.5	2.1 ± 4.0	0.48 ± 1.79	1.9 ± 3.9
Osmolality	A	291.1 ± 9.4 *	286.2 ± 8.9	–	–	286.7 ± 7.2
(mOsm/kg)	M	289.4 ± 8.0 *†	288.8 ± 7.7	–	–	286.8 ± 6.7

* Main effect of time: significantly different ($p < 0.05$) with respect to reference category (T0 group A).

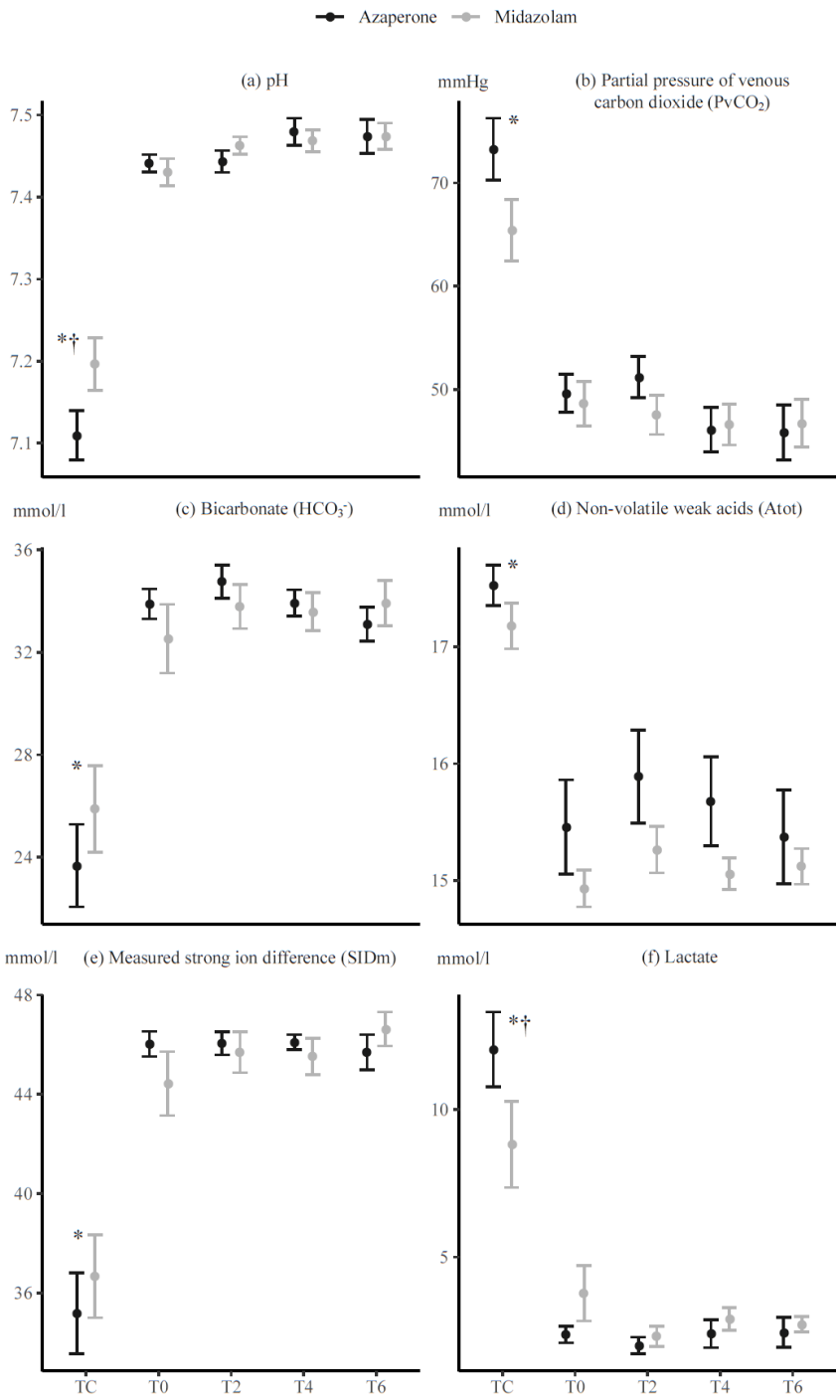
† Interaction effect of group x time: statistically different ($p < 0.05$) from group A at the same time point.

Table 5. Mean ± SD for clinical chemistry analyte concentrations used to calculate, or interpret, dependent acid-base parameters in white rhinoceros captured and transported with either azaperone (group A) or midazolam (group M). Time points: capture (TC), start of transport (T0), two (T2), four (T4) and six (T6) hours of transport.

Variable (unit)	Group	Time points				
		TC	T0	T2	T4	T6
Lactate	A	12.04 ± 4.21 *	2.38 ± 0.93	2.01 ± 0.93	2.41 ± 1.57	2.54 ± 1.67
(mmol/l)	M	8.82 ± 5.07 *†	3.77 ± 3.23	2.32 ± 1.17	2.91 ± 1.35	2.71 ± 0.91
Sodium	A	135 ± 4	134 ± 4	134 ± 4	134 ± 4	135 ± 3
(mmol/l)	M	133 ± 4 †	135 ± 3	134 ± 3	134 ± 4	135 ± 4
Potassium	A	5.0 ± 0.4 *	4.1 ± 0.4	3.4 ± 0.3 *	3.1 ± 0.3 *	3.1 ± 0.3 *
(mmol/l)	M	4.7 ± 0.5 *†	4.4 ± 0.4	3.6 ± 0.3 *	3.2 ± 0.3 *	3.0 ± 0.2 *†
Chloride	A	95 ± 5 *	92 ± 4	92 ± 4	91 ± 5	92 ± 5
(mmol/l)	M	95 ± 5 *	93 ± 4	93 ± 5	91 ± 5	91 ± 5
Calcium	A	1.53 ± 0.08 *	1.40 ± 0.08	1.41 ± 0.08	1.38 ± 0.06	1.40 ± 0.08
(mmol/l	M	1.47 ± 0.05 *	1.38 ± 0.04	1.37 ± 0.05	1.36 ± 0.08	1.38 ± 0.08
Magnesium	A	1.26 ± 0.11 *	1.01 ± 0.09	0.95 ± 0.08	0.89 ± 0.08 *	0.84 ± 0.06 *
(mmol/l)	M	1.25 ± 0.12 *	1.04 ± 0.08	0.95 ± 0.04	0.88 ± 0.05 *	0.89 ± 0.13 *
Phosphorus	A	1.46 ± 0.21 *	0.99 ± 0.23	0.97 ± 0.22	0.97 ± 0.24	0.92 ± 0.24
(mmol/l)	M	1.42 ± 0.15 *	1.05 ± 0.24	0.90 ± 0.29	0.85 ± 0.26 †	0.83 ± 0.30
Albumin	A	26.6 ± 1.5 *	24.6 ± 1.0	25.6 ± 1.5 *	25.8 ± 1.1 *	25.0 ± 0.7
(g/l)	M	27.4 ± 1.4 *	24.6 ± 1.8	26.2 ± 1.3 *	26.3 ± 1.5 *	26.1 ± 1.1 †
Globulin	A	63.4 ± 7.0 *	57.9 ± 8.8	59.5 ± 8.6	57.9 ± 8.3	57.6 ± 9.2
(g/l)	M	60.0 ± 4.1 *	53.4 ± 3.8	5..2 ± 3.6	54.2 ± 2.7	55.1 ± 3.7
Glucose	A	8.5 ± 2.7 *	6.3 ± 2.3	6.5 ± 1.9	7.1 ± 1.3	7.6 ± 1.1
(mmol/l)	M	10.1 ± 2.8 *	7.5 ± 2.4	7.2 ± 1.6	8.1 ± 1.8	8.0 ± 1.7
Urea	A	3.31 ± 0.37	3.44 ± 0.40	–	–	3.70 ± 0.41 *
(mmol/l)	M	3.28 ± 0.51	3.40 ± 0.47	–	–	3.82 ± 0.42 *
Creatinine	A	145 ± 22	143 ± 24	–	–	139 ± 26
(µmol/l)	M	149 ± 30	150 ± 29	–	–	148 ± 29
BHB	A	0.19 ± 0.08	0.30 ± 0.11	–	–	0.45 ± 0.15 *
(mmol/l)	M	0.16 ± 0.02	0.29 ± 0.14	–	–	0.56 ± 0.27 *
CK	A	242.6 ± 158.7	617.7 ± 270.7	–	–	1928 ± 1622 *
(U/l)	M	239.6 ± 133.2	495.1 ± 322.4	–	–	1682 ± 1253 *
AST	A	55.2 ± 12.7	57.0 ± 15.8	–	–	77.4 ± 33.9 *
(U/l)	M	51.6 ± 10.5	51.6 ± 7.1	–	–	72.8 ± 19.1 *

* Main effect of time: significantly different ($p < 0.05$) with respect to reference category (T0 group A).

† Interaction effect of group x time: statistically different ($p < 0.05$) from group A at the same time point.



* Main effect of time: significantly different ($p < 0.05$) with respect to reference category (T0 group A).

† Interaction effect of group x time: statistically different ($p < 0.05$) from group A at the same time point.

Figure 7: Mean ± SD pH, $PvCO_2$, HCO_3^-, BE, SIDm, Atot and lactate concentrations in white rhinoceros captured and transported with either azaperone or midazolam. Time points: capture (TC), start of transport (T0), two (T2), four (T4) and six (T6) hours of transport.

4.4. DISCUSSION

During capture, compared to reference intervals for horses, rhinoceros experienced respiratory acidosis combined with a (metabolic) lactic- and non-volatile weak acid acidosis. Blood pH normalised by the start of transport and rhinoceros developed a mild metabolic- and strong ion alkalosis towards the end of transport. Midazolam provided a safer alternative to azaperone causing a less severe lactic acidosis, and milder electrolyte changes, during rhinoceros capture.

Because there are no published reference intervals for acid-base variables in resting rhinoceros, we used intervals from horses to interpret our findings, which is a limitation of the study. Furthermore, these equine reference intervals were not generated using the EPOC system and should be regarded with caution (244). Nevertheless we believe that repetitive blood sample collection within individual rhinoceros has allowed us to detect the trend of acid-base variables over time and identify major changes in acid-base status.

Respiratory acidosis is a common finding in rhinoceros immobilised with the potent opioid etorphine (33,34,228). During capture, we expected respiratory acidosis to be pronounced as $PvCO_2$ values were greatly elevated compared to the equine reference interval of 36.3 – 54.0 mmHg (242) and to the values measured during rhinoceros transport. Increases in blood carbon dioxide partial pressures are indicative of impaired alveolar ventilation and are thought to be the result of respiratory neuronal depression (50) and thoracic muscular rigidity (31) caused by the etorphine. Similar to horses, ventilation-perfusion mismatching and shunting during prolonged lateral recumbency likely also contributed to the observed hypercapnia (35,245) together with an increased metabolic carbon dioxide production that is often observed in etorphine-immobilised white rhinoceros (34). By the start of transport $PvCO_2$ had decreased to normal equine reference values, which were maintained throughout transport. Partial reversal of the etorphine by administering the opioid agonist-antagonists butorphanol and diprenorphine (37) and the change in body position,

from lateral recumbency to standing, rapidly improved alveolar ventilation (231,246,247) and reduced metabolism (36). These respiratory changes likely had the greatest compensatory effects on the pH from TC to T0 but increases in HCO_3^- and BE at this time indicated that a blood intracellular chemical buffer response had also taken place.

In the traditional Henderson-Hasselbalch approach, the calculated AG indicates the difference in charge between unmeasured anions and unmeasured cations and an increase in AG (i.e. increase in anions) is often used as an assumption or indirect measure of increased blood lactate (an anion) concentrations (241). Compared to reference intervals from resting horses (< 2 mmol/l)(241) and ground immobilised white rhinoceros (4.6 mmol/l)(222), blood lactate concentrations, and thus AG, were markedly elevated during capture. Hyperlactataemia is the result of an increased lactate production during oxygen debt and leads to hydrogen ion generation and acidosis (147). Hyperlactataemia was not unexpected, as rhinoceros darted from a helicopter likely experience high levels of muscular activity, increased oxygen consumption and hypoxia prior to and during immobilisation (34). Recovery from the hyperlactataemia occurred by the start of transport indicating that most of the excess lactate had been rapidly utilised by the muscles and, or, recycled by the liver and other organs (147). Generated hydrogen ions were likely also buffered by extracellular and intracellular chemical buffer systems, rapidly attenuating the acidosis together with the respiratory changes after the immobilisation was reversed.

Additionally, a pronounced increase in HCO_3^- and BE from TC to T0 may indicate that a renal compensatory response was initiated. Within hours of respiratory and, or, metabolic acidosis, the kidneys will start excreting hydrogen ions and generate HCO_3^-. This process may lead to a secondary metabolic alkalosis, which was observed in our rhinoceros. The elevation of HCO_3^- and BE was sustained during transport because the renal response to acidosis is slow and takes up to a few days to be of maximal effectiveness (248). Activation of the hypothalamic-pituitary-adrenal (HPA) axis in response to stress could have contributed to the mild metabolic alkalosis, which is

often seen in patients with hyperadrenocorticism (249,250). Alternatively, increased catabolism of organic acids, associated with the herbivore diet of the rhinoceros, could have resulted in the formation of excess HCO_3^- (251).

By using the traditional Henderson-Hasselbalch bicarbonate based approach to acid-base status interpretation, rhinoceros in this study experienced a respiratory and metabolic (lactic) acidosis during capture, from which they recovered quickly, followed by a mild metabolic alkalosis during transport. The traditional Henderson-Hasselbalch approach only uses one independent ($PvCO_2$) and two dependent variables (pH, HCO_3^-) for the assessment of acid-base status and changes in protein or electrolyte concentrations are not considered (241). The Stewart's quantitative strong ion difference based approach uses three independent ($PvCO_2$, SIDm and Atot) and one dependent variable (pH) for the assessment of acid-base status (252). This approach has been used in transported *Bos indicus* steers (253) and was evaluated from venous blood in horses (241,254) to more accurately describe acid-base and electrolyte abnormalities associated with exercise.

The rhinoceros' SIDm was mildly lower during capture (tendency towards acidosis), but mildly higher during transport (tendency towards alkalosis), than published equine reference intervals (240,241). Changes in SIDm were caused by changes in blood lactate and Cl^- concentrations. Chloride concentrations decreased from TC to T0, and remained at lower concentrations throughout transport. This decrease likely resulted from renal compensation of the acidosis where HCO_3^- is reabsorbed and exchanged for Cl^- (248). Sweating and glucocorticoid release associated with a stress response might have also played a role (255). However, despite being higher at capture compared to transport, measured electrolyte concentrations remained largely within normal equine reference intervals (240–242).

In the simplified strong ion approach, Atot represents the total plasma concentration of non-volatile weak acids (238). Plasma proteins and phosphates are weak acids that are not fully dissociated at physiological pH and are thus able to buffer hydrogen ions

(252). The Atot were higher at capture than during transport. Increased Atot results in a pH decrease and is often a consequence of haemoconcentration (241). Captured rhinoceros, particularly if wild, exhibit a fight or flight response causing severe systemic arterial hypertension after darting with etorphine-based drug combinations (41,54). This acute increase in blood pressure may lead to a shift of plasma from the intravascular compartment into interstitial spaces and cause haemoconcentration and a total weak acid acidosis (256). The higher plasma osmolality at capture compared to transport was unlikely due to a concurrent total body water loss, because sodium, creatinine and urea concentrations did not change. The increased, but normal (270 – 300 mOsm/kg)(241), plasma osmolality was caused by higher plasma glucose concentrations at capture compared to transport. This increase in plasma glucose concentrations likely represented a metabolic effect of a stress response to capture, which causes an increase in hepatic gluconeogenesis, glycogenolysis and a decrease in insulin sensitivity (257).

The last variable used in the Stewart's quantitative strong ion difference based approach is SIG. Strong ion gap is the difference in charge between all unmeasured strong anions and all unmeasured strong cations. It is more specific for detecting changes in unmeasured strong ions (such as lactate) than AG as it also considers non-volatile weak acids (252). Unsurprisingly, SIG was decreased in rhinoceros at capture reflecting hyperlactataemia and lactic acidosis. Another strong ion is BHB and thus, any increase in BHB will decrease SIG. Our rhinoceros experienced a significant increase in BHB concentrations towards the end of transport indicating lipolysis in response to a negative energy balance or exercise (258). However, SIG remained within the normal range for horses (-1 – +6 mmol/l)(241).

By using the Stewart's quantitative strong ion difference based approach to acid-base status interpretation, rhinoceros in this study experienced a strong ion acidosis combined with a non-volatile weak acid acidosis during capture, from which they recovered quickly, followed by a mild strong ion alkalosis during transport.

The acidosis observed at capture was significantly less severe in rhinoceros captured with etorphine-midazolam than etorphine-azaperone. These differences in blood pH between the two groups were likely caused by differences in lactate concentrations and associated calculated variables (AG, BE and SIG). Rhinoceros captured with etorphine-midazolam, due to the midazolam's muscle relaxant effects, likely experienced less muscular rigidity and thus, less hyperlactataemia and acidosis. Lower osmolality in rhinoceros of this group, was likely the result of lower K^+ concentrations during capture associated with a smaller decrease in pH. As muscle contraction is associated with K^+ release, the lower K^+ concentrations possibly also resulted from the muscle relaxing effect of the benzodiazepine (259). However, no differences between the midazolam and azaperone group were detected in the muscle-leakage enzymes CK and AST. Both CK and AST concentrations significantly increased from capture throughout transport indicating that myocyte degeneration and skeletal muscle damage occurred in both groups (260), a finding that is common in transported animals (68,89).

To sum up, capture caused the greatest disruption to the acid-base status of the rhinoceros, which appeared to be quickly compensated for after capture, and remained relatively stable during transport. For capture, co-administration of midazolam with the etorphine caused less of an acid-base imbalance therefore providing a safer and effective alternative to azaperone for the capture of white rhinoceros. Lactate production, and thus lactic acidosis, was reduced in rhinoceros captured with etorphine-midazolam compared to etorphine-azaperone, likely due to the benzodiazepine's muscle relaxing effect. Skeletal muscle contraction and perfusion should be objectively investigated in future studies to confirm this assumption. Rigidity of thoracic muscles adversely affects the adequacy of respiratory excursions and $PvCO_2$ (31). A tendency, but not statistically significant difference, in $PvCO_2$ was noted between the two groups indicating that rhinoceros captured with etorphine-midazolam may have ventilated better. Arterial blood samples would help

to better detect associated differences in blood oxygenation and any respiratory benefits of using midazolam. Due to the rhinoceros' conscious state in the crates and their wild nature, arterial anaerobic blood sample collection was not possible in this study. However, venous pH, $PvCO_2$ and HCO_3^- are known to be reliable indicators of acid-base status and are therefore a good surrogate where it is not possible to collect arterial samples (261,262). Because of logistical factors, we transported rhinoceros on six different translocation events and collected venous blood samples from two different venipuncture sites, the cephalic (TC) and auricular vein (T0 – T6). Different environmental conditions during these events might have influenced our results and cannot be excluded. However, the choice of venipuncture site has recently been shown to not influence rhinoceros haematological and biochemical variables as long as the same anticoagulant is used (263). We therefore believe that sample collection from different sites would have had minimal effects on the result from our rhinoceros.

4.5. CONCLUSION

Capture caused the main acid-base imbalance in wild white rhinoceros bulls by inducing respiratory acidosis and lactic acidosis. The Stewart's quantitative strong ion difference-based approach further identified a non-volatile weak acid acidosis during capture and is therefore important to include in acid-base interpretations if protein or electrolyte imbalances are suspected. The acidosis was quickly compensated for post-capture and during transport rhinoceros developed a mild metabolic- and strong ion alkalosis.

Rhinoceros captured with etorphine-midazolam suffered less from lactic acidosis than rhinoceros captured with etorphine-azaperone. Midazolam might therefore represent a better alternative to azaperone for the capture of white rhinoceros.

CHAPTER 5: HAEMATOLOGICAL AND IMMUNOLOGICAL RESPONSES TO CAPTURE AND TRANSPORT IN WILD SOUTHERN WHITE RHINOCEROS BULLS (*CERATOTHERIUM SIMUM SIMUM*) SEDATED WITH EITHER AZAPERONE OR MIDAZOLAM

This chapter is in preparation to be submitted as a research paper for publication.

Pohlin F, Hoojiberg EH, Buss P, Huber N, Viljoen FP, Blackhurst D, Meyer LCR. Haematological and immunological responses to capture and transport in wild southern white rhinoceros bulls (*Ceratotherium simum simum*) and their modulation by midazolam compared to azaperone.

ABSTRACT

Assessing stress in wildlife by measuring haematological and immunological variables is becoming more popular as characteristic changes can be quantified and related to a stress response and its effects. The aim of this study was to (1) investigate the stress response to capture and transport and associated characteristic haematological and immunological changes in wild white rhinoceros bulls and (2) investigate whether midazolam compared to azaperone mitigated these changes.

Twenty-three wild white rhinoceros bulls were transported 280 km within the Kruger national park (KNP) for management purposes unrelated to the study. Rhinoceros were captured with a combination of etorphine, 3 to 4 mg, combined with either azaperone (n=11) or midazolam (n=12), at five times the etorphine dose in mg. Azaperone or midazolam, respectively, were re-administered every two hours during transport at 25 times the etorphine dose in mg. Serial blood samples were collected from an auricular intravenous catheter at capture (TC), the start of transport (T0) and after 6 hours of transport (T6). Changes in haematological and immunological variables over time and between groups were compared using general mixed effects models.

Increases in plasma adrenaline and serum cortisol concentrations indicated that rhinoceros mounted a stress response to capture and transport. In line with this finding, rhinoceros demonstrated characteristic changes in the erythron, leukon, oxidative status and acute phase response. Packed cell volume decreased from TC to T6 indicating that stress-haemoconcentration occurred at capture. Neutrophils progressively increased and lymphocytes and eosinophils progressively decreased from TC to T6, resulting in an increase in neutrophil to lymphocyte (N:L) ratio; a characteristic leukocyte response to circulating glucocorticoids. Rhinoceros experienced a decrease in unsaturated fatty acids and an increase in lipid peroxidation products indicating oxidative stress. A reduction in albumin and iron may suggest an acute phase response. Although plasma adrenaline and serum cortisol concentrations

did not differ between the two groups, midazolam appeared to influence some immunological responses to stress, particularly in the leukon, in a manner that remains to be investigated further.

5.1. INTRODUCTION

Translocation is the deliberate human-mediated movement of individuals or populations of wild animals from one location to another (1). Hundreds of white rhinoceros are translocated each year for conservation purposes (6,23,38). Despite the widespread use and importance of this practice, rhinoceros translocations often result in morbidity and even mortality (38,177,201). Although the direct causes for these mortalities are often related to external factors, such as novel pathogens, vulnerability to these factors is likely exacerbated by the immunological effects of translocation-induced stress (9,10,174). The term stress is an ambiguous concept in biology and biomedicine and is often defined as a threat to homeostasis (62,163). A more integrated definition states that stress is a constellation of events, consisting of an unexpected stimulus (stressor), that precipitates a reaction in the brain (stress perception), which activates physiological systems in response (stress response) (3,42). The two most studied physiological systems that initiate a stress response are the autonomic nervous system (ANS) and the hypothalamic-pituitary-adrenal (HPA) axis (42,45). The response of the ANS to a stressor results in an almost immediate (milliseconds) increased release of the catecholamine neurohormone adrenaline from the adrenal medulla (45,163). Stimulation of the HPA axis results in a slower (minutes), but more sustained, release of the glucocorticoid steroid hormone cortisol from the adrenal cortex (45,155,163). These hormones induce cellular changes in various tissues and organs, provide information about the presence of a stressor, and also have significant effects on immune cell distribution and function (3). Specifically, these latter effects include a decrease in lymphocytes and eosinophils, an increase in neutrophils (154), a reduction in leukocyte coping capacity (LCC) (163), which is a measure for neutrophil

function (3), oxidative stress (264) and the mounting of an acute phase response (166). The N:L ratio and LCC have been identified as specific stress response indicators and are therefore increasingly being used in wildlife research (155,163).

Rhinoceros are frequently tranquilised during capture and transport, components of translocation, to reduce stress perception (9,10,31). Azaperone, a butyrophenone, is most commonly used in rhinoceros and functions both, as an opioid synergist during capture, and as a short duration tranquiliser for the transport (29,31,32). Its behavioural calming effects are mediated primarily by blockade of dopamine receptors in the central nervous system (184). Midazolam is starting to be used more often in rhinoceros translocation as it is believed to have greater anxiolytic effects than azaperone (191,192). It is a benzodiazepine derivative which modulates the gamma-aminobutyric acid $(GABA)_A$ receptor in the central nervous system producing powerful anxiolytic, amnestic, hypnotic, and sedative effects (194). Benzodiazepines also bind to peripheral benzodiazepine receptors (PBR), or translocator proteins (18 kDa), which are widely expressed throughout the body (196). Interestingly, PBR densities are particularly rich in steroidogenic tissues, specifically in the adrenal gland, and may therefore have a direct modulating effect on the stress response and associated immunological changes (196,197).

The aim of this study was to investigate the stress response to capture and transport and associated characteristic haematological and immunological changes in wild white rhinoceros bulls. These changes included changes in (1) the erythron & thrombon; (2) leukon; (3) stress response indicators (adrenaline, cortisol, N:L ratio, LCC); (4) oxidative status and (5) acute phase response. Furthermore, we compared azaperone with midazolam in mitigating these responses.

5.2. MATERIALS AND METHODS

Twenty-three sub-adult wild white rhinoceros bulls were road-transported 280 km within the KNP (24859944.5099S, 31835911.1799E; altitude 317 m), South Africa, for reasons unrelated to the study. Four animals (three on one occasion) were captured

and transported at a time, resulting in six translocations taking place over a three week period in July 2018 (southern hemisphere wintertime). All procedures were performed according to the Standard Operating Procedure for the Capture, Transport and Maintenance in Holding Facilities of Wildlife as approved by the South African National Parks (SANParks) Animal Use and Care Committee (AUCC). International Air Transport Association (IATA) compliant transport crates were used and practical guidelines for transport of live wild animals (181) and rhinoceros (24,29) followed. The study was approved by the University of Pretoria Animal Ethics and Research Committee (V067-17) and SANParks AUCC (009/17).

5.2.1. Capture and transport

Rhinoceros were darted remotely from a helicopter into the gluteal muscle using 3.0 ml plastic darts with a 60 mm uncollared needle (Dan-Inject®; International S.A., Skukuza, South Africa). Two different immobilisation protocols were used: either etorphine (etorphine hydrochloride 9.8 mg/ml, Captivon®; Wildlife Pharmaceuticals, Karino, South Africa) combined with azaperone (azaperone tartrate 50 mg/ml, Wildlife Pharmaceuticals) (group A, n = 11), or etorphine combined with midazolam (midazolam hydrochloride 50 mg/ml, Dazonil®; Wildlife Pharmaceuticals) (group M, n = 12). Etorphine doses were based on standardised estimated weight categories: 1250 – 1500 kg = 3 mg; 1500 – 1750 = 3.5 mg; 1750 – 2000 = 4 mg. Azaperone or midazolam were administered at five times the etorphine dose in mg. Once immobilised, rhinoceros were positioned in lateral recumbency and a blood sample immediately collected from the cephalic vein (TC). The auricular skin was aseptically prepared and a 16 gauge 20-cm over-the-wire intravenous catheter (Arrow®, PA 19605 USA) inserted into an auricular vein using the Seldinger technique. Heart rate, respiratory rate, and body temperature were monitored throughout the 30 minute procedure and oxygen was delivered at a constant rate of 10 l/min by nasal insufflation. Once the catheter was in place, butorphanol (5 mg for every mg of etorphine; butorphanol

tartrate 50 mg/ml, Wildlife Pharmaceuticals) was administered intravenously to partially antagonise the μ-opioid receptor effects of the etorphine (232) and allow for loading of the rhinoceros into the transport crate. An intravenous bolus of diprenorphine (3 mg for every mg of etorphine; diprenorphine hydrochloride 12 mg/ml Activon®; Wildlife Pharmaceuticals) was administered once the animal was in the crate to further antagonise the immobilising, but not the sedative, effects of the etorphine (37). Additionally, azaperone (group A) or midazolam (group M) were re-administered intramuscularly at 25 times the etorphine dose in mg. Once all four rhinoceros (three rhinoceros on one occasion) had been captured and loaded into the transport crates, a venous blood sample was collected from the auricular catheter (T0) at the start of transport. Azaperone (group A) or midazolam (group M) was re-administered intramuscularly at a standard dose of 25 times the etorphine dose, in mg, at the start of transport, and two and four hours later. The destination was reached after six hours, a final blood sample was collected (T6) and the auricular catheter removed. Naltrexone (80 mg; naltrexone hydrochloride 50 mg/ml, Trexonil®; Wildlife Pharmaceuticals) was administered intravenously to fully antagonise any residual etorphine effects prior to releasing the rhinoceros back into the wild.

5.2.2. Blood sample collection and analysis

Complete blood cell count (erythron, thrombon, leukon). Blood directly collected into ethylenediaminetetraacetic acid (EDTA) tubes (BD Vacutainer; Becton and Dickinson, Plymouth, UK) was stored in a cooler box with ice packs during transport and analysed at the release site with the fully-automated Abaxis® VetScan HM5 differential haematology analyser (Abaxis Global Diagnostics, Griesheim, Germany). One level of commercial quality control material was run each day of sample analysis and results were within the manufacturer's target range. The device measured and, or, calculated: haematocrit, red blood cell count (RBC), haemoglobin concentration (HGB), mean cell volume (MCV), mean cell haemoglobin (MCH), mean cell haemoglobin concentration (MCHC), red blood cell distribution width (RDW),

plateletcrit (PCT), platelet count (PLT), mean platelet volume (MPV), platelet distribution width (PDW) and white blood cell count (WBC). Packed cell volume was determined manually. The calculated haematocrit of the Abaxis was compared to the manual packed cell volume (PCV) as reference, using different settings, and if there was a discrepancy larger than 5%, the analysis was repeated. The Abaxis "cow" setting demonstrated the best match. In order to examine cell morphology, and because automated leukocyte counts have not been validated for this species (265,266), blood smears were made by using the wedge method (267) and examined at a later point by an experienced clinical pathologist. The relative proportion of each WBC type (differential count) was measured by light microscope examination of 100 leukocytes in a modified Romanowsky stained blood smear; immature neutrophils (BANDS%), mature neutrophils (SEG%), lymphocytes (LYM%), monocytes (MON%), and eosinophils (EOS%) were counted. Absolute neutrophil (NEU) and lymphocyte (LYM) counts were calculated by multiplying the sum of BANDS% and SEG% (NEU), and LYM% (LYM), from the 100-cell count, with the total Abaxis WBC count. The N:L ratio was calculated by dividing NEU by LYM.

Leukocyte coping capacity. Leukocyte coping capacity measurements were carried out in the field as described by Huber et al. 2017 (268) immediately after blood collection into lithium-heparinised blood tubes (BD Vacutainer; Becton and Dickinson). Briefly, 10 µl of blood were mixed with 90 µl luminol (5-amino-2,3-dihydrophthalazine-1,4-dione; VWR International, Stockholm, Sweden) and 10 µl phosphate-buffered saline (PBS, pH 7.4) (unstimulated LCC measurement). A portable chemiluminometer (Junior LB 9509, EG & G Berthold, Germany) was used to measure blood chemiluminescence (expressed in relative light units (RLU)). A second tube was prepared in parallel and 10 µl of 10−5 mol/l phorbol 12-myristate 13-acetate (PMA; VWR International, Stockholm, Sweden) were added instead of the 10 µl PBS (stimulated LCC measurement). Chemiluminescence for each tube was measured for 30 seconds, every 5 minutes, for a total of 80 minutes. When not in the

chemiluminometer samples were incubated in a metal bead bath (Minitübe, Germany) at 37 °C.

Two variables were used for statistical analysis: the area under the curve (AUC) and "corrected" LCC (cLCC). The AUC represents the integral of the LCC response curve over 80 minutes. To counter individual variances between rhinoceros, the differences between PMA unstimulated and stimulated LCC measurements were used to calculate AUC. "Corrected" LCC (cLCC) represents the LCC per neutrophil calculated by correcting the AUC by the absolute neutrophil count.

Adrenaline. Immediately after collection, EDTA blood tubes were centrifuged in a cooled centrifuge. Plasma was pipetted into cryovials and immediately snap-frozen in liquid nitrogen. Samples were subsequently stored at -80°C for two weeks and shipped to the Analytical Technical Laboratory of the Faculty of Health Sciences, North-West University, using dry-ice. Adrenaline concentrations were determined using high performance liquid chromatography (HPLC) as described by De Villiers et al. (1987) (269) using the Agilent 1200 HPLC system (Agilent Technologies, Santa Clara, CA, USA). Briefly, plasma (900 µl) was mixed with 50 mg activated acidic aluminium oxide, 500 µl Tris buffer (pH 8.7) and 20 µl 3,4-dihydroxybenzylamine (internal standard, 7.5µg/ml). After centrifuging and washing with distilled water, the supernatant was discarded and 200 µl of M perchloric acid added. The mixture was rested on ice for 30 minutes and then centrifuged. The resulting acidic extract (25 µl) was used for HPLC analysis. All chemicals were obtained from Merck (Pty) Ltd (Johannesburg, South Africa) and Sigma-Aldrich Pty (Ltd) (Johannesburg, South Africa).

Oxidative stress biomarkers. Duplicate snap-frozen EDTA plasma samples were shipped on dry-ice to the chemical pathology laboratory of the Faculty of Health Sciences, University of Cape Town. Plasma triglyceride concentrations were determined using enzymatic colorimetric kits (KAT Medicals, Calicom Trading, South Africa) and

phospholipids (WAKO Chemicals, Neuss, Germany) in a Labsystems Multiskan MS analyzer (AEC Amersham Co.). Concentrations of conjugated dienes (CD) and thiobarbituric acid reactive substances (TBARS) were measured by spectrophotometric methods using a GBC UV/VIS analyser (Wirsam Scientific and Precision Equipment, South Africa) (CD) or the Labsystems Multiskan MS Analyzer (AEC Amersham Co.) (TBARS). Conjugate dienes were measured at 234 nm after appropriate dilution in cyclohexane (Spectrosol) as described by Pryror & Castle (1984) (270) and Esterbauer et al. (1989) (271). Thiobarbituric acid reactive substances were measured at 532 nm after being prepared as described by Nduhirabandi et al. 2011 (204). Conjugate dienes and TBARS measurements were corrected per absolute fatty acid concentration (the sum of triglycerides and phospholipids) to examine the effect of the fatty acids on reactive oxygen species (ROS) production.

The antioxidant capacity of the plasma was assessed by the oxygen radical absorbance capacity (ORAC) method described by Cao et al. (1993) (205) and Huang et al. (2002) (206). Fluorescein (3,6-dihydroxyspiro(isoberyofuran-1(3H),9(9H)-xanthen)-3-one disodium) and 2,2'-azobis(2-methyl-propionamidine)dihydrochloride were prepared in phosphate buffer to a working solution of 95.7 nmol/l and 32.1 µmol/l, respectively. Fifty µl of trolox standards (6-OH-2,5,7,8-tetromethylchromane-2-carboxylic acid) and deproteinised plasma were added. The resulting fluorescence was measured over time using the Varian Cary Eclipse fluorescence spectrophotometer (Varian Australia Pty Ltd) at an excitation wavelength of 485 nm and emission wavelength of 520 nm.

Acute phase reactants and cortisol. Blood directly collected into sodium-citrate (CTAD) and serum tubes (BD Vacutainer; Becton and Dickinson) was stored in a cooler box with ice packs during transport and centrifuged at the release site. Serum and plasma were aliquoted and stored at -80°C until analysis at the clinical pathology laboratory of the Onderstepoort Veterinary Academic Hospital. Fibrinogen was determined from the CTAD plasma with the modified Clauss method on an ACL Elite automated coagulometric analyser (Instrumentation Laboratory, Bedford, MA, USA). Serum

haptoglobin was determined by the haemoglobin-binding method using a commercial kit (PHASE Haptoglobin Assay, Tridelta Development Limited) on a Cobas Integra 400 Plus automated biochemistry analyser (Roche Diagnostics Ltd.). Serum iron, cholesterol, total serum protein (TSP), albumin and globulin (calculated as TSP – albumin) were measured using commercially available kits on the Cobas Integra 400 Plus. Serum cortisol concentrations were assessed by a chemiluminescent immunoassay using the Immulite/Immulite 1000 Cortisol® following manufacturer's instructions (Siemens Healthcare). All analysers were maintained and kits were calibrated according to manufacturer's instructions; two levels of commercial quality control material were analysed before each assay run and results were within the laboratory's predetermined target ranges.

5.2.3. Statistical analysis

Variables were divided into five multivariate datasets for the interpretation of results: (1) Erythron (PCV, RBC, HGB, PCV, MCV, MCH, MCHC, RDW) & thrombon (PCT, PLT, MPV, PDW), (2) leukon (WBC, BANDS%, SEG%, LYM%, MON%, EOS%, NEU, LYM), (3) stress response indicators (epinephrine, cortisol, N:L ratio, AUC, cLCC), (4) oxidative stress status (triglycerides, phospholipids, CD, TBARS, ORAC) and (5) acute phase reactants (fibrinogen, haptoglobin, iron, TSP, albumin, globulin, cholesterol). Statistical analysis was performed with the software R version 3.3.1 (243). Data were assessed for normality by calculating descriptive statistics and plotting of histograms. Mean ± standard deviation (SD) were calculated for each analyte per sample point and group and interval plots were generated for descriptive purposes. A general linear mixed model (fixed factors: time and group; random factors: rhinoceros; interactions: time and time x group) was used to compare changes over time and between groups. Start of transport (T0) and group A were used as reference category to (1) better differentiate the effects of capture (TC to T0) from the effects of transport (T0 to T6) and because (2) azaperone is the drug that is currently most commonly added to the etorphine for rhinoceros capture and transport. Pearson's correlations were

performed to investigate correlations between stress-associated variables. The Bonferroni correction for multiple correlations was applied. Differences were considered significant when $p \leq 0.050$.

5.3. RESULTS

All rhinoceros survived capture and transport. The time from capturing the first rhinoceros (TC) to the start of transport (T0) was 162 ± 84 (mean ± SD) minutes. Mean ± SD for measured variables at TC, T0 and T6 are shown in Table 6 for the different groups.

Table 6. Mean ± SD for measured variables in white rhinoceros captured and transported with either azaperone (group A) or midazolam (group M). Time points: capture (TC), start of transport (T0), and six hours of transport (T6).

Variable	Unit	Group	Time point		
			TC	T0	T6
Erythron					
PCV	%	A	46 ± 3 *	37 ± 5	34 ± 3 *
		M	45 ± 3 *	37 ± 4	36 ± 2 *
RBC	10^{12}/l	A	7.53 ± 0.36 *	6.35 ± 0.46	5.84 ± 0.56 *
		M	7.84 ± 0.52 *	6.64 ± 0.97	6.39 ± 0.52 *
HGB	g/l	A	155 ± 8 *	127 ±8	117 ±9 *
		M	158 ± 8 *	131 ± 17	127 ± 9 *
MCV	fl	A	69 ± 5 *	70 ± 4	70 ± 4
		M	68 ± 5 *	69 ± 4	69 ± 5
MCH	pg	A	20.6 ± 1.5 *	20.1 ± 1.6	20.1 ± 1.4
		M	20.2 ± 1.3 *	19.8 ± 1.1	19.9 ± 1.2
MCHC	g/l	A	301 ± 7 *	287 ± 9	288 ±11
		M	300 ± 10 *	289 ± 11	288 ± 7
RDW	%	A	22.2 ± 1.1 *	21.4 ± 1.1	21.5 ± 1.0
		M	22.4 ± 0.8 *	21.6 ± 0.7	21.5 ± 0.6
Thrombon					
PCT	%	A	0.09 ± 0.04	0.08 ± 0.04	0.07 ± 0.04
		M	0.08 ± 0.02	0.07 ± 0.03	0.09 ± 0.04 †

Table 6 continued.

Variable	Unit	Group	Time point		
			TC	T0	T6
PLT	10^9/l	A	149 ± 59	128 ± 59	108 ± 64
		M	132 ± 33	103 ± 47	127 ± 33 †
MPV	fl	A	6.4 ± 0.6	6.2 ± 0.4	6.4 ± 0.3
		M	6.2 ± 0.3	6.3 ± 0.3	6.3 ± 0.3
PDW	%	A	27.6 ± 1.8	26.9 ± 1.3	27.0 ± 1.1
		M	26.9 ± 1.4	27.2 ± 1.5	27.2 ± 1.3
Leukon					
WBC	10^9/l	A	14.40 ± 2.61	15.15 ± 3.20	16.83 ± 2.18 *
		M	12.29 ± 1.79 †	10.98 ± 3.09 †	15.96 ± 1.80 *†
BANDS%	%	A	5 ± 4 *	3 ± 2	2 ± 2
		M	3 ± 3 *	2 ± 2	2 ± 1
SEG%	%	A	37 ± 8 *	61 ± 12	75 ± 14 *
		M	44 ± 10 *†	53 ± 13	81 ± 6 *†
LYM%	%	A	35 ± 9 *	20 ± 7	14 ± 7 *
		M	30 ± 7 *†	24 ± 9	11 ± 5 *
MON%	%	A	10 ± 3	8 ± 3	7 ± 4
		M	8 ± 3 †	10 ± 3	5 ± 3 †
EOS%	%	A	13 ± 4 *	7 ± 6	2 ± 4 *
		M	15 ± 5 *	11 ± 6 †	0 ± 0 *†
NEU	10^9/l	A	5.98 ± 1.00 *	9.89 ± 3.39	13.10 ± 3.18 *
		M	5.85 ± 1.60 *†	6.21 ± 2.53 †	13.31 ± 1.79 *†
LYM	10^9/l	A	5.08 ± 1.81 *	3.01 ± 0.97	2.30 ± 1.18
		M	3.59 ± 0.86 *	2.47 ± 0.92	1.79 ± 0.84
Indicators of a stress response					
Epinephrine	nmol/l	A	9.28 ± 9.77 *	2.14 ± 4.73	1.51 ± 3.16
		M	6.15 ± 7.99 *	1.29 ± 2.84	1.16 ± 2.50
Cortisol	mmol/l	A	52.2 ± 21.1 *	107.6 ± 52.7	64.8 ± 35.6 *
		M	47.2 ± 16.8 *	122.2 ± 30.3	96.5 ± 50.2 *
N:L ratio	N:L ratio	A	1.33 ± 0.59	3.77 ± 2.25	8.54 ± 8.14 *
		M	1.78 ± 0.84	3.05 ± 2.28	9.85 ± 7.34 *
AUC	RLU	A	15515 ± 4170*	40012 ± 25996	61676 ± 23375*
		M	19730 ± 14413*	24580 ± 12259	51020 ± 29402*
cLCC	RLU	A	2643 ± 310	3984 ± 1774	5184 ± 3338
		M	3469 ± 2215	4937 ± 4187	3990 ± 2612

Table 6 continued.

Variable	Unit	Group	Time point		
			TC	T0	T6
Oxidative status					
Triglycerides	mmol/l	A	0.36 ± 0.09 *	0.46 ± 0.13	0.27 ± 0.10 *
		M	0.37 ± 0.08 *	0.47 ± 0.16	0.34 ± 0.14 *
Phospholipids	mmol/l	A	0.11 ± 0.05 *	0.16 ± 0.07	0.16 ± 0.05
		M	0.11 ± 0.04 *	0.13 ± 0.06	0.13 ± 0.05
CD	µmol/l	A	0.20 ± 0.10 *	0.13 ± 0.04	0.19 ± 0.08 *
		M	0.21 ± 0.11 *	0.15 ± 0.09	0.21 ± 0.11 *
TBARS	µmol/l	A	0.00 ± 0.00	0.00 ± 0.00	0.31 ± 1.03
		M	0.51 ± 1.19	0.42 ± 1.44	0.22 ± 0.72
ORAC	µmol/l	A	1394 ± 319 *	1104 ± 432	1289 ± 644
		M	1305 ± 275 *†	1374 ± 348	1228 ± 445 †
Acute phase reactants					
Fibrinogen	g/l	A	2.25 ± 0.34 *	1.80 ± 0.47	1.91 ± 0.33
		M	2.24 ± 0.26 *	1.65 ± 0.55	1.64 ± 0.51
Haptoglobin	g/l	A	0.68 ± 1.38 *	0.47 ± 0.94	0.51 ± 0.96
		M	1.15 ± 1.24 *	0.84 ± 0.96	0.85 ± 0.95
Iron	µmol/l	A	21.6 ± 1.3 *	17.3 ± 3.1	11.0 ± 2.6 *
		M	20.7 ± 1.9 *	17.3 ± 2.3	12.1 ± 2.8 *
Total serum protein	g/l	A	90.0 ± 6.3 *	83.0 ± 8.8	82.6 ± 9.1
		M	87.4 ± 4.4 *	78.0 ± 4.1	81.3 ± 3.2
Albumin	g/l	A	26.6 ± 1.5 *	24.6 ± 1.0	25.0 ± 0.7
		M	27.4 ± 1.4 *	24.6 ± 1.8	26.1 ± 1.1 †
Globulin	g/l	A	63.4 ± 7.0 *	57.9 ± 8.8	57.6 ± 9.2
		M	60.0 ± 4.1 *	53.4 ± 3.8	55.1 ± 3.7
Cholesterol	mmol/l	A	2.80 ± 0.49 *	2.59 ± 0.46	2.33 ± 0.52 *
		M	2.71 ± 0.59 *	2.49 ± 0.61	2.41 ± 0.59 *†

* Main effect of time: significantly different ($p < 0.05$) with respect to reference category (T0 group A).
† Interaction effect of group x time: statistically different ($p < 0.05$) from group A at the same time point.

Abbreviations: packed cell volume (PCV), red blood cell count (RBC), haemoglobin (HGB), mean cell volume (MCV), mean cell haemoglobin (MCH), mean cell haemoglobin concentration (MCHC), red blood cell distribution width (RDW), plateletcrit (PCT), platelet blood count (PLT), mean platelet volume (MPV), platelet distribution width (PDW), white blood cell count (WBC), percentage immature

neutrophils (BANDS%), percentage mature neutrophils (SEG%), percentage lymphocytes (LYM%), percentage monocytes (MON%), percentage eosinophils (EOS%), absolute neutrophil count (NEU), absolute lymphocyte count (LYM), neutrophil to lymphocyte ratio (N:L ratio), area under the curve (AUC), corrected leukocyte coping capacity per neutrophil (cLCC), conjugate dienes (CD), thiobarbituric acid reactive substances (TBARS), oxygen radical absorbance capacity (ORAC).

Erythron and thrombon. Figure 8 represents changes in a) PCV, b) RDW and c) PLT. The main changes in the erythron occurred from TC to T0. Packed cell volume, RBC and HGB decreased from TC to T0 ($p < 0.001$) and from T0 to T6 ($p = 0.002$, $p < 0.001$ and $p = 0.002$, respectively). The red cell indices MCH, MCHC and RDW decreased ($p < 0.001$), and MCV increased ($p = 0.004$), from TC to T0, but did not change significantly thereafter. There were no significant differences in red cell indices between the groups. No significant changes in the thrombon were observed between different time points. At T6, however, PCT and PLT were higher in midazolam-sedated than in azaperone-sedated rhinoceros ($p = 0.030$ and $p = 0.036$, respectively).

Leukon. Figure 9 represents changes in a) WBC, b) NEU and c) LYM. White blood cell count increased from T0 to T6 ($p = 0.018$). There were more BANDS% at TC than at T0 ($p = 0.034$) and during transport. Neutrophils increased from TC to T0 (SEG% and NEU $p < 0.001$) and T0 to T6 (SEG% $p = 0.001$ and NEU $p < 0.001$). Lymphocytes decreased from TC to T0 (LYM% and LYM $p < 0.001$) and T0 to T6 (LYM% $p = 0.019$). Similarly, EOS% decreased from TC to T0 ($p = 0.001$) and T0 to T6 ($p = 0.003$). There were no significant changes in MON% over time.

White blood cell counts were lower in group M compared to group A at all time points (TC $p = 0.035$, T0 $p < 0.001$, T6 $p = 0.001$). In group M, SEG% and NEU were higher at TC ($p = 0.010$ and $p = 0.002$, respectively) and T6 ($p = 0.010$ and $p < 0.001$) than in group A; however, NEU were lower at T0 ($p < 0.001$). Group M had lower LYM% at TC than group A ($p = 0.033$). Finally, EOS% and MON% decreased to lower concentrations in group M compared to group A ($p = 0.019$ and $p = 0.043$, respectively, at T6).

Stress response indicators. Indicators for a stress response are shown in Figure 10. At TC, plasma adrenaline concentrations were above the range of detection (> 5 nmol/l) in 12 animals (group A, n= 7; group M, n= 5). At T0 and T6, plasma adrenaline concentrations were detected only in four of these animals (two from each group). Serum cortisol concentrations increased from TC to T0 ($p < 0.001$) and decreased from T0 to T6 ($p = 0.002$).The N:L ratio increased markedly from T0 to T6 ($p = 0.013$). The AUC increased from TC to T0 ($p = 0.005$) and T0 to T6 ($p = 0.013$). However, there were no significant changes in cLCC over time indicating that changes in ROS production were likely associated with changes in neutrophil numbers and not neutrophil function. Therefore, AUC was not included in further statistical analyses. There were no significant differences between the groups in plasma adrenaline, serum cortisol, N:L ratio and cLCC. Results of the Pearson correlation showed that there were also no significant correlations between N:L ratio, cLCC, adrenaline and cortisol concentrations (Table 7).

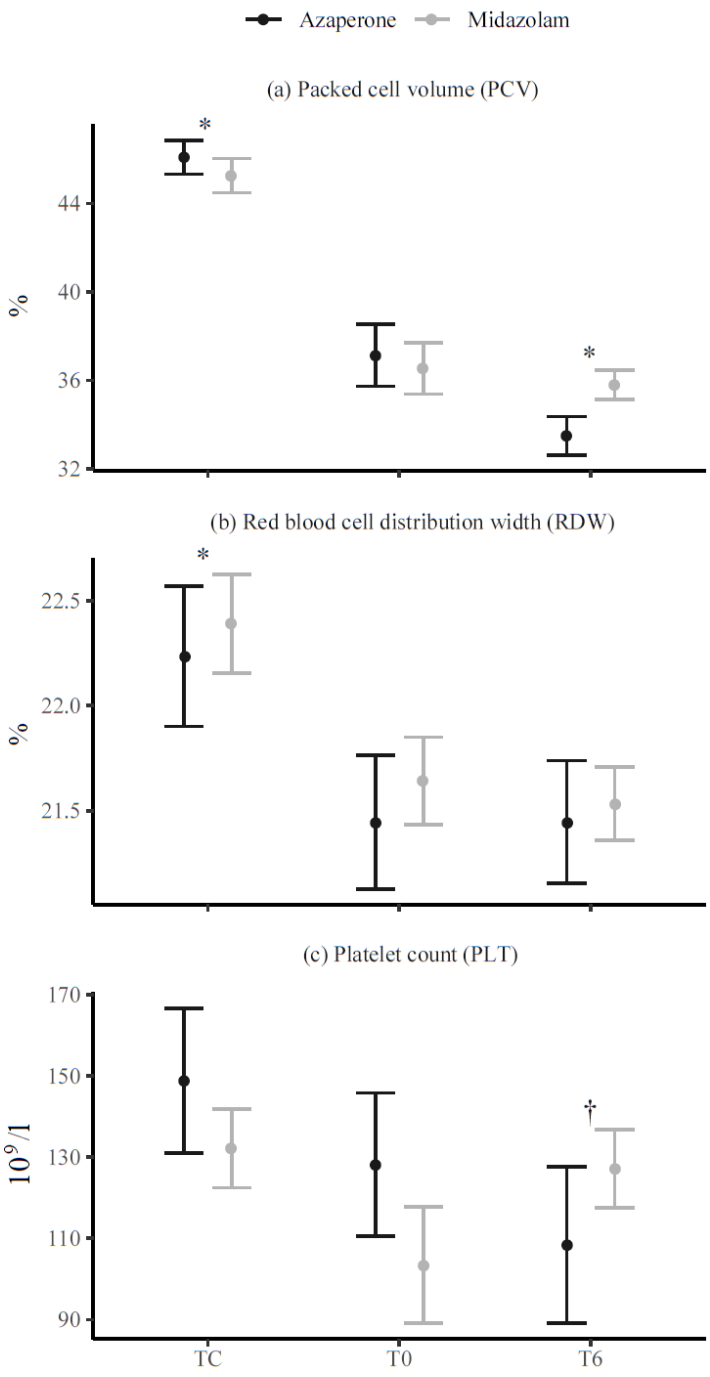
Table 7. Association among neutrophil to lymphocyte ratio (N:L ratio), corrected leukocyte coping capacity (cLCC), plasma adrenaline and serum cortisol measurements in 23 white rhinoceros bulls during capture and transport. Pearson correlation coefficients (r) and *p* values are shown.

	N:L ratio	**cLCC**	**Adrenaline**	**Cortisol**
N:L ratio		$p = 1.000$	$p = 0.152$	$p = 1.000$
cLCC	r = - 0.069		$p = 0.658$	$p = 1.000$
Epinephrine	r = - 0.269	r = - 0.194		$p = 0.130$
Cortisol	r = - 0.010	r = 0.125	r = - 0.276	

Oxidative status. Figure 11 represents changes in a) triglycerides, b) CD and c) ORAC. Plasma triglyceride and phospholipid concentrations increased from TC to T0 ($p = 0.021$ and $p = 0.028$, respectively). Triglyceride concentrations decreased from T0 to T6 ($p < 0.001$), but phospholipid concentrations did not change. Conjugate dienes

decreased from TC to T0 ($p = 0.004$) but increased from T0 to T6 ($p = 0.014$). There were no statistically significant changes in TBARS concentrations. The trend of ORAC over time differed between the two rhinoceros groups. In group A, ORAC decreased from TC to T0 ($p = 0.011$) and did not change significantly thereafter. Group M did not experience this decrease and had lower concentrations at TC and T6 than group A ($p = 0.023$ and $p = 0.034$).

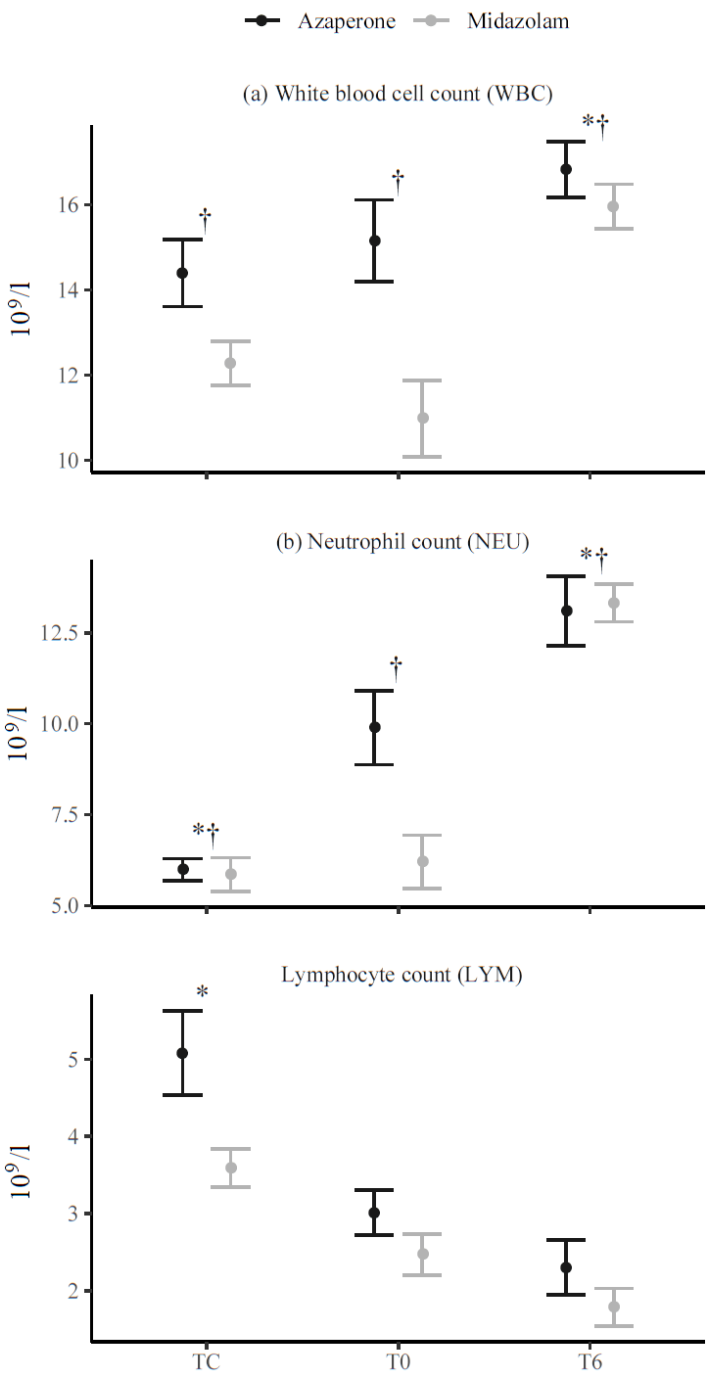
Acute phase reactants. Figure 12 represents changes in a) fibrinogen, b) iron, c) albumin and d) cholesterol. Fibrinogen and haptoglobin concentrations were higher at TC than at T0 ($p = 0.002$ and $p = 0.022$, respectively) and remained stable during transport. Serum iron and cholesterol concentrations gradually decreased from TC to T0 ($p < 0.001$ and $p = 0.002$, respectively) and T0 to T6 ($p < 0.001$). Total serum protein, albumin and globulin decreased from TC to T0 ($p < 0.001$), but did not change thereafter. At T6, group M had slightly higher cholesterol and albumin concentrations than group A ($p = 0.020$ and $p = 0.043$, respectively).



* Main effect of time: significantly different ($p < 0.05$) with respect to reference category (T0 group A).

† Interaction effect of group x time: statistically different ($p < 0.05$) from group A at the same time point.

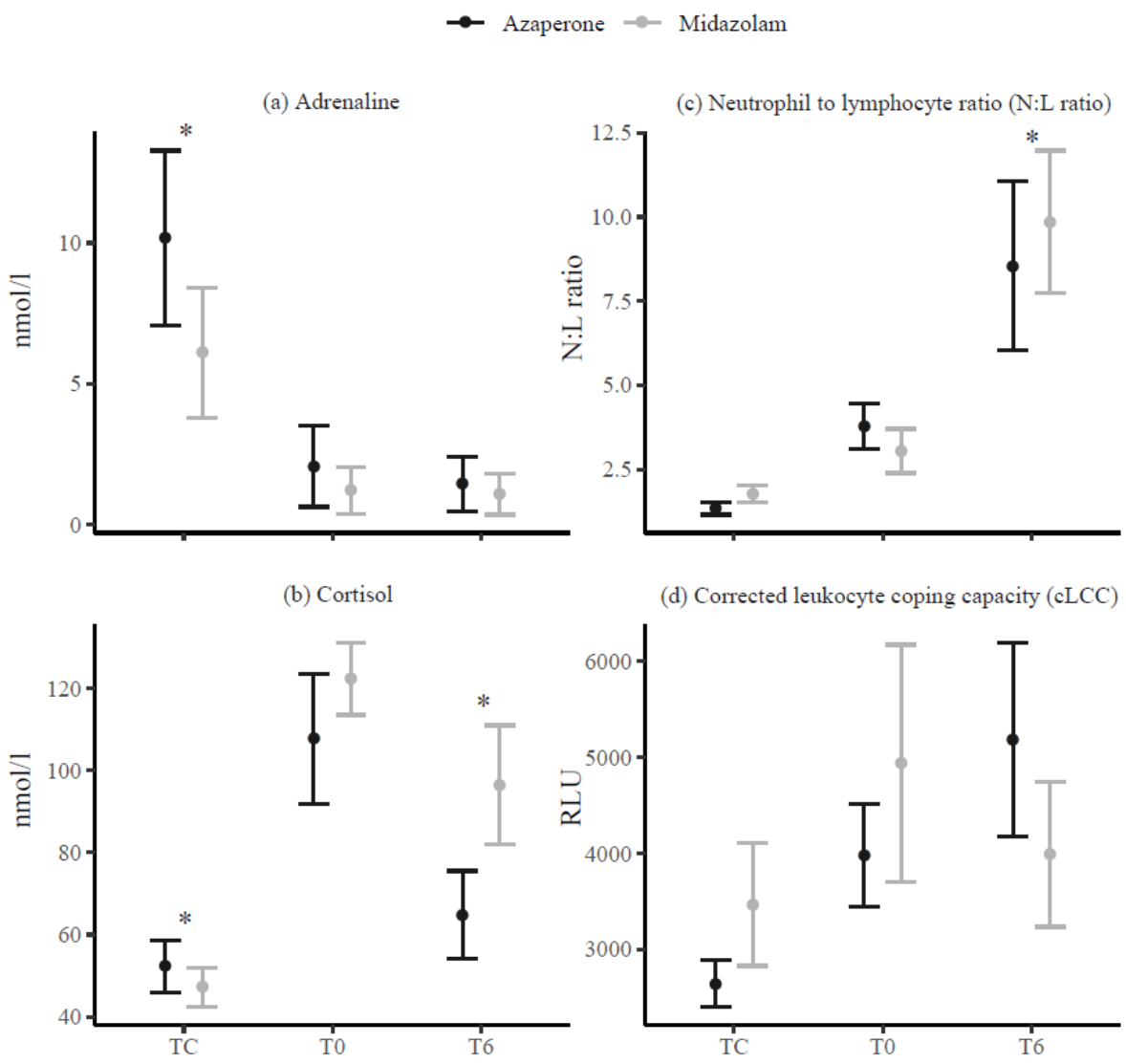
Figure 8: Mean ± SD of selected variables from the erythron and thrombon: PCV, RDW, PLT in white rhinoceros captured and transported with either azaperone or midazolam. Time points: capture (TC), start of transport (T0), and six (T6) hours of transport.



* Main effect of time: significantly different ($p < 0.05$) with respect to reference category (T0 group A).

† Interaction effect of group x time: statistically different ($p < 0.05$) from group A at the same time point.

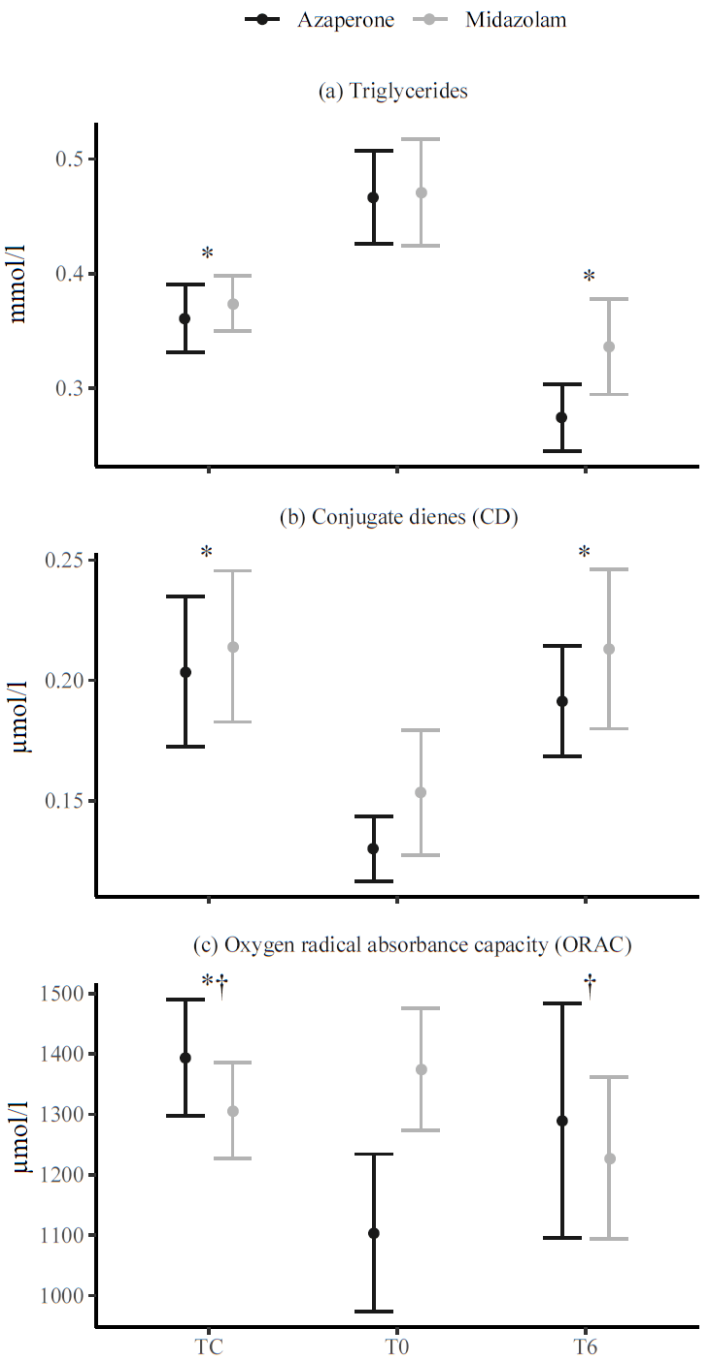
Figure 9: Mean ± SD of selected variables from the leukon: WBC, NEU, LYM in white rhinoceros captured and transported with either azaperone or midazolam. Time points: capture (TC), start of transport (T0), and six (T6) hours of transport.



* Main effect of time: significantly different ($p < 0.05$) with respect to reference category (T0 group A).

† Interaction effect of group x time: statistically different ($p < 0.05$) from group A at the same time point.

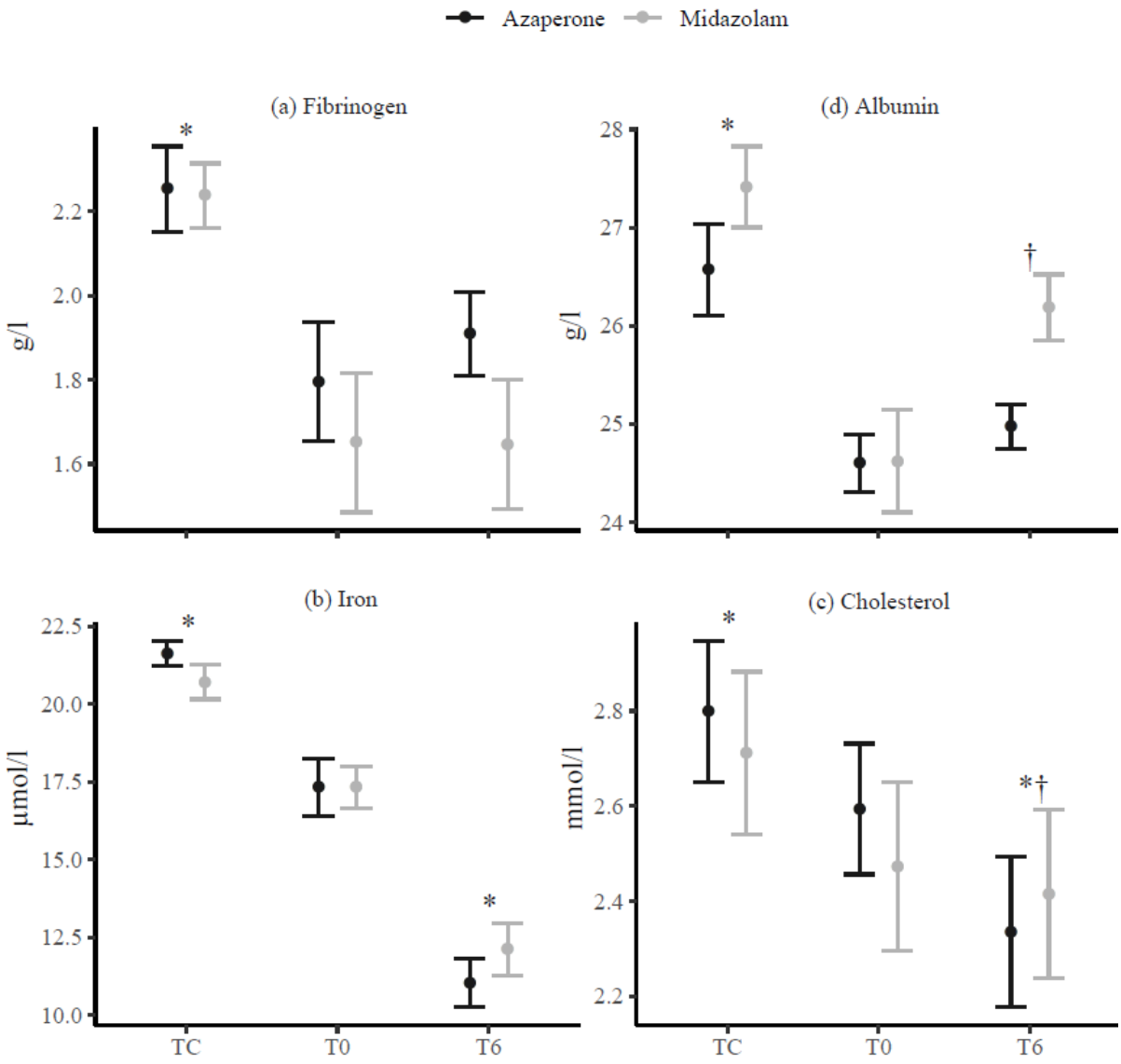
Figure 10: Mean ± SD of stress response indicators: adrenaline, cortisol, N:L ratio and cLCC in white rhinoceros captured and transported with either azaperone or midazolam. Time points: capture (TC), start of transport (T0), and six (T6) hours of transport.



* Main effect of time: significantly different ($p < 0.05$) with respect to reference category (T0 group A).

† Interaction effect of group x time: statistically different ($p < 0.05$) from group A at the same time point.

Figure 11: Mean ± SD of selected oxidative stress biomarkers: triglycerides, CD, ORAC in white rhinoceros captured and transported with either azaperone or midazolam. Time points: capture (TC), start of transport (T0), and six (T6) hours of transport.



* Main effect of time: significantly different ($p < 0.05$) with respect to reference category (T0 group A).

† Interaction effect of group x time: statistically different ($p < 0.05$) from group A at the same time point.

Figure 12: Mean ± SD of selected acute phase reactants: fibrinogen, iron, albumin and cholesterol in white rhinoceros captured and transported with either azaperone or midazolam. Time points: capture (TC), start of transport (T0), and six (T6) hours of transport.

5.4. DISCUSSION

Rhinoceros mounted a stress response to capture and transport with increases in plasma adrenaline and serum cortisol, and characteristic changes to the erythron, leukon, oxidative status and acute phase response. The sedative drug used had an influence on some of these changes, especially on the leukon response. The stress response magnitude did not differ between groups A and M suggesting the administered dosage of midazolam, compared to azaperone, did not meaningfully alter HPA function.

5.4.1. Erythron and thrombon

Rhinoceros immobilised with etorphine based drug-combinations are known to exhibit severe tachycardia and systemic hypertension resulting from the effects of the potent opioid (36,54) combined with a capture-induced sympathetic dominance (41,53). Due to the elevated plasma adrenaline concentrations at capture, our rhinoceros likely also experienced hypertension, which for logistical reasons, we were not able to measure during the translocation. We observed a higher ratio of red blood cells to the plasma volume (measured as PCV, RBC, HGB) at capture compared to transport. Increased hydrostatic pressure from the sympathetic-induced tachycardia and hypertension might have caused movement of fluid from the vessels into the extravascular space (256,272). This effect has been linked to acute psychological stress in humans and is referred to as stress-haemoconcentration (256,273). It is important to note that during stress-haemoconcentration, the number and mass of red blood cells remains constant (256).

The function of the rhinoceros' spleen is not well known. Similar to horses, the rhinoceros' spleen likely represents a reservoir of red blood cells, which rapidly enter the circulation during a fight or flight response in order to enhance oxygen transport capacity (272,274). These red blood cells would be older and smaller in size than normal circulating red blood cells (275), which could explain the higher RDW and MCHC, and lower MCV at capture compared to transport.

Although we observed no changes in the thrombon over time, PLT, and thus PCT, were lower in azaperone-sedated compared to midazolam-sedated rhinoceros at the end of transport (T6). An explanation for this difference between the two groups could be that, after the capture-stress wore off, the alpha-1-adrenergic-blocking effects of the azaperone became more prominent and caused thrombocyte sequestration in the spleen (184). More research investigating the function of the rhinoceros' spleen is necessary to confirm these assumptions.

5.4.2. Leukon

In recent years, evaluation of the leukon has become more popular with wildlife researchers as characteristic changes in blood leukocyte counts can be quantified and related to a stress response (155). These characteristic changes include an initial rapid increase followed by a sustained decrease in lymphocyte numbers and a sustained increase in neutrophil numbers (3). In our rhinoceros, lymphocyte numbers at TC may have been higher than in non-chased, non-immobilised rhinoceros, as a result of adrenaline-induced hypertension, which leads to T lymphocyte activation (276) and shift of cells from the marginating to the circulating blood pool (154). Moreover, adrenaline-induced release of immature neutrophils from the bone marrow and spleen may have caused the increase of this white blood cell-type observed at TC (277). Plasma cortisol concentrations increased from capture to the start of transport and likely caused the observed sustained decrease in lymphocytes and eosinophils, and increase in mature neutrophils over time. In response to glucocorticoids, circulating lymphocytes and eosinophils adhere to the vascular endothelium and transmigrate from the circulation into other tissues, such as lymph nodes, spleen, bone marrow and skin, where they are sequestered (155). Neutrophils in contrast, migrate from the bone marrow into the blood and shift from the marginating to circulating blood pool (154,155).

Rhinoceros sedated with midazolam had, at all times, lower WBC than rhinoceros sedated with azaperone. In human leukocytes, PBRs have been identified on the

plasma membrane and are suggested to play a role in neuroendocrine-immunomodulation (197). Monocytes and lymphocytes in particular appear to express an abundance of these receptors (278). During an initial stress response, circulating monocytes and lymphocytes produce proinflammatory cytokines, which assist in attracting neutrophils (161,279). In midazolam-sedated rhinoceros, the release of neutrophils from the bone marrow and redistribution from the marginating to the circulating pool appeared to be somewhat delayed and neutrophil counts only started increasing after transport had already started. This delay could have resulted from a monocyte and lymphocyte inhibitory effect of the midazolam, reducing the cells' capacity to attract neutrophils (278). Moreover, in midazolam compared to azaperone sedated rhinoceros, monocyte and lymphocyte concentrations were lower at capture; their function, however, remains to be investigated.

To sum up, midazolam influenced white blood cell responses to capture and transport in white rhinoceros and could therefore represent a potential hazard in the development of disease after transport. On the other hand, a reduction in leukocyte function could also have benefits, such as an anti-inflammatory effect (280). It is therefore crucial to perform careful post-release monitoring and disease risk analysis to closer investigate these assumptions. Leukocyte function and proinflammatory cytokines should also be investigated further.

5.4.3. Stress response indicators

The N:L ratio is commonly measured as an indicator of a stress response (155). Similar to reports in other transported wild mammals (97,141,159), N:L ratio gradually increased in the studied rhinoceros. The increase in N:L ratio is believed to be proportional to the magnitude of glucocorticoid release (155). However, we found no correlation between N:L ratio, adrenaline and cortisol concentrations. The reason for this discrepancy could be that the increase in N:L ratio occurs over a different time scale than the hormonal responses to a stressor (155). In the rhinoceros of this study, adrenaline concentrations were elevated only following capture, and not during

transport, suggesting a rapid and short duration release of the catecholamine (45). Cortisol concentrations followed trends described in transported domestic animals (220,223); they increased from capture to the start of transport and had decreased to nearly capture concentrations prior to release. Unlike the hormonal responses to stress, leukocyte responses may only occur after a few hours (155). In horses, elevations in N:L ratio were found to occur four hours after cortisol injection (281). We observed a similar time span in our rhinoceros, where N:L ratio increased from the start to the end of transport, supporting the assumption that the stress-associated variables did not correlate due to different time scales. Despite the differences in leukocyte differential counts between the two rhinoceros groups, N:L ratio did not differ. This finding agrees with the fact that plasma adrenaline and serum cortisol concentrations also did not differ between the two groups.

The second commonly used immunological indicator for a stress response is LCC. Leukocyte coping capacity quantifies the capacity of a neutrophil to produce ROS in response to a chemical stimulus (162). In the rhinoceros of this study, LCC per neutrophil (cLCC) did not change over time. This study is not the first where neutrophil function has been investigated in rhinoceros. Kruger et al. (2011) measured neutrophil function in white rhinoceros at capture (anaesthetic induction) and immediately after loading into the transport crate (282). Similar to our study, they did not find any significant differences between capture and loading samples in most animals. These results do not necessarily mean that capture and transport had no effect on the potential of circulating neutrophils to produce free radicals. McLaren et al. (2003) measured neutrophil function in transported and non-transported wild European badgers and found that cLCC was greater in non-transported compared to transported individuals (162). Comparison of cLCC measurements with a non-captured, or at least a captured but non-transported control group, would be necessary to more accurately interpret the effect of transport on neutrophil function in rhinoceros.

In agreement with other studies (163,165,268), we did not find any correlations between cLCC, adrenaline and cortisol concentrations. This lack of correlation has been attributed to individual variations, differing physiological strategies to cope with stress and the different time scales of variables involved in the stress response (163). Furthermore, we did not find any significant differences in cLCC between the two rhinoceros groups. In horses, midazolam has been shown to induce a dose-dependent reduction on peripheral blood neutrophil function (283). The midazolam dose we used (0.010 – 0.015 mg/kg) was much lower than the starting dose (0.06 mg/kg) used in Massoco and Palermo-Neto's (2003) study (283), which may be the reason for the lack of effect. Future studies in rhinoceros need to investigate dose-dependent effects of midazolam on stress response indicators.

5.4.4. Oxidative status

Although cLCC did not change over time, the increase in AUC from capture throughout transport indicated a relative increase in ROS generation associated with the increase in neutrophil numbers. Investigating the generation of ROS from the respiratory burst of neutrophils, and other cells, is important because ROS can damage lipids, DNA and proteins and cause oxidative stress (284). Oxidative stress is defined by the imbalance between the production of ROS and the endogenous antioxidant mechanisms, which counteract the effects of ROS (285). Unsaturated fatty acids, such as phospholipids and triglycerides, are particularly vulnerable to oxidation by ROS, leading to a process known as lipid peroxidation (284,285). During lipid peroxidation, a hydrogen ion is removed from the unsaturated fatty acid and the remaining lipid carbon radical undergoes molecular rearrangement to form a CD (285). Following a complex sequence of propagative reactions, lipid hydroperoxide is formed, which then decomposes to malondialdehyde (MDA), a reactive aldehyde, and other products (286). Lipid peroxidation can be identified at different stages by measuring: (1) losses of unsaturated fatty acids (e.g. triglycerides and phospholipids), (2) increases of primary peroxidation products (e.g. CD), or (3) increases of secondary

peroxidation products (e.g. MDA) (286). In our rhinoceros, the concentrations of unsaturated fatty acids were lower, and CD higher, at capture compared to the start of transport, indicating that lipid peroxidation occurred at this time point. Rhinoceros appeared to recover from the oxidative stress by the start of transport, but towards the end of transport triglycerides decreased and CD increased again. Plasma MDA concentrations, measured as TBARS, did not change significantly throughout capture and transport. However, MDA is only a minor product of lipid peroxidation and is quickly metabolised; it is therefore not an ideal biomarker for oxidative status (286). The increase in lipid peroxidation at capture could be caused by the hypoxia (287) that is commonly seen in etorphine-immobilised rhinoceros (33,34); the increase in lipid peroxidation towards the end of transport could be caused by the relative increase in ROS generation coupled with the glucocorticoid induced increase in neutrophil numbers. However, other glucocorticoid induced effects, for example, the modulation of gene expression, likely contributed to the development of oxidative stress (288). Increases in oxidative stress have been associated with prolonged elevated secretion of glucocorticoids and thus, chronic stress (288,289). Chronic stress can increase susceptibility to disease, reduce reproductive capacity and lead to maladaptation after release (9). Whether the rhinoceros in this study started to experience chronic stress is unclear and remains subject of future studies that also implement comprehensive post-release monitoring.

Another method to assess free radical activity is the measurement of plasma antioxidants (286), which we did by using the (indirect) ORAC method. Oxygen radical absorbance capacity is an integrated variable, which reflects the complex interaction amongst antioxidant substances and their effect on the oxygen status of a sample (285). Interestingly, the trend of ORAC over time differed between the two rhinoceros groups. Azaperone-sedated rhinoceros experienced a decrease in ORAC after capture indicating that radical-scavenging antioxidants were consumed in response to ROS generation (286). In midazolam-sedated rhinoceros ORAC did not drop at this time point, perhaps because of the delayed increase in neutrophil

numbers. However, at capture and at the end of transport ORAC was lower in midazolam-sedated than in azaperone-sedated rhinoceros. Whilst butyrophenones have been linked to increased ROS production and cytotoxicity (290), midazolam has been found to inhibit mitochondrial ROS production in endothelial, neural and inflammatory cells (283,291). The finding that, at TC and T6, midazolam-sedated rhinoceros appeared to produce more ROS than azaperone-sedated rhinoceros is surprising and could be linked to the doses we used in this study. To better understand the modulation of oxidative burst potential by these drugs in rhinoceros, dose- and time-dependent effects on ROS production need to be investigated in the future.

5.4.5. Acute phase reactants

Another core part of the innate immune system in response to a stressor is the acute phase response (166). Rising adrenaline and glucocorticoid concentrations and associated release of pro-inflammatory cytokines and ROS drastically alter liver metabolism and lead to an increase (positive) or decrease (negative) of specific acute phase reactants (APRs) (292). In white rhinoceros, fibrinogen and haptoglobin have been identified as positive, and iron and albumin as negative APRs (203). In the rhinoceros of this study, fibrinogen and haptoglobin concentrations did not increase; instead, they decreased from capture to transport. The unexpected changes in these proteins were similar to those seen for total protein, albumin and globulins, which were all likely relative changes caused by fluid shifts associated with stress-haemoconcentration (256). Acidosis, which is a common finding in immobilised rhinoceros (33,34), is known to increase fibrinogen breakdown and could have contributed to the observed decrease in this variable (293). In the black and white rhinoceros of chapter 3, haptoglobin concentrations also did not change. These results suggest that, as in other species, fibrinogen and haptoglobin are probably not major APRs in rhinoceros, meaning that the increase in concentrations is not marked and their response is slow and prolonged in duration and elevations can be observed in

more chronic processes (166). Although fibrinogen and haptoglobin gave no indication that rhinoceros mounted an acute phase response to capture and transport, the negative APRs albumin and iron indicated otherwise and decreased. In contrast to albumin, iron concentrations are unlikely to be affected by haemoconcentration and might therefore present a more reliable indicator of an acute phase response than the large proteins (> 69 kDa) when fluid shifts are expected (256,294). Lastly, cholesterol concentrations decreased. Cholesterol is not a traditional acute phase reactant, but multifactorial reduction in reverse cholesterol transport occurs during an acute phase response resulting in a decrease in this variable (218). Further research is required to fully elucidate minor and major APRs, and their role during a stress response, in the white rhinoceros.

After six hours of transport, albumin and cholesterol concentrations were higher in midazolam-sedated compared to azaperone-sedated rhinoceros indicating that the acute phase response might have been modulated in rhinoceros that received midazolam. Similar results have been reported in rats with induced systemic polyarthritis and attributed to an immunomodulating effect of the benzodiazepine (280). However, peripheral vasodilation and haemodilution caused by the alpha-1-adrenergic-blocking effects of azaperone might have also occurred at this time (295,296). Nevertheless, it seems important to further investigate potential immunomodulating effects of the midazolam in rhinoceros.

5.5. SUMMARY AND CONCLUSION

Increases in plasma adrenaline and serum cortisol concentrations indicated that rhinoceros mounted a stress response to capture and transport, which was associated with characteristic haematological and immunological changes. Red blood cell to plasma volume decreased from TC to T6 indicating that stress-haemoconcentration resulting from the effects of the circulating catecholamines (i.e. hypertension and, possibly, splenic contraction) occurred at capture. In response to the circulating glucocorticoids, neutrophils progressively increased and lymphocytes and

eosinophils progressively decreased from TC to T6. Rhinoceros experienced a decrease in unsaturated fatty acids and an increase in lipid peroxidation products indicative of oxidative stress. A reduction in serum iron concentrations suggested an acute phase response.

It is important to understand that these immunological changes have a protective purpose in an acute situation and prepare the immune system for challenges that may be imposed by the stressor (3,155). In chronic situations, which persist from days to months, these adaptive immunological responses may become harmful and increase susceptibility to disease (3,9). In our rhinoceros, midazolam appeared to mitigate immunological responses to stress, particularly in the leukon, but not the stress response per se. Several studies have identified an increased risk of developing disease (e.g. pneumonia, orthopox virus infection) in patients exposed to benzodiazepines (297–299). This risk, together with the fact that wildlife translocation has already been linked to chronic stress and morbidity (9,10,174), raises questions about the repetitive use of midazolam during capture and transport in white rhinoceros. Further research is necessary to investigate if rhinoceros develop an increased risk to disease after translocation when midazolam is used. Dose- and time-dependent immunomodulating effects of this drug need to be explored, as well as a potential anti-inflammatory effect. Particularly during immobilisation, favourable effects of the midazolam, such as muscle relaxation, might outweigh any negative side-effects (190). In order to establish guidelines for balanced and safe use of midazolam in the capture and transport of white rhinoceros, both favourable and unfavourable effects of this drug need to be investigated further.

CHAPTER 6: DISCUSSION AND CONCLUDING REMARKS

The research described in this thesis covered the exploration of the effects of capture and transport on:

1) Selected clinical chemistry analytes in the black and white rhinoceros.
2) Electrolyte and acid-base status in white rhinoceros bulls.
3) Haematological and immunological indices in the black (APRs and oxidative stress) and white rhinoceros (erythron, thrombon, leukon, LCC, APRs and oxidative stress).

Changes in these analytes were compared between white rhinoceros bulls sedated with either midazolam or azaperone.

Black and white rhinoceros transported over a long time experienced total body water loss, mobilisation of energy reserves, muscular damage and stress-induced immunomodulation. White rhinoceros bulls experienced respiratory acidosis combined with a lactic- and non-volatile weak acid acidosis during capture, from which they recovered quickly, followed by a mild metabolic- and strong ion alkalosis during transport. Increases in plasma adrenaline and serum cortisol concentrations confirmed that rhinoceros mounted a stress response to capture and transport. The stress response induced characteristic haematological and immunological changes including stress-haemoconcentration, a progressive increase in N:L ratio, the mounting of an acute phase reaction and oxidative stress.

Based on these results, similar to Fisher et al (2009) (68), we identified the following challenges to animal welfare associated with rhinoceros capture and transport: (1) life-threatening acid-base abnormalities associated with the unique challenges of rhinoceros capture; (2) fear and the mounting of a stress response to capture and the novelty of transport; (3) stress-induced immunomodulation; and (4) skeletal muscle

fatigue, energy imbalance and dehydration that likely become more relevant with transport time.

Acidaemia is a well-documented adverse effect associated with the use of etorphine and azaperone, and the mounting of a fight or flight response during a pursuit, in rhinoceros capture (33–35,44). We measured acid-base responses to capture and transport only in the white rhinoceros bulls translocated within the KNP (chapter 4), but elevated serum lactate concentrations at capture in the white rhinoceros translocated from South Africa to Botswana (chapter 3) suggest that these animals also experienced metabolic (lactic) acidosis. The black rhinoceros (chapter 3) were captured with a different method, in bomas, and did not experience as marked an elevation in serum lactate concentrations as the white rhinoceros. Arterial blood samples should be collected in future studies, and blood gas analysis performed, to identify this possible advantage of low-dose etorphine capture in boma housed rhinoceros. To determine the value of temporary captivity as part of the translocation process, possible negative aspects, such as maladaptation, also need to be taken into consideration.

Acidaemia can result in catecholamine release, together with an impaired myocardial and vascular responsiveness to the catecholamines, causing a reduction in cardiac function, cardiac arrhythmias and systemic hypotension (300). Neurologically, acidaemia can cause lethargy, seizures, stupor, coma, and ultimately death (248). Acidaemia becomes even more lethal when it is combined with hypoxaemia, which is a well-known complication in etorphine-immobilised rhinoceros (33–35). It is therefore not surprising that mortalities have been associated with rhinoceros capture (24,52). By combining the etorphine with midazolam, instead of azaperone, we achieved a reduction of the metabolic (lactic) acidosis in the KNP white rhinoceros bulls (chapter 4). This reduction was likely attributed to the muscle relaxing effects of the benzodiazepine (190). We did not measure arterial blood gases and arterial blood pressure in these rhinoceros and therefore do not know if etorphine-induced

hypoxaemia and hypertension were also reduced with this combination. Further studies need to investigate these cardiopulmonary effects associated with the administration of midazolam in etorphine-immobilised white rhinoceros.

The white rhinoceros bulls recovered quickly from the acidosis at capture and developed a mild (metabolic) alkalosis during transport. This alkalosis could be a result of a slow renal response to the preceding acidosis combined with the effects of a stress response (248,250). Alternatively, white rhinoceros could naturally have a slightly higher pH than other species, because of their herbivore diet (251). This assumption needs to be tested in non-stressed and non-immobilised animals.

Stress has been identified as the main cause of mortality during rhinoceros translocation (24). Although we found no change in serum cortisol concentrations between capture and release samples of black and white rhinoceros transported over a long time (chapter 3), serial blood sample results from the white rhinoceros bulls indicated otherwise (chapter 5). Following capture and journey commencement, after an immediate short rise in plasma adrenaline concentrations and a gradual initial increase in cortisol concentrations, white rhinoceros bulls showed a decline in cortisol concentrations (chapter 5). These results indicate that (1) we likely missed peak plasma concentrations in the rhinoceros that were transported over a long time by only taking capture and release samples, and (2) capture likely represents the main stressor to the rhinoceros during capture and transport (68). This finding is in agreement with domestic animal studies that identified "handling and loading" as the main source of "stress" during transport (68). The mounting of a stress response is not necessarily bad for the animal and has an adaptive purpose aiming to promote immediate survival (3). On the scale of seconds to a few minutes energy is mobilised and diverted to the exercising muscle; immune function modulated to fight the stressor; reproductive physiology, appetite and feeding behaviour inhibited; and cerebral perfusion and glucose utilisation increased to sharpen cognition (3,46). The magnitude and duration of stress-induced increases in catecholamine and

glucocorticoid hormones have major effects on immune cell distribution and function, stress response physiology (e.g. responsiveness to catecholamines or glucocorticoid secretion), and behavioural coping (3,9).

Although we were able to obtain a blood sample during the peak phase of the cortisol stress response, comparison to baseline cortisol concentrations was not possible because it requires blood sample collection within a few minutes of the onset of the stressor (in this case the helicopter pursuit) (155). Moreover, there are no reference intervals for any of the measured variables in resting, non-stressed, and non-immobilised wild rhinoceros, which could have been used to aid interpretation of results. The use of remote sampling devices or the collection of resting samples from habituated animals in captivity could possibly fill that gap and facilitate the interpretation of stress-related variables in the future. However, due to high intraindividual variations, particularly in stress response indicators, comparing once-off measurements to population-based reference intervals can be of limited value and interpreting the trend of measured variables within individuals, as done in this thesis, is recommended (139). The use of leukocyte profiles for assessing a stress response to capture and transport was of particular value in our rhinoceros, because of the time-lag in the leukocyte response to stress (155).

Stress-induced immunomodulation, to varying degrees, was observed in all of our rhinoceros. The stress response induced a gradual decrease in blood lymphocyte and eosinophil numbers, a gradual increase in neutrophil and blood leukocyte numbers (chapter 5), the mounting of an acute phase response and the generation of ROS (chapter 3 and 5). These immunological responses prepare the rhinoceros for challenges (e.g. wounding or infection) that might be caused by the stressor (e.g. attack by a predator; in this case the helicopter pursuit) and have a protective function (154,155). However, with time this protective immunological function of the stress response might become detrimental to the rhinoceros and start causing problems (3,9,10). The point at which the stress response itself becomes harmful is not known in

rhinoceros translocation, but presumably varies in a circadian and seasonal manner, and between individuals (301).

During translocation, animals are exposed to repetitive acute stressors, such as capture, the initiation of transport, and the release into a novel environment, and prolonged stressors, such as transport, temporary captivity, and adaptation to a novel environment, and are likely to develop a chronic stress response (9,10). Chronic exposure to elevated glucocorticoid concentrations and their effects may cause wear and tear (decreased capacity to cope with environmental change) and an increased likelihood to develop pathology (301). Translocated individuals often show an increased susceptibility to disease, an increased risk of predation, a decreased reproductive capacity, anorexia and dispersal from the release site (9). Most of these complications have also been documented in translocated black and white rhinoceros and attributed to chronic stress (9,38,112,177,302).

A possible indicator for the mounting of a chronic stress response in our rhinoceros was the development of oxidative stress. Oxidative stress, measured as lipid peroxidation and oxidative damage to RNA or DNA, has been directly linked to chronic stress exposure in women (289) and is currently emerging as a cumulative indicator of chronic stress and animal welfare in wildlife (303). If this is true, the increased lipid peroxidation observed in the black rhinoceros in chapter 3 potentially indicates the chronicity of a stress response, which could have been associated with boma confinement and should be investigated further.

The white rhinoceros bulls in chapter 5, after an initial increase in lipid peroxidation, which was quickly resolved, experienced another increase in lipid peroxidation after six hours of transport, suggesting that the threshold where the stress response becomes harmful might have been reached. Further research investigating different immune cell subpopulation numbers and function (e.g. CD4+ and CD8+ T cells, natural killer cells) as well as inflammatory cytokine concentrations (interferon γ, tumour necrosis factor) should be assessed to better identify and understand chronic immunological responses to translocation stress (3). New, non-invasive markers of

oxidative stress could be implemented in post-release monitoring and investigated together with spatial, behavioural, hormonal, and disease measurements to identify animals with chronic stress and increased risk of developing morbidity after translocation. These measurements could be used to further investigate the effectiveness and clinical importance of applied interventions that aim to mitigate translocation stress.

Regardless of the mounting of an acute or chronic stress response, factors like **muscular fatigue, energy imbalance and dehydration** became important with increasing transport time in both black and white rhinoceros, representing stressors themselves.

All animals, including the ones transported for only six hours, experienced a pronounced increase in CK and AST over time indicating that myocyte metabolic dysfunction and skeletal muscle damage occurred (chapter 3 and 4) (260). These findings are also common in transported domestic and other wild animals (95,124,141,220) and have been attributed to tiredness and fatigue associated with a prolonged state of muscle contraction due to standing and might be intensified in rhinoceros because of their heavy weight (68). Intense exercise during the capture, repeated intramuscular administration of the tranquilising drugs, and dehydration likely further contribute to this issue (48,221,239).

In domestic animals, it is believed that increases in muscle enzymes tend to be indicative of knocks and bruises (68). Rhinoceros are generally sedated during transport and the drugs used for sedation, including the benzodiazepines, are known to affect neuromuscular processing related to balance control (304). These effects mean that the risk of losing balance to sudden perturbations during transport, and being knocked and bruised, are probably high in transported rhinoceros.

Interventions that might alleviate muscle injury could be: (1) providing padding to the transport crate, (2) motivating the rhinoceros to stand more comfortably (e.g. providing food might motivate animals to stand more put in order to eat), and, or (3)

using drugs, or doses, that cause less ataxia and allow the animal to have better footing and lay down when required. These interventions need to be systematically investigated in future research.

Another factor that became more important over time was energy metabolism. Glucose concentrations did not differ between "capture" and "after transport" samples in black and white rhinoceros transported over a long time (chapter 3). In the white rhinoceros bulls, glucose peaked at capture, likely in response to elevated adrenaline concentrations from a fight or flight response, but did not change throughout a six hour transport (chapter 4). Although glucose concentrations gave no indication that rhinoceros entered a negative energy balance, increasing BHB and NEFA concentrations (white rhinoceros chapter 3 and 4) over time demonstrated that there was mobilisation of lipid stores from the adipose tissue to generate energy (258). The black rhinoceros in chapter 3 only showed an increase in plasma triglyceride concentrations, which could also indicate the mobilisation of lipid stores for energy, or could be the result of oxidative stress. Again, the effect of temporary captivity on these clinical chemistry analytes should be investigated in more detail. To prevent rhinoceros from entering a negative energy balance during transport, further research on nutritional planning (i.e. type and amount of food) prior to, and during translocation, is also necessary.

Finally, black and white rhinoceros transported over a long time (over 19 hours) (chapter 3) developed dehydration. Long periods of time without access to water (and food) have been identified as a major concern in domestic animal transports (69). Rhinoceros may be exposed to prolonged thirst and dehydration during long transport even if water is offered, as they are usually reluctant to drink during transport from unknown sources (M. Hofmeyr, pers. comm.). It is important to note that there are interactions between the thermal conditions of a journey and the animals' resistance to the effects of water deprivation (68). In rhinoceros, this resistance appears to be high under natural conditions, because these animals usually

only drink once a day to every second day (29,211). However, if thermal conditions are very hot, rhinoceros are likely to develop dehydration more easily (68,69). Current recommendations state that rhinoceros should be captured when temperatures are lower than 25°C and transported during colder months of the year (24,29). Even if translocations take place during cold and dry seasons, it can still become very hot in the middle of the day, and especially if crates are not adequately ventilated. Therefore, it is important to monitor thermal conditions, especially in the crates during transport, and further investigate the effects of changing thermal conditions on the rhinoceros' resistance to water deprivation. Currently there are no recommended limits for transport-duration, or water deprivation times, for rhinoceros. Importantly, these should be established.

To provide a logical and comprehensive framework for good animal welfare, the Farm Animal Welfare Council of the United Kingdom established the concept of the five freedoms, defining ideal states for an animal's physical and mental condition, which also apply during transport (179). These freedoms include: (1) freedom from hunger and thirst; (2) freedom from discomfort; (3) freedom from pain, injury, or disease; (4) freedom to express normal behaviour; and (5) freedom from fear and distress (73,179). Later, from these five freedoms, five domains of potential animal welfare compromise have been established, namely: nutrition, environment, health, behaviour, and mental state (178). To a certain degree, it appears to be possible to comply with most of these aspects during rhinoceros translocation.

By administering midazolam, instead of azaperone, we attempted to reduce stressors associated with capture and transport and enhance the freedom from fear and distress, or mental state (chapter 5). However, we were not able to demonstrate a stress reducing effect of this drug at the given dose. Instead, we identified a possible immunosuppressive effect of the benzodiazepine. Further studies are required to find out if this effect is dose-dependent, of prolonged duration, and if it leads to an increase in the rhinoceros' susceptibility to disease after transport. In rats, midazolam reversed

behavioural deficits associated with chronic stress and mitigated stress-induced hyperthermia (191,193). These effects remain to be investigated in transported rhinoceros as the benefit of behavioural coping might be more important than the immunological side effects.

A main limitation of this study was the lack of control animals, which would have helped in 1) better differentiating the effects of capture versus transport, and 2) the effects of tranquiliser-administration during transport. We tried to differentiate the effects of capture from the effects of transport by taking a "start transport (T0)" blood sample. This differentiation worked well with clinical variables that change quickly (e.g. electrolytes), but was only of limited value for slow-changing variables (e.g. N:L ratio). Ideally, a set of control animals should have been immobilised, sampled and then released without any form of transport. Samples would then have had to be collected from these animals after release at the same intervals as for the transported animals without re-immobilising them. Clearly, this would have been extremely difficult and could not be done. Moreover, for ethical reasons, we decided against a non-tranquilised but transported control group, because the risk of injury would have been too high. Nevertheless, we believe that this study allowed us to identify major challenges to animal welfare associated with capture and transport in rhinoceros.

To sum up, rhinoceros experienced respiratory and metabolic (lactic) acidosis during capture and a mild metabolic alkalosis during transport. The mounting of a stress response to capture and transport was associated with characteristic immunological changes. Skeletal muscle fatigue, energy imbalance and dehydration occurred over time. Midazolam reduced the metabolic acidosis during capture, but was associated with immunosuppression.

Based on these results, we recommend the use of midazolam, instead of azaperone, for etorphine-based capture of white rhinoceros. During transport, there appears to be no benefit in using midazolam over azaperone. Further studies investigating

cardiopulmonary, immunological, and behavioural differences between these drugs during rhinoceros translocation are required.

To improve animal welfare during long transports, future research needs to investigate other interventions that might enhance one, or more, of the "five freedoms", or "domains" during rhinoceros translocation. Offering fluids and, or, nutritional supplements could increase the freedom of hunger and thirst during rhinoceros transport (nutritional domain). Padding of the transport crates, and better monitoring and regulating of environmental conditions within them, could increase the freedom of discomfort (environmental domain) and the use of midazolam could improve behavioural coping during transport (behavioural domain) and therefore be advised despite its immunological effects. Animal health is an important part of animal welfare and improving animal welfare during transport will increase the freedom of disease during, and after, rhinoceros transport (health domain) (72).

The ultimate goal of this thesis was to help improve the outcome of rhinoceros translocation and contribute towards conservation of the species. The information gained from this thesis, importantly, has paved the way for further studies that can now be aimed at reducing the stressors, and their consequences, that are induced by capture and transport in rhinoceros, thereby improving animal welfare and the success of rhinoceros conservation translocations.

CHAPTER 7: FUTURE RESEARCH DIRECTIONS

The results of this thesis, in particular the work done on the physiological effects of long road transport in black and white rhinoceros and the modulation of these effects by using of midazolam instead of azaperone, point the way for several new studies. New avenues of investigation could include:

1. Establishment of limits for transport-duration, or water and food deprivation times, in black and white rhinoceros.
2. Exploration of the value of temporary confinement as part of rhinoceros translocation.
3. Exploration of immunological responses to chronic stress by investigating characteristic changes in leukocyte subpopulation numbers and function and inflammatory cytokine concentrations.
4. Investigation of nutritional feasibility and planning (e.g. type and amount of food, potential side-effects) for rhinoceros during transport.
5. Investigation of the effects of changing thermal conditions on the rhinoceros' resistance to water deprivation.
6. Identification of effective methods of fluid administration (e.g. route of administration, type of fluid, and possible parenteral feeding) during long transport in rhinoceros
7. Systematic investigation of midazolam use in black rhinoceros.
8. Further investigation of cardiopulmonary effects when midazolam is used instead of azaperone for the capture of white rhinoceros to determine arterial blood pressure and arterial blood gases.
9. Further investigation of immunological effects associated with the use of midazolam in white rhinoceros to further characterise white cell function and the susceptibility to disease after transport.

10. Exploration of possible behavioural benefits of using midazolam, instead of azaperone, for the transport of white rhinoceros
11. Post-release monitoring should be included in future studies to better evaluate the clinical relevance of measured physiological responses to capture and transport in black and white rhinoceros and their modulation by implemented interventions.

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APPENDICES

PUBLICATIONS

1) **Pohlin F**, Hoojiberg EH, Meyer LCR. A review on the effects of transport on animal welfare in wild mammalian species. Review article; submitted to the Journal of Zoo and Wildlife Medicine (in review).
2) **Pohlin F**, Hofmeyr M, Hooijberg EH, Blackhurst D, Reuben M, Cooper D, Meyer LCR. Challenges to animal welfare associated with capture and long road transport in boma-adapted black (*Diceros bicornis minor*) and semi-captive white (*Ceratotherium simum simum*) rhinoceroses. J Wildlife Dis 2020; 56(2):000-000 (in press).
3) **Pohlin F**, Buss P, Hooijberg EH, Meyer LCR. Midazolam alters acid-base status less than azaperone during the capture and transport of wild Southern white rhinoceros (*Ceratotherium simum simum*). Research article; submitted to the Journal of Veterinary Anesthesia and Analgesia (in review).
4) **Pohlin F**, Hooijberg EH, Buss P, Huber N, Viljoen FP, Blackhurst D, Meyer LCR. Haematological and immunological responses to capture and transport stress in wild white rhinoceros bulls (*Ceratotherium simum simum*) and their modulation by midazolam compared to azaperone. Research article; in preparation.

DOI: 10.7589/2019-02-045

Journal of Wildlife Diseases, 56(2), 2020, pp. 000–000

CHALLENGES TO ANIMAL WELFARE ASSOCIATED WITH CAPTURE AND LONG ROAD TRANSPORT IN BOMA-ADAPTED BLACK (*DICEROS BICORNIS*) AND SEMI-CAPTIVE WHITE (*CERATOTHERIUM SIMUM SIMUM*) RHINOCEROSES

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ABSTRACT: Capture and transport are part of translocation and expose animals to a variety of stressors that can lead to morbidity and mortality. We aimed to establish a better understanding of the physiologic responses to capture and transport in black (*Diceros bicornis*) and white (*Ceratotherium simum simum*) rhinoceroses in Southern Africa. Fourteen adult black rhinoceroses were transported 600 km by vehicle and 32 white rhinoceroses (24 adults and 8 juveniles) were transported 1,300 km by vehicle. The black rhinoceroses had been wild-caught and boma-adapted over 6 wk prior to the translocation and were only sedated to allow for loading into the transport crates. The white rhinoceroses originated from a game farm and were chemically immobilized from a helicopter and then loaded. Paired blood samples were collected from animals at loading (capture) and after transport and evaluated for changes in clinical chemistry analytes, acute phase reactants, and oxidative stress biomarkers. The Wilcoxon rank sum test was used to compare changes in measured analytes from capture and after transport. All rhinoceroses survived capture and transport. Rhinoceroses experienced total body water loss, mobilization of energy reserves, and muscular damage. Alterations in acute phase reactants suggested that animals mounted a stress response. Oxidative stress was observed in black rhinoceroses. We identified the following challenges to animal welfare during transport: hydration status, energy balance, skeletal muscle fatigue, and stress-induced immunomodulation. Measures to mitigate these challenges, such as administration of fluids, need to be included in the planning of future translocations.

Key words: Energy balance, fatigue, hydration, rhinoceros, stress, translocation, transport.

INTRODUCTION

The Southern-central black rhinoceros (*Diceros bicornis minor*) is listed as critically endangered, and the Southern white rhinoceros (*Ceratotherium simum simum*) as near threatened, by the International Union for Conservation of Nature (IUCN) Red List of Threatened Species (Emslie 2011, 2012). The main reasons for these assessments are the continued and increased poaching threat and the increasing illegal demand for rhinoceros horn associated with the increased involvement of organized international criminal syndicates in rhinoceros poaching (Emslie et al. 2016). Translocation for population reintroduction or reinforcement, or metapopulation management, represents an essential tool for the management of these species and is an integral part of national and international rhinoceros conservation plans (Knight 2017). Translocation involves capture, temporary captivity, transport, and release into a novel environment, exposing the animals to a variety of stressors such as prolonged periods of

CONGRESS ORAL PRESENTATIONS RELATED TO THIS THESIS

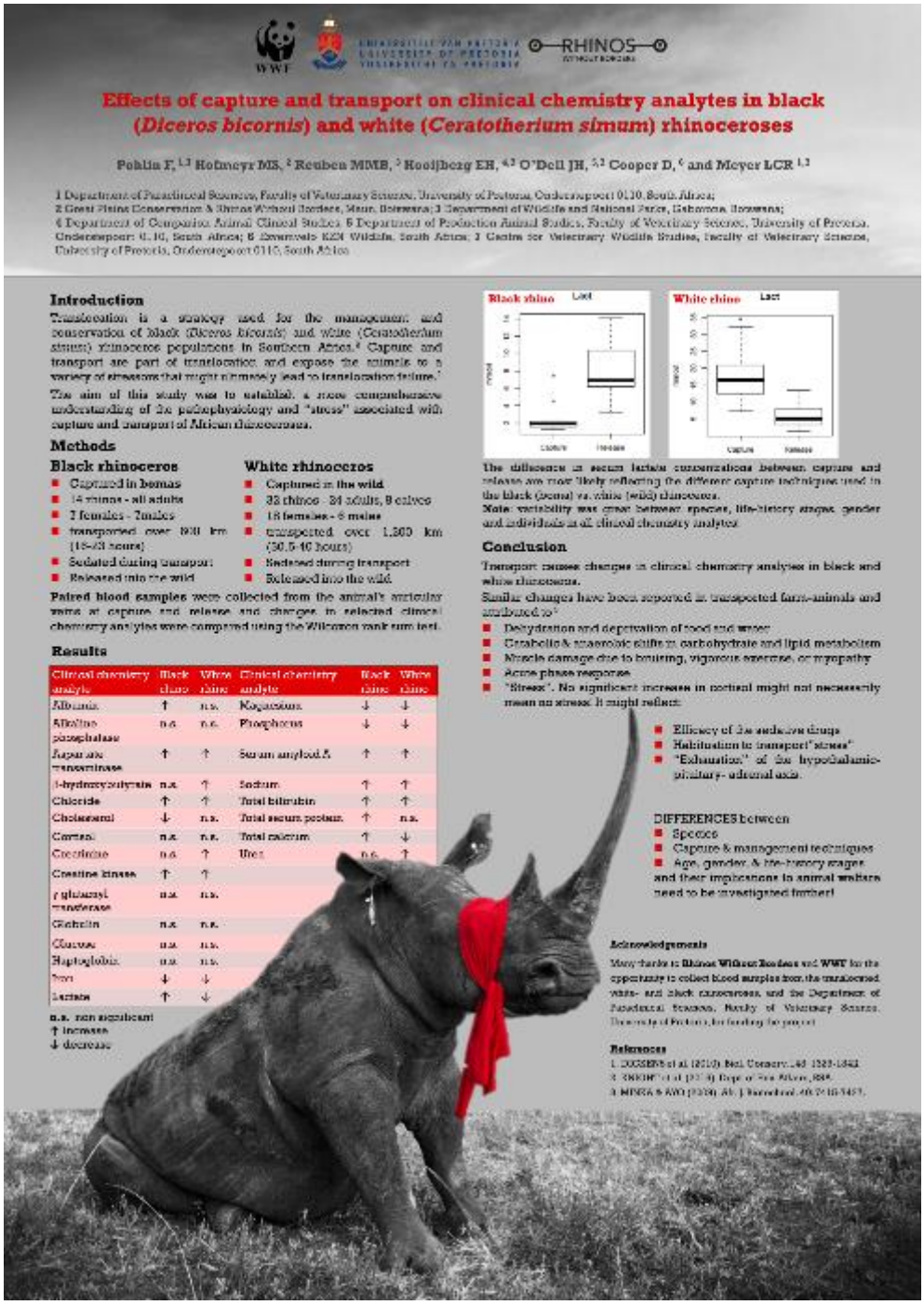
1) **Pohlin F**, O'Dell JH, Hooijberg EH, Cooper D, Leeming R, Flamand J, Meuffels J, Meyer LCR. Effects of transport on clinical chemistry analytes in black rhinoceros (*Diceros bicornis*) translocated in South Africa. Wildlife Group of the South African Veterinary Association (SAVA) Annual Congress, Muldersdrift, South Africa, March 2018.

2) **Pohlin F**, Hofmeyr M, Reuben M, Hooijberg EH, Meyer LCR. Effects of capture and transport on clinical chemistry analytes in white rhinoceros (*Ceratotherium simum*) translocated for over 30 hours. Joint AAZV/ EAZWV/ Leibniz-IZW Zoo and Wildlife Health Conference, Prague, Czech Republic, October 2018. Murray Fowler International Conference awardee.

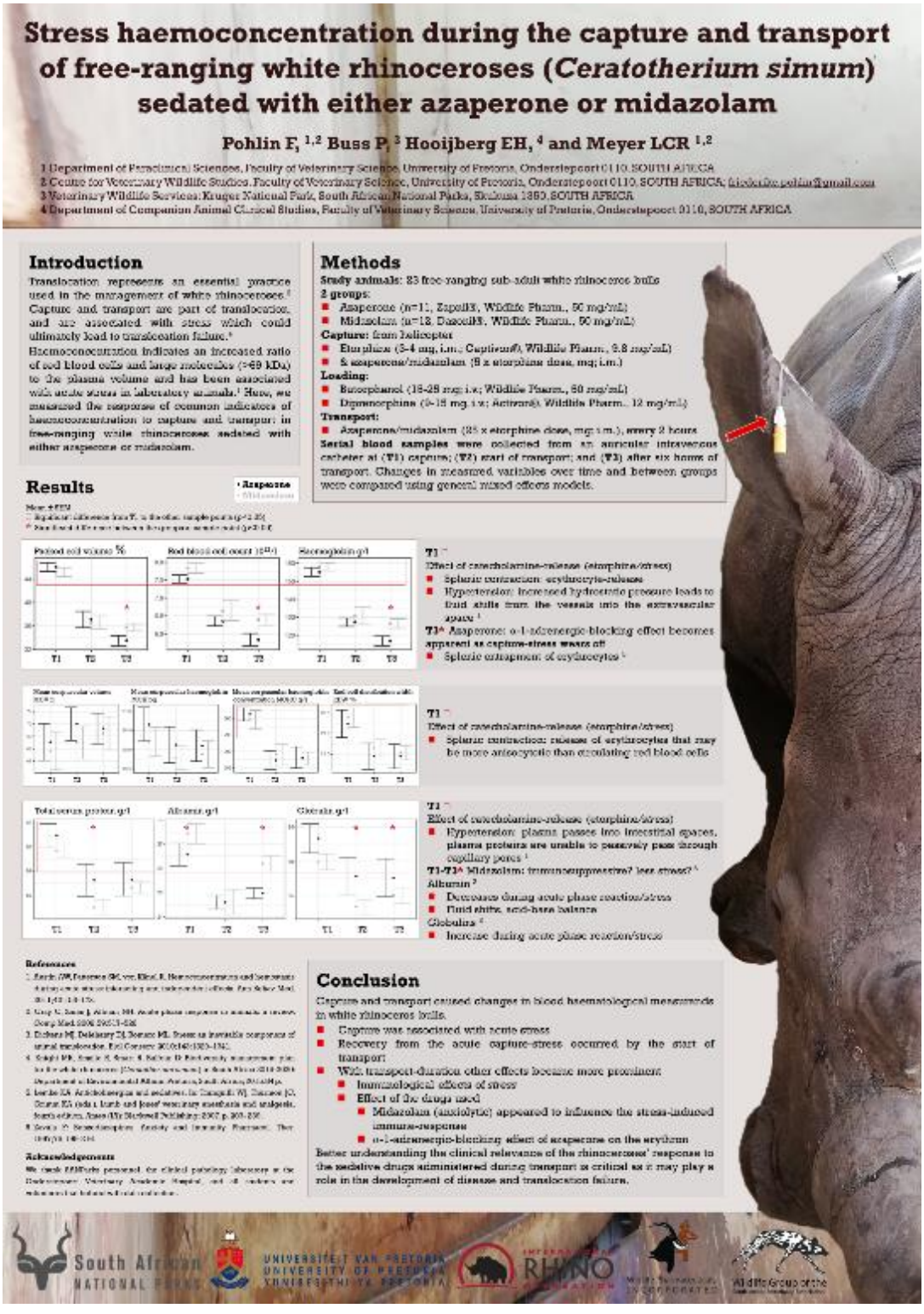
3) **Pohlin F**, Buss P, Hooijberg EH, Meyer LCR. Using haematological measurands to assess translocation-stress in white rhinoceros (*Ceratotherium simum*) sedated with either azaperone or midazolam. Wildlife Group of the SAVA Annual Congress, Muldersdrift, South Africa, March 2019.

CONGRESS POSTER PRESENTATIONS RELATED TO THIS THESIS

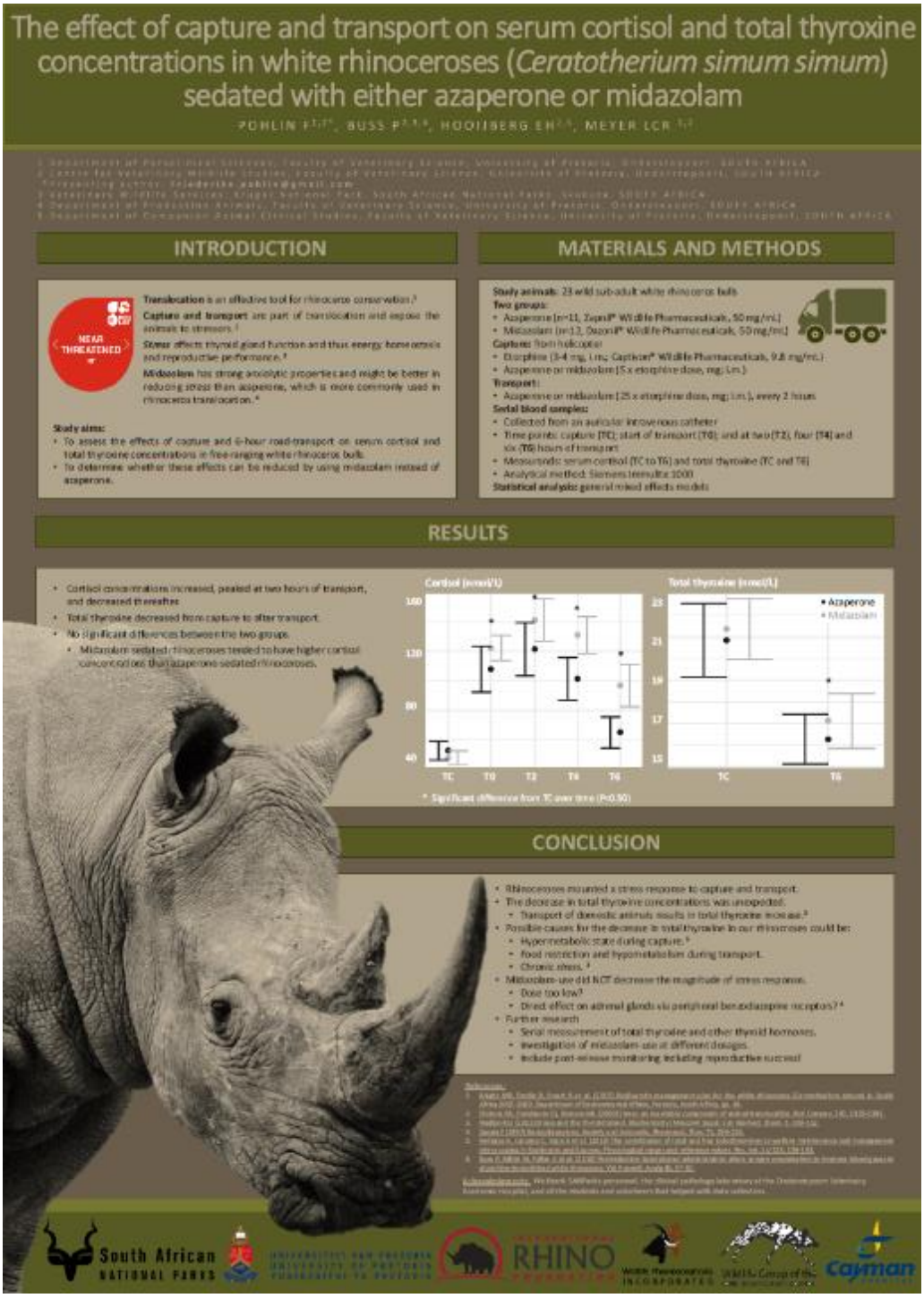
1) **Pohlin F**, Hofmeyr M, Reuben M, Hooijberg EH, O'Dell JH, Cooper D, Meyer LCR. Effects of capture and transport on clinical chemistry analytes in black (*Diceros bicornis*) and white (*Ceratotherium simum*) rhinoceros. Faculty day, Faculty of Veterinary Sciences, University of Pretoria, Onderstepoort, South Africa, August 2018. Winner of the best poster award.



2) **Pohlin F**, Buss P, Hooijberg EH, Meyer LCR. Stress haemoconcentration during the capture and transport of free-ranging white rhinoceros (*Ceratotherium simum*) sedated with either azaperone or midazolam. Joint Leibniz-IZW/ EAZWV/ ECZM Zoo- and Wildlife Health Conference, Kolmården, Sweden, June 2019.



3) **Pohlin F**, Buss P, Hooijberg EH, Meyer LCR. The effect of capture and transport on serum cortisol and total thyroxine concentrations in white rhinoceros (*Ceratotherium simum simum*) sedated with either azaperone or midazolam. Conference of the International Society of Wildlife Endocrinology (ISWE), Skukuza, Kruger National Park, South Africa, October 2019. Cayman Chemical travel grant awardee. Winner of the best poster award.



OTHER PRESENTATIONS OR INITIATIVES RELATED TO THIS THESIS

1) Online presentation on "translocation stress in wildlife" at the joint Southern Africa Wildlife Disease Association Student Chapter (SAWDASC)/ Zoo and Wildlife Medicine Study Group (ZWMSG) online journal club, 2017 (repeated 2018).
2) Oral presentation on "translocating wildlife" at the Conservation of Exotics, Zoo- and Wildlife Symposium, Ghent University, Merelbeke, Belgium, 2017.
3) "Run rhino run" flash tattoo (small tattoos for a good cause), raising public awareness for rhinoceros poaching and funds for this project by DEFF INK tattoo, Berlin, Germany 2018.

KOBEN 2018

4) Oral presentation on "rhinoceros translocation" at the rhinoceros conservation fundraiser „Studierende gegen Wilderei - Ein Abend für den Nashornschutz", Vetmeduni Vienna, Austria, 2018.
5) Oral presentation on "physiological responses to capture and transport in black and white rhinoceros" at the Wildlife Disease and Conservation Evening of the SAWDASC at the University of Cape Town, Cape Town, South Africa, 2019.
6) Presentation evening to the general public on "illegal wildlife trafficking and rhinoceros poaching" at UFO, Bruneck, Italy, 2019.
7) Interactive children workshop on "illegal wildlife trafficking and rhinoceros poaching" at the Paul-Troger middle school (grade 6 to 8), Welsberg-Taisten, Italy, 2020.

Congratulations to Neil Aldridge for winning the 1st price in the World Press 2018 Photo Contest, category: environment, singles. This photo shows a young southern white rhinoceros, immobilised and blindfolded, which is about to be released into the wild in the Okavango Delta, Botswana, after being captured and transported from South Africa for protection from poachers (chapter 3).

ANIMAL ETHICS CERTIFICATES

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

PROJECT TITLE	**Pharmacological management of stress and its pathophysiological consequences during the transport of the free-ranging white rhinos *(Ceratotherium simum)***
PROJECT NUMBER	**V067-17**
RESEARCHER/PRINCIPAL INVESTIGATOR	**F Pohlin**

STUDENT NUMBER (where applicable)	**U_17310441**
DISSERTATION/THESIS SUBMITTED FOR	**PhD**

ANIMAL SPECIES	**White rhinos *(Ceratotherium simum)***	**White rhinos (Ceratotherium simum)**
NUMBER OF SAMPLES	**3-6 (Pilot study)**	**50 Experiment**
Approval period to use animals for research/testing purposes		**June 2017- June 2018**
SUPERVISOR	**Prof. L Meyer**	

KINDLY NOTE:

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

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

S4285-15

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

PROJECT TITLE	**Pharmacological management of stress and its pathophysiological consequences during the transport of the free-ranging white rhinos *(Ceratotherium simum)***
PROJECT NUMBER	**V067-17 (Amendment 1)**
RESEARCHER/PRINCIPAL INVESTIGATOR	**F Pohlin**

STUDENT NUMBER (where applicable)	**U_17310441**
DISSERTATION/THESIS SUBMITTED FOR	**PhD**

ANIMAL SPECIES	**White rhinos *(Ceratotherium simum)***	
NUMBER OF SAMPLES	**32**	
Approval period to use animals for research/testing purposes		**January 2018 – January 2019**
SUPERVISOR	**Prof. L Meyer**	

KINDLY NOTE:

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

APPROVED (* with condition)	Date 7 February 2018
CHAIRMAN: UP Animal Ethics Committee	Signature

CONDITION

Please submit a detailed progress report of the pilot study as well as an incident report if any occurred

S4285-15

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Veterinary Science
Animal Ethics Committee

5 August 2019

Approval Certificate
Annual Renewal
(Extension 2)

AEC Reference No.:	V067-17
Title:	Pharmacological management of stress and its pathophysiological consequences during the transport of free-ranging rhinoceroses in Southern Africa
Researcher:	Mrs F Pohlin
Student's Supervisor:	Prof LCR Meyer

Dear Mrs F Pohlin,

The **Annual Renewal** as supported by documents received between 2019-06-06 and 2019-07-29 for your research, was approved by the Animal Ethics Committee on its quorate meeting of 2019-07-29.

Please note the following about your ethics approval:

1. The use of species is approved:

Species and Samples	Number
White rhinoceros (*Ceratotherium simum*)	88

2. Ethics Approval is valid for 1 year and needs to be renewed annually by 2020-08-05.
3. Please remember to use your protocol number (V067-17) on any documents or correspondence with the AEC regarding your research.
4. Please note that the AEC may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.

Ethics approval is subject to the following:

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

We wish you the best with your research.
Yours sincerely

Prof V Naidoo
CHAIRMAN: UP-Animal Ethics Committee

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Fakulteit Veeartsenykunde
Lefapha la Diseanse tša Bongakadiruiwa

To develop and manage a system of national parks that represents the biodiversity, landscapes, and associated heritage assets of South Africa for the sustainable use and benefit of all.

South African NATIONAL PARKS

ANIMAL USE AND CARE COMMITTEE: APPLICATION FOR APPROVAL

A. PROJECT DETAILS

Project Title:	Pharmacological management of stress and its pathophysiological consequences during the transport of free-ranging white rhinoceros (*Ceratotherium simum*).		
Researcher	Friederike Pohlin	SANParks Reference No.	009/17

B. SCIENTIFIC REVIEW STATEMENT

(Every application should be supported by a declaration that it has undergone prior scientific review through at least one of the SANParks Research Nodes.)

This research protocol has been reviewed by the Savannah and / or Arid Research Centres SANParks and has been judged to be of national importance, designed in accordance with accepted scientific practices and norms and is in the opinion of the reviewers likely to be successful in achieving its objective.

Name:	Designation:	Signature:	Date:
HHendricks	Snr GM: CSD		30/8/2017

Ps. As per Research Approval process.

Note: In accordance with the South African National Standard (SANS 10386-2008): "The Care and Use of Animals for Scientific Purposes", an animal is regarded as being "live, sentient non-human vertebrate, including eggs, foetuses and embryos, that is, fish, amphibians, reptiles, birds and mammals, including domestic animals, purpose-bred animals, farm animals, wildlife and higher invertebrates such as advanced members from the Cephalopoda and Decapoda".

This form should be submitted with the SANParks standard Research Project Application, and (where relevant) the following supporting documents: CVs of practitioners in support of competence to handle or treat animals, notices of approval of other ethics committees, diagrams or references illustrating the equipment and/or techniques to be applied.

For Administrative Purposes			
Submission Date	27th July, 2017	APPROVED	DISAPPROVED
AUCC approval / Disapproval Date	30/8/2017	Signature	
Reason for Decision	The study fully meets ethical standards		

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