Comparison of the acute stress response in southern white rhinoceros (*Ceratotherium simum simum*) immobilised with four different drug protocols

by

Dr Luke Sotiris Michaelides

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Supervisor

Prof Emma H Hooijberg

BVSc, CertGP (SAP), PhD, Dipl. ECVCP

Department of Companion Animal Clinical Studies and Centre for Veterinary Wildlife Research

Faculty of Veterinary Science

University of Pretoria

Co-supervisor

Prof Leith CR Meyer

BSc, Hon Wildlife Management, BVSc, PhD

Department of Paraclinical Sciences and Centre for Veterinary Wildlife Research

Faculty of Veterinary Science

University of Pretoria

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Faculty of Veterinary Science
Animal Ethics Committee

10 June 2021

Approval Certificate
New Application

AEC Reference No.:	REC057-21
Title:	Acute stress response in southern white rhinoceros (Ceratotherium simum simum) immobilized with four different drug combinations
Researcher:	Dr LS Michaelides
Student's Supervisor:	Prof LCR Meyer

Dear Dr LS Michaelides,

The **New Application** as supported by documents received between 2021-04-21 and 2021-06-04 for your research, was approved by the Animal Ethics Committee on its quorate meeting of 2021-06-04.

Please note the following about your ethics approval:

1. The use of species is approved:

Species	**Number**
White Rhinoceros	8
Samples EDTA tube 4mL	192 (6 mL per rhino in total)
Li heparin tube 4mL	64 (32ml per rhino in total)
Serum tube 4mL	96 (48ml per rhino in total)

2. Ethics Approval is valid for 1 year and needs to be renewed annually by 2022-06-10.
3. Please remember to use your protocol number (REC057-21) on any documents or correspondence with the AEC regarding your research.
4. Please note that the AEC may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.
5. **All incidents** must be reported by the PI by email to Ms Marleze Rheeder (AEC Coordinator) within 3 days, and must be subsequently submitted electronically on the application system within 14 days.
6. The committee also requests that you record major procedures undertaken during your study for own-archiving, using any available digital recording system that captures in adequate quality, as it may be required if the committee needs to evaluate a complaint. However, if the committee has monitored the procedure previously or if it is generally can be considered routine, such recording will not be required.

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.

Room 6-13, Arnold Theiler Building, Onderstepoort
Private Bag X04, Onderstepoort 0110, South Africa
Tel +27 12 529 8434
Fax +27 12 529 8321
Email: marleze.rheeder@up.ac.za

Fakulteit Veeartsenykunde
Lefapha la Diseanse tša Bongakadiruiwa

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And lastly, to my dad, I wish that I could have shared this experience with you. I hope that I've been able to make you proud. This is for you Pops.

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ACTH – Adrenocorticotrophic hormone

AUC – Area under curve

AUCC – Animal Use and Care Committee

CBG – Corticoid binding globulin

CRH – Corticotrophin-releasing hormone

EDTA – Ethylenediaminetetraacetic acid

GABA – Gamma-aminobutyric acid

HCT – Haematocrit

HPA-axis – Hypothalamic-pituitary-adrenal axis

HPLC – High performance liquid chromatography

IUCN – International Union for Conservation of Nature and Natural Resources

KNP – Kruger National Park

LCC – Leucocyte coping capacity

LC/MS – Liquid chromatography mass spectrometry

N: L ratio – Neutrophil: lymphocyte ratio

MHPG – 3-methoxy-4-hydroxyphenylglycol

$PaCO_2$ – Partial pressure of carbon dioxide

PBRs – Peripheral benzodiazepine binding receptors

PCV – Packed cell volume

PMA - Phorbol myristate acetate

PaO_2 – Partial pressure of oxygen

RBC – Red blood cell

ROS – Reactive oxygen species

SANParks – South African National Parks

SNS – Sympathetic nervous system

SPSS – Statistical Package for the Social Sciences

VWS – Veterinary Wildlife Services

Summary

Efforts made to ensure conservation of the southern white rhinoceros (*Ceratotherium simum simum*) include dehorning and translocation, which necessitate chemical immobilisation. Rhinoceros experience high levels of stress during capture, and numerous immobilisation drug combinations have been proposed, with each claimed to be less stressful and safer than others. The purpose of this study was to evaluate the differences in the effects of four commonly-used drug protocols on the acute stress response in white rhinoceros.

Eight sub-adult males were captured in the southern Kruger National Park area and housed in individual bomas. The rhinoceros were chemically immobilised with four etorphine-based drug protocols every two weeks for a 10-week period, using a cross-over design. The protocols used were etorphine + sterile water (control), etorphine + azaperone, etorphine + medetomidine, and etorphine + midazolam. All animals received intravenous butorphanol fifteen minutes after going down into lateral recumbency. Venous blood samples were collected at three time points during immobilisation (0, 20 and 40 min). The following were measured: noradrenaline, adrenaline and their metabolites, dopamine, serotonin, cortisol, glucose, neutrophil: lymphocyte ratio, haematocrit and leukocyte coping capacity. Differences in biomarkers between drug protocols were analysed using a linear-mixed model.

All rhinoceros mounted an acute stress response, as shown by high catecholamines, blood pressure and heart rate. Noradrenaline was lower (p = 0.008) with the midazolam protocol compared to the etorphine-only control and medetomidine protocols and was also lower with the azaperone protocol compared to the medetomidine protocol (p = 0.008), but not compared to the etorphine-only control protocol. There were no other differences in biomarkers between the azaperone and midazolam protocols, and the control protocol. With the medetomidine protocol, glucose was higher compared to etorphine alone (p = 0.003), cortisol was lower compared to the azaperone protocol (p=0.027), neutrophil: lymphocyte ratio was higher than with azaperone and midazolam protocols (p < 0.001) and haematocrit was lower compared to all protocols (p < 0.001).

We found that the addition of azaperone and midazolam yielded a minimal dampening in the stress response when compared to the etorphine control, although noradrenaline was lower with the addition of midazolam. The medetomidine protocol showed the most differences in the stress biomarkers, with the highest N: L ratio and glucose concentration, and significantly lowered haematocrit, which may negatively affect tissue oxygenation. These differences seen with medetomidine may be related to the direct effect of the drug on some of the biomarkers.

The study found that the sedative and tranquilizing drugs currently in use have a limited ability to alter the stress response in rhinoceros chemically immobilised with etorphine, highlighting the need for

research into the addition of other anxiolytic drugs to reduce etorphine-induced effects during rhino immobilisation procedures.

Chapter 1: Introduction

1.1 Background

The southern white rhinoceros (*Ceratotherium simum simum*) has undergone severe population loss over the last decade, with the major contributing factor being the increase in poaching of the animals for their valuable horns (1, 2). They are classified as Near Threatened on the International Union for Conservation of Nature (IUCN) Red List for Threatened Species with the current population estimated to be at approximately 17000-18000 animals, and declining (3, 4). A large proportion of these animals are confined to private game reserves and the Kruger National Park in South Africa (5). This population decline has led to increased efforts to try and conserve the species by various methods, including dehorning to deter poaching (6) and translocation to augment declining subpopulations (7, 8). White rhinoceros have been shown to undergo high levels of stress during capture, handling and translocation, which can affect the success of conservation efforts by negatively impacting their welfare (9) and, in some cases, causing death (8).

In recent years, many studies of the physiological stress response in wildlife have been conducted to improve conservation outcomes (10). Although stress caused by capture, handling and translocation is unavoidable, it can be mitigated by the use of certain appropriate immobilisation drugs that have good anxiolytic properties (11). In this study, I evaluated whether the addition of sedatives and tranquilisers to etorphine-based drug protocols resulted in differences in the acute stress response of white rhinoceros during chemical immobilisation.

1.2 Aim and Objectives

Aim: To evaluate whether there is a difference in the acute stress response of white rhinoceros during chemical immobilisation, with the addition of the sedatives, midazolam or medetomidine, or the tranquiliser, azaperone, to etorphine-based drug protocols.

Objectives:

- To characterise the acute stress response during chemical immobilisation in white rhinoceros by measuring blood levels of a suite of stress biomarkers (catecholamines and their metabolites, serotonin, cortisol, glucose, the neutrophil: lymphocyte (N: L) ratio and haematocrit), heart rate and blood pressure, and evaluating the leukocyte coping capacity (LCC) at standardised time points over a 40-minute period following the induction of chemical immobilisation.
- To compare stress biomarker levels, heart rate, blood pressure and the LCC response in white rhinoceros during chemical immobilisation with four different drug combinations.
- To evaluate associations between stress biomarker levels, heart rate, blood pressure and the LCC response in white rhinoceros during chemical immobilisation.

1.3 Hypotheses

- H_0: Stress biomarker levels, heart rate, blood pressure and LCC responses do not differ between drug protocols when sedative or tranquilizing drugs are added to an etorphine-based protocol for the chemical immobilisation of white rhinoceros.

  H_1: Stress biomarker levels, heart rate, blood pressure and LCC responses differ between drug protocols when sedative or tranquilizing drugs are added to an etorphine-based protocol for the chemical immobilisation of white rhinoceros.

- H_0: Stress biomarker levels, heart rate, blood pressure and LCC responses are not correlated in white rhinoceros following chemical immobilisation.
- H_1: Stress biomarker levels, heart rate, blood pressure and LCC responses are correlated in white rhinoceros following chemical immobilisation

1.4 Benefits arising from this project

- Gain experience in the collection of data and use of various biomarkers to evaluate the acute stress response in white rhinoceros.
- Gain experience using the LCC technique to evaluate the acute stress response in white rhinoceros.
- Determination of the effectiveness of the addition of azaperone, midazolam or medetomidine to an etorphine-based chemical immobilisation protocol to mitigate the acute stress response in white rhinoceros.
- Results from this study will contribute to a larger PhD study investigating the safety of various drug combinations used in the chemical immobilisation of white rhinoceros.
- Improve the conservation of white rhinoceros by potentially reducing their stress response to chemical immobilisation.
- Fulfilment of the requirements of the principal investigator's MSc degree.

Chapter 2: Literature review

2.1 Physiological stress response

Stress is perceived as a disruption to the normal state of homeostasis that the body is in at any point in time (12). These disruptions to homeostasis or aversive stimuli acting on the body are commonly referred to as stressors (7). Stressors can be internal factors such as poor oxygen delivery to tissues or hypoglycaemic conditions, or can be in the form of external factors such as changes in environmental temperatures, changes in physical activity or the presence of a potentially harmful agent, such as bacteria and viruses (13).

The body responds to these potential threats through various normal physiological mechanisms, involving the nervous, endocrine and immune systems (12). These mechanisms result in behavioural and physiological adaptations known as allostasis, and commonly referred to as the stress response. These behavioural and physiological changes are not static, but change depending on the nature and duration of the stressor. An organism is therefore in a constant state of flux ("allostasis = stability through change") (14). The stress response in vertebrates is co-ordinated by two main pathways - the Sympathetic Nervous System (SNS) and the Hypothalamic-Pituitary-Adrenal axis (HPA-axis) (15-17). The SNS causes catecholamines to be released almost instantaneously after activation, resulting in changes in the body that are commonly described as the flight or fight response (18, 19). The HPA-axis on the other hand, has a slightly slower response, occurring within minutes, and involves focusing the body's resources on essential organ systems, such as the brain, cardiovascular and pulmonary systems. These systems become up-regulated at the expense of growth, reproduction, the immune system and the antioxidant system, allowing the body to channel its resources to help fight the stressor that is being imposed on it. Activation of the HPA-axis results in the release of glucocorticoids, with cortisol being the primary glucocorticoid often measured to indicate stress in mammals (20, 21).

Stress can range in duration from acute to chronic. Acute stress is normally a beneficial adaptive response that enables the body to cope with the potentially harmful stimulus (22) and involves the behavioural and physiological actions the body undergoes in response to a stressor (23). Animals can, however, become chronically stressed if the body is unable to adequately deal with a stressor or if repeated acute stress responses take place after being brought about by numerous acute stressors (24). This build-up of stress over time, or an extreme stress response, can often be detrimental to the performance and survival of the animal (22). The normal adaptive stress responses brought about to try to protect the animal can become harmful to an animal, potentially even leading to the formation of pathological changes (25). It is difficult to define the duration of acute stress, except in the context of chronic stress - animals become chronically stressed if they are unable to deal with a stressor or if repeated acute stress responses take place. Stress is therefore acute for a variable duration of time before it chronically affects the animal. During an extreme acute stress response, increased activation

of the SNS takes place, resulting in a marked release of catecholamines, especially noradrenaline, from the adrenal medulla, in what is known as adrenergic overload (26). Excessive levels of noradrenaline can directly alter the cardiac myocytes, resulting in pathological changes that can result in heart failure (26). Additionally, negative cardiovascular changes such as hypertension, arrhythmias, increased cardiac output and potential infarcts forming in the myocardium due to increased catecholamine levels can occur (18).

Stress can be measured in many different ways. Clinical variables such as heart rate (HR), respiration rate and blood pressure (BP) can be used to measure stress. Other methods of measuring stress include the determination of cortisol, glucose and catecholamine concentrations and the neutrophil: lymphocyte ratio (N: L), as well as a relatively new technique which involves measuring the leukocyte coping capacity (LCC). All of these biomarkers change over different periods of time and need to be evaluated accordingly. Changes in catecholamine concentrations appear almost instantly after a stressful insult, while changes in glucose and cortisol concentrations also take place within a matter of minutes (27). The LCC and N: L ratio change after a slightly longer period of time (27), but all of these markers provide valuable information regarding short term stress that animals undergo after specific stress insults.

2.2 Biomarkers used to measure stress

2.2.1 Catecholamines and serotonin

Catecholamines, which include dopamine, adrenaline and noradrenaline, are derivatives of phenylethylamine and are found mostly in the peripheral nervous system and medulla of the adrenal gland (28). During a stress response, the SNS releases acetylcholine, which binds to nicotinic receptors on chromaffin cells in the adrenal medulla, leading to the release of adrenaline and noradrenaline from the adrenal medulla into the blood (29). These catecholamines bind to adrenoreceptors, which mediate their effects; alpha and beta-agonist drugs are also able to stimulate these adrenoreceptors (29). Adrenaline and noradrenaline play a vital role in the stress response, acting on alpha-1 adrenoreceptors to activate the phosphoinositide cascade and acting on alpha-2 receptors to inhibit adenylate cyclase (30). The results are contraction of smooth muscle in blood vessels suppling the kidney and skin, smooth muscle relaxation of the intestines, as well as platelet aggregation (30). Catecholamines and beta-agonist drugs also stimulate beta-adrenoreceptors, which in turn activates adenylate cyclase (30). This activation increases heart rate, breakdown of adipose tissue, and glycogenolysis and gluconeogenesis in skeletal muscle and the liver (30). All of these changes serve to divert energy to vital organs to help protect the body during a stressful incident (31).

The catecholamines adrenaline and noradrenaline are broken down into various metabolites, including metanephrine, normetanephrine and 3-methoxy-4-hydroxyphenylglycol (MHPG) (32).

Metanephrine and normetanephrine are metabolites of adrenaline and noradrenaline respectively, while MHPG is one of the major metabolites of noradrenaline in blood and urine and has been used extensively in humans to assess the functional status of the noradrenergic system (32, 33). These breakdown products of catecholamines have not been studied extensively in non-human animals but may be beneficial in assessing the stress response over a longer period of time as they are often present at a later stage, and are more stable in blood samples, than their already metabolised precursors (34).

Dopamine and serotonin are important neurotransmitters involved in many nervous system functions. The catecholamine dopamine plays a role as both a hormone and neurotransmitter (35). Dopamine is involved in many nervous system functions, including motor control, motivation, sexual behaviour, reward and arousal (36). Administration of opioids causes hyperpolarisation of interneurons, reducing gamma-aminobutyric acid- (GABA-) mediated input to dopamine cells, with a resultant increase in dopamine concentrations (37). Increases in dopamine concentrations have also been seen with the administration of benzodiazepines, like diazepam, which disinhibit dopamine neurons (38). Alpha-2 agonists have been shown to decrease dopamine concentrations in humans, with decreases seen in various regions of the brain (39), possibly attributable to the effects of these drugs on dopamine cell firing (40). Azaperone decreases dopamine concentrations and concurrently causes dose-dependent sedation, due to its central and peripheral dopamine-receptor blocking characteristics (41).

Serotonin, also referred to as 5-hydroxytryptamine or 5HT, plays a major role in many nervous system functions, such as cognition and behaviour (42). Serotonin is also involved in anxiety, the complex response to stress where the stressor is not present or clearly identifiable, with many of the anxiolytic drugs having a significant relationship with serotonin (42). Anxiety behaviour has been reported in many invertebrates, including crayfish, who when faced with social or physical stress, started avoiding light exposure, with this avoidance behaviour associated with increased concentrations of serotonin in their brains (43). Rodents and fruit flies have also been found to exhibit anxiety-like behaviour in response to stress, which was reduced by diazepam, an anxiolytic benzodiazepine drug (44). Medetomidine has been shown to cause dose-dependent decreases in serotonin concentrations (45), while azaperone antagonises serotonin at high doses (46). Various opioids have been found to inhibit the reuptake of serotonin, causing resultant increases in plasma and synaptic cleft concentrations of serotonin (47).

Due to the fact that catecholamines are vital components of the stress response, their changes in the blood can be observed very rapidly, with changes in the catecholamine concentrations being noted within seconds of activation of the stress response (48). Because of these rapid changes, determining baseline levels can be challenging, although measuring adrenaline can be very useful in monitoring the short-term response after a stressful insult (27). Catecholamines have previously been measured

in studies conducted on immobilised white rhinoceros to evaluate the stress response in these animals (48). A study by Pohlin et al. in 2020 showed that the concentration of adrenaline was above the detection limit in 12 of 26 rhinoceros when the animals were captured through immobilisation, but decreased to below the detection limit in nearly all of the study subjects a few hours later after the immobilisation was reversed and they were already placed in crates for translocation (28). Adrenaline was subsequently detected in only 4 of these 12 rhinoceros after 6 hours of transport in the crates, indicating that an acute stress response occurred at capture but subsequently decreased (48).

2.2.2 Glucocorticoids: Basal cortisol

Glucocorticoids alter the physiological processes in the body in order to protect the body in a stressful situation (10). Cortisol is a lipophilic glucocorticoid hormone produced in the cortex of the adrenal glands and it is commonly measured to evaluate stress in mammals (49, 50). In response to a stressor, the HPA-axis is activated, with the resultant release of cortisol from the adrenal glands (48). Cortisol causes the up-regulation of the essential organ systems such as the brain, cardiovascular and pulmonary systems at the expense of growth, reproduction, the immune system and the antioxidant system, allowing the body to channel its resources to help fight the stressor that is being imposed on it (15).

Glucocorticoids, primarily cortisol and corticosterone in vertebrates, are commonly measured in blood (10). Another method of measuring a stress response that is becoming increasingly popular is through analysing cortisol, or its metabolites, in faecal samples, allowing for non-invasive sampling (50). Other ways of measuring glucocorticoids include in urine and various body cells. These non-invasive methods were less commonly used in the past (10), but have been used more regularly in recent times (51).

Interpreting changes in glucocorticoids is challenging as their concentrations vary greatly between species and between animals of a particular species. Factors that can cause variations in glucocorticoid levels include age, sex, body condition, abundance of food, immune status, reproductive or breeding status, external environment, social status, threat of predation, capture, handling, transport and human presence (10). It is thus important to measure the basal cortisol levels of individual animals to have a reference point from which to compare changes.

Changes in cortisol concentrations are species-specific and often occur within minutes following exposure to a stressor (52, 53). The determination of cortisol levels in multiple blood samples collected over the course of minutes to hours following exposure to a stressor can be used to evaluate the stress response (27). With acute stress, cortisol levels generally return to baseline levels within a few hours (53). In addition to assessing acute stress, cortisol levels can also be used to monitor stress

responses over a longer period of time, making it a valuable tool to measure both short- and long-term stress responses (27).

2.2.3 Glucose

Glucose is a carbohydrate that circulates in the blood and serves as an energy source for peripheral tissues (54). Blood glucose concentrations are influenced by a variety of hormones, with the most important being insulin (54). Insulin affects glucose by reducing glucose production and accelerating glucose uptake and utilisation to decrease blood glucose levels, but glucose is also affected by other hormones such as glucagon, cortisol, adrenaline, noradrenaline and growth hormone, with these hormones playing a major role in increasing blood glucose concentrations following a stressful insult to the animal (54).

After insult from a stressor, changes in blood glucose concentrations are brought about by increases in catecholamines and cortisol, which lead to mobilization of energy stores via the processes of glycogenolysis and gluconeogenesis, and the reduction of energy usage by non-vital tissues to optimize energy usage to protect the animal after a stressful insult (27). These changes result in a rapid increase in blood glucose levels over a period of minutes (27). However, blood glucose levels can also be measured over longer periods of time, weeks to months, to evaluate the physiological response to persistent stressors such as starvation and long periods of inappetence (27).

Glucose can be used to measure both acute and chronic stress. Persistent hyperglycaemia can be an indication of disease severity and chronic stress (55), while increases in glucose have also been observed in acute settings, such as after excessive handling and experimental procedures (56). This highlights the usefulness of glucose as an indicator of stress.

2.2.4 Neutrophil: lymphocyte (N: L) ratio

Many studies have shown that the immune system is strongly linked to the stress response (15). Changes in different variables of the immune system can therefore be used to monitor stress in wildlife. One of the more acknowledged variables that is used to analyse changes brought about by stressors in organisms is the use of leukocyte profiles, with an important aspect being the N: L ratio. Various studies have shown that higher stress levels are associated with an increased number of neutrophils and a decreased number of lymphocytes in the vasculature i.e. an increase in the N: L ratio (15). Leukocytes are activated due to cortisol binding to glucocorticoid receptors and catecholamines binding to adrenoreceptors on the leukocytes (53). These changes cause lymphocytes and eosinophils that are in circulation to adhere to the endothelium of the blood vessels and migrate from the circulation to various organs, including lymph nodes, bone marrow, spleen and

skin, where they are sequestered (48). In contrast, activated neutrophils migrate from organs into the blood circulation, and move from a marginating pool along the endothelium into a central pool. The net effect is an increased blood N: L ratio (48). These changes allow for the ratio to be used as an indicator of response to a stressful insult. Although cortisol is needed for the activation of leukocytes, the N: L ratio does not change in proportion with the cortisol concentrations measured at the same time (57). This is because leukocytes do not respond immediately to stress and first need to be activated by cortisol, whereas cortisol concentrations change within minutes of the presence of a stressor (53). Results need to therefore be interpreted independently of each other but can be used together to track the changes in response to a stressor over a longer period of time.

Changes in the N: L ratio are variable and species dependent, with results varying between studies (27). The general consensus is that the N: L ratio increases approximately 1-4 hours after a stressful insult, with changes in the ratio sometimes persisting for months to years after the insult (27). A study on white rhinoceros found that the N: L ratio increased slowly over time, with increases in the ratio being found over a period of approximately 6 hours (48).

2.2.5 Leukocyte coping capacity (LCC)

The LCC assay works on the principle that an animal produces an immune response to a potential stressor, and that this immune response can be diminished in the face of overwhelming or persistent stress (58). During this immune response, leukocytes are activated, with the mechanism of leukocyte activation due to cortisol binding to glucocorticoid receptors and catecholamines binding to adrenoreceptors on the leukocytes (53). The LCC is analysed by measuring the ability of the animal's leukocytes to produce reactive oxygen species (ROS) in response to stress (59) by using phorbol myristate acetate (PMA) to activate ROS production via the protein kinase C pathway (60). Animals that are able to produce more ROS, in what is known as a respiratory burst, will have a higher LCC and be more likely to respond better physiologically to a stressor (58). Over time, as the stressor persists, the oxidative burst decreases, leading to a corresponding decrease in LCC over time (58). The decrease in LCC indicates a bigger or more persistent stressor that is affecting an animal (58). Leukocyte coping capacity is a useful tool for evaluating the effect of stress in animals because leukocytes are continuously associated with changes in the HPA-axis, endocrine factors in the plasma and blood biochemistry levels (61), allowing changes to the LCC to be picked up quickly after a stressful event (58).

A 2003 study by McLaren et al. (58) used LCC to analyse stress in transported badgers (*Meles meles*), showing that under more stressful conditions the leukocytes produce less free radicals (i.e., ROS). Similar results were found in a study of migrating garden warblers (*Sylvia borin),* as the LCC decreased with increased stress in the form of increased handling time (62), which was also the case

in a similar study on European roe deer (*Capreolus capreolus)* (63). Atlantic salmon (*Salmo salar L.)* that experienced stress during confinement for a period of two hours also showed a marked reduction in ROS production (64). The study on the European roe deer also showed that the LCC did not correlate with other traditional indicators of stress such as cortisol levels, N: L ratios, behavioural aspects, heart rate and body temperature, and were not an adequate measure of stress in a short time frame (63). Similar results were seen during a study on Scandinavian brown bears (*Ursus arctos*), with LCC results not correlating with other more commonly used indicators of stress such as heart rate, N: L ratio, cortisol and blood glucose levels (65).

Apart from changes due to stressors, neutrophil oxidative burst has also been shown to be affected by anaesthetic agents (66). Natural opioids like morphine, and benzodiazepines like midazolam, decrease ROS production in humans. The benzodiazepines in particular have strong suppressive effects on neutrophil function (67). On the other hand, synthetic opioids and alpha-2 agonists like medetomidine have not been shown to significantly affect neutrophil function (68). The effect of different immobilisation agents used in wildlife, on the LCC in particular, has not yet been studied.

Leucocyte coping capacity responds to stressors more slowly than catecholamines and cortisol, with decreases in the oxidative burst generally seen after approximately 10 minutes (27). Leucocyte coping capacity continues to decrease for approximately 2 hours after a stressful insult (27), allowing for samples to be taken during this period of time to evaluate the LCC response during a stressful insult to an animal. In a 2020 study on rhinoceros undergoing immobilisation and transportation by Pohlin et al., it was found that duration of transport did not have significant effects on the LCC (48). In another study involving white rhinoceros, no changes were seen in LCC from the time of immobilisation to when animals were loaded for transportation around 20 to 30 minutes later (69). On the other hand, a study on badgers *(Meles meles)* by McLaren et al. showed that the LCC did decrease in animals that were transported compared to animals that were not transported (58). The study by Pohlin et al. in 2020 surmised that the LCC was probably decreased at capture and then did not recover during transportation, but speculated that changes in LCC would possibly be seen in animals that were only immobilised and not transported (48).

2.2.6 Haematocrit (HCT)

The haematocrit (HCT), also commonly referred to as the packed cell volume (PCV), is the percentage of red blood cells in the blood (70). Increases in HCT may be caused by dehydration or may indicate the increased production of red blood cells (71). In animals with a contractile spleen, splenic contraction after stimulation by catecholamines such as adrenaline is another cause of an increased HCT (71). Decreases in the HCT may indicate over-hydration due to excessive fluid administration, splenic relaxation after the administration of various anaesthetic agents or indicate

anaemia, which may be caused by haemorrhage, haemolysis or as a result of the decreased production of red blood cells (71). Studies in humans have shown that stress can cause an increase in haematocrit levels, although many studies have also found no changes (71). Measuring the magnitude of the decrease in HCT caused by the administration of various anaesthetic agents may be beneficial in assessing the magnitude of the acute stress response induced by different anaesthetic agents.

2.2.7 Response times and duration of response of biomarkers

Table 1 below summarizes the response times of the various stress biomarkers and the duration of their response (27, 71):

Table 1. A summary of the response times and response duration of acute stress biomarkers.

	Biomarker					
	Catecholamines	**Cortisol**	**Glucose**	**N: L**	**LCC**	**HCT**
Response time after insult	Within seconds	2-5 minutes	Within minutes	Approximately an hour	10 minutes	Within minutes
Duration of response after insult	Minutes to hours	Generally for a few hours, but response can be for up to a few months	Weeks to a month	Months to years	2 hours	Minutes to hours

2.3 Anaesthesia in white rhinoceros

Rhinoceros often have to undergo chemical immobilisation for procedures such as translocations, treatments, dehorning and sample collection (72). Many different drug combinations have been used for this purpose, with the favoured drug protocols changing over time as more research is conducted.

2.3.1 Etorphine

The basis of the majority of the drug combinations that are used in the chemical immobilisation of rhinoceros is an opioid. Etorphine hydrochloride, carfentanil, thiafentanil and butorphanol are some of the opioids that have been used in rhinoceros (73), with etorphine being most commonly used,

particularly in white rhinoceros. Etorphine is a semi-synthetic alkaloid derivative of thebaine, and is commonly used in combination with other drugs such as sedatives and neuroleptic tranquilizers to immobilize wildlife (74). It is a lipophilic compound, 1000 – 3000 times more potent than morphine as an analgesic, with a stronger binding capacity to opioid receptors than morphine (73). Etorphine works by acting as an agonist on mu-, kappa- and delta-opioid receptors, with sedation and analgesia mostly brought about as a result of etorphine binding to the kappa receptors, while some analgesia, catatonic rigidity and respiratory depression are the primary effects seen from binding to the mu receptors (75). Effects from administration of etorphine typically take between two and twelve minutes to occur and are characterised by rapid immobilisation, analgesia, sedation and muscle relaxation (73, 74). Etorphine reaches maximum efficacy at approximately 20-30 minutes (73).

White rhinoceros are highly sensitive to the effects of opioids, with common side effects of etorphine administration being hypertension, tachycardia and respiratory compromise (73, 74, 76). Other side effects often seen in white rhinoceros include muscle tremors and limb paddling, hypomotility of the gastrointestinal tract, mydriasis, increased salivation, hypoxaemia, and hypercapnia (73, 77, 78). Respiratory depression is one of the major effects of opioid administration in rhinoceros and is worsened by the fact that rhinoceros have heavy thoracic muscles, affecting their ability to maintain an adequate respiratory rate and rhythm (73, 79). Heavier animals tend to struggle even more, with arterial blood supply to the ventral muscles severely decreased and venous drainage often completely occluded, leading to an increase in lactate and a higher chance of muscle spasms (72). The additional side effects of muscle tremors and limb paddling increase the risk of hyperthermia (73).

A study by Buss et al. (2018) found that hypoxaemia and hypercapnia were two of the most significant respiratory side effects that white rhinoceros immobilised with etorphine experienced (80). These changes are thought to occur as a result of respiratory depression, inadequate gas exchange in the lungs and greater oxygen use caused by tremors and hypermetabolism (80).

Hypertension occurs as a result of an elevated cardiac output and peripheral vascular resistance. If animals are maintained in a relatively light state of anaesthesia, the presence of hypertension may also be related to sympathetic stimulation (73). Buss et al. (2016) hypothesized that the cardiovascular response of increased arterial blood pressure and tachycardia with etorphine use could be as a result of the hypoxia caused by the drug-induced respiratory compromise (76). In humans, hypoxia leads to an increase in systolic blood pressure, as well as an elevated cardiac output and heart rate, due to the activation of arterial chemoreceptors (76). Comparable results were seen when etorphine and acepromazine were administered to horses, with the cardiovascular findings attributed to catecholamines being released from postganglionic neurons, leading to a sympathetic response (76, 81, 82). Other odd-toed ungulates like Grevy's zebra and Mongolian horses have exhibited similar cardiovascular blood pressure effects when immobilised with opioids (83), highlighting the fact that opioid-induced activation of arterial chemoreceptors can lead to tachycardia

with a subsequent increase in cardiac output (84). It has also been suggested that etorphine may directly activate the sympathomedullary axis by binding to opioid receptors within the sympathetic and parasympathetic nervous systems (85). Increases in circulating catecholamines were also seen in humans when morphine was administered, suggesting that opioids may directly stimulate the sympathetic nervous system (86). Dose-dependent increases in both adrenaline and noradrenaline were also seen with increasing doses of the potent opioid fentanyl (87). In summary, etorphine appears to result in activation of the sympathetic nervous system in response to hypoxaemia caused by respiratory compromise, but also has direct effects (85, 86).

2.3.2 Azaperone

Butyrophenones are one of the more commonly-used adjuncts with etorphine in rhinoceros immobilisation, with the tranquilizer azaperone often being used as an opioid synergist (88). The combination of azaperone with etorphine was the preferred combination put forward at a symposium on white rhinoceros in 1993 (72). Effects of azaperone are usually seen within 15-20 minutes of administration, with some effects lasting up to six hours (73). When used alone, total intramuscular doses of 60 mg of azaperone have been shown to keep rhinoceros calm in crates while being transported, with total doses of between 200 and 400 mg in adult animals providing deeper sedation but subsequently also causing animals to become recumbent (73).

Many of the effects of azaperone are brought about by the drug's antagonistic effect on alpha-1 adrenoreceptors (89, 90). The central nervous system effects, generally only present after higher doses of azaperone are administered, are primarily due to the inhibitory effect on the central dopamine receptors (91, 92). The inhibition of vascular alpha-adrenoreceptors causes peripheral vasodilation and a decrease in vasomotor tone, resulting in hypotension (89). This reduction in blood pressure is a key reason for combining azaperone with etorphine in immobilisation procedures, as it reduces the hypertension that is brought about by etorphine (76). A slight elevation of cardiac output is also often seen as a result of a reduction in peripheral resistance and an increase in heart rate (73).

The administration of azaperone also causes a decrease in dopamine concentrations, due to its central and peripheral dopamine-blocking effects (41). High doses of azaperone also cause decreases in other neurotransmitters like serotonin (46).

A study by Lees et al. (1976) found that the administration of azaperone to ponies inhibited various effects of adrenaline, such as increases in mean arterial pressure, PCV and haemoglobin concentration, as well as hyperkalaemia; all effects mediated for the most part by alpha-adrenoreceptors (89). These changes were attributed to the blocking effect of azaperone on vascular alpha-receptors (89). Azaperone on its own causes a decrease in haemoglobin concentrations and PCV (73).

Additional beneficial effects of azaperone include minimal changes to the partial pressure of carbon dioxide in arterial blood ($PaCO_2$), partial pressure of oxygen in arterial blood (PaO_2) and pH in horses (73). Azaperone has also been thought to aid in counteracting the respiratory depression induced by opioid drugs (73, 93), although recent studies have shown the opposite, with etorphine and azaperone drug combinations having a greater negative effect on ventilatory function than etorphine alone (94).

Azaperone is often used in combination with etorphine for rhinoceros immobilisation in order to decrease the induction time during immobilisation procedures, with shorter induction times seen compared to when etorphine was administered alone, along with a similar quality of immobilisation (92). The shorter induction time is vital as it allows veterinarians to access immobilised animals sooner, reducing the potential risks associated with immobilisation procedures (92).

Azaperone can exacerbate some of the negative side effects of etorphine, such as increasing muscle tremors, ear twitching and rigid limbs (75). These effects can however be mitigated with the administration of butorphanol once the animal is recumbent (78).

A study by de Lange et al. (2017) analysed the effects of the etorphine and azaperone drug combination on muscle tremor intensity in white rhinoceros (78). The muscle tremors were caused by the release of catecholamines by the immobilising drugs, with hypoxaemia and acidaemia also being seen. The tremors were however reduced with the addition of butorphanol and oxygen insufflation (78).

2.3.3 Midazolam

Benzodiazepines are another group of drugs that have been used with etorphine to maintain immobilisation in southern white rhinoceros (95). Some of the more commonly used benzodiazepine drugs include midazolam, diazepam and zolazepam, while other commonly used benzodiazepines in human medicine include clonazepam, alprazolam, oxazepam and temazepam (96). Benzodiazepines are centrally acting muscle relaxants that are commonly used to reduce muscle spasms, by amplifying the effects of the inhibitory neurotransmitter gamma-aminobutyric acid (GABA) (97). Other important effects brought about by the benzodiazepines are sedation and a reduction in anxiety (98). The potent anxiolytic effects of midazolam and other benzodiazepines are due to enhancement of the inhibitory effects of GABA on neurons, making them less responsive to catecholamines, dopamine and serotonin and this class of drugs may potentially be useful in mitigating some of the negative effects of immobilisation-induced stress (98).

Midazolam, an imidazobenzodiazepine, has been used in conjunction with etorphine to improve muscle relaxation and sedation in white rhinoceros (73). Midazolam has a fast onset of action due to inherent lipid solubility characteristics, which allows for rapid entry into the brain, making it useful in

an immobilisation setting (99). It is more potent than diazepam (98), with a shorter half-life and duration of activity due to faster hepatic and urinary excretion compared to other benzodiazepines (100).

Midazolam may improve deep ventilation due to its strong muscle relaxation effects, potentially combating the oxygen depletion that is a result of the muscle tremors and hyperthermia that is brought about by opioid administration (101). Midazolam does however need to be used with caution as it can amplify the respiratory depression that is brought about by opioid administration (73). Respiratory depression was noted after midazolam administration in humans and is thought to be caused by a direct depression of the central nervous system and the resulting decrease in ventilatory response to CO_2 (102). A reduction in respiratory rate was also seen in a study by Smith et al. (1991) in pigs after midazolam was administered (99).

Midazolam is however beneficial as it has been shown to produce less acidaemia during the capture process than azaperone (103) (both administered with etorphine), and could be used to decrease some of the risks associated with rhinoceros capture. A study by van Zijll Langhout et al. (2016) also found etorphine and midazolam to be an effective alternative combination for immobilizing white rhinoceros as the combination provided good muscle relaxation (104). This same study did however show that it took longer for animals to become recumbent when darted with midazolam and etorphine than azaperone and etorphine, possible owing to a slower absorption rate of the midazolam when compared to azaperone (104).

Analysis of blood gases revealed no significant changes in pigs receiving midazolam alone (99). Severe hypoxaemia, hypercapnia and respiratory acidosis were however observed in white rhinoceros receiving midazolam, although these effects may be due to the administration of etorphine that these animals received concurrently with midazolam (105). Respiratory rate, heart rate, PaO_2, $PaCO_2$, pH, blood pressure and other cardiopulmonary variables were outside the normal reference intervals described for rhinoceros (106), but did show an improvement after intravenous butorphanol administration (105). This improvement was greater than in animals immobilised with etorphine and azaperone, possibly due to the profound muscle relaxation effects and slower absorption rate of midazolam (105).

Studies have been conducted to compare the addition of azaperone or midazolam to etorphine immobilisation protocols in white rhinoceros, with similar adrenaline and cortisol concentrations observed between the protocols (48). Rhinoceros immobilised with midazolam and etorphine had lower white blood cell counts than those immobilised with azaperone (both in combination with etorphine) (48). Peripheral benzodiazepine binding receptors (PBRs) have been found on the cell membrane in human leukocytes and may play a role in neuroendocrine immune-modulation (107). There are many of these receptors on monocytes and lymphocytes (108) and during a stress

response, these leukocytes normally produce pro-inflammatory cytokines which help to attract neutrophils (109). In animals sedated with midazolam, increases in neutrophils were more delayed than with azaperone, potentially due to midazolam inhibiting the monocytes and lymphocytes via PBR binding (48). Although there were differences in the leukocyte counts between the azaperone and midazolam protocols in the Pohlin et al (2020) study, the N: L ratio was very similar in both groups, showing that midazolam and azaperone had similar effects on the acute stress response in this study (48).

2.3.4 Medetomidine

Medetomidine is an alpha-2 adrenoreceptor agonist that has been used successfully in drug combinations to immobilize many wildlife species (45). It is a highly selective and specific full agonist at both pre- and postsynaptic alpha-2 adrenoreceptors (110). Medetomidine acts on adrenergic nerve terminals to modulate the release of the catecholamine noradrenaline (110), causing a dose-dependent decrease in noradrenaline release (111). This reduction in noradrenaline leads to many of the effects seen after medetomidine administration, including cardiovascular effects such as biphasic blood pressure changes and bradycardia, as well as other effects such as analgesia, sedation, hypothermia and mydriasis (111).

The benefits of medetomidine during immobilisation include the strong sedative and analgesic effects, as well as the ability to reduce anxiety levels and provide good muscle relaxation (45, 112). Anxiolytic and sedative effects are brought about by binding of the alpha-2 agonists to supraspinal autoreceptors in the pons, while binding to heteroreceptors in the dorsal horn of the spinal cord brings about the analgesic effects seen with medetomidine (113).

Medetomidine and other alpha-2 agonists have both stimulatory and inhibitory effects on catecholamine secretion. Alpha-2 agonists generally inhibit noradrenaline release by acting on alpha-2 adrenoreceptors to inhibit the sympathetic outflow in the central nervous system (114). However, medetomidine also acts on imidazoline receptors, causing a concurrent release of norepinephrine through indirect mechanisms related to the effects on these I1 imidazoline receptors (114). The suppression of catecholamines seen with the administration of some of the alpha-2 agonists like medetomidine is therefore usually quite small. Studies in various species have shown decreases in catecholamine concentrations following the administration of alpha-2 agonists, including horses (115, 116), goats (117), dogs (118), cats (114) and humans (119), with decreases commonly seen in the plasma concentrations of both adrenaline and noradrenaline.

The small suppression of catecholamines by alpha-2 agonists can also be attributed to the effects of high doses of alpha-2 agonists on the cardiovascular system (114). Alpha-2 agonists act on peripheral alpha-2 adrenoreceptors in both arteries and veins to cause vasoconstriction, resulting in an initial

hypertensive state (114). The arterial baroreceptor reflex is activated, causing a marked bradycardia, as well as other cardiovascular effects such as a decrease in cardiac output (120, 121). The initial increase in blood pressure is usually followed by a longer-lasting normotensive or slightly hypotensive state (120).

A study by Grimsrud et al. (2012) of medetomidine administration in horses showed that there was a decrease in heart rate and respiratory rate after administration (122). Medetomidine has also been shown to suppress the ability to thermoregulate under sedation due to the depression of noradrenergic receptors in the hypothalamus (110, 121). Respiratory drug effects include a reduction in respiration rate (121), as well as contributing to the respiratory depression that is accompanied by hypercapnia and hypoxaemia when medetomidine is administered in conjunction with opioids or benzodiazepines (123). Alpha-2 agonists like medetomidine also inhibit the transmission of nerve impulses in the sympathetic nervous system, potentially reducing stress and the etorphine-induced catecholamine release and resultant effects (124-126).

Plasma cortisol concentrations are regulated in the central nervous system by the release of corticotrophin-releasing hormone (CRH) and adrenocorticotrophic hormone (ACTH), as well as peripherally in the adrenal cortex (114). Medetomidine has age and species-specific effects on cortisol concentrations with both increases and decreases reported (114). Mild increases were seen in calves and larger increases seen in adult cattle and sheep (127). On the other hand, changes in cortisol concentrations were varied in dogs, with no significant changes seen in some studies (118), while other studies showed that premedication with medetomidine resulted in a reduction or delayed increase in plasma cortisol concentration (128) and sedation with xylazine also resulted in a reduction in the increase of cortisol (129).

Alpha-2 adrenergic receptor agonists like medetomidine and xylazine have also been shown to induce hyperglycaemia in various species (114, 118, 127, 130). This mechanism of hyperglycaemia is through the inhibition of insulin secretion by the action of these agonists on the alpha-2-receptors in the B-cells of the pancreas (114). In cats anaesthetized with medetomidine, hyperglycaemia was found to be associated with acute stress and linked to the increase in the plasma concentration of glucocorticoids and catecholamines (131). The study conducted on horses by Grimsrud et al. (2012) also found an increase in glucose concentrations after medetomidine was administered (122).

Administration of alpha-2 agonists has been shown to cause a decrease in packed cell volume (PCV) (132). Various different mechanisms have been attributed to this, including a potential fluid shift from the extravascular to the intravascular space and the splenic sequestration of red blood cells (RBCs) (132, 133). The spleen accounts for a large proportion of the circulating RBCs, which are released by splenic contraction as a result of sympathetic release associated with a stressor or exercise (133). A study conducted by Kullman et al. (2014) analysed the effects of alpha-2 agonists administration on

the spleen and PCV in horses (132). The study used ultrasonography to measure the splenic thickness over time after administration of alpha-2 agonists and found that the spleen increased in thickness after the drugs were administered, with a concurrent decrease in the PCV, supporting the mechanism of RBCs being sequestered in the spleen (132). Another study in horses also found a decrease in PCV and total protein concentration after the administration of medetomidine (122).

Medetomidine was used with etorphine and butorphanol to immobilize rhinoceros for various procedures in a study by Citino (2008) and was shown to improve muscle relaxation and analgesia, as well as not having a detrimental effect on cardiovascular variables (134).

2.3.5 Butorphanol

Butorphanol tartrate is a synthetic opioid that is included in rhinoceros immobilisation procedures due to its mixed agonistic-antagonistic effects (73). Butorphanol acts as a full agonist at kappa-opioid receptors and is a partial agonist or antagonist at mu-opioid receptors (135). Butorphanol is believed to reduce the effects of etorphine at the mu-receptors, consequently improving respiration (136). When butorphanol was added to etorphine and azaperone in the dart combination, beneficial effects such as decreased lactate levels and improved pH were seen, potentially reducing metabolic issues that can lead to the occurrence of acidosis and capture myopathy (137).

A 2018 study by Buss et al. on the administration of butorphanol following etorphine induction found that PaO_2 and $PaCO_2$ values improved due to decreases in metabolic oxygen consumption as a result of decreases in muscle tremors (80). These changes are thought to be as a result of the action of butorphanol as an antagonist which decreases the sympathetic nervous system effects caused by etorphine (80). Another study also found that the administration of butorphanol and oxygen following etorphine induction led to a decrease in metabolic acidosis and hypoxaemia, and were also associated with lower catecholamine concentrations and less muscle tremors (138).

Butorphanol has a potency three to five times that of morphine and also has less respiratory and cardiovascular side effects (93). When compared to drug protocols using solely etorphine, respiratory rates and pulse oximetry values are generally improved when butorphanol is added (73).

The use of oxygen insufflation and the intravenous administration of butorphanol has been shown to correct hypoxaemia in captive and free-ranging white rhinoceros immobilised with etorphine (138, 139). A study conducted by Meyer et al. (2018) showed that a lower dose of butorphanol combined with diprenorphine and oxygen insufflation brought about similar results (140).

Butorphanol has been combined with etorphine and azaperone in the dart combination to induce standing immobilisation and improve ventilation (141), and has been given to already darted animals, where it has been shown to beneficially decrease the heart rate and $PaCO_2$ (75, 76, 141). All of these

benefits have made butorphanol an integral part of any immobilisation process in rhinoceros, along with the use of oxygen supplementation and positioning the animal in a sternal position to improve pulmonary gas exchange and blood oxygenation (75, 140).

2.3.6 Reversal agents

Various drugs can also be used to antagonise and reverse the effects of the opioids used to immobilise rhinoceros. Diprenorphine has been used extensively in the past as the reversal agent for etorphine (142). Animals typically wake up within 60-90 seconds after administration (143), but the animal still remains in a mild state of sedation for up to six hours after waking up (88). This can be beneficial if animals are being transported, but is often a problem when rhinoceros calves and cows are darted together as the cows might wander away from their calves because of their state of mild sedation, leaving the calves vulnerable to predation (144). Animals should also be monitored for adverse effects such as head pressing and recumbency (73).

Although marketed as the specific reversal agent for etorphine, diprenorphine is not always used to reverse the effects of etorphine, with naltrexone currently more commonly used (88). Naltrexone is an opioid-antagonist with no agonistic effects (73). Administration of naltrexone causes complete reversal of etorphine due to its prolonged half-life of up to ten hours, making it a favourable reversal drug (88).

Nalorphine is another drug that also causes incomplete reversal of etorphine's effects (142, 143), and was often used to partially reverse the effects of etorphine in recumbent animals (143).

Naloxone is an opioid antagonist with no agonistic effects and can also be used to reverse etorphine, although it has a short half-life of about 30 minutes and re-narcotization occurs (142).

2.3.7 Summary of anaesthetic agents

Azaperone, midazolam and medetomidine have all been used in combination with etorphine in various rhinoceros immobilisation procedures, with all of these drugs having the potential to alter stress responses during chemical immobilisation. The acute stress response that occurs during chemical immobilisation procedures with these drug protocols has however not been comprehensively studied or compared between the different drug protocols. Our study therefore aims to fill this gap in knowledge by evaluating whether the addition of the sedatives, midazolam or medetomidine, or the tranquiliser, azaperone, to etorphine-based drug protocols reduces the acute stress response of white rhinoceros during chemical immobilisation.

Chapter 3: Materials and methods

3.1 Experimental design

3.1.1 Study design

This was a prospective cross-over study.

3.1.2 Study background

The study involved eight sub-adult male white rhinoceros (*Ceratotherium simum simum*) and four different immobilisation protocols. The study took place in the Kruger National Park, South Africa (24.9948◦ S, 31.5969◦ E; altitude 317m). 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). The study was approved by the University of Pretoria Animal Ethics and Research Committee (REC 011-21, REC 057-21) and SANParks AUCC (reference number 011-20). Besides the stress response data collected for this study, physiological and blood gas data were collected from these rhinoceros for the purpose of several other studies.

3.1.3 Study population

Eight sub-adult male southern white rhinoceros (*Ceratotherium simum simum*) were selected as study subjects. These specific animals were chosen by park authorities to allow for a homogenous study group. All animals were between the ages of four to seven years and in good physical condition.

3.2 Experimental procedure

3.2.1 Initial capture and boma acclimatisation

The study subjects were darted remotely from a helicopter and transported to the SANParks Veterinary Wildlife Services (VWS) adaptation bomas (25 m x 50 m) where they were kept for six weeks to acclimatize before the study commenced. Each rhinoceros was weighed upon arrival for prospective monitoring of body weight and condition and to enable accurate estimations of weight for drug dose calculations once the study commenced. At initial capture and release into the bomas, a physical examination was performed and blood samples were collected for haematology and serum clinical chemistry analysis to evaluate the health of the individual animals before the study commenced. Before release into the bomas, a long-acting tranquillizer, zuclopenthixol acetate, (Clopixol Acuphase® Lundbeck (Pty) Ltd, Randpark Ridge, 50 mg/animal) was administered to each rhinoceros intramuscularly. The captured rhinoceros were kept with an adult rhinoceros cow, already habituated to captivity, to facilitate adaptation and acclimatization to the boma setting. The rhinoceros

were fed a mixture of 50 % tef (*Eragrostis tef*) and 50 % lucerne (*Medicago sativa*). Each animal was evaluated daily by caregiver staff for food consumption, volume and consistency of faeces, demeanour and behaviour, and indications of sickness or injuries. Treatments and other interventions were provided by SANParks veterinarians.

Once a rhinoceros was fully adapted to captivity, it was moved to the study rhinoceros-holding bomas (20 m x 25m), for the duration of the study. Each animal was kept alone in a boma. Management was as for the adaptation phase.

3.2.2 Immobilisation procedure

During the study period, the rhinoceros were darted every 2 weeks with a different immobilisation protocol. A two-week period between immobilisations was used to allow for adequate drug washout and for welfare reasons. Immobilisation was carried out in the morning when environmental temperatures were cooler than the body temperature of the rhinoceros. Between two and three animals were immobilised per day of each immobilisation week. The day of immobilisation and order of immobilisation for each animal were not kept constant.

Darting was carried out within the precincts of the bomas by a VWS veterinarian and dart placement sites were the hump or rump muscle. Immobilizing drugs were administered using 3 millilitre Dan-Inject darts with a 2 X 60 mm uncollared needle, fired by a carbon dioxide-powered dart-gun (Dan-Inject, International S.A., Skukuza, South Africa). The immobilisation dosages were calculated by an experienced veterinarian from VWS and were based on the estimated body weights of the animals according to the VWS protocol which can be found in Appendix 1. The doses were etorphine 2.5-3.0 mg (Captivon®), azaperone 12.5-15.0 mg (Stressnil® Janssen Pharmaceutical Halfway house 40 mg/mL), midazolam 12.5-15.0 mg and medetomidine 6.25-7.5 mg (Wildlife Pharmaceuticals, Nelspruit, South Africa, 50 mg/mL). Each study candidate was given butorphanol (50 mg/mL; Wildlife Pharmaceuticals, Nelspruit, South Africa) intravenously at a dose of 10 mg/mg of etorphine.

The capture crew ensured that the animals went down in a safe area, administered eye ointment and placed a blindfold and earplugs to prevent any visual or auditory stimulation during the study.

The immobilisation was antagonised using naltrexone hydrochloride (Trexonil®; Wildlife Pharmaceuticals 50mg/ml) at 20 times the dose of etorphine injected via the auricular vein, with medetomidine antagonised using atipamezole hydrochloride (Antisedan®, Zoetis South Africa Pty Ltd, Bute Lane, Sandton, 5 mg/ml) at five times the dose of medetomidine via the auricular vein.

The treatment groups were as follows:

1. Treatment EB - Etorphine (2.0 µg/kg) (E) IM then butorphanol (20 µg/kg) (B) IV 15 minutes after positioning in lateral recumbency.

2. Treatment EAzaB - Etorphine (E) (2.0 µg/kg) and azaperone (Aza) (dose 10 µg/kg) IM then butorphanol (20 µg/kg) (B) IV at 15 minutes after positioning in lateral recumbency.

3. Treatment EMedB - Etorphine (E) (2.0 µg/kg) and medetomidine (5 µg/kg) IM then butorphanol (20 µg/kg) (B) IV at 15 minutes after positioning in lateral recumbency.

4. Treatment EMidB - Etorphine (E) (2.0 µg/kg) and midazolam (Mid) (10 µg/kg) IM then butorphanol (20 µg/kg) (B) IV at 15 minutes after positioning in lateral recumbency.

The study followed a crossover design, where each rhinoceros was given all four of the treatments over the study period, in a random order. The allocation of immobilisation options was done by lottery. This meant a mix of different immobilisation combinations occurred in the group at each trial, with each animal being its own control.

The depth of immobilisation was recorded for every animal during each immobilisation. Table 2 shows the scoring system that was used to determine the quality of immobilisation.

Table 2. Scoring system used to determine the depth of immobilisation

Level	**Description**
Level 1	No effect
Level 2	Cannot handle safely (standing)
Level 3	Can handle safely (standing)
Level 4	Recumbent, ear movements, tail curled
Level 5	Recumbent, fully relaxed, no ear movements, tail relaxed
Level 6	Recumbent, excessive depth, dilated pupils, bradycardia, respiratory rate < 3 BPM

3.2.3 Blood sample collection and analysis

Venous blood samples were collected as soon as the animals went down into sternal recumbency. The first venous blood sample (time = 0 minutes) was taken from the auricular vein, with further samples taken at 20 and 40 minutes from a superficial carpal vein once the animals were positioned in lateral recumbency, with the leg used varying depending on which side the animal went down on. Blood was drawn using an 18 G 1.5-inch needle and vacutainers. All variables were measured from

the 0-, 20- and 40-minute samples, apart from the LCC which was only determined from the 0- and 40-minute samples.

3.2.3.1 Catecholamines and cortisol

Blood samples were collected into ethylenediaminetetraacetic acid (EDTA) tubes (BD Vacutainer; Becton and Dickinson, Plymouth, UK) and immediately stored in a cooler box with ice blocks to prevent degradation of the catecholamines. The samples were taken within 2 minutes to the laboratory and centrifuged at 4,000 rpm for 5 minutes in a centrifuge cooled to 4°C. Plasma was transferred into cryovials, which were immediately snap-frozen in liquid nitrogen. The samples were stored in the liquid nitrogen until all the immobilisation work had been completed for the day, before being transferred to a -80°C freezer for storage at the VWS laboratory until the study was complete. Upon completion of the study, these samples were transported on dry ice to the Analytical Technical Laboratory of the Faculty of Health Sciences, North-West University, South Africa, for analysis.

Catecholamines and cortisol were measured using a liquid chromatography mass spectrometry (LC/MS) method, on an Ultivo Triple Quadrupole LC/MS System, controlled by the MassHunter Software from © Agilent Technologies, Inc. (Santa Clara, CA 95051 US). A Kinetix C18 analytical high performance LC column (2.1 x 1000 mm, particle size 2.6 µm, pore size 100 Å, surface area 200 m^2/g; Phenomenex, Torrance, CA, USA) was used. Calibration curves were set up and the limit of detection determined for each analyte from a dilution series using commercially available standards. A full description of the analytical procedures is supplied in Appendix 2.

3.2.3.2 Glucose

Blood was collected into serum tubes (BD Vacutainer) and left to clot at room temperature for 60 minutes before undergoing centrifugation. The serum obtained was aliquoted and stored at –80°C until completion of the study, before being transported on ice packs to the Clinical Pathology Laboratory of the Onderstepoort Veterinary Academic Hospital, University of Pretoria, for analysis. Serum glucose was measured using the hexokinase method on a Roche Cobas Integra 400 Plus analyser (Roche, Switzerland).

3.2.3.3 Neutrophil: lymphocyte ratio and haematocrit

Blood was collected in an EDTA tube and an automated blood count, including total leukocyte count, was performed within 8 hours of collection in the VWS laboratory using the Abaxis HM5 haematology analyser. The automated HCT result was used for this study. Blood smears were made from the EDTA blood within 8 hours of collection and stained with a modified Romanowsky

quick stain. A 200-cell manual leukocyte differential count was performed and the percentages and total numbers of neutrophils and lymphocytes and the N: L ratio were determined.

3.2.3.4 LCC

Immediately after blood collection into lithium-heparinized blood tubes (BD Vacutainer), LCC measurements were carried out using the technique described by Huber et al. (15). Luminol was added and the chemiluminescence of phorbol 12-myristate 13-acetate (PMA)-stimulated and unstimulated samples (control) were measured using a portable chemiluminometer (Junior LB 9509 Portable Luminometer, Berthold Technologies, Germany). The chemiluminescence of stimulated and unstimulated samples was measured every 5 minutes for 80 minutes. The area under the response curve (AUC) was calculated from PMA-stimulated samples and corrected for the PMA-unstimulated measurements. The resulting AUC was further corrected for the absolute neutrophil count at each time sample point (final AUC) to examine the effect of ROS production per neutrophil and to control for a potential mass effect (15).

3.2.3.5 Heart rate (HR) and blood pressure (BP)

Heart rate and mean arterial blood pressure (BP) were measured for the purposes of other studies, and data from the 20 min and 40 min time points are included here. Heart rate was measured manually using a stethoscope and watch. Arterial BP was monitored using an IntraTorr BP monitor via a 22-gauge catheter inserted into an auricular artery.

3.3 Statistical analysis

3.3.1 Changes over 40 minutes

Evaluation of changes in variables over the 40 min time period of immobilisation was performed, for all protocols together, and for each protocol individually. For variables where data was recorded for three time points a Friedman's test (non-parametric alternative to repeated measures ANOVA) with a post-hoc Conover test was performed. For LCC, HR and BP, with only two time points, a Wilcoxon test for paired samples was used. Non-parametric tests were used as the group size was small (n=8) for each individual protocol time point. A $P<0.05$ was considered significant.

3.3.2 Differences between protocols

Biomarker data were assessed for normality by calculating descriptive statistics, plotting histograms, and performing the Anderson-Darling test in commercial software (MINITAB Statistical Software, Release 13.32, Minitab Inc, State College, Pennsylvania, USA). Data distributions were described

using the mean and standard deviation when normally distributed and the median and absolute range when the normality assumption appeared to be violated. Non-normal data were transformed using the natural logarithm prior to statistical analysis. For variables that had data collected at 0 minutes (T0), 20 minutes (T20) and 40 minutes (T40), biomarker data were compared among the four immobilisation protocols using linear mixed-effects models incorporating a random-effect term for each individual animal and fixed-effect terms for the day of the week that an animal was immobilised and the daily immobilisation order, to account for the cross-over design with repeated measurements and potential confounding, respectively. Immobilisation protocol was included as a main effect and post hoc comparisons between treatment groups were adjusted using Bonferroni correction of P values. To compare biomarkers that were measured only at two time points (LCC, HR, BP), a repeated-measures ANOVA with post-hoc Bonferroni correction (normally distributed date) or a Friedman's test (non-normal data) with a post-hoc Conover test was performed. The time from darting to sternal recumbency and therefore T0 were compared between the 4 protocols using a Friedman's test with a post-hoc Conover test. A P-value of < 0.05 was considered significant.

Correlations between biomarkers were estimated using Spearman's rho (r) due to the violation of the normality assumption for some data distributions. Where biomarkers were only measured twice, the difference in the results between the two time points was calculated. These differences were correlated with the mean values of biomarkers measured three times during the study to account for the different measurement schedules. Statistical analyses were performed in commercially available software (IBM SPSS Statistics Version 28, International Business Machines Corp., Armonk, NY, USA and MedCalc® Statistical Software version 20.111, MedCalc Software Ltd, Ostend, Belgium) and significance was set as $P < 0.05$.

Chapter 4: Results

4.1 Study subjects

The eight sub-adult male southern white rhinoceros all remained in good health for the duration of the study period, with all of the animals displaying normal eating, drinking and defecating behaviour. All rhinoceros were also weighed after each immobilisation trial in order to monitor their health status and to provide more accurate estimations of drug dosages needed for the subsequent trials. The animals' mean body mass was 1,312 ± 111 kg, with mass ranging from 1,084 – 1,467 kg. Most of the rhinoceros maintained a fairly constant weight during the study period, with only one animal losing weight (1 kg lost) and the highest gain being 102 kg over the 10-week period.

4.2 Immobilisation procedure

Drug dosages were consistent with the estimates set out in the dosing table used by SANParks. Etorphine was administered at 2.5-3.0 mg per rhinoceros, azaperone at 12.5-15.0 mg per rhinoceros, medetomidine at 6.25-7.50 mg per rhinoceros, and midazolam at 12.5-15.0 mg per rhinoceros. Butorphanol was given at 25-30 mg per rhinoceros depending on the etorphine dose, with a second dose given if butorphanol was mistakenly administered subcutaneously which occurred on one occasion.

Table 3 shows the drug combinations that were used to immobilize each rhinoceros every trial week. The study design aimed to immobilise eight rhinoceros every two weeks, but because of technical issues and veterinary concerns during some immobilisation procedures, there were some animals where immobilisation data were not collected during the designated study period. These animals were then immobilised with the outstanding drug combination two weeks after their last immobilisation, making the total duration of the trial ten weeks. Trial weeks were two weeks apart to allow for washout of the drugs before the next immobilisation. Animals that failed their original protocol are denoted with FAIL in bold next to the immobilisation protocol, with their repeat being done in trial week 5.

Table 3. Summary of the different drug combinations that each rhinoceros received for each trial week over the duration of the study.

	Trial week				
Rhinoceros number	1	2	3	4	5
1	**EAzaB – FAIL**	EMedB	EMidB	EB	EAzaB
2	**EB – FAIL**	EMidB	EAzaB	EMedB	EB
3	**EMidB - FAIL**	EAzaB	EB	EMedB	EMidB
4	EAzaB	EB	EMedB	EMidB	
5	EMedB	EAzaB	EB	EMidB	
6	EB	EMedB	EAzaB	EMidB	
7	EMedB	EMidB	**EAzaB – FAIL**	EB	EAzaB
8		EMidB	EB	EMedB	EAzaB

The reasons for rhinoceros failing original protocols were as follows:

Rhinoceros 1 (Trial 1 = EAzaB) – No physiological data obtained due to technical errors with the equipment.

Rhinoceros 2 (Trial 1 = EB) – LCC measurements were not plausible and an error in the sample preparation or measurement was suspected.

Rhinoceros 3 (Trial 1 = EMidB) – Rhinoceros had extreme tremoring and an elevated HR early on during immobilisation, but stabilized after butorphanol was given. After 45 minutes the rhinoceros spontaneously woke up and had to be manually restrained, preventing the collection of some data (for the purposes of another study).

Rhinoceros 7 (Trial 3 = EAzaB) – Error when collecting BP data (for the purposes of another study).

Rhinoceros 8 (Trial 1) – Was captured later than the other rhinoceros due to the difficulty in finding suitable study subjects as a result of the severe population decline caused by the ongoing poaching crisis. The animal was still being adapted to the bomas when the trial started and could not be darted during the first trial week as it was still in the 6-week habituation period.

Body temperature was measured throughout the immobilisation procedure and remained fairly constant, with only slight decreases observed. Temperatures ranged from 34.6°C to 39.2°C, with a mean and standard deviation of 37.4 ± 0.89°C. The rhinoceros were immobilised at various times of

the morning with ambient temperatures ranging from 10.7°C – 29.0°C, with a mean and standard deviation of 21.28 ± 5.77°C.

The depth of immobilisation was recorded for all immobilised animals. Animals were found to be in the 4th and 5th levels for all of the immobilisation protocols.

The median induction times (with minimum and maximum) from darting to sternal recumbency were: etorphine 15:23 (13:00; 26:22), etorphine + azaperone 9:06 (6:20; 10:25), etorphine + midazolam 10:01 (8:30, 15:34), etorphine + medetomidine 9:32 (7:45; 12:53). Induction times were significantly longer for etorphine, and significantly shorter for etorphine + azaperone versus the other three protocols (Friedman's P-value < 0.0001; Conover P-value <0.05).

4.3 Effects of drug protocols on stress biomarkers

There were significant differences in HCT, N: L, noradrenaline, cortisol, glucose and BP between some immobilisation protocols. Results of the comparison of biomarkers between protocols are shown in Table 4 (linear mixed effects model) and Table 5 (repeated measures ANOVA for LCC, BP, HR). The results for the analysis of changes in stress biomarkers during the 40-minute immobilisation period are presented in Table 6, and illustrated as clustered multiple comparison graphs in Figures 1-3.

Table 4. Results of the linear mixed effects model showing descriptive statistics and comparison of stress biomarkers.

Biomarker	Time (min)	Treatment Group				P-value
		Etorphine	Etorphine + Azaperone	Etorphine + Midazolam	Etorphine + Medetomidine	
Adrenaline (ng/mL) *	0	25.9 (11.9)	29.3 (5.7)	28.3 (10.1)	26.1 (2.7)	0.556
	20	24.1 (3.5)	25.1 (9.1)	26.9 (9.3)	21.3 (4.1)	
	40	20.7 (2.8)	24.6 (6.2)	23.4 (5.7)	20.9 (6.0)	
	Overall	23.4[a] (20.4, 26.3)	25.5[a] (22.3, 28.6)	25.6[a] (22.6, 28.6)	24.5[a] (21.2, 27.7)	
Noradrenaline (ng/mL) **	0	24.4 (14.4, 39.1)	26.2 (15.8, 37.1)	18.3 (12.6, 37.3)	21.5 (7.7, 43.9)	**0.008**
	20	26 (21.4, 60.0)	24.7 (16.6, 32.7)	19.8 (10.4, 37.6)	26.7 (16.8, 54.0)	
	40	27.5 (15.8, 50.8)	28.9 (6.8, 39.0)	23.5 (9.4, 42.7)	25.5 (14.2, 55.2)	
	Overall	26.4[a] (21.1, 33.0)	22.8[a,b] (18.2, 28.7)	20.1[b] (16.1,25.1)	27.3[a] (21.7, 34.3)	
Metanephrine (ng/mL) *	0	0.88 (0.29)	0.96 (0.15)	0.77 (0.33)	0.89 (0.21)	0.138
	20	0.98 (0.49)	1.07 (0.45)	0.98 (0.30)	0.88 (0.45)	
	40	0.70 (0.25)	1.06 (0.31)	0.92 (0.32)	0.81 (0.54)	
	Overall	0.86[a] (0.72, 1.01)	1.05[a] (0.90, 1.20)	0.91[a] (0.76, 1.05)	0.81[a] (0.65, 0.97)	
Normetanephrine (ng/mL) *	0	0.73 (0.25)	0.94 (0.34)	0.64 (0.46)	0.64 (0.22)	0.550
	20	0.76 (0.33)	0.88 (0.21)	0.82 (0.40)	0.55 (0.24)	
	40	0.74 (0.18)	0.68 (0.28)	0.72 (0.28)	0.61 (0.18)	
	Overall	0.74[a] (0.61, 0.87)	0.79[a] (0.66, 0.92)	0.70[a] (0.58, 0.83)	0.67[a] (0.53, 0.81)	
MHPG	0	7.9 (6.94, 14.13)	8.32 (2.81, 14.15)	6.68 (1.82, 19.69)	10.26 (2.65, 13.67)	0.886

Biomarker	Time (min)	Treatment Group				P-value
(ng/mL) **	20	5.4 (3.33, 28.13)	9.23 (6.23, 30.54)	5.36 (4.20, 16.90)	8.04 (2.76, 16.82)	
	40	8.18 (4.34, 18.16)	7.62 (1.58, 22.4)	10.12 (2.60, 12.37)	8.7 (5.03, 10.05)	
	Overall	7.60[a] (6.04, 9.56)	8.42[a] (6.57, 10.79)	7.43[a] (5.89, 9.36)	7.53[a] (5.82, 9.74)	
Dopamine	0	0.33 (0.16, 0.56)	0.33 (0.18, 0.45)	0.29 (0.11, 0.79)	0.17 (0.11, 0.61)	0.435
(ng/mL) **	20	0.33 (0.12, 0.84)	0.28 (0.13, 0.70)	0.28 (0.07, 0.34)	0.23 (0.10, 0.83)	
	40	0.26 (0.14, 0.40)	0.26 (0.05, 0.47)	0.23 (0.14, 0.42)	0.17 (0.15, 0.39)	
	Overall	0.27[a] (0.22, 0.37)	0.27[a] (0.20, 0.33)	0.25[a] (0.18, 0.30)	0.22[a] (0.16, 0.27)	
Serotonin	0	850 (550)	890 (356)	948 (470)	711 (301)	0.116
(ng/mL) *	20	1004 (407)	1318 (470)	1145 (473)	967 (431)	
	40	1129 (264)	1110 (341)	1072 (198)	818 (246)	
	Overall	984[a] (783 (1186)	1119[a] (908, 1329)	1050[a] (848, 1253)	834[a] (621, 1048)	
Cortisol	0	15.4 (5.6)	17.4 (6.0)	13.4 (4.6)	11.8 (3.9)	**0.027**
(ng/mL) *	20	11.6 (5.6)	15.3 (9.2)	13.1 (6.5)	12.6 (6.9)	
	40	14.7 (4.6)	15.1 (5.2)	13.4 (6.6)	14.0 (4.0)	
	Overall	13.7[a,b] (11.3, 16.1)	17.1[a] (14.6, 19.7)	13.7[a,b] (11.3, 16.1)	11.3[b] (8.7, 14.0)	
Glucose	0	5.79 (4.65, 7.61)	6.11 (4.13, 8.03)	6.22 (4.86, 7.39)	6.49 (5.76, 7.72)	**0.003**
(mmol/L) **	20	6.49 (4.89, 10.07)	8.12 (5.15, 15.24)	8.59 (5.44, 15.07)	8.11 (7.05, 10.72)	

Biomarker	Time (min)	Treatment Group				P-value
	40	6.05 (4.57, 8.08)	7.19 (4.91, 12.07)	7.40 (4.66, 14.25)	10.21 (7.68, 11.87)	
	Overall	6.19[a] (5.34, 7.17)	6.93[a,b] (5.95, 8.07)	7.29[a,b] (6.30, 8.46)	8.06[b] (6.91, 9.41)	
HCT (%) *	0	47.6 (4.9)	45.9 (3.5)	46.8 (2.8)	39.6 (3.5)	**<0.001**
	20	44.8 (3.3)	45.1 (2.6)	47.7 (8.9)	37.2 (4.3)	
	40	42.0 (3.5)	42.1 (4.0)	42.6 (3.2)	34.3 (3.2)	
	Overall	44.6[a] (42.2, 47.0)	45.3[a] (42.7, 47.8)	45.9[a] (43.5, 48.4)	36.1[b] (33.6, 38.7)	
N: L **	0	2.15 (1.20, 2.50)	1.85 (1.60, 2.30)	1.75 (1.10, 2.00)	2.15 (1.30, 4.20)	**<0.001**
	20	2.30 (1.20, 2.80)	2.25 (1.80, 3.10)	2.00 (1.20, 3.30)	2.30 (1.50, 3.60)	
	40	2.50 (1.30, 3.10)	2.25 (1.90, 3.10)	2.10 (1.30, 3.10)	2.35 (1.50, 4.30)	
	Overall	2.15[a,b] (1.85, 2.50)	2.04[a] (1.75, 2.38)	1.91[a] (1.65, 2.23)	2.39[b] (2.05, 2.79)	

* Data presented are the group mean (standard deviation)

** Data presented are the group median (minimum, maximum) and statistical analysis performed on the natural logarithm transformation of the data due to apparent violation of the normality assumption

HCT = Haematocrit. N: L = neutrophil lymphocyte ratio MHPG = 3-Methoxy-4-hydroxyphenylglycol. LCC = leukocyte coping capacity

Superscripts [a,b] denote differences and similarities between the four immobilisation protocols. Results were different between protocols with a different superscript. Results did not differ between protocols with the same superscript.

Table 5. Comparison of leukocyte coping capacity (LCC), mean arterial blood pressure (BP) and heart rate (HR) between protocols.

Biomarker	Etorphine	Etorphine + azaperone	Etorphine + midazolam	Etorphine + medetomidine	P-value
LCC**	1957 (956–3269)[a]	1954 (1330, 3117)[a]	2001 (1173–4343)[a]	2868 (1146–5431)[a]	0.104
BP (mmHg)*	144 (27)[a]	129 (31)[a]	135 (32)[a]	149 (27)[a]	0.078
HR (beats per minute)*	73 (19)[a]	82 (23)[a]	46 (9)[b]	75 (15)[a]	**<0.001**

* Data presented are the group mean (standard deviation)

** Data presented are the group median (minimum, maximum)

Superscripts [a,b] denote differences and similarities between the four immobilisation protocols. Results were different between protocols with a different superscript. Results did not differ between protocols with the same superscript.

The catecholamine noradrenaline was significantly lower with the EMidB protocol compared to EB and EMedB. There was no difference in noradrenaline between EAzaB or EMedB, and the EB control. There were no differences in the concentrations of the other catecholamines or their metabolites (adrenaline, metanephrine, normetanephrine, MHPG, dopamine) between immobilisation protocols (Table 4). Adrenaline decreased over the immobilisation time, for all protocols combined and for the EMedB protocol alone (Table 6, Fig 1A). There were no significant changes in noradrenaline, metanephrine, normetanephrine, MHPG or dopamine concentrations over the 40-minute period (Table 6, Fig 1B-E and Fig 2A).

There was also no difference in serotonin concentrations between immobilisation protocols (Table 4). Over the immobilisation period, serotonin showed a significant increase from T0 to T20 and then decrease to T40, for all protocols combined, and specifically for the EAzaB and EMedB protocols (Table 6, Fig 2B.

Cortisol was lower in the EMedB protocol compared to EAzaB, but there were no differences in EAzaB, EMidB or EMedB compared to the EB control (Table 4). There were no significant changes in cortisol over the immobilisation period (Table 6, Fig 2C).

Glucose concentrations were significantly higher in the EMedB group compared to the EB group, but were not different between the EB, EAzaB and EMidB protocols (Table 4). Glucose showed a significant increase from T0 to T20 and decrease from T20 to T40 for all of the protocols excepting

the EMedB protocol, where there was a significant increase from T0 all the way through to T40 (Table 6, Fig 2D).

Haematocrit results were similar for all drug combinations with the exception of animals immobilised with EMedB where the haematocrit was significantly lower than the other immobilisation combinations, with a group mean of 36.1% (Table 4). The haematocrit showed many changes over the immobilisation period, with a significant decrease over the 40 min period for all protocols (Table 6, Figure 2E.

With regards to the N: L ratio, none of the drug protocols (EAzaB, EMidB and EMedB) differed compared to the etorphine control protocol (Table 4). There was a significant increase in the N: L ratio from T0 to T20 to T40 for all of the protocols, with significant increases at T20 and T40 vs T0 for the EAzaB and EMedB protocols, and a significant increase at T40 vs T0 and T20 for the EB protocol (Table 6, Fig 3A).

There was no difference in LCC between the different protocols and no changes over time.

Heart rate was significantly lower in the EMidB group compared to other groups (Table 5). There were no significant changes in HR over the immobilisation period, except for the EMidB protocol, where there was a significant increase from T20 to T40 (Table 6, Fig 3C). There was no difference in BP between the different drug protocols, but there was a significant decrease from T20 to T40 for the EMedB protocol (Table 6, Fig 3D).

Table 6. Changes of acute stress biomarkers over the 40-minute immobilisation period, for all immobilisation protocols combined, and individually, using the Friedman's or Wilcoxon paired tests. P-values are reported, and where significant differences were found, the pattern is further described based on post-hoc testing where applicable. A P-value of <0.05 was considered significant.

Biomarker	All protocols P-value	Etorphine P-value	Etorphine + azaperone P-value	Etorphine + midazolam P-value	Etorphine + medetomidine P-value
Adrenaline*	<0.001 Significant decrease from T0 to T20 and T40	0.062	0.085	0.234	<0.001 Significant decrease from T0 to T20 and T40
Noradrenaline*	0.320	0.204	0.096	1.000	0.234
Metanephrine*	0.368	0.637	0.818	0.637	0.444
Normetanephrine*	0.886	0.896	0.204	0.444	0.715
MHPG*	0.614	0.714	0.346	0.346	0.444
Dopamine*	0.386	0.444	0.204	0.715	0.444
Serotonin*	<0.001 Significant increase from T0 to T20 and decrease from T20 to T40	0.085	<0.001 Significant increase from T0 to T20 and decrease from T20 to T40	0.444	0.018 Significant increase from T0 to T20 and decrease from T20 to T40

Biomarker	All protocols P-value	Etorphine P-value	Etorphine + azaperone P-value	Etorphine + midazolam P-value	Etorphine + medetomidine P-value
Cortisol*	0.169	0.234	0.896	0.715	0.204
Glucose*	<0.001 Significant increase from T0 to T20 and decrease from T20 to T40	0.715	<0.001 Significant increase from T0 to T20 and decrease from T20 to T40	<0.001 Significant increase from T0 to T20 and decrease from T20 to T40	<0.001 Significant increase from T0 to T20 to T40
HCT*	<0.001 Significant decrease from T0 to T20 to T40	<0.001 Significant decrease from T0 to T20 to T40	<0.001 Significant decrease at T40 vs T0 and T20	0.001 Significant decrease at T40 vs T0 and T20	<0.001 Significant decrease from T0 to T20 to T40
N:L*	<0.001 Significant increase from T0 to T20 to T40	0.003 Significant increase at T40 vs T0 and T20	<0.001 Significant increase at T20 and T40 vs T0	<0.001 Significant increase from T0 to T20 to T40	0.003 Significant increase at T20 and T40 vs T0
LCC**	0.017 Significant increase from T0 to T40	0.109	0.641	0.250	0.148
HR**	0.728	0.219	0.938	0.047 Significant	0.563

Biomarker	**All protocols** **P-value**	**Etorphine** **P-value**	**Etorphine + azaperone** **P-value**	**Etorphine + midazolam** **P-value**	**Etorphine + medetomidine** **P-value**
				increase from T20 to T40	
BP**	0.837	0.219	0.109	0.383	0.008 Significant decrease from T20 to T40

* Friedman's test with Conover test as post-hoc test

** Wilcoxon paired-sample test

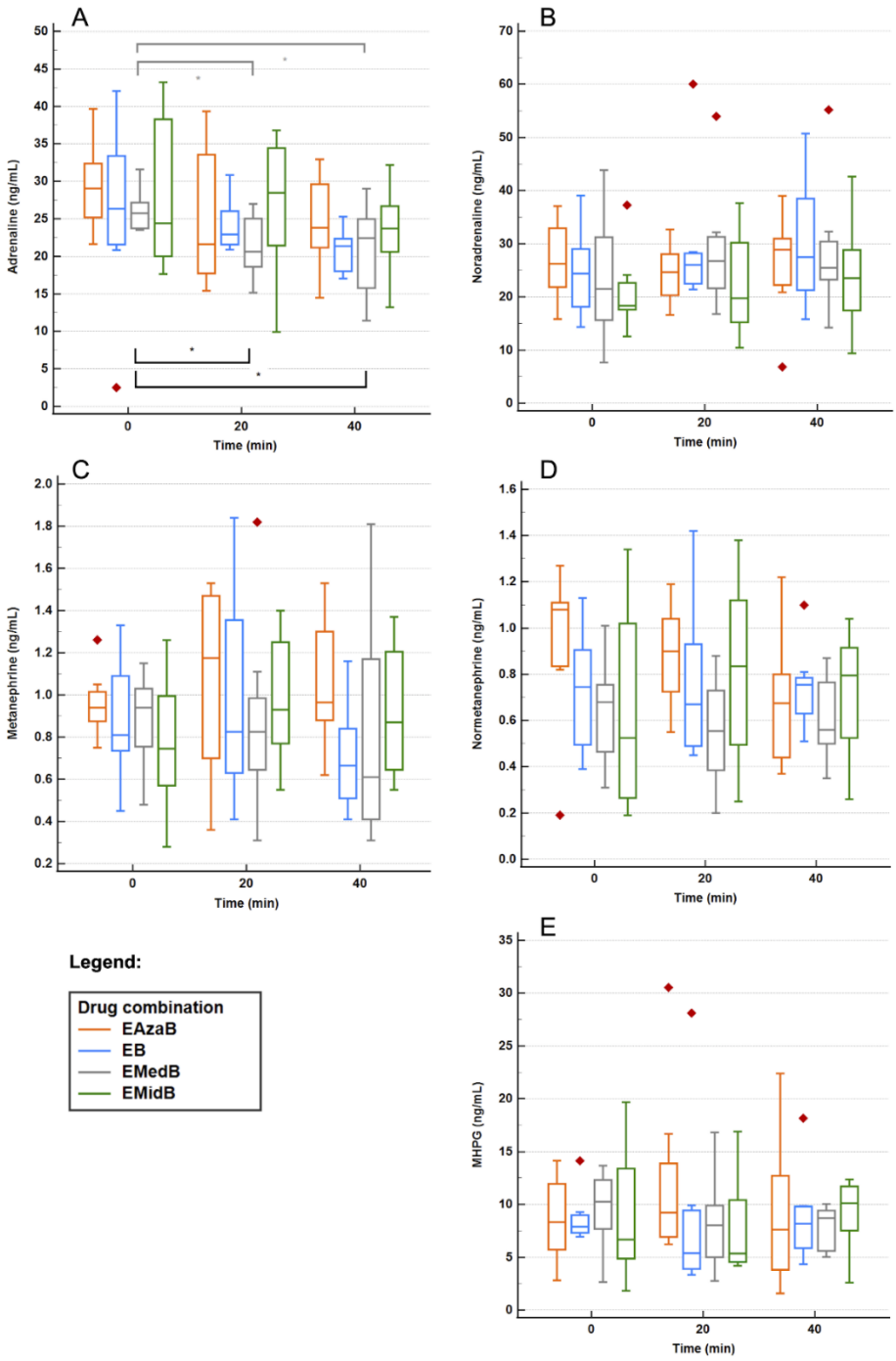

Figure 1. Clustered multiple comparison graphs showing results of stress biomarkers in white rhinoceros for four immobilisation protocols at 0, 20 and 40 min after sternal recumbency.

Data are presented as box and whisker plots; red diamonds represent outliers. Black connector bars with * denote significant differences in the biomarker results for all protocols combined; coloured connector bars with * denote differences in biomarkers for that protocol. A: adrenaline; B: noradrenaline; C: metanephrine; D: normetanephrine; E: MHPG (3-methoxy-4-hydroxyphenylglycol)

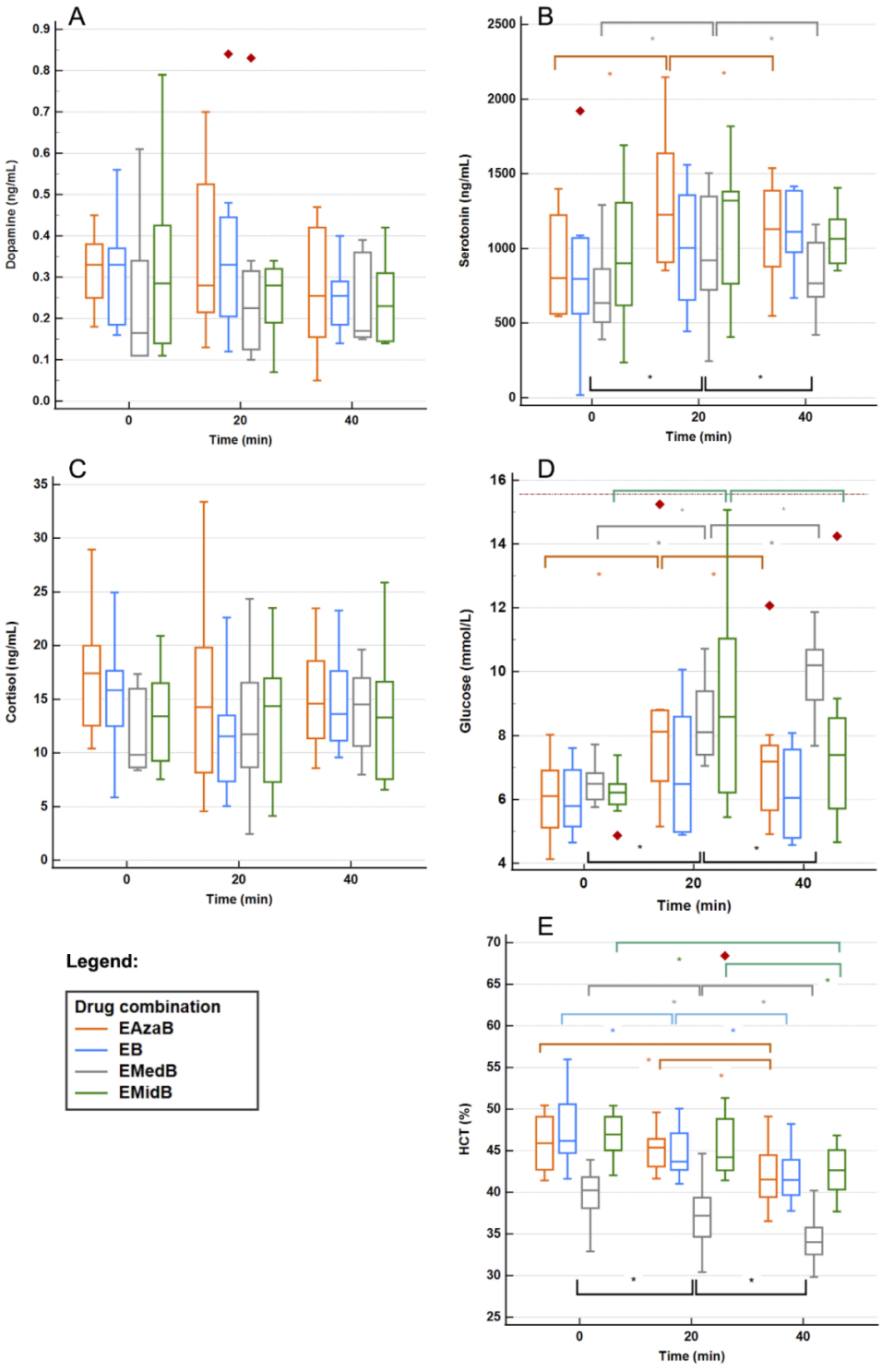

Figure 2. Clustered multiple comparison graphs showing results of stress biomarkers in white rhinoceros for four immobilisation protocols at 0, 20 and 40 min after sternal recumbency.

Data are presented as box and whisker plots; red diamonds represent outliers. Black connector bars with * denote significant differences in the biomarker results for all protocols combined; coloured connector bars with * denote differences in biomarkers for that protocol. A: dopamine; B: serotonin; C: cortisol; D: glucose; E: HCT (haematocrit)

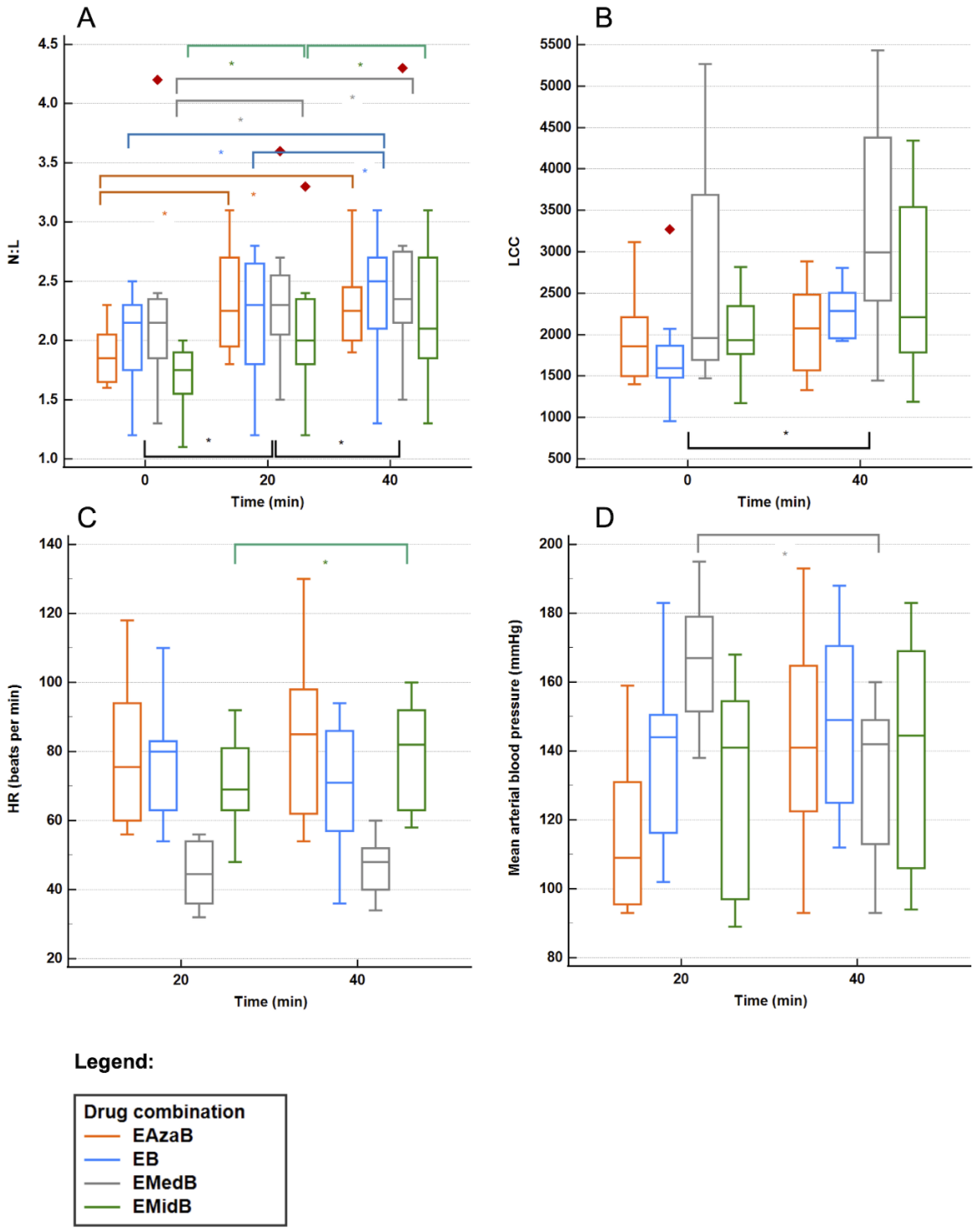

Figure 3. Clustered multiple comparison graphs showing results of stress biomarkers in white rhinoceros for four immobilisation protocols at 0, 20 and 40 min after sternal recumbency.

Data are presented as box and whisker plots; red diamonds represent outliers. Black connector bars with * denote significant differences in the biomarker results for all protocols combined; coloured connector bars with * denote differences in biomarkers for that protocol. A: N: L (neutrophil to lymphocyte ratio); B: LCC (leukocyte coping capacity); C: HR (heart rate); D: mean arterial blood pressure

4.4 Associations between stress biomarkers over time

There were significant correlations between many of the stress biomarkers and duration of the trial (Table 7). Significant positive correlations were present between trial duration and noradrenaline, adrenaline, normetanephrine, and serotonin, with all these biomarkers increasing with time as the study progressed. Metanephrine and glucose showed a negative correlation with study duration.

There were fewer correlations between the stress biomarkers and the immobilisation order used on the day (Table 7), with the only significant correlations being negative correlations seen with the N: L (P = 0.004), HR (P = 0.021) and normetanephrine (P = 0.031). These biomarkers all decreased as the immobilisation order increased, meaning that animals immobilised later in the day showed decreased values for these biomarkers.

Table 7. Correlation data of the various stress biomarkers using Spearman's rho. Values in bold indicate significant correlations.

Biomarker	Duration of trial		Daily immobilisation order	
	Spearman's rho	Significance (P-value)	Spearman's rho	Significance (P-value)
N: L	-0.199	0.051	-0.293	**0.004**
HCT	0.118	0.254	-0.105	0.309
BP	-0.158	0.219	-0.065	0.614
HR	0.164	0.195	-0.288	**0.021**
Dopamine	-0.153	0.136	-0.184	0.073
Noradrenaline	0.220	**0.031**	-0.012	0.904
Adrenaline	0.552	**<0.001**	-0.128	0.213
MHPG	0.057	0.583	0.036	0.729
Metanephrine	-0.316	**0.002**	-0.157	0.127
Cortisol	-0.063	0.544	0.090	0.382
Normetanephrine	0.385	**<0.001**	-0.221	**0.031**
DHEA	0.180	0.080	-0.150	0.145
Serotonin	0.290	**0.004**	-0.021	0.837
Glucose	-0.240	**0.019**	0.071	0.492

Biomarker	Duration of trial		Daily immobilisation order	
	Spearman's rho	Significance (P-value)	Spearman's rho	Significance (P-value)
LCC – NEU AUC	-0.078	0.540	0.025	0.847

There were also significant correlations between some of the stress biomarkers (Table 7). Haematocrit was negatively correlated with the N: L ratio and noradrenaline, and positively correlated with adrenaline, metanephrine, cortisol and serotonin. The N: L decreased as cortisol increased. There was a negative correlation between BP and glucose. Metanephrine and noradrenaline were negatively correlated.

Table 8. Correlation data between the various stress biomarkers using Spearman's rho. Only significant correlations are shown.

Variable 1	Variable 2	Spearman's rho	Significance (P-value)
HCT	N: L	-0.434	<0.001
	Noradrenaline	-0.213	0.037
	Adrenaline	0.209	0.041
	Metanephrine	0.222	0.030
	Cortisol	0.215	0.035
	Serotonin	0.207	0.043
Serotonin	Adrenaline	0.272	0.007
	Glucose	0.235	0.021
	Normetanephrine	0.230	0.024
Normetanephrine	Adrenaline	0.272	0.007
	NEU AUC	0.366	0.039
	Blood pressure	-0.371	0.044
Glucose	Metanephrine	0.229	0.025
	Blood pressure	0.526	0.003
Noradrenaline	Metanephrine	-0.219	0.032

Variable 1	Variable 2	Spearman's rho	Significance (P-value)
Cortisol	N: L	-0.310	0.002

Chapter 5: Discussion

5.1 Introduction

This study used a variety of stress biomarkers to evaluate whether the addition of various sedative and tranquilizing drugs to etorphine-based drug protocols reduced the magnitude of the acute stress response in southern white rhinoceros during chemical immobilisation. Capture by darting and the subsequent handling and manipulation of the animal into sternal or lateral recumbency is expected to result in a stress response, as a normal, adaptive process (12). This stress response is expected to be influenced by different sedative and tranquilizing drugs that are added to immobilising drug protocols.

Some significant differences in some of the measured biomarkers, namely noradrenaline, glucose, cortisol, HCT, N: L ratio and BP, were seen between the different drug protocols. Noradrenaline was lower ($P = 0.008$) with the midazolam protocol compared to the etorphine-only control and medetomidine protocols, and was also lower with the azaperone protocol compared to the medetomidine protocol ($P = 0.008$), but not compared to the etorphine-only control protocol. There were no other differences in biomarkers between the azaperone and midazolam protocols, and the control protocol. With the medetomidine protocol, glucose was higher compared to etorphine alone ($P = 0.003$), cortisol was lower compared to the azaperone protocol ($P = 0.027$), neutrophil: lymphocyte ratio was higher than with azaperone and midazolam protocols ($P < 0.001$) and haematocrit was lower compared to all protocols ($P < 0.001$).

The various sedative and tranquilizing drugs used in this study had minimal effects on reducing the side effects of etorphine administration. Other drugs with better anxiolytic properties should be evaluated in the future to improve the safety of chemical immobilisation procedures for white rhinoceros.

5.2 Limitations

This study has several limitations, including the lack of reference values for unrestrained animals, the potential adaptation of animals to the procedures over the time period of the study and the effect of other stressors outside of chemical immobilisation, including captivity and close proximity to humans.

A major limitation of this study was the lack of normal or reference values for unrestrained white rhinoceros for all of the biomarkers. Although having reference values would have been highly beneficial, it was not possible due to the nature of the project and the absence of unstressed, unrestrained animals in the study area, the Kruger National Park. Obtaining blood samples from rhinoceros that are not immobilised is not possible unless animals are habituated and trained for conscious blood collection. This was not the case in our study. Values for respiratory and

cardiovascular variables for non-immobilised, non-restrained white rhinoceros are available and findings for HR and BP could be compared with these (106). “Normal” values or reference intervals for the majority of the blood biomarkers, derived from unrestrained animals, are however not available (78). Having access to baseline values would be highly beneficial in determining the true magnitude of the acute stress response exhibited by immobilised animals. That there is a stress response is logical based on knowledge of the effect of a stressor, and also based on literature. Other studies that measured catecholamines in immobilised and transported rhinoceros found a significant decrease over time indicating that the stress response decreased - i.e. that there was a stress response present at initial measurement (straight after lateral recumbency) (48, 78). Future studies which aim to determine reference values in healthy, captive, non-chemically immobilised rhinoceros would be beneficial. However, these values may still differ from those found in wild animals due to a different stress factors in the wild compared to in captivity.

The administration of butorphanol in our study and the benefits thereof cannot be overlooked. Butorphanol is an important adjunct in white rhinoceros immobilisation and is used because it is able to partly reverse some of the negative effects induced by etorphine through its activation of mu-opioid receptors (136). Butorphanol greatly reduces respiratory compromise and hypoxaemia brought about by etorphine, making it an integral part of any immobilisation process in rhinoceros. Other important interventions in our study and many other immobilisation protocols are firstly the use of oxygen supplementation, and secondly, positioning the animal in a sternal position to improve blood oxygenation (75, 140).

All animals received butorphanol fifteen minutes after going down into lateral recumbency, between the first and second venous blood samples. It is possible that butorphanol may have had its own separate effects on the stress biomarkers, but since all animals received butorphanol, and since the statistical model analysed results from all blood sample time points together, butorphanol administration should not have confounded the results. The exceptions are BP and HR, where no T0 values were taken, and the dynamics and general lack of differences of the response in HR and BP may be due more to the administration of butorphanol than to the sedative or tranquiliser used in the protocol.

Our study aimed primarily to compare stress responses between different drug protocols. As with any stress study, stressors unrelated to the independent variables could also influence results. One concern was that the order of immobilisation on each day would affect results. Rhinoceros situated in neighbouring bomas, and next in the queue to be immobilised, may have been disturbed by the activity and become agitated, or even accustomed to the disturbances. However, few significant correlations between the stress biomarkers and the immobilisation order used on the day were found. Only the N: L ratio, HR and normetanephrine decreased the later in the day an animal was

immobilised. Immobilising animals at various times of the day did not seem to significantly affect the magnitude of the acute stress response.

Another concern was the potential adaptation of the rhinoceros to the bomas and the immobilisations with a resultant decrease in stress over time, or on the other hand, an increasing stress level related to confinement and anticipation of the procedure, leading to an increase in stress over time. There were significant positive correlations between the trial duration and noradrenaline, adrenaline, normetanephrine and serotonin. These findings suggest that animals potentially became more stressed over time. The potential confounding effect of an increase in stress over time would have been minimised by the cross-over study design and randomization of trials.

Another potential confounding factor was varying induction times between the different immobilisation protocols. We expected the induction times to vary between the different drug protocols, but as this was something that we could not fully control, we tried to standardise our sample collection by taking our first samples as soon as the animals were safely immobilised and in sternal recumbency. Although the induction times ranged from on average 09:06 minutes for the azaperone protocol to 15:23 minutes for the etorphine control protocol, T0 was always when the animals had reached a similar depth of immobilisation.

However, even with a standardised T0, it is possible that some of the drugs had not come into full effect by the time the first sample was taken. This would have been seen as changes in biomarkers between T0 and T20, and could have accounted for some of the findings presented in Table 6. During routine rhinoceros immobilisation, sedatives or tranquilisers are added to etorphine in the dart, and not given at a separate time point. This procedure was mimicked in this study. Administration of the drugs used here at different time points to etorphine, based on their known onset of action, may alter the stress response differently to that seen here, and could be investigated in future.

5.3 Characterization of the acute stress response in white rhinoceros immobilised with etorphine-based protocols

The etorphine and butorphanol protocol was used as the control protocol because etorphine forms the basis of the majority of the drug protocols that are used to immobilise southern white rhinoceros. Results from the other additional drug protocols were compared to results from the etorphine protocol, in order to evaluate whether the addition of the sedatives, midazolam or medetomidine, or the tranquiliser, azaperone, reduces the acute stress response of white rhinoceros during their immobilisation.

Although reference intervals for catecholamines in unstressed rhinoceros do not exist, reference values for other species are available. Human upper reference limits for plasma adrenaline and

noradrenaline using LC-MS have been reported as 0.18 ng/mL and 0.76 ng/mL, respectively (145). Healthy horses had a mean plasma adrenaline concentration of 0.03 ng/mL and mean noradrenaline of 0.07 ng/mL, using an HPLC method (146), while healthy dogs had median values of 0.13 ng/mL for adrenaline and 0.27 ng/mL for noradrenaline, also using HPLC (147). Adrenaline and noradrenaline concentrations measured in the rhinoceros in this study were several orders of magnitude higher than these values, with average values of 23.4 ng/mL and 26.4 ng/mL respectively for the etorphine protocol (EB), suggesting a catecholamine response. Adrenaline decreased significantly from T0 to T20 and T40 (Table 6 and Fig 1A), highlighting the immediate release of adrenaline that takes place in response to stress (148). No significant increases in noradrenaline were seen, potentially due to the fact that noradrenaline is constantly being released into the circulation in small amounts (148). The catecholamine metabolites metanephrine and normetanephrine were present in much lower concentrations than the catecholamines themselves, as expected (148). MHPG, the major metabolite of noradrenaline, was present in concentrations of about one quarter of those of noradrenaline, similar to what has been seen in other species (149). Although many opioids have effects on dopamine concentrations in humans, there were no significant changes in dopamine over the immobilisation period in this study (37). There was a significant increase from T0 to T20 and decrease from T20 to T40 for the neurotransmitter serotonin over all protocols ($p<0.001$), with increases in serotonin seen after administration of opioids (47). The decrease in serotonin seen from T20 to T40 may be a result of serotonin concentrations normalising after the initial stressful insult of immobilisation has subsided.

For the etorphine drug protocol, cortisol concentrations remained fairly constant during the immobilisation process (Table 6), with an average of 13.7 ng/mL. The cortisol concentrations in these rhinoceros were similar to but slightly lower than those recorded for free-living animals chased and darted with etorphine combinations from a helicopter (average of 18 ng/mL). This is probably because chased animals experience more stress compared to the animals in this study that were darted in bomas (48).

After insult from a stressor, changes in glucose concentrations in the blood are brought about by increases in catecholamines and cortisol. Here, based on statistical analysis (Table 6), glucose concentrations initially increased from T0 to T20 as expected, and then decreased from T20 to T40. Although potential positive correlations could be expected between glucose and the catecholamines and cortisol, no significant correlations were seen in our study. This may be due to the fact that cortisol concentrations remained fairly constant throughout the immobilisation process, therefore not correlating with the increases seen in glucose concentrations.

HCT decreased significantly from T0 to T20 to T40 ($p < 0.001$) for the etorphine protocol alone and all the other protocols. Potential reasons for a relatively high HCT at T0 include the presence of hypertension and tachycardia (see below) which cause fluid shifting from the intravascular to the

extravascular space (150, 151). Another mechanism is the release of sequestered red blood cells due to splenic contraction as a result of catecholamine release - a process known as stress haemoconcentration (48, 133, 150, 151).

The N: L ratio is expected to increase during stress, due to an increased concentration of neutrophils and a decreased concentration of lymphocytes in the vasculature (15). This change was seen in the present study, with an increase in the N: L ratio over the immobilisation period for all protocols (Table 6). The increase in N: L ratio is also expected to be proportional to the increase in glucocorticoid concentration (57). This proportional relationship between the N: L ratio and cortisol was however not seen in a study on rhinoceros conducted by Pohlin et al. in 2020, and also not found in this study. In fact, the opposite was found in this study, with a negative correlation seen between cortisol and the N: L ratio. This may be due to the fact that leucocytes in the blood do not change immediately in response to stress and first need to be activated by cortisol, which takes time (27). Cortisol concentrations on the other hand change within minutes of the presence of a stressor (53), potentially explaining the negative correlation between cortisol concentrations and the N: L ratio seen here. Therefore, these variables need to be interpreted independently of each other, but can be used together to track the changes in response to a stressor over a longer period of time (53).

Although expected to decrease, the LCC increased over the immobilisation period, when data from all protocols was combined. Generally, as a stressor persists over time, the oxidative burst decreases, leading to a decrease in LCC over time (58). The LCC responds to stressors slower than catecholamines and cortisol, with decreases in the oxidative burst generally seen from ten minutes to approximately two hours after a stressful insult (27). LCC results are however not always seen that soon, with no changes seen in LCC from the time of immobilisation to when animals were loaded for transportation around 20 to 30 minutes later in another rhino immobilisation study (69). The 40-minute time period in our study may therefore not have been long enough to see the expected changes in the LCC. More research into this method is needed for it to be used successfully as a measure of the acute stress response in white rhinoceros.

Tachycardia and hypertension are two of the most common cardiovascular findings in white rhinoceros immobilised with etorphine based drug protocols (73, 74, 76). These side effects are likely brought about by the drug-induced respiratory depression in combination with the effects of opioid-induced sympathetic stimulation (48, 78, 80). Based on the fact that unrestrained resting white rhinoceros have a HR ranging from 32 – 42 bpm and a mean arterial pressure (MAP) ranging from 108 – 135 mm Hg, the animals in our study all showed varying degrees of tachycardia and hypertension, with HRs of around 70 bpm and BPs ranging from 130 – 160 mmHg (106). The lack of changes in HR and BP overall over 40 min (Table 6) can be attributed to the fact that both measurements for these variables were taken post-butorphanol administration. Butorphanol is known to partly reduce tachycardia and hypertension in etorphine immobilised white rhino (76).

5.4 Differences in the acute stress response between drug protocols

5.4.1 Azaperone

The addition of azaperone to the immobilisation protocol yielded similar results to the etorphine-control protocol for all of the blood biomarkers. For serotonin, an initial significant increase from T0 to T20 was seen, followed by a decrease from T20 to T40. This latter decrease is potentially linked to the ability of azaperone to antagonize serotonin (37). The general lack of change seen with the addition of azaperone to the immobilisation protocol indicates that azaperone does not modify the acute stress response significantly compared to etorphine alone.

In white rhinoceros immobilisation, azaperone is currently the most commonly used adjunct with etorphine, due to its effect in reducing the hypertension that is brought about by etorphine (73, 76, 93). A reduction in hypertension was however not seen in this study, with no significant difference in BP in animals immobilised with the azaperone drug protocol compared to etorphine alone, and no change over time in BP with the azaperone protocol.

Our findings showed that azaperone had no clear beneficial effect on reducing the magnitude of the acute stress response or the hypertension induced by immobilisation with etorphine.

5.4.2 Midazolam

The addition of midazolam yielded many similar results when compared to the etorphine-control protocol, with only a few differences seen. Noradrenaline concentrations were significantly lower than the etorphine protocol, likely due to the fact that midazolam and other benzodiazepines are anxiolytics (73). The lower noradrenaline concentrations are an indication that the addition of midazolam may reduce the magnitude of the acute stress response. However, significant changes would also be expected in some of the other stress biomarkers, like cortisol, which was not the case in this study. Reasons why these effects did not occur may be linked to etorphine's profound effects on the sympathetic nervous system or other direct pharmacological effects of etorphine on the adrenal gland or other regions in the body associated with stress (152).

Other studies in white rhinoceros have shown that midazolam does reduce the activation of the HPA-axis to a greater extent than azaperone when animals are immobilised and transported (48). Our study did not find differences in cortisol, or catecholamines, between the azaperone and midazolam protocols, suggesting that a similar stress response is seen with both drugs during the immobilisation with etorphine.

Haematocrit values were high initially, most likely due to initial stress-induced contraction of the spleen in response to the administration of the immobilising drugs (48, 133, 150, 151). The haematocrit then

decreased over time, similarly to the other protocols, attributable to probable splenic relaxation and erythrocyte sequestration or a fluid shift from the extravascular to the intravascular space, rather than an actual decrease in the stress response. Hypertension was still seen with the midazolam protocol, indicating no beneficial effect of the drug on BP.

Lymphocytes and monocytes have peripheral benzodiazepine binding receptors (PBRs) which affect their ability to attract neutrophils, generally causing lowered N: L ratios when benzodiazepines are administered (48). Although expected to therefore be lower, the N: L ratio was not statistically different to the other drug protocols (Table 6).

Although noradrenaline concentrations were decreased when compared to the etorphine-control protocol, the lack of changes in other biomarkers suggests that midazolam does not significantly reduce the magnitude of the acute stress response when compared to etorphine alone.

5.4.3 Medetomidine

Medetomidine yielded the most statistically different results, but there were no differences in catecholamine concentrations compared to etorphine alone. This finding is unexpected because the release of catecholamines are usually inhibited by the administration of alpha-2 agonists, with these drugs acting on pre-synaptic alpha-2 adrenoreceptors inhibiting the sympathetic outflow in the central nervous system (114). Studies in various species have shown decreases in catecholamine concentrations following the administration of alpha-2 agonists, with decreases commonly seen in the plasma concentrations of both adrenaline and noradrenaline (114-119). A reason for the lack of a difference in catecholamine concentrations may be due to the action of medetomidine on imidazoline receptors. Medetomidine is different to some of the other alpha-2 agonists as it not only inhibits norepinephrine release through the action on alpha-2 adrenoreceptors, but may also cause a concurrent release of norepinephrine through indirect mechanisms related to the effects on I1 imidazoline receptors (114).

A significant decrease in serotonin concentrations was seen in this study from T20 to T40. Medetomidine administration has been shown to cause dose-dependent decreases in serotonin (45), although the decrease seen may also be attributable to serotonin concentrations normalizing after the initial insult from the stress of immobilisation. The significant increase from T0 to T20 was however slightly unexpected, but may be due to the initial strong effects of etorphine, with potent opioids known to cause increases in serotonin concentrations (47).

Medetomidine has age and species-specific effects on cortisol concentrations (114), with mild increases seen in calves and larger increases seen in adult cattle and sheep (127). Changes in cortisol concentrations were varied in dogs. No significant changes were seen in some canine studies (118), while other studies showed that premedication with medetomidine resulted in a reduction or

delayed increase in plasma cortisol concentration (128). Sedation with other alpha-2 adrenoreceptor agonists like xylazine also resulted in a reduction in cortisol concentrations (129). In our study, cortisol concentrations were not different from the etorphine control protocol, although they were significantly lower than that seen with the azaperone protocol.

Glucose concentrations were highest in animals immobilised with medetomidine, and were significantly higher than in rhinoceros immobilised with the etorphine control protocol. One would expect elevated glucose concentrations to be linked to an increase in catecholamines and cortisol, because an insult from a stressor leads to increases in catecholamines and cortisol and then to increased blood glucose. The increase in blood glucose occurs due to mobilization of energy stores via the processes of glycogenolysis and gluconeogenesis, and the reduction of energy usage by non-vital organs. The aim is to optimize energy usage to protect the animal after a stressful insult (27). In our study, this was not the case, as catecholamine and cortisol concentrations were not different between the medetomidine protocol and most other protocols. However, the increased glucose concentrations with the medetomidine protocol are likely due to the direct effect of medetomidine as it has been shown to induce hyperglycaemia in various species by acting on alpha-2-receptors in the B-cells of the pancreas with subsequent inhibition of insulin secretion (114).

Haematocrit was significantly lower with the medetomidine protocol compared to the other drug protocols. This finding is consistent with other studies that reported a decrease in HCT or PCV after alpha-2 agonist administration, including in a study conducted by Kullman et al. in 2014 on the effects of alpha-2 agonist administration on the spleen and PCV in horses, a species that shares a common ancestor with rhinoceros (132). The study used ultrasonography to measure the splenic thickness over time after administration of alpha-2 agonists and found that the spleen increased in thickness after these drugs were administered. This increase in thickness occurred concurrently with a decrease in the PCV, supporting the mechanism that RBCs were being sequestered into the spleen (132). An important consequence of a lower haematocrit during immobilisation may be that it adversely affects tissue oxygenation as there are less red blood cells to carry oxygen to the tissues.

Medetomidine administration has been shown to cause a biphasic response in BP, with an initial increase followed by a normotensive or slightly hypotensive state (120). In our study, animals immobilised with the medetomidine protocol were hypertensive at T20, then showed a decrease in BP over the next 20 minutes to T40 (Table 6). The medetomidine protocol was the only protocol where this decrease was seen. It is possible that the well-described initial increase in BP was missed as no BP readings were taken prior to T20. Overall BP values were not different when compared to the other drug protocols, suggesting that medetomidine does not significantly reduce etorphine-induced hypertension over a 40-min period.

Animals immobilised with the medetomidine protocol showed the most changes in stress biomarkers; with these effects mostly directly related to the pharmacological actions of the drug.

5.5 Additional comments

The drug combinations and dosages used in the study were the same as those routinely used by the SANParks veterinary team to immobilise white rhinoceros in a routine field setting. These standardised dosing tables have been calculated according to weight and have been used effectively to provide safe and stable immobilisation in free-ranging white rhinoceros. Our study did not try to create new combinations and dosages, but rather aimed to determine which of the existing protocols mitigated the acute stress response.

Regardless of the different immobilisation protocol used, the depth of immobilisation did not differ much, with all animals found to be in the 4th and 5th levels (Table 2). This similar depth of immobilisation can also be attributed to the accuracy of standardised drug dosages already in place. Different depths of anaesthesia could have potentially altered the results, but as animals were maintained at a similar depth of anaesthesia due to accurate dosages used, we believe that this did not affect our results.

Although it is difficult to determine the magnitude of the acute stress response with the lack of unstressed, unrestrained animals as a comparison group, our results suggest that the rhinoceros in our study mounted an acute stress response to the immobilisation process. The highest concentrations of adrenaline occurred at the first sampling time point and increases in the N: L ratio were seen over the immobilisation period, similar to what was seen in other studies on immobilised white rhinoceros (48). These results align with what is expected during an acute stress response, as stress-induced activation of the sympathetic nervous system and HPA-axis results in a rapid release of catecholamines (15).

Many of the changes in stress biomarkers can be attributed to the direct pharmacological effect of the various drugs. As has been highlighted in various areas of the dissertation, the drugs therefore directly modify the stress response. The drugs however also indirectly modify the stress response by directly causing physiological changes like hypoxia, hypercapnia and acidosis which also activate physiological stress responses in rhino (78). Additionally, other factors unrelated to the immobilising drugs, but still related to the immobilisation procedure, such as the act of being darted, lying in lateral recumbency, and the presence of humans, also act as stressors. The stress response that is measured in an immobilised rhinoceros is therefore dependant on the complex interaction of the direct effect of drugs, the indirect effect of drugs, and non-drug factors. All of these factors contribute to the stress response, and it not possible to evaluate the individual contribution of each to the stress response. In this study, the global effect of a drug combination on stress biomarkers was therefore evaluated.

Although a few of the stress biomarkers analysed in our study showed differences between the different drug protocols, there were many biomarkers that did not yield any significant differences. These included HR, LCC, the catecholamine dopamine and the monoamine neurotransmitter serotonin. Metanephrine, one of the major breakdown products of adrenaline, as well as normetanephrine and MHPG, the major breakdown products of noradrenaline, also showed no differences between the drug protocols. A reason for no significant differences for some of the biomarkers, especially the catecholamine metabolites and the LCC, could be because the 40-minute sampling period was not enough to yield differences. Another possible reason could be because these additional drugs do not mitigate the stress responses brought about by immobilisation with etorphine.

Another reason for the rather minimal effect of the additional sedatives and tranquilisers may be due to the fact that etorphine seems to have a very fast onset of action. The relatively slower onset of action of the sedatives and tranquilisers, albeit only differing marginally by a few minutes, may thus limit their effectiveness in preventing the acute stress response caused by etorphine. Although not practical in a wild immobilisation setting, administering these sedatives and tranquilisers ten to fifteen minutes before administering etorphine may make them more effective. This could potentially be evaluated in a study with captive rhinoceros to determine if these drugs may be of any use in decreasing the magnitude of the acute stress response induced by immobilisation with etorphine.

Future studies could also look at investigating the use of more potent anxiolytic benzodiazepines, such as the human drug clonazepam. These would however have to have a fast onset of action to be effective in an immobilising setting and be compatible with the other immobilising drugs, meaning extensive studies may be needed.

One of the minor objectives of this study was to evaluate whether the LCC would be a good marker of the acute stress response after positive initial findings in other studies (48). Research has shown that animals that have a higher LCC are more likely to respond better physiologically to a stressor (58). Over time as the stressor persists, the oxidative burst then decreases, leading to a decrease in LCC (58). Anaesthetic agents can also influence the LCC (66), but we found no significant differences between the drug protocols with regards to the LCC.

Chapter 6: Conclusions

Etorphine has been and will likely continue to be used for the immobilisation of white rhinoceros. It does however have marked side effects, highlighting the need for additional drugs to be added to rhino immobilisation protocols to try and improve their safety. Our study provided new insight into how chemical immobilisation using etorphine with various types of central nervous system-depressant drugs affected the acute stress response in southern white rhinoceros.

In our study, immobilisation was successful with all of the different drug protocols that were tested. However, a few significant differences were seen between the protocols. The medetomidine protocol showed the most differences in the stress biomarkers during the immobilisation process, with the highest N: L ratio and glucose concentrations, and significantly lowered haematocrits, which may negatively affect tissue oxygenation.

We found that the addition of azaperone and midazolam yielded minimal improvements in the stress response when compared to the etorphine control, although noradrenaline was lower with the addition of midazolam. Combining these drugs or medetomidine with etorphine may have beneficial effects on respiratory, cardiovascular and neuromuscular functions, but this would need to be confirmed with further study.

Future research to determine reference values for the various stress biomarkers would be highly beneficial as this will allow the true magnitude of the acute stress response to be measured. Additionally, comparing research findings with reference values in resting conscious rhinoceros will aid in determining how clinically relevant the acute stress response is.

Since the sedative and tranquilizing drugs used in this study had minimal effects on reducing the side effects of the acute stress response induced by etorphine immobilisation, other drugs with better anxiolytic properties, and quicker onsets of action should be considered. For captive-held animals, future studies could also potentially investigate the timing of the administration of anxiolytic drugs, as their use as a premedication may be more beneficial than when co-administered with etorphine, due to the general slower onset of action of anxiolytics, compared to etorphine.

This is the first study to compare the acute stress response when various different drug protocols are used to chemically immobilise white rhinoceros, and it shows that the current sedative and tranquilizing drugs used have a limited ability to alter this stress response in rhinoceros induced by immobilisation with etorphine. We hope that our findings will enable a better understanding of the acute stress response of rhinoceros during chemical immobilisation and facilitate the selection and development of the safest drug protocols for chemical immobilisation procedures.

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Appendix documents

Appendix 1: Summary of immobilisation dosages for white rhinoceros used in the study based on the estimated body weight categories and VWS protocol.

Animal weight (kg)	**Etorphine (0.002 mg/kg)**	**Azaperone (0.01 mg/kg)**	**Midazolam (0.01 mg/kg)**	**Medetomidine (0.005 mg/kg)**	**Butorphanol (10 x etorphine dose, mg)**	**Naltrexone (20 x etorphine dose, mg)**
500 – 750	1.5	7.5	7.5	3.75	30	30
750 - 1000	2.0	10.0	10.0	5.0	40	40
1000 - 1250	2.5	12.5	12.5	6.25	50	50
1250 – 1500	3.0	15.0	15.0	7.5	60	60
1500 – 1750	3.5	17.5	17.5	8.75	70	70
1750 – 2000	4.0	20.0	20.0	10	80	80

Appendix 2: LCMS method for the detection and quantification of a variety of biochemical messengers in Rhinoceros plasma samples

1. Chemicals, reagents, materials and instruments

1.1. Chemicals and reagents

L-Noradrenaline hydrochloride, 3-methoxy-4-hydroxyphenylglycol hemipiperazinium salt (MHPG), normetanephrine hydrochloride, metanephrine hydrochloride, adrenaline (epinephrine) bitartrate salt, dopamine hydrochloride, serotonin creatinine sulphate, hydrocortisone (cortisol), dehydroepiandrosterone (DHEA) and ethyl 4-hydroxy-2-quinolinecarboxylate (EHQC) as internal standard were obtained from Sigma-Aldrich Pty (Ltd) (Johannesburg, South Africa). Chemicals used for the mobile phase were HPLC grade deionized water, HPLC grade methanol (MeOH) and formic acid (99%). Chemicals used for the preparation of the standards and samples were methanol, acetonitrile (ACN) glacial acetic acid (99%) and formic acid (99%); all the chemicals were obtained from Merck (Pty) Ltd (Johannesburg, South Africa).

1.2. Materials

The analytical HPLC column used was a Kinetix C18 (purchased from Phenomenex, Torrance, CA, USA, 2.1 x 1000 mm, a particle size of 2.6 µm, pore size of 100 Å and a surface area of 200 m^2/g.

1.3. Instrumentation

Ultivo Triple Quadrupole LC/MS System, controlled by the MassHunter software from © Agilent Technologies, Inc. (Santa Clara, CA 95051 US).

2. Methods (standards, internal standard, mobile phase, instrument setup and sample preparation)

2.1. Standard solutions

Dissolve approximately 1 mg of each the analytes (except DHEA; 2 mg) separately in 10ml 10 % methanol solution. To protect the analytes from light, 10 ml amber volumetric flasks were used. This will form the stock solution of each analyte.

From this stock solution a range of 6 concentrations was prepared to setup a standard calibration curve.

2.2. Internal standard solution

Prepare an internal standard stock solution of the internal standard; ethyl 4-hydroxy-2-quinolinecarboxylate (EHQC), with a concentration of 100 µg/ml using a solvent mix of 0.1 M formic acid in 100% acetonitrile. Prepare a working internal standard solution with a final concentration of 250 ng/ml by appropriate dilution from the internal standard stock solution using the same solvent mixture as for the stock solution. The working solution will also be used for the preparation of the different biological sample matrixes.

2.3. Mobile phase

A gradient mobile phase consisting of A: 0.1 % formic acid/HPLC grade water and B: 0.1 % formic acid/Methanol were prepared. Table 1 shows how the gradient mobile phase was applied.

Table 1: Mobile phase gradient setup

Step	Time (min)	A (%)	B (%)
1	Start. Cond. min	92.0 %	10.0 %
2	1.00 min	92.0 %	10.0 %
3	5.00 min	0.0 %	100.0 %
4	11.00 min	0.0 %	100.0 %
5	12.00 min	92.0 %	8.0 %

2.4. LCMS/MS Instrument settings

LC instrument settings:	
Flow rate	0.300 ml/min
Injection volume	1 µl
Run time	15 minutes (3 minutes posttime)

Mass spectrometer settings:	
Source parameter	**Positive value**
Gas temperature (ºC)	350
Gas flow (L/min)	13
Nebulizer	60
Capillary voltage (V)	4000
Analyte setup:	

Analytes	Transition Precursor -> Product (m/z)	Dwell (ms)	Fragmentor (V)	Collision Energy (V)	Polarity	Scan	RT (min)
Noradrenaline	170.1 -> 152.1	100	51	5	+	MRM	1.824
Normetanephrine	184.1 -> 166.2	100	71	5	+		1.843
Metanephrine	198.1 -> 180.1	100	51	5	+		1.892
MHPG	183.1 -> 121.0	100	56	40	-		2.584
Dopamine	154.1 -> 65.0	100	71	40	+		1.877
Serotonin	177.1 -> 115.0	100	66	40	+		2.213
Cortisol	363.2 -> 120.9	100	106	40	+		9.377
DHEA	289.2 -> 90.9	100	76	68	+		10.005
EHQC I.Std	218.1 -> 116.0	100	86	40	+		6.387

3. Sample preparation

3.1. Plasma samples

To 100µl of plasma sample and 100µl of the Internal standard solution (Methanol: 0.1%Formic acid: Internal standard, EHQC) was added. The mixture was placed on ice for 20 minutes to complete protein precipitation. Samples were centrifuged at 14 000 rpm for 20 minutes at 4ºC. 1 µl of the supernatant was injected onto the LC-MS system.

4. *Linearity / calibration curve and detection limit (sensitivity)*

The linearity was done by preparing 7 standard concentrations as described in section 3.1. Six replicates of each standard were injected to establish linear regression for each analyte. The linear regression value (coefficient of determination, R^2) for the calibration curve must not be less than 0.95 for endogenous biomolecules [1].

The limit of detection (LOD) can be defined as the minimum concentration where the signal-to-noise ratio was at least 10:1 and 3:1 greater than the average background noise of an unspiked blank (only containing the internal standard), at the retention time of each analyte, respectively [2].

5. Linearity / calibration curve and detection limit (sensitivity)

The calibration curves constructed was evaluated by means of its linear regression value. Linearity was excellent over the respective calibration ranges (table 2), with corresponding coefficient of determination (R^2) for each of the analytes.

Table 2: Linear regression line equation and coefficient of determination

Analytes	Concentration range (ng/ml)	y = mx + c (Determined by MassHunter, line forced through origin)	Coefficient of Determination (R^2)
Noradrenaline	3.125; 6.25; 12.5; 25.0; 50.0; 100.0; 200.0	y = 10,952*x + 663,678	= 0,98
Normetanephrine		y = 55,891*x + 73,06	= 0,99
Metanephrine		y = 173,79*x + 243,037	= 0,99
MHPG		y = 0,141*x + 0,52	= 0,99
Dopamine		y = 25,686*x + 20,909	= 0,99
Serotonin		y = 9,422*x + 47,732	= 0,99
Cortisol		y = 2,020*x + 35,145	= 0,99
DHEA	6.25; 12.5; 25.0; 50.0; 100.0; 200.0; 400.0	y = 1,423*x + 42,606	= 0,98

The limit of detection (LOD) = 2.0 ng/ml.

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Appendix 3: 1 min PPT presentation for Faculty of Veterinary Science Faculty Day 20 October 2022

Access available via: https://youtu.be/SWuzyrywlPc

Appendix 4: 1 min PPT presentation for UP Postgraduate Research Day 2022

Access available via: https://youtu.be/Soed1AbMgRc