Comparison of etorphine-midazolam and etorphine-azaperone for African buffalo (*Syncerus caffer*) immobilisation

By

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Abstract

Objective To compare physiological and time variables of etorphine-azaperone (EA) to etorphine-midazolam (EM) immobilisation in African buffalo bulls.

Study design Randomised crossover study

Animal population A group of ten adult buffalo bulls of mean ± standard deviation 49 ± 19 months old and weighing 353 ± 97 kg.

Method Each buffalo was administered, by dart, EA and EM once, one week apart. Once recumbent, the buffalo was instrumented, and physiological variables were recorded at 5-minute intervals, starting at 5 minutes until 20 minutes after recumbence. Opioid antagonist (naltrexone 20 mg mg^{-1} etorphine dose) was administered at 40 minutes. Induction (dart placement to recumbent) and recovery (naltrexone administration to standing) times were recorded. Arterial and jugular venous blood samples were analysed for gases, acid-base status, and electrolytes at 5 and 20 minutes using a portable blood gas analyser. The arterial to end-tidal carbon dioxide gradient and alveolar to arterial partial pressure of oxygen gradient were calculated. Data were compared between combinations using a general linear mixed model and reported as mean (± standard deviation). Significant findings were set to $p < 0.05$.

Results The doses of etorphine, azaperone and midazolam administered were 0.015 ± 0.001, 0.15 ± 0.01 and 0.16 ± 0.02 mg kg^{-1}, respectively. Induction times for buffalo immobilised with EA was 326 ± 304 seconds (5.4 ± 5.1 minutes) and not different to 247 ± 162 seconds (4.1 ± 2.7 minutes) for EM. The overall mean heart rate for the buffalo immobilised with EA was 113 ± 27 beats $minute^{-1}$, which was significantly faster compared to 79 ± 22 beats $minute^{-1}$ for EM ($p < 0.001$). The respiratory rate, when buffalo were immobilised with EA, was 18 ± 4 breaths $minute^{-1}$ which was significantly slower compared to 23 ± 7 breaths $minute^{-1}$ for EM ($p < 0.001$). The overall

mean arterial blood pressure for buffalo immobilised with EA was 102 ± 25 mmHg, which was significantly lower compared to 163 ± 18 mmHg for EM ($p < 0.001$). The overall mean arterial partial pressure of oxygen for buffalo immobilised with EA was 37 ± 12 mmHg which was not different compared to 43 ± 8 mmHg for EM ($p = 0.062$). The potassium concentrations of both arterial and venous samples of the EM immobilised buffalo were 4.0 ± 0.4 mmol L^{-1} and within expected reference intervals, but significantly different compared to 3.7 ± 0.3 mmol L^{-1} for EA immobilised buffalo ($p < 0.01$). The other measured electrolytes (sodium, chloride, and ionised calcium) were not different between EA and EM buffalo nor between arterial and venous blood samples within a combination. The majority of the buffalo demonstrated a negative arterial to end-tidal carbon dioxide gradient with alveolar to arterial partial pressure of oxygen gradients greater than 20 mmHg in both EA and EM, however, there were no significant differences between drug combinations. Recovery times were 71 ± 27 seconds (1.2 ± 0.5 minutes) for EA and not different to 86 ± 44 seconds (1.4 ± 0.7 minutes) for EM immobilisation.

Conclusion and clinical relevance EM produced an equally effective and reliable immobilisation as EA in buffalo bulls. However, systemic arterial hypertension was a concern in EM and both combinations caused clinically relevant hypoxaemia. However, treatment of this hypoxaemia is questioned as no clinical variables indicated that life-threatening interventions were required. We speculate that an early compensatory response to severe hypoxaemia was most likely present. Astute monitoring is advised with both drug combinations, especially if supplemental oxygen is administered.

List of abbreviations

%	percentage
°C	degree(s) Celsius
α	alpha
β	beta
BE(b)	base excess (blood)
BE(ecf)	base excess (extracellular fluid)
Ca^{2+}	calcium ion
cAMP	cyclic adenosine monophosphate
CaO_2	arterial oxygen content
Cl^-	chloride ion
CNS	central nervous system
CO_2	carbon dioxide
Crea	creatinine concentration (whole blood)
δ	delta
DAP	diastolic arterial blood pressure
EA	etorphine-azaperone
EM	etorphine-midazolam
FIO_2	fraction inspired oxygen
f_R	respiratory frequency
γ	gamma
Glu	glucose concentration (whole blood)
g dL^{-1}	gram(s) per decilitre
GPCR	G protein-coupled receptors
H^+	hydrogen ion
Hb	haemoglobin
HCO_3^-	bicarbonate ion
HR	heart rate
Ht	haematocrit
iCa	ionised calcium
IP_3	inositol 1, 4, 5-triphosphate
IV	intravenous(ly)
K^+	potassium ion
κ	kappa
kg	kilogram(s)
kPa	kilopascal(s)
Lac	lactate
MAP	mean arterial blood pressure
µmol L^{-1}	micromole(s) per litre
mg kg^{-1}	milligram(s) per kilogram
mL	millilitre(s)
mL dL^{-1}	millilitre(s) per decilitre
mm	millimetre(s)
mmHg	millimetres mercury
mmol L^{-1}	millimole(s) per litre
µ	mu
Na^+	sodium ion
n	number
O_2	oxygen
p	probability value

$PaCO_2$	arterial partial pressure of carbon dioxide
PAO_2	alveolar partial pressure of oxygen
$P(A\text{-}a)O_2$	alveolar to arterial partial pressure of oxygen gradient
PaO_2	arterial partial pressure of oxygen
$P(a\text{-}E')CO_2$	arterial to end-tidal carbon dioxide gradient
PB	mean ambient barometric pressure
$PE'CO_2$	end-tidal carbon dioxide
PH_2O	water vapour pressure (standard 47 mmHg)
PIO_2	partial pressure of inspired oxygen
$PvCO_2$	venous partial pressure of carbon dioxide
PvO_2	venous partial pressure of oxygen
RAP	right atrial filling pressure
RQ	respiratory quotient
SaO_2	arterial haemoglobin oxygen saturation
SAP	systolic arterial blood pressure
SD	standard deviation
SvO_2	venous haemoglobin oxygen saturation
T0	time point 0 minutes (time of first handling)
T5	time point 5 minutes from time of first handling
T10	time point 10 minutes from time of first handling
T20	time point 20 minutes from time of first handling
T40	time point 40 minutes from time of first handling

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Chapter 1 - Introduction and literature review

1.1 Introduction

The African buffalo (*Syncerus caffer*), one of the world's most dangerous animals, are highly sought after in wildlife conservancies and breeding programmes, especially if the individual has a disease-free status or is a hunting-trophy animal (McNamee 2011). Veterinarians are required to chemically capture (immobilise) buffalo for various management procedures such as translocation, horn morphometry, testing for disease and general health evaluation. Drugs used alone or in various combinations to immobilise African buffalo are reported in textbooks and in descriptive studies, however, comparative studies are lacking (Hattingh et al. 1984; Oosthuizen et al. 2009; Kock et al. 2012; Napier & Armstrong 2014; Wolfe 2015; Couch et al. 2017). These drugs and their dosages have been derived over decades based on clinical experience which has been shared amongst wildlife and zoo veterinarians (Napier & Armstrong 2014). It could be speculated that dosages for African buffalo have been extrapolated from other species from the same taxonomic subfamily of *Bovinae*, that includes bison, antelopes (four-horned and spiral-horned species), and water buffalo (McMahon & Bradshaw 2008). However, opioid drugs have different binding affinities in different species and opioid receptor density and distribution within the brain, spinal cord and peripheral tissue vary among species (Schumacher 2008; Feng et al. 2012). Therefore, extrapolating drug effects and dosages from other species could be equivocal and lead to harm of the animal and personnel. In Africa, the use of etorphine alone or, more commonly, in combination with a sedative is a popular choice of drug to immobilise African buffalo (Oosthuizen et al. 2009; Kock et al. 2012).

1.2 Literature review

Drugs used in African buffalo immobilisation

During chemical immobilisation, the capture team must consider the risks of morbidity and mortality during the immobilisation period. A focus on drug related risks will be discussed later in the review. Furthermore, other aspects of immobilisation such as location, weather, time of day and equipment must also be considered during the pre-capture risk analysis (Caulkett & Arnemo 2015). However, these aspects are covered in the referenced literature and will not be discussed further. Consideration of all these factors is important to ensure safety of the personnel and well-being of the animal. The characteristics of an ideal drug to use during immobilisation are 1) high therapeutic index to allow for error in estimated body mass, 2) non-irritant to tissue, 3) short induction times, 4) analgesic properties, 5) predictable effects in variety of species, 6) solution should be stable at room temperature storage, 7) miscible and stable in combination with other drugs 8) high concentration formulations to allow for small volumes, and 9) able to be antagonised (Fowler 2008; Caulkett & Arnemo 2015). The end-goal of administering an ideal drug is to produce an effective and reliable state of immobilisation. The drugs are preferably delivered to African buffalo by using a remote system such as a projectile dart because of their aggressive demeanour. African buffalo are unlike Asian water buffalo (*Bubalus bubalis*) which are usually domesticated and allow hand-injection (Fowler 2008). Caulkett and Arnemo (2015) reported that an increase in drug dose of up to 50% is required to achieve effective immobilisation when using a remote delivery system versus hand-injection. Furthermore, the total volume of the drugs becomes limited by the capacity of the payload chamber of the dart during remote delivery. Hence, high concentration (mg mL^{-1}) formulations of injectable drugs allow a smaller volume of drug that can be added to the payload chamber to ensure an effective dose is administered to the animal.

African buffalo immobilisation dates back to the early 1960s with one of the earliest literature reports from Harthoorn and Lock (1961) where he compared different peripheral muscle relaxants (gallamine triethiodide and D-tubercurarine) as the sole immobilisation drug. The use of these paralytics was less successful in larger hoofed animals compared to smaller hoof stock (Harthoorn & Bligh 1965; Harthoorn 1966). Therefore, the use of a novel drug at that time, compound M99, emerged as a successful drug for immobilisation. The M99 compound, later named etorphine hydrochloride, is an oripavine alkaloid that is synthetically derived from thebaine, a natural occurring compound, found in opium (Bentley & Hardy 1963; Blane et al. 1967). Etorphine is classed as a potent opioid agonist and has been administered to African buffalo, as well as other potent opioid agonists (Kalema-Zikusoka et al. 2005; Oosthuizen et al. 2009; Napier & Armstrong 2014; Szabo et al 2015).

Potent opioids are a group of drugs that encompasses most of the characteristic for an ideal immobilisation drug. Potent opioid drugs are used either alone or in combination with a sedative drug (neuroleptanalgesia). The potent opioids are favoured because of their predictable effects that result in a reliable state of immobilisation and can be antagonised. Etorphine is the most commonly used potent opioid agonist for immobilisation of herbivorous wildlife, however, thiafentanil, another type of potent opioid agonist is gaining popularity because it is thought to result in a faster state of immobilisation (Oosthuizen et al. 2009; Kock et al. 2012; Napier & Armstrong 2014). Despite the high potency of etorphine, high doses are usually required to reliably immobilise *Bovinae* species when administered alone (Kock et al. 2012; Wolfe 2015). Therefore, it has become common practice to use neuroleptanalgesic drug combinations to achieve a reliable immobilisation (Curro 2007; Oosthuizen et al. 2009; Napier & Armstrong 2014). These drug combinations are thought to allow reduction of the etorphine dose which results in a decrease in the severity of its side effects that, amongst others, include respiratory depression, systemic hypertension, and skeletal muscle rigidity (Schumacher 2008).

Currently, etorphine-azaperone is a widely used drug combination with etorphine-xylazine and etorphine-medetomidine also being recommended to immobilise water buffalo and African buffalo (Curro 2007; McMahon & Bradshaw 2008; Kock et al. 2012; Wolfe 2015). Furthermore, in the last decade, benzodiazepine agonists have gained popularity in the large hoof stock species owing to good sedative and minimal cardiopulmonary effects (Malik 2011; Bodh 2015; Rina et al. 2018). However, the benzodiazepine agonists, to date, have been administered by the intravenous (IV) route as a premedication before the induction of anaesthesia in domesticated water buffalo and yak (Nain et al. 2010; Kumar et al. 2014; Rina et al. 2018). The reason for this practice is perhaps related to the current available drug formulations having a low concentration which make hand-injection the only option to deliver drugs from this class. Considering the drugs that have been administered to buffalo, the etorphine and azaperone combination are of interest as well as the novel high-concentration formulation of midazolam in combination with etorphine.

Etorphine hydrochloride

Etorphine hydrochloride [molecular formula: $C_{25}H_{34}ClNO_4$; 7α-(1-(R)-hydroxy-1-methylbuthyl)-6,14-endoethenotetrahydro-oripavine hydrochloride] is a potent (or sometimes described as an ultrapotent) mu (μ) opioid receptor agonist which also has agonist activity on kappa (κ) and delta (δ) opioid receptors (Blane et al. 1967; Waldhoer et al. 2004; Koyyalagunta 2006; Law & Loh 2013). These opioid receptors are widely distributed within the mammalian central nervous system (CNS) and to a lesser extent throughout the periphery which includes the synovium, gastrointestinal tract, heart, and leukocytes (Pathan & Williams 2012; KuKanich & Wiese 2015). Etorphine is regarded as being at least 1000 times more potent than morphine sulphate with the ability to cause catatonia and when administered at low doses, it can immobilise game animals (Blane et al. 1967; Koyyalagunta 2006). Etorphine (ligand – first

messenger) binds to the membrane-bound opioid receptors which are G protein-coupled receptors (GPCR) associated with a heterotrimeric G_i-protein (comprised of $G_i\alpha$, Gβ, Gγ subunits) (Koyyalagunta 2006; Chong & Johnson 2017). This binding activates the G_i-protein cascade which ultimately results in the exchange of guanosine diphosphate (GDP), which is attached to the $G_i\alpha$ subunit, for guanosine triphosphate (GTP). Once bound, the $G_i\alpha$-GTP complex causes the subunit Gβγ complex to dissociate and move into the cellular cytosol. The activation of the G_i-protein also leads to inhibition of adenylyl cyclase and results in a decreased formation of cyclic adenosine monophosphate (cAMP; second messenger) (Koyyalagunta 2006; Al-Hasani & Bruchas 2011; Pathan & Williams 2012; Heidemann 2013; Whittem et al. 2015; Chong & Johnson 2017). The $G_i\alpha$-GTP complex interacts with the G protein gated inwardly, rectifying potassium channels resulting in enhancement of postsynaptic receptor-operated K^+ efflux which causes hyperpolarisation of nociceptive neurons and increased activation thresholds (Inturrisi 2002; Al-Hasani & Bruchas 2011; KuKanich & Wiese 2015; Chong & Johnson 2017). The Gβγ-complex binds directly with the presynaptic neuron voltage-gated Ca^{2+} channels resulting in a decrease in intracellular calcium (Ca^{2+}) with a decreased release of the excitatory neurotransmitters substance P and glutamate (Inturrisi 2002; Al-Hasani & Bruchas 2011; KuKanich & Wiese 2015; Chong & Johnson 2017). Physiological effects which result from the administration of non-potent opioids are species dependent but ultimately can include hypoalgesia (decreased perception of pain), respiratory depression, systemic hypertension, bradycardia, increased vagal tone, gastrointestinal dysmotility and increased bladder tone (KuKanich & Wiese 2015). Most opioids undergo Phase I and Phase II hepatic biotransformation with the majority of metabolites being excreted *via* urine, however, biliary excretion also occurs (Koyyalagunta 2006; KuKanich & Wiese 2015).

Azaperone

Azaperone [molecular formula: $C_{19}H_{22}FN_3O$; 1-(4-fluorophenyl)-4-(4-pyridin-2-ylpiperazin-1-yl)butan-1-one] is a short-acting butyrophenone derivative, which is a multipotent receptor blocker which mainly antagonises dopamine (D2) and α_1-adrenoceptors, and to a less extent dopamine (D1), serotonin (5-HT), histamine (H1) and muscarinic (M1) receptors (Lamont & Grimm 2014; Rankin 2015; Dugdale et al. 2020). Butyrophenone derivatives also have a GABA-mimetic activity in the reticular activating system which contributes to the sedative effect (Dugdale et al 2020). The D2 receptors are also GPCR associated with a heterotrimeric G_i-protein, with blockade leading to no intracellular effects common to this GPCR (Rankin 2015). The α_1-adrenoceptors (predominant α-adrenoceptor on vascular smooth muscle) are GPCR, however they are associated with the heterotrimeric G_q-protein and when activated leads to an increase in inositol 1, 4, 5-triphosphate (IP_3 – second messenger) which results in vascular smooth muscle contraction and thus vasoconstriction (Klabunde 2012; Heidemann 2013). Butyrophenone derivatives have minimal cardiopulmonary depression, although through α_1-adrenoceptor antagonism a reduction in IP_3 leads to a decrease in vascular tone which could result in vasodilation (Klabunde 2012; Heidemann 2013; Rankin 2015; Dugdale et al 2020). This vasodilation, especially in the peripheral vasculature, results in a decrease in arterial blood pressure and contributes to increased heat loss and dissipation of heat from the core to the periphery. The heat loss could result in hypothermia, because the antidopaminergic effects cause poikilothermia. However, the poikilothermia could, contrariwise, result in excessive heat gain, especially during high ambient temperatures or more importantly high environmental heat loads (Rankin 2015; Dugdale et al. 2020).

Butyrophenone derivative overdose can lead to prolonged hypotension, tachycardia, increased muscle rigidity, extrapyramidal excitation, hyperthermia, suppression of oestrus cycle

(reduction of follicular stimulating and luteinising hormones) and seizures (Rankin 2015; Dugdale et al. 2020). Two syndromes have been observed with butyrophenone administration, neuroleptic malignant syndrome, and central anticholinergic syndrome (Dugdale et al. 2020). Neuroleptic malignant syndrome is characterised by hyperthermia, altered consciousness (seizures, excitation, restlessness), muscle rigidity (due to extrapyramidal excitation) and autonomic instability (tachycardia, sweating, labile blood pressure) (Dugdale et al. 2020). Whereas, central anticholinergic syndrome is characterised by depression or excitation, restlessness, muscular incoordination, convulsions, coma, tachycardia, xerostomia, and urinary incontinence (Dugdale et al. 2020). Differential diagnoses for these two syndromes are malignant hyperthermia, early stages of tetanus, serotonin syndrome, rhabdomyolysis, clinical hypocalcaemia tetany, and capture myopathy (Paterson 2014; Dugdale et al. 2020). Butyrophenone derivatives follow the same metabolic and eliminatory processes as opioids. Azaperone is used in wild ungulate species for tranquilisation during translocation and as a synergistic drug with etorphine to immobilise buffaloes (Oosthuizen et al. 2009; Kock et al. 2012; Lamont & Grimm 2014; Napier & Armstrong 2014; Caulkett & Arnemo 2015; Wolfe 2015).

Midazolam hydrochloride

Midazolam [molecular formula: $C_{18}H_{14}Cl_2FN_3$; 8-chloro-6-(2-fluorophenyl)-1-methyl-4H-imidazo(1,5-a)(1,4)benzodiazepine hydrochloride] is an imidazobenzodiazepine agonist, which causes centrally-mediated muscle relaxation, anxiolysis, anticonvulsant effects and hypnotic sedation (Olkkola & Ahonen 2008; Rankin 2015). Midazolam binds to a specific benzodiazepine receptor binding site on gamma-aminobutyric acid A ($GABA_A$) receptors (a pentameric structure of homologous subunits with a central ion-pore) which results in a positive allosteric modulation of this receptor which enhances the effects of GABA. This modulation

results in an increase of chloride conductance which hyperpolarises the postsynaptic cell membranes (Olkkola & Ahonen 2008; Rankin 2015). The lack of direct agonistic activity on the GABA receptor generates a wide safety margin regarding CNS depression. Midazolam is less of a tissue irritant than its sister compound diazepam, which has been solubilised in propylene glycol and this leads to discomfort and tissue irritation when administered intramuscularly (Dugdale et al 2020). Midazolam has a unique characteristic, where one of the imidazole rings closes (becomes unionised) at body pH, the drug becomes lipid-soluble from an open-ring (ionised) water-soluble state at low pH (Olkkola & Ahonen 2008; Rankin 2015). Multiple, mostly inactive metabolites, except α_1-hydroxymidazolam which has mild activity, are formed from hepatic metabolism, which are excreted *via* urine and bile (Dugdale et al. 2020). The advantages of using midazolam are minimal respiratory and cardiovascular depression which maintains tissue oxygen (O_2) delivery (Rankin 2015).

The use of benzodiazepine agonists in domestic and free-ranging ruminants is not unusual, because they are frequently included as a premedication drug or as a co-induction drug to induce general anaesthesia (Caulkett 2003; Stegmann 2004; Nain et al. 2010; Malik et al. 2011; Kumar et al. 2014; Bodh et al. 2015; Dugdale et al. 2020). Previously, midazolam could not be used in darts because of its concentration (5.0 mg mL^{-1}) in available formulations being too low, which resulted in a large administration volume that cannot fit into capture darts (limited payload volume). However, midazolam is now under investigation in a high concentration (50.0 mg mL^{-1}) formulation and it could be added to a dart to deliver a therapeutic dose. Midazolam has been used in Nubian ibex, water buffalo and yak and was reported to be an effective sedative (Nain et al. 2010; Lapid & Shilo-Benjamini 2015; Rina et al. 2018). Bodh et al. (2015) reported that midazolam-butorphanol had a superior sedative effect when compared to midazolam being administered alone in water buffalo. Midazolam, theoretically compared

to azaperone, has less cardiovascular side effects and better muscle relaxation properties which could be advantageous in etorphine-based drug combinations for immobilisation.

Effects of the drugs of interest in Bovinae immobilisation

Immobilisation of African buffalo is routinely performed by wildlife veterinarians, however, as mentioned previously, no formal species-specific investigations on the physiological effects and reliability of etorphine drug combinations have been conducted. The drugs of interest have been administered to species of the same subfamily taxonomic group (*Bovinae*). However, the focus of these investigations reported minimal clinical and physiological effects of the administered drugs (Grootenhuis et al. 1976; Hattingh et al. 1984; Bryant et al. 2019).

Since the initial use of etorphine in the 1960s to capture wild ungulates, there has been a paradigm shift where immobilisations became more effective and mortalities less compared to using other drugs (gallamine triethiodide and D-tubercurarine) available at that time (Harthoorn & Bligh 1965). Harthoorn and Bligh (1965) reported the first clinical effects from etorphine administration, which appeared 3 to 4 minutes after dart placement with slight ataxia followed by a hackney gait (ridged goose-stepping gait often with opisthotonos). The animals typically transition from standing into sternal recumbence, but lateral recumbence is rare. However, the initial effective dosages of etorphine that were used pre-1980s were much larger compared to those recommended currently (Fahlman 2008). This difference in earlier and current recommended dosages highlights two important points 1) species-specific dosage studies should be conducted, and 2) enough physiological variables should be collected to document pharmacodynamic properties that these drugs have on animals. Interestingly, higher doses did not appear to translate in higher mortality due to the greater therapeutic index of potent opioid immobilisation, but the overall safety of etorphine, especially when combined with other drugs is largely unknown. Williams and Riedesel (1987) stated that the effective empirical dose of

etorphine that are prescribed now days to immobilise wild ungulates was determined by trial and error. Following on the administration of etorphine to wild ungulates, the development of etorphine combinations over the next couple of decades surfaced. Hattingh et al. (1984) used an etorphine-xylazine combination in African buffalo. They reported that the mean ± standard deviation (SD) time from dart placement to recumbence was 6.2 ± 1.8 minutes. The buffalo's heart rate (HR) decreased from 104 ± 28 to 54 ± 19 beats minute^{-1} and the respiratory rate (f_R) decreased from 31 ± 11 to 21 ± 8 breaths minute^{-1} from the initial measurement once handled to the final recorded value during immobilisation. The partial pressures of oxygen (PaO_2) and carbon dioxide ($PaCO_2$) were 57 ± 15 mmHg and 45 ± 8 mmHg, respectively with a pH of 7.37 ± 0.06. In the absence of more literature on African buffalo to draw conclusions, a review of these drugs administered to other species was conducted.

Etorphine-azaperone has been administered to ungulate species not found in the *Bovinae* subfamily that include white rhinoceros (*Ceratotherium simum*) blesbok (*Damaliscus pygargus phillipsi*) and giraffe (*Giraffa camelopardalis* sp.) (Buss et al. 2016; Gaudio et al. 2020; Vitali et al. 2020). However, these animals do not make good models for species from the *Bovinae* subfamily due to difference in body size and shape, behavioural temperament and total drug doses required to achieve reliable immobilisation. Thus, only these drugs administered to species from the subfamily *Bovinae* were reviewed further.

Woolf et al. (1973) used etorphine alone in eland (*Taurotragus oryx*) on two separate occasions and proposed that a tranquiliser should be added when aiming to immobilise a large ungulate. Grootenhuis et al. (1976) used a combination of etorphine and azaperone in eland, despite anecdotal recommendations at that time, for reasons unknown, to not use azaperone in eland. This study only reported drug dosages without mention of immobilisation characteristics or physiological effects (Grootenhuis et al. 1976). Bryant et al. (2019) compared thiafentanil-

azaperone (TA) and thiafentanil-etorphine-azaperone (TEA) immobilisation in Asian water buffalo. The mean ± SD induction time of TEA and TA were 6.6 ± 2.1 minutes and 5.9 ± 5.1 minutes, respectively. The overall HR for TEA and TA were 61 ± 8 beats minute^{-1} and 64 ± 27 beats minute^{-1}, respectively. An overall f_R of 14 ± 5 breaths minute $^{-1}$ for TEA and 15 ± 5 breaths minute $^{-1}$ for TA were observed.

Physiological characteristics have been reported for the administration of midazolam as premedication drug in bovid species (Nain et al. 2010; Malik et al. 2011; Kumar et al. 2014; Muchalambe et al 2018). Nain et al. (2010) reported physiological and sedation characteristics in Asian water buffalo calves after IV administration of midazolam at 0.2 mg kg^{-1}. The calves became sternally recumbent at 34.0 ± 15.9 minutes. The calves experienced moderate muscle relaxation and a significant increase in f_R but no significant change in HR. Malik et al. (2011) used a midazolam-butorphanol (0.2 mg kg^{-1} and 0.05 mg kg^{-1}, respectively) combination in subadult Asian water buffalo which resulted in moderate sedation. However, some buffalo never became recumbent. The median time for recumbence was 46.5 minutes. Compared to baseline readings obtained before drug administration, the HR rose significantly, and the mean arterial pressure (MAP) rose, but non-significantly. Kumar et al. (2014) administered midazolam (0.3 mg kg^{-1}) IV, as a premedication, to Asian water buffalo calves which became sternally recumbent 1.27 minutes later. The HR and f_R prior to ketamine induction were 52 ± 3 beats minute^{-1} and 16 ± 2 breaths minute^{-1}, respectively. Muchalambe et al. (2018) compared midazolam to xylazine premedication prior to propofol induction in domestic cattle. For midazolam only, the mean ± SD for sternal recumbence was 14.8 ± 0.9 minutes, with a HR and f_R fluctuating within expected reference intervals. These reported clinical effects of midazolam as premedication drug demonstrates the possible benefit of utilising a benzodiazepine in wild ruminant immobilisation because minimal cardiovascular derangements and effective sedation were observed.

Knowledge gaps and problem statement

Formal drug dose and comparative drug studies for African buffalo immobilisation have not been conducted and thus the limited data published in textbooks and review articles appear to originate from clinical experience gained over decades (Kock et al. 2012; Napier & Armstrong 2014; Wolfe 2015). Furthermore, the physiological effects of etorphine in combination with sedative drugs, as recommended for large ungulate capture, in African buffalo have not been formally investigated. By determining and comparing physiological variables, an informed approach to safer and more reliable immobilisation can be performed. To address the shortfalls, we investigated the immobilisation quality and physiological effects of etorphine in combination with either azaperone or midazolam in boma-confined African buffalo bulls.

1.3 Aim and objective

The aim of the study was to compare the commonly used etorphine-azaperone to a novel etorphine-midazolam drug combination for the immobilisation of African buffalo bulls. Objectively, we achieved this aim by evaluating the immobilisation characteristics (induction time, degree of chemical restraint and muscle relaxation) and determined physiological variables (HR, f_R, arterial blood pressure and arterial and venous blood gases) over time during etorphine-azaperone immobilisation in African buffalo, and then compared these values to etorphine-midazolam immobilisation in the same study population.

1.4 Hypothesis

The primary hypothesis was:

> H0: An etorphine-azaperone combination will result in no difference in recumbence time and physiological variables compared to an etorphine-midazolam combination for immobilisation in African buffalo bulls.
>
> H1: An etorphine-azaperone combination will result in significantly different recumbence time and physiological variables compared to an etorphine-midazolam combination for immobilisation in African buffalo bulls.

Chapter 2 - Materials and methods

2.1 Study design

A randomised crossover prospective, blinded comparative study was conducted after approval by the animal ethics committee of the University of Pretoria (Certificate number: V050-18). The study took place at the Wildlife Pharmaceuticals Wildlife Research Facility (Registration number: RF17/15918), Ngongoni Game Farm, Tipperary Conservancy, Nelspruit, Mpumalanga, RSA (25°31'29.75" S, 31°6'56.55" E; altitude was 829.0 meters above sea level).

Animals and housing

Ten free-ranging African buffalo bulls (mean ± SD: 47 ± 19 months old; 353 ± 97 kg; 2.5 / 5 Body Condition Score) were captured and translocated to an outdoor holding facility (boma) two weeks prior to commencement of the study to allow habituation. The sample size was based on previous research experience using a similar study design in zebra (*Equus zebra*) (Stemmet et al. 2019). The boma consisted of five adjoining pens, each connected *via* a lockable sliding gate. Nine buffalo were housed in the two largest pens which consisted of a roofed (15 x 4 metres) and unroofed (15 x 5 metres) portion. The largest buffalo was housed alone in a smaller adjoining pen (8 x 5 metres) to prevent conflict with smaller bulls but had continuous visual interaction with the others through the pole-wall division. Lucerne (*Medicago sativa*), hay (*Eragrostis curvula*) and water were available *ad libitum* and supplementary concentrate cubes (Alzu GP100 cubes) were provided twice daily. Food and water were withheld 36 hours and 12 hours, respectively prior to immobilisation.

Drug combination

All buffalo received each drug combination once (none were excluded from completing the study), at random (balanced, one-block design: http://www.randomization.com) order at a one-week interval, as follows:

- EA - etorphine (0.015 mg kg^{-1}; Captivon 9.8 mg mL^{-1}; Wildlife Pharmaceuticals, White River, Mpumalanga, South Africa), azaperone (0.15 mg kg^{-1}; 100.0 mg mL^{-1}; Wildlife Pharmaceuticals).
- EM - etorphine (0.015 mg kg^{-1}; Captivon 9.8 mg mL^{-1}), midazolam (0.15 mg kg^{-1}; 50.0 mg mL^{-1}; Wildlife Pharmaceuticals)

Published recommended dosages for each drug were used (Napier & Armstrong 2014; Wolfe 2015; Dugdale et al. 2020). Drug doses were based on an estimated body weight determined by experienced wildlife veterinarians. The primary investigator was blinded to the drug combination administered. Each drug was drawn up into separate syringes and injected sequentially into a 3 mL plastic dart (Dan-inject 3 mL; 2.0 mm x 40 mm collard needle; Dan-inject International, Kolding, Denmark) using a 20-gauge 75 mm spinal needle. The dart was filled, to capacity, with sterile water (Kyron Laboratories Ltd., Benrose, Gauteng, South Africa).

Image 1 Immobilisation team collecting data on an African buffalo in the boma where they were housed.

2.2 Experimental procedures

Pre-immobilisation phase

The buffalo were darted in the shoulder muscles from a 5-8 metre distance using a carbon dioxide-powered injection rifle (Model JM; Dan-inject International, Kolding, Denmark) set to 4-6 Bar pressure. Once the buffalo was darted, the induction time (time from dart placement and discharge to when the buffalo became recumbent without attempts to stand) was recorded and quality was scored using a subjective descriptive scale (Table 1). Once recumbence was achieved, the remaining buffalo were moved to the adjacent pen. When deemed safe, the immobilised buffalo was approached and placed into sternal recumbence (and remained in sternal) on a purpose-built rubber stretcher. A 5-minute period was allowed to instrument and prepare the buffalo for data collection.

Image 2

Experienced wildlife vet darting an African buffalo.

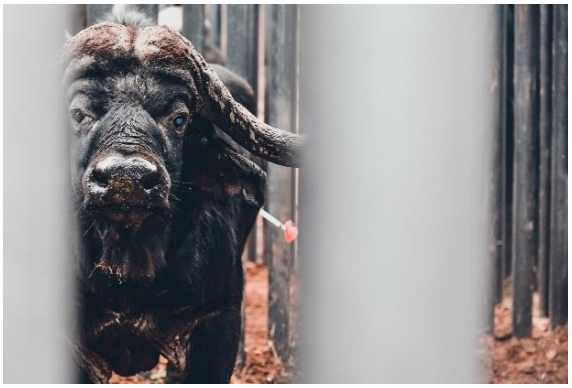

Image 3

Darted African buffalo bull that has been darted in the muscles of the shoulder.

Table 1 Description of the descriptive scoring system used to categorise the quality of induction into recumbence, quality of immobilisation and quality of recovery in boma-confined buffalo immobilised using etorphine-azaperone and etorphine-midazolam by remote dart delivery system.

Score	Description	Classifier
Induction		
0	Animal does not become recumbent, although it can be approached and touched without running. No tail flicking observed. Head pressing may be observed.	Standing immobilisation
1	Slight ataxia observed followed by < 2 attempts to lie down in sternal recumbence. No signs of CNS excitation observed without falling or stumbling. Smooth transition from sternal to lateral recumbence. Time to reach sternal recumbence (3-4 min) and lateral recumbence (up to 5 min).	Excellent
2	Moderate ataxia observed with > 2 attempts required to lie in sternal recumbence. Minimal signs of CNS excitation observed. Moderate stumbling observed. Time to reach sternal recumbence (> 4 min) and lateral recumbence (> 5 min).	Good
3	Severe ataxia with numerous attempts to lie down is observed. Moderate signs of CNS excitation are observed. Severe stumbling and falling is observed before the animal becomes recumbent. Moderate risk of injury. Time to reach sternal recumbence (> 6-8 min) and lateral recumbence (> 10 min).	Fair
4	Severe ataxia without animal becoming recumbent. Moderate to severe CNS excitation is observed. Repeated stumbling and falling is observed with animal requiring a second dart. Time to reach sternal recumbence (> 10-15 min) and lateral recumbence (> 30 min)	Poor

Table 1 continues on the next page…

Immobilisation

1	Minimal immobilisation achieved, animal posing a risk to ground crew and high risk of self-injury. No anaesthetic plane reached. Minimal immobilisation achieved, animal posing a risk to ground crew and high risk of self-injury. No anaesthetic plane reached.	Limited effect
2	Animal struggles during manipulation, spontaneous motor activity noted. Animal responds to painful stimuli with both anal and palpebral reflexes still present.	Deep sedation
3	Reduced muscle rigidity to smooth complete relaxation. Anal reflex diminishes but retained slow palpebral. Loses pedal reflex. No reaction to painful stimuli. Animal can be handled safely. *Moderate level- no involuntary tail movements and tongue easily extractable.*	Light to moderate anaesthetic level
4	All characteristics of 3 present. Palpebral reflex and jaw tone diminished.	Surgical anaesthesia
5	Anaesthetic plane is too deep, all reflexes absent with evidence of hypoxia ARO cardiopulmonary depression.	Excessive level of anaesthesia

Recovery

1	Transition from lateral to sternal occurs with minimal ataxic movements. Stands within 1 to 2 attempts, which are calm. Slight ataxia observed as starts to walk. Recumbence to recovery < 10 min post reversal.	Excellent
2	Transition from lateral to sternal occurs with moderate ataxic movements. Standing requires > 2 attempts, which are relatively coordinated. Moderate ataxia observed as start to walk. Imbalance and in coordination are observed. Recumbence to recovery 10-15 min post reversal.	Good
3	Frequent and severely ataxic attempts to move from lateral to sternal. Numerous erratic attempts required to stand. Stumbling and falling is observed. Markedly ataxic gait when walking. Recumbence to recovery > 20 min post reversal.	Fair
4	Animal remains recumbent for > 30 min. No response to stimuli and no attempts to raise observed.	Poor

Immobilisation phase

The time to first handling (dart placement and drug discharge until first handling) was recorded and regarded as time zero (T0) for subsequent data collection time points. First handling was used as indicator for level of immobilisation as to ensure safety to personnel during this phase. The buffalo was instrumented to measure the following physiological variables: HR, invasive systolic (SAP), diastolic (DAP) and mean (MAP) arterial blood pressure, f_R and end-tidal carbon dioxide ($P_E'CO_2$) using a multiparameter monitoring machine (GE Datex Ohmeda CardioCap 5 Multiparameter; GE Healthcare, Chicago, Illinois, USA). The physiological variables were continuously monitored and recorded every 5 minutes (T5, T10, T15 and T20, respectively) from T0. Immobilisation quality was subjectively scored at T20 (Table 1).

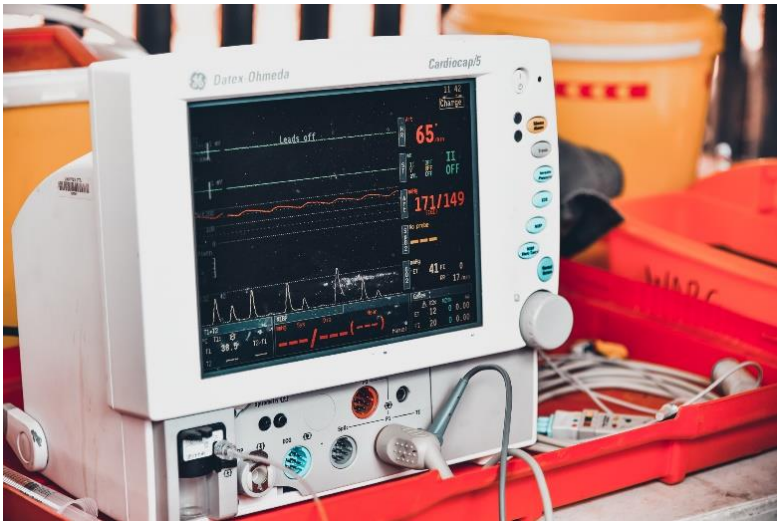

Image 4

Physiological variables being displayed on the multi-parameter monitor in the field.

An auricular artery was aseptically cannulated (22 gauge, 25mm, Jelco IV Catheter; Smiths Medical, Lower Pemberton, Ashford, UK) and an electronic strain gauge transducer (Medex Abbott; Biometrix, Overbroek, Gronsveld, The Netherlands) was attached and zeroed to atmospheric air pressure at the level of the right scapulohumeral joint to measure invasive arterial blood pressure. A polyvinyl chloride cuffed endotracheal tube (8 mm internal diameter; Kruuse, Havretoften, Langeskov, Denmark) was placed into the ventral nasal meatus to

measure f_R and $PE'CO_2$ via the side-stream respiratory gas analyser (sampling rate: 200 mL minute^{-1}). Respiratory rate was confirmed by visual observation of abdominal excursion and turbulent air flow at nares. The HR was measured with a stethoscope (Littmann Classic; 3M, Saint Paul, Minnesota, USA) and correlated to the value obtained from the multiparameter monitor arterial pulse waveform. Rectal temperature was measured in degrees Celsius (°C) using an electronic thermometer (Thermoval Rapid; Hartmann, Randburg, Gauteng, South Africa).

Arterial and venous blood samples were drawn from the auricular cannula (2 mL waste and 1 mL sample) and jugular vein (needle puncture), respectively at T5 and T20. The blood samples were collected anaerobically into heparinised syringes (A-Line syringes; Becton Dickinson; Franklin Lakes, New Jersey, USA) and analysed immediately using a patient-side blood gas analyser (EPOC Blood Analysis System; Siemens Healthineers, Erlangen, Germany). The mean ± SD ambient temperature was 23.3 ± 2.4°C and barometric pressure was 703 ± 2 mmHg, which were recorded from the blood gas analyser at each T5 and T20 analysis and then averaged. Blood gas variables for arterial and venous samples included partial pressures of oxygen (PaO_2 and PvO_2) and carbon dioxide ($PaCO_2$ and $PvCO_2$), oxygen saturation (SaO_2 and SvO_2), pH, bicarbonate (HCO_3^-), base excess extracellular [BE(ecf)], base excess blood [BE(b)], sodium (Na^+), potassium (K^+), chloride (Cl^-), ionised calcium (iCa), haematocrit (Ht), haemoglobin concentration (Hb), glucose (Glu), lactate (Lac), creatinine (Crea).

At T20, after all variables had been recorded and samples taken, all instruments and cannula were removed from the buffalo. The buffalo, while still positioned on the purpose-built rubber stretcher (of known mass), was moved, and weighed using a crane operated hanging scale (HIS; AE Adam, Oxford, Connecticut, USA). This was followed by horn morphometry and body measurements were taken for presale evaluation that were not relevant to the study.

Post-immobilisation phase

At the end of all procedures, the buffalo was removed from the rubber stretcher and placed in sternal recumbence. The pure opioid antagonist naltrexone (20 mg mg^{-1} etorphine; Trexonil 40 mg mL^{-1}; Wildlife Pharmaceuticals) was administered IV at a standardised time of 40-minutes (T40). The recovery time was recorded as the time from administering the antagonist until the buffalo began to walk. The quality of recovery was subjectively scored (Table 1). Food and water were provided once data collection had been completed on all the buffalo. The buffalo were observed for any signs of renarcotisation or untoward effects of the study procedures over a 24-hour period following immobilisation.

Image 5 African buffalo bull being placed on rubber stretcher (known weight) to be weighed by crane operating hanging scale

2.3 Rescue intervention

The welfare and safety of the African buffalo bulls were always a priority. Any buffalo bull which was not regarded as clinically healthy from observational evaluation (oculonasal discharge, coughing, depressed mentation, lethargic) and poor subjective body condition scoring were excluded from the study.

Potential major complications that could arise during the study procedures:

1. Inadequate immobilisation where buffalo do not become recumbent or cannot be approached safely within 20 minutes of darting.
2. Regurgitation of rumen fluid
3. Severe life-threatening hypoxaemia
4. Severe acidosis
5. Failure to stand after 25 minutes from naltrexone administration

If any of these major complications occurred, then the following rescue interventions were provided:

1. The capture of that buffalo was excluded from further data collection and naltrexone was administered using a drop-out dart. The buffalo was re-immobilised during a third data collection session, if required. The records of the failed immobilisation attempt could be added to the data if the researchers believed it was a drug combination problem and not an administration problem.
2. The buffalo was treated for an aspiration pneumonia by receiving a long acting antibiotic and non-steroidal anti-inflammatory drug, at the discretion of the wildlife veterinarian. The buffalo might have required a longer washout period and only if there were no untoward clinical signs would it be included in the study. A buffalo could be

replaced, if pneumonia develops, these interventions were at the discretion of the wildlife veterinarian.

3. Detected as a PaO_2 of < 30 mmHg. Other physiological parameters (HR and arterial blood pressure and f_R) were evaluated by an experienced specialist veterinary anaesthesiologist. Either O_2 insufflation was administered as a rescue intervention and the buffalo was not excluded; or the animal was reversed immediately, and the incomplete data set that was captured was included in the analysis.
4. A blood pH of < 7.01 was considered a severe acidosis and the buffalo was closely monitored for cardiovascular inefficiency by an experienced specialist veterinary anaesthesiologist to determine if the animal required immediate reversal and exclusion from the study. A pH of < 6.8 was considered life-threating and the buffalo was reversed immediately and its data was included in the analysis.
5. If the buffalo was safe to approach, then a clinical examination was performed. Otherwise, a second dose of naltrexone and flumazenil (in the etorphine-midazolam group) were administered by drop-out dart into the gluteal muscle group.

If any of these major complications occurred, then the event was reported to the Animal Ethics Committee.

2.4 Data collection and analysis

Statistical analysis

The drug dosages were retrospectively corrected for body mass; total drug administered (mg) divided by body mass (kg). The arterial to end-tidal carbon dioxide gradient ($P(a\text{-}E')CO_2$) was calculated by $P(a\text{-}E')CO_2 = PaCO_2 - PE'CO_2$. The alveolar to arterial partial pressure of oxygen gradient was calculated using the following formula: $P(A\text{-}a)O_2 = PAO_2 – PaO_2$, where $PAO_2 = PIO_2 – (PaCO_2 \div RQ)$ [RQ: respiratory quotient = 1.0 in ruminants (Zeiler & Meyer 2017); PIO_2: partial pressure of inspired oxygen] and $PIO_2 = FIO_2$ x (PB – PH_2O) [FIO_2: fraction inspired oxygen, PB: mean ambient barometric pressure measured from the blood gas analyser, PH_2O: water vapour pressure was standardised at 47 mmHg] (Ewart 2020). The arterial oxygen content (CaO_2) was calculated using the formula: $CaO_2 = 1.34$ x Hb x $SaO_2 + PaO_2$ x 0.003 (1.34: Huffner's constant; 0.003 = millilitres of O_2 carried by 1 decilitre of blood per 1 mmHg of partial pressure oxygen) (Haskins 2015).

Time and physiological data were normally distributed based on examining histograms, descriptive statistics, and the Anderson-Darling test for normality. Data were reported as mean ± standard deviation (SD). Times to events (induction, first handling and recovery) were compared using a student t-test. Subjective quality scores during induction, immobilisation and recovery were reported descriptively as mean (minimum to maximum) values. Physiological data collected over time were compared between drug combinations using a general linear mixed model (Interactions: time, drug combination, drug combination x time; Fixed variables: time, drug combination; Random variable: buffalo). Model fits were assessed by visually inspecting residual plots to assess linearity, homogeneity of variances, normality, and outliers. Significant values were compared using Bonferroni correction for multiple pairwise comparisons. Physiological variables were averaged over time for each drug combination for

textual reporting of an overall value. Commercially available software (MiniTab 18.1.0; MiniTab Incorporated, State College, Pennsylvania, USA) was used for analysis and the level of significance was set to $p < 0.05$.

Image 6 A blood sample being analysed on the patient-side blood gas analyser

Image 7 The whole immobilisation team monitoring and collecting data on an African buffalo bull.

Chapter 3 - Results

Actual doses were calculated as 0.015 ± 0.001 mg kg^{-1} etorphine combined with either 0.15 ± 0.01 mg kg^{-1} azaperone or 0.16 ± 0.02 mg kg^{-1} midazolam.

3.1 Times and scores

The mean induction time for EA was 326 ± 304 seconds (5.4 ± 5.1 minutes) and not different for EM, which was 247 ± 162 seconds (4.1 ± 2.7 minutes). The time to first handling for EA was 459 ± 370 seconds (7.7 ± 6.2 minutes) and not different for EM, which was 442 ± 384 seconds (7.4 ± 6.4 minutes). The mean (range) induction score was 1 (1 to 3) for EA and 2 (1 to 3) for EM. The two biggest bulls had induction scores of 3 for both EA and EM. Early signs of drug effect in buffaloes using both combinations were salivation, ataxia, swaying, and drooping of the lower lip that were observed before the buffalo either stumbled and fell or calmly transitioned into sternal recumbence. During the immobilisation phase, no buffalo attempted to stand, regardless of the drug combination administered. The overall immobilisation score was 3 (2 to 3) for both drug combinations. Palpebral and anal reflexes were always present and none of the buffalo developed skeletal muscle rigidity. However, pronounced tail twitching (n = 1; EA), thoracic limb tremors (n = 1; EM), jaw trembling or chattering (n = 1; EA, and n = 1; EM) and reactivity to people speaking nearby (n = 2; EA only) were observed.

During the post-immobilisation phase, the recovery time for EA immobilisation was 71 ± 27 seconds (1.2 ± 0.5 minutes) and not different compared to 86 ± 44 seconds (1.4 ± 0.7 minutes) for EM immobilisation. The mean recovery score was 1 (1 to 1) for buffaloes recovering from both drug combinations. All buffaloes were ataxic for the first few seconds of walking. Two buffaloes, however, fell on recovery on the EM combination but stood up immediately. All

buffaloes recovered completely and no renarcotisation occurred within 24 hours after antagonist administration.

3.2 Clinical variables

The overall mean HR for the buffalo during the EA immobilisation was 113 ± 27 beats minute^{-1}, which was significantly faster compared to 79 ± 22 beats minute^{-1} for EM immobilisation ($p < 0.001$: Figure 1). The arterial blood pressure for buffalo during EM immobilisation was profoundly elevated and significantly higher compared to using EA ($p < 0.001$: Table 2). The overall MAP was 102 ± 25 mmHg and 163 ± 18 mmHg for EA and EM, respectively. The f_R for buffalo during the EA immobilisation was 18 ± 4 breaths minute^{-1} which was significantly slower compared to 23 ± 7 breaths minute^{-1} for EM immobilisation ($p < 0.001$: Figure 2). Furthermore, a shallow breathing pattern with intermittent deep breaths was regularly observed, regardless of the drug combination. The $P_E'CO_2$ during EA immobilisation was 60 ± 7 mmHg (8.1 ± 0.9 kPa) and was significantly higher compared to 56 ± 7 mmHg (7.6 ± 0.9 kPa) for EM immobilisation ($p < 0.03$). The mean rectal temperature during EA immobilisation was 38.9 ± 0.7°C and was significantly lower compared to 39.4 ± 0.5°C for EM immobilisation ($p < 0.005$), although not clinically relevant.

Figure 1 Heart rates (HR) (a) and mean arterial blood pressures (MAP) (b) over time in etorphine-azaperone (EA) and etorphine-midazolam (EM) immobilised African buffalo bulls ($p < 0.01$ between combinations). The plot represents the mean and 95% confidence interval.

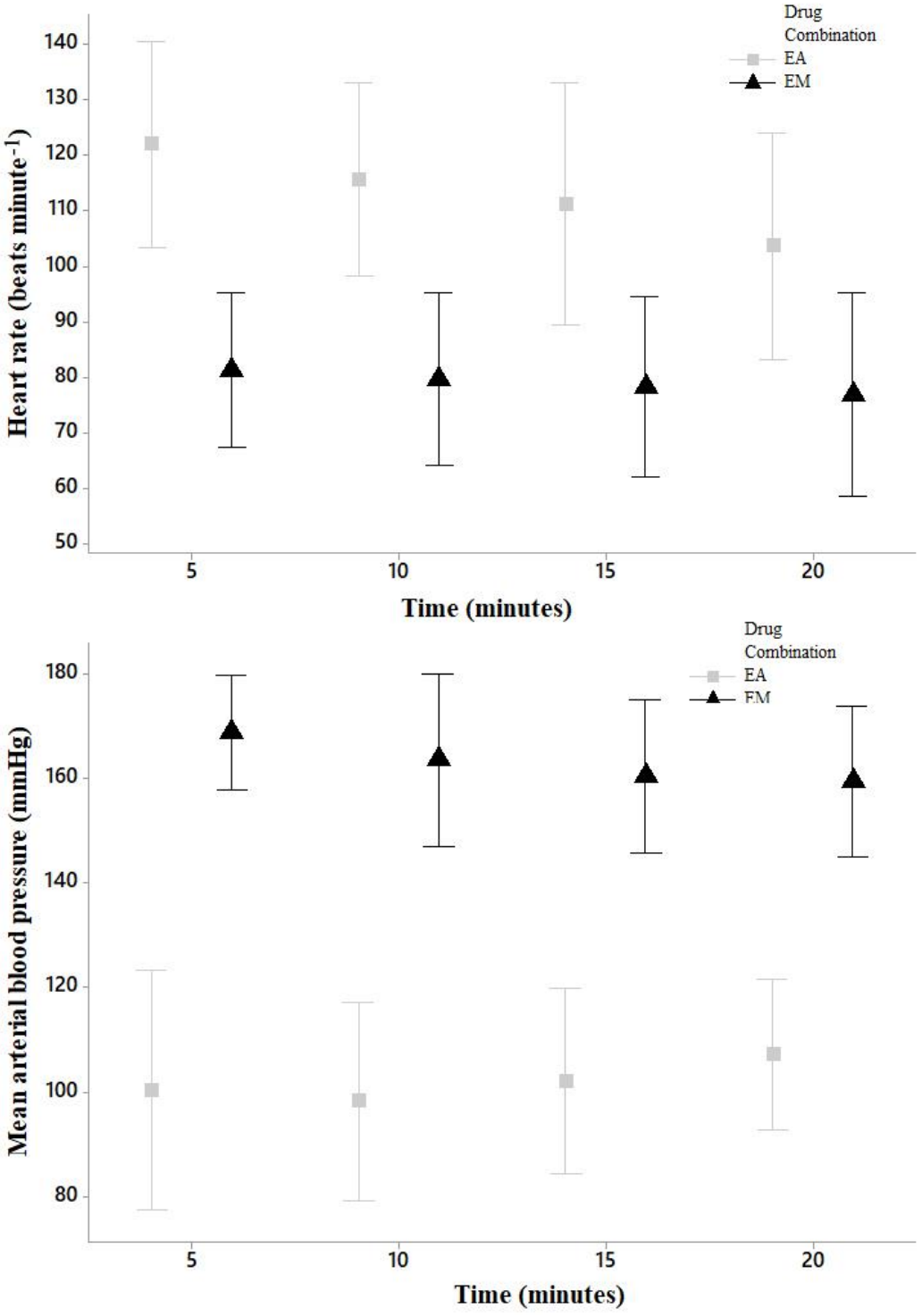

Figure 2 Respiratory rates (f_R) (a) and end-tidal carbon dioxide ($P_E'CO_2$) values (b) over time in etorphine-azaperone (EA) and etorphine-midazolam (EM) immobilised African buffalo bulls ($p < 0.01$ between combinations). The plot represents the mean and 95% confidence interval.

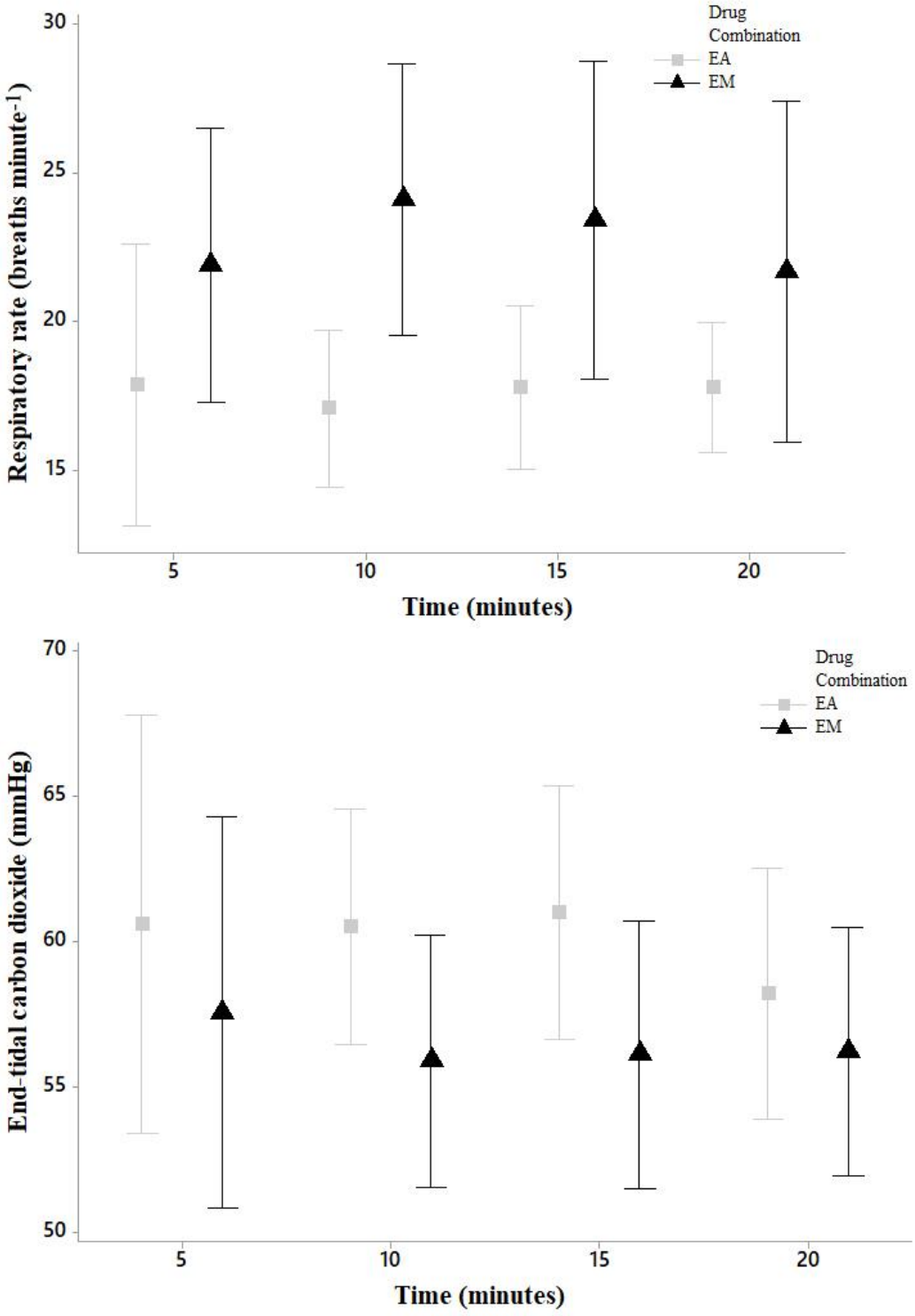

Table 2 Physiological variables of etorphine-azaperone (EA) and etorphine-midazolam (EM) immobilised African buffalo (*Syncerus caffer*) over a 20-minute period, post-induction. Variables are reported as mean ± standard deviation at 5-minute intervals.

Variable	Combination	5 minutes		10 minutes		15 minutes		20 minutes	
		Mean	± SD	mean	± SD	mean	± SD	mean	± SD
Heart rate	EA	122†	26	116†	24	111†	30	104†	29
(beats minute^{-1})	EM	81†	20	80†	22	78†	23	77†	26
SAP	EA	120†	27	117†	29	121†	26	127†	24
(mmHg)	EM	194†	21	187†	29	186†	28	189†	23
DAP	EA	86†	33	85†	27	88†	25	94†	19
(mmHg)	EM	15†	11	147†	17	141†	17	141†	16
MAP	EA	100†	30	98†	26	102†	25	107†	20
(mmHg)	EM	169†	13	163†	22	160†	19	159†	19
Respiratory rate	EA	18†	7	17†	4	18†	4	18†	3
(breaths minute^{-1})	EM	22†	6	24†	6	23†	7	22†	8
$P_{E'}CO_2$	EA	61†	10	61†	6	61†	6	58†	6
(mmHg)	EM	58†	9	56†	6	56†	6	56†	6
(kPa)	EA	8.2†	1.4	8.2†	0.8	8.2†	0.8	7.8†	0.8
	EM	7.8†	1.2	7.6†	0.8	7.6†	0.8	7.6†	0.8
Temperature	EA	39.2†	0.6	39.0†	0.6	38.9†	0.7	38.7†	0.8
(°C)	EM	39.4†	0.5	39.4†	0.5	39.4†	0.5	39.3†	0.6

SAP: systolic arterial blood pressure, DAP: diastolic arterial blood pressure, MAP: mean arterial blood pressure, $P_{E'}CO_2$: partial pressure of end-tidal carbon dioxide, °C: degrees Celsius, mmHg: millimetres of mercury, kPa: kilopascals, †: statistical difference between treatments, statistical difference reported as p-value < 0.05.

3.3 Arterial and venous blood gas and biochemistry

The arterial blood gas analysis revealed severe hypoxaemia with a PaO_2 of 37 ± 12 mmHg (5 ± 1.6 kPa) for EA immobilised buffalo, which was not different compared to 43 ± 8 mmHg (5.8 ± 1.0 kPa; $p = 0.062$) for EM immobilised buffalo (Table 3). The $P(A\text{-}a)O_2$ gradients were greater than 20 mmHg and not different between the drug combinations at T5 and T20. Moderate hypercapnia where $PaCO_2$ was 57 ± 6 mmHg (7.7 ± 0.6 kPa) and not different to 56 ± 3 mmHg (7.5 ± 0.5 kPa) for EA and EM, respectively. The majority of $P(a\text{-}E')CO_2$ gradients were negative and not different between the drug combinations. The CaO_2 in both drug combinations were not different and less than 11.1 mL dL^{-1}. Potassium ($p < 0.01$) was the only significantly different electrolyte between EA and EM arterial and venous blood samples, whereas Ht, Hb, HCO_3^-, BE(ecf), BE(b) were significantly different between EA and EM on the venous blood samples only (all $p < 0.01$). The biochemical variables are presented in Table 4.

Table 3 Partial pressures and gas gradients of arterial and venous blood gases in etorphine-azaperone (EA) and etorphine-midazolam (EM) immobilised African buffaloes (*Syncerus caffer*). Variables are reported as mean ± standard deviation at 5- and 20-minutes post-induction.

Variable	Combination	Arterial blood gas analysis				Venous blood gas analysis			
		5 minutes		20 minutes		5 minutes		20 minutes	
		mean	± SD	mean	± SD	mean	± SD	Mean	± SD
PCO_2	EA	57	6	57	3	56	4	58	3
(mmHg)	EM	56	4	55	3	56	4	57	3
(kPa)	EA	7.7	0.8	7.7	0.4	7.6	0.5	7.8	0.4
	EM	7.6	0.5	7.4	0.4	7.6	0.5	7.7	0.4
PO_2	EA	37	10	37	14	39	10	40	8
(mmHg)	EM	42	9	45	7	40	7	45	10
(kPa)	EA	5	1.4	5	1.9	5.3	1.4	5.4	1.1
	EM	5.7	1.2	6.1	0.9	5.4	1.0	6.1	1.6
SO_2 (%)	EA	62.9	14.6	70.3	11.1	67.6	12.3	70.1	11.9
	EM	71.3	13.9	76.3	9.6	69.2	12.2	75.3	11.6
Calculated gradients and arterial oxygen content									
$P(a\text{-}E')CO_2$	EA	-4	11	-1	6				
(mmHg)	EM	-1	10	-1	7				
(kPa)	EA	-0.5	1.5	-0.1	0.8				
	EM	-0.1	1.4	-0.1	1				
$P(A\text{-}a)O_2$	EA	44	8	43	14				
(mmHg)	EM	40	7	39	6				
(kPa)	EA	6.0	1.1	5.8	1.9				
	EM	5.4	1.0	5.3	0.8				
CaO_2	EA	9.1	3.8	9.5	3.5				
(mL dL^{-1})	EM	11.1	3.1	11.0	2.2				

PCO_2: arterial or venous partial pressure of carbon dioxide, PO_2: arterial or venous partial pressure of oxygen, SO_2: arterial or venous haemoglobin oxygen saturation in percentage, $P(a\text{-}E')CO_2$: partial pressure gradient between arterial and end-tidal carbon dioxide, A-a gradient: partial pressure gradient between alveolar and arterial oxygen, CaO_2: arterial oxygen content, mmHg: millimetres of mercury, kPa: kilopascals, mL dL^{-1}: millilitre per decilitre.

Table 4 Biochemical variables for arterial and venous blood gases in etorphine-azaperone (EA) and etorphine-midazolam (EM) immobilised African buffaloes (*Syncerus caffer*). Variables are reported as mean ± standard deviation at 5- and 20-minutes post-induction.

Variable	Combination	Arterial blood gas analysis				Venous blood gas analysis			
		5 minutes		20 minutes		5 minutes		20 minutes	
		Mean	± SD	Mean	± SD	Mean	± SD	Mean	± SD
pH	EA	7.359	0.038	7.365	0.022	7.360	0.030	7.364	0.023
	EM	7.354	0.046	7.376	0.021	7.343	0.035	7.364	0.026
HCO_3^-	EA	32.0	1.8	32.8	2.3	31.4*	1.3	32.9*	1.6
(mmol L^{-1})	EM	31.2	2.6	32.1	1.6	30.3*	2.3	32.3*	2.0
BE(ecf)	EA	6.5	1.9	7.4	2.6	6.0*	1.4	7.6*	1.8
(mmol L^{-1})	EM	5.7	3.2	7.0	1.6	4.6*	2.7	6.9*	2.3
BE(b)	EA	5.4	1.8	6.3	2.6	4.9*	1.4	6.5*	1.7
(mmol L^{-1})	EM	4.4	3.0	5.8	1.5	3.3*	2.5	5.6*	2.0
Na^+	EA	139	3	138	5	142	2	143	2
(mmol L^{-1})	EM	139	6	139	6	142	3	143	3
K^+	EA	3.8†	0.3	3.7†	0.3	3.7†	0.2	3.7†	0.3
(mmol L^{-1})	EM	4.2†	0.4	4.0†	0.4	4.0†	0.4	4.0†	0.5
iCa	EA	1.06	0.07	1.04	0.06	1.08	0.05	1.09	0.06
(mmol L^{-1})	EM	1.05	0.06	1.04	0.07	1.08	0.06	1.08	0.06
Cl^-	EA	99	2	98	4	103	1	102	1
(mmol L^{-1})	EM	99	5	99	5	103	2	104	3
Ht	EA	31	6	29	8	30†	6	27†	6
(%)	EM	34	6	31	5	35†	6	33†	5
Hb	EA	10.4	2.1	9.8	2.7	10.3†	2.1	9.2†	1.9
(g dL^{-1})	EM	11.5	2.1	10.6	1.6	12.0†	2.2	11.2†	1.6
Glucose	EA	5.7	1.2	5.5	1.1	5.6	1.2	5.6	1.1
(mmol L^{-1})	EM	5.9	1.5	5.6	1.5	5.8	1.5	5.7	1.4
Lactate	EA	2.79	1.64	2.12	1.05	2.85	1.68	2.05	1.15
(mmol L^{-1})	EM	3.57	2.30	2.54	1.74	3.90	2.21	2.49	1.78
Creatinine	EA	119	25	117	17	121	17	123	18
(µmol L^{-1})	EM	127	14	123	14	122	15	124	14

HCO_3^-: bicarbonate concentration, BE(ecf): base excess of the extracellular fluid, BE(b): base excess of the blood, Na^+: sodium concentration, K^+: potassium concentration, iCa: ionised calcium concentration; Cl^-: chloride concentration, Ht: haematocrit, Hb: haemoglobin concentration, mmol L^{-1}: millimoles per litre; g dL^{-1}: grams per decilitre, µmol L^{-1}: micromoles per litre, *: statistical difference over time, †: statistical difference between treatments, statistical difference reported as *p*-value < 0.05.

3.4 Limitations

During the study only African buffalo bulls were immobilised. Speculation could be made that buffalo cows might demonstrate different physiological changes compared to the bulls, especially during the oestrus cycle. The respiratory gases were sampled in the nasopharynx and not from the proximal trachea. This was done because ruminant species are prone to active regurgitation during manipulation of the laryngeal area when animal is in a light plane of anaesthesia or immobilisation (Riebold 2015). This can lead to possible aspiration pneumonia, and thus it was advocated to not attempt tracheal intubation. However, this could have resulted in CO_2 from the rumen contaminating the respiratory gases sampled and give a false elevation of $P_{E'}CO_2$ and contribute to the negative $P(a\text{-}E')CO_2$ gradient. Further investigation is required to determine if the negative $P(a\text{-}E')CO_2$ gradient is from higher cardiac output compared to a lower alveolar minute ventilation or rumen CO_2 contamination. The alveolar minute ventilation was not investigated as endotracheal intubation was not performed to allow spirometry *via* large animal pitot-tube to determinate the tidal volume, minute volume and total thoracic compliance. Thus, only speculations on hypoventilation can be made with the aid of f_R and $P_{E'}CO_2$. The buffalo were maintained in sternal recumbency and further investigation is needed to determine the ratio of ventilation to perfusion utilising electrical impedance tomography technology. Due to buffalo muscles being darker (higher myoglobin concentration) and the binding and storage of O_2 within the cardiac and skeletal muscle to myoglobin, only speculations could be made on the importance of the O_2 utilisation within the muscles during chemical immobilisation. Near-infrared spectroscopy is needed to investigate the movement and utilisation of O_2 bound to the myoglobin. The drug combinations used in this study were performed on boma-confined buffaloes. Minimal exertional exercise and stress were present, compared to immobilisation of free-ranging buffaloes from vehicle or helicopter. Thus, further investigation is required in free-ranging African buffalo which have not been boma-habituated.

The allocated time of 20-minutes for data collection might have been inadequate to allow for some physiological changes to be detected. Over a longer time, the lactate levels might have increased substantially, and decompensation of auto-compensatory mechanisms could have emerged requiring intervention. However, the majority of buffalo immobilisation are short duration procedures, usually less than 30 minutes. Oxygen supplementation was not administered to any of the buffalo even though severe hypoxaemia was observed. However, no clinical indications were present which prompted treatment of the hypoxaemia. Without the administration of supplemental O_2, only speculation can be made with regards to the possible suppression of the hypoxic drive.

Chapter 4 - Discussion and conclusion

4.1 Discussion

Boma-confined buffalo bulls were effectively immobilised with both EA and EM drug combinations. Physiological and immobilisation characteristics were similar between the two drug combinations, although marked systemic hypertension was observed in buffaloes administered EM compared to EA. Clinically relevant hypoxaemia and hypercapnia were evident with both drug combinations.

Ideally, an immobilisation combination should result in a rapid induction time where the transition from dart placement to recumbence is achieved within less than 10 minutes (Fowler 2008). A prolonged induction time during free-ranging animal immobilisation is not desirable because it could result in 1) an animal fleeing out of sight or into the bush, 2) succumbing to cardiopulmonary side effects from the drugs, 3) becoming susceptible to predation, 4) hyperthermia, 5) self-injury, or 6) capture myopathy (Fowler 2008; Schumacher 2008). Effective immobilisation ideally produces minimal excitation, excellent muscle relaxation, adequate analgesia, and minimal cardiopulmonary depression (Schumacher 2008). Both drug combinations in this study achieved rapid induction with calm transition from standing to sternal recumbence. The demeanour and level of activity changed (becoming more stressed and excited) over the course of the day as subsequent buffalo were darted and the remaining buffalo moved to adjacent holding pens before approaching the recumbent buffalo. This changed demeanour and increased responsiveness to the study procedures could have resulted in the greater range in induction times. The overall immobilisation quality for both drug combinations was adequate, with no attempts to stand and sufficient relaxation to allow manipulation of the buffalo. No additional drugs were administered during the immobilisation

phase to any of the buffalo. Furthermore, the two largest buffalo bulls had longer times to recumbence and first handling than the others, possibly due to underdosing of the drugs or injectate being deposited in a fascial plain (between subcutis and muscle) or subcutaneously due to thicker cutaneous layer. This could have led to a poor absorption of the drugs and delayed onset of action (Zeiler & Meyer 2017). Due to safety concerns to personnel, entry into the pen was only done when the buffalo was recumbent, and the other buffalo moved into the adjacent pen. When deemed safe to approach the recumbent buffalo, T0 was marked as time to first handling. This allowed monitoring to occur exactly 5-minutes after first handling for all buffalo. This demonstrates time differences between individual buffalo as not all buffalo became recumbent at the same time from dart placement and discharge. Thus, a safe standardised time was allocated to be time to first handling.

Rapid recovery with minimal ataxia after administration of the antagonists are important to prevent injury and predation after immobilisation, especially in free-ranging animals (Fowler 2008). Although rapid recoveries were observed with both EA and EM, a notable concern was that two buffaloes fell during recovery on the EM drug combination. However, the falling might not be solely related to drug effect as Kock et al. (2012) stated that an IV administration of 15-25 mg of midazolam can sedate a buffalo for approximately 20 minutes, despite unknown pharmacokinetics; whereas our buffalo received midazolam intramuscularly and were recovered after 40 minutes. We speculate that alternative reasons for the bulls falling during recovery could be due to 1) severe hypoxaemia (decreased O_2 delivery to muscles), 2) prolonged recumbence with (partial) occlusion of blood supply to the major muscle groups, 3) systemic hypertension resulting in fatigue and ataxia, or 4) delayed absorption and duration of drug effect if the injectate was deposited in a fascial plain. However, muscles are relatively resistant to ischaemic injury and during muscle hypoxia, vascular endothelium nitric oxide synthase (eNOS) is released and other vasodilatory mediators which locally regulates blood

flow to the muscles by causing vasodilation and decrease precapillary sphincter tone (Harriman 1977; Joyner & Casey 2013; Stephenson 2020b). The local regulatory effects of skeletal muscle also ensure tissue perfusion pressure by activating muscle ischaemia receptors which will lead to an increase in arterial pressure (Stephenson 2020b). The severe systemic hypertension increases left cardiac ventricular workload leading to increased O_2 consumption which can result in fatigue (Stephenson 2020a). The severe systemic hypertension also ensures an adequate muscle perfusion pressure (muscle perfusion pressure = MAP – muscle intercompartmental pressure), as a minimum of 30-40 mmHg is needed to maintain muscle perfusion and O_2 delivery (Dugdale et al. 2020). However, greater perfusion pressure does not equate to greater blood flow as the vascular resistance within muscles has a contributing effect on blood flow (blood flow to muscle = muscle perfusion pression ÷ vascular resistance of muscle) (Stephenson 2020b). Apart from fatigue and possible ischaemia of the muscles leading to stumbling and ataxia on recovery, Polania Gutierrez and Munakomi (2020) described that delayed action of drugs when the drug is inadvertently deposited subcutaneously or between fascial planes could also be a cause. Transition from recumbence to standing should be calm, rapid and excitement free (Schumacher 2008). Minimal ataxia and stumbling with return of the swallowing reflex is warranted to safeguard the buffalo from injury and aspiration pneumonia during the early and late (3-5 days) recovery periods and this could translate into a decreased risk of predation. Despite effective immobilisation with both combinations, marked systemic hypertension was observed with EM drug administration.

Severe systemic hypertension, defined as MAP > 120 mmHg, is a common side effect when using potent opioid agonists (Heard et al. 1990; Heard et al. 1996; Schumacher 2008; Haskins 2015; Meyer et al 2015; Buss et al. 2018; Dugdale et al. 2020). Midazolam, unlike azaperone, has minimal effects on the cardiovascular system and thus the severe systemic hypertension was speculated to be directly induced by etorphine. This severe systemic hypertension is caused

by the binding of etorphine to the μ opioid GPCR which activates the G_i-protein, resulting in a decrease in cAMP which leads to smooth muscle contraction of peripheral vascular system, thus increasing the peripheral vascular resistance (Heard et al. 1990; Heard et al. 1996; Al-Hasani & Bruchas 2011; Pathan & Williams 2012; Heidemann 2013; Whittem et al. 2015; Chong & Johnson 2017). This G_i-protein signalling pathway is similar to that of the α_2-adrenoceptors which also result in peripheral vasoconstriction and systemic hypertension. Therefore, binding of a potent opioid to the scant density of μ opioid receptors found in the vasculature could result in activation of the G_i-protein signalling pathways similar to that of activating the sympathetic nervous system. The severe systemic hypertension in the EM resulted in a baroreceptor reflex to maintain a lower HR compared to EA. However, a lower systemic arterial blood pressure and elevated HR were observed in the EA drug combination. This effect was likely attributed to the α_1-adrenoceptor antagonism of azaperone which inhibited vasoconstriction and in combination with hypoxia-induced vasodilation resulted in an increase in HR to maintain the systemic arterial blood pressure (Klabunde 2012; Dugdale et al. 2020). Another possible cause for the lower systemic arterial blood pressure is the antagonism of the D1 and D2 peripheral GPCR (Cocker 1994; Beaulieu & Gainetdinov 2011; Posner & Burns 2013). The D1 receptor can lead to vasodilation when stimulated, although antagonism leads to inactivation of the heterotrimeric G_s-protein which results in inhibition of the intracellular pathway. Antagonism of the D2 receptor leads to inactivation of the heterotrimeric G_i-protein which allows for release of noradrenaline (Cocker 1994; Beaulieu & Gainetdinov 2011). However, the binding of azaperone to the α_1-adrenoceptors competes with the binding of noradrenaline. This competitive binding action could result in an increase in circulating noradrenaline which could bind to β-adrenoceptors. The binding to β-adrenoceptors causes central vasodilation and an increased risk of causing relative hypotension. We speculate that the etorphine-induced hypertension is counteracted by azaperone by the inactivation of the

α-adrenoceptors, despite GPCR mediated vasoconstriction through μ opioid receptor activation. Furthermore, vasodilation could have occurred at the muscles due to the local regulation of vascular tone during hypoxemic conditions (Joyner & Casey 2013; Stephenson 2020b). The elevation in HR could also be because of an indirect (hypoxaemia or hypercapnia) increase in sympathetic stimulation (Muir 2015a). The β-adrenoceptors are divided into β_1 (cardiomyocytes), β_2 (smooth muscles of arteries and bronchioles) and β_3 (adipose tissue) which are all GPCR associated with heterotrimeric G_s-protein (Klabunde 2012; Muir 2015b; Stephenson 2020c). The activation of the β_1-adrenoceptors *via* noradrenaline and adrenaline results in an increase in chronotropy, dromotropy and inotropy of the heart (Stephenson 2020c). However, the activation of κ and δ opioid receptors, found in heart muscle tissue, by etorphine may inhibit the actions of β-adrenoceptor stimulation in the heart by the G_i-protein activation (Barron 1999; Wong & Shan 2001; Pepe et al. 2004). The presence of acidaemia has direct effect on β-adrenoceptors which decreases myocardial inotropy (Biais et al. 2012). However, respiratory acidosis has negative inotropic effect and metabolic acidosis impairs the positive inotropic effect of β-adrenoceptor stimulation and decreases myocardial contraction (Biais et al. 2012). This inhibition of the sympathetic stimulation on the heart will reduce cardiac injury by decreasing the cardiac workload and O_2 consumption during periods of hypoxia, called hypoxic-preconditioning (Wong & Shan 2001). Furthermore, the increase in circulating catecholamines will stimulate vacant α_1- and α_2-adrenoceptor GPCR (G_q-protein and G_i-protein, respectively) and result in a greater smooth muscle contraction of the peripheral vascular system as seen in the EM combination (no azaperone-induced α_1-adrenoceptor antagonism). However, this is not the case with the α_1-adrenoceptor antagonistic effect of azaperone which results in the inhibition of smooth muscle contraction of peripheral vascular system and the blockade of catecholamine binding to α_1-adrenoceptors. It has been demonstrated that crosstalk between μ opioid receptor and α_2-adrenoceptor occur which

modify the activity of one another (Root-Bernstein et al. 2019). The possibility of receptor redundancy between μ opioid receptor and α_2-adrenoceptor can exist because both are G_i-protein coupled receptors. This redundancy follows the notion, as seen with the clinical effects of potent opioid on GPCR, that when the one receptor (μ opioid receptor) activates the G_i-protein, the other receptor (α_2-adrenoceptor) has little to no effect on activating the same G_i-protein to stimulate the same intracellular pathways. The myocardium is influenced not only by the potent opioid but also by hypoxaemia and hypercapnia. The myocardium receives approximately 5% of the left ventricular output and has an O_2 extraction ratio of 70-80% (Muir 2015b). The coronary blood flow is influenced by 1) extravascular compression of intramural blood vessels, 2) HR, and 3) contractile state of the myocardium (Muir 2015b). The coronary blood flow to the left ventricle occurs during diastole and to the right ventricle during systole. The coronary perfusion pressure is the difference between diastolic arterial pressure and right atrial filling pressure (RAP). When the RAP increases either by drug effect (etorphine) or fluid boluses, the coronary perfusion pressure will decrease resulting in a reduction of coronary blood flow (Muir 2015b). However, high diastolic arterial pressure and local adenosine and nitric oxide induced vasodilation (reduced coronary vascular resistance) will ensure adequate coronary perfusion and blood flow (Muir 2015b; Stephenson 2020b). As mentioned previously, the left ventricular myocardium perfuses during diastole and thus the HR is the primary determinant of diastolic filling time (Stephenson 2020a). Normal compensatory mechanism of the heart to hypoxia and ischaemia is activation of epicardial chemoreceptors which initiates a coronary chemoreflex (Bezold-Jarisch reflex) leading to bradycardia and vasodilation which could lead to hypotension (Muir 2015b); however, this was not detected during the study. We speculate that the persistent hypertension observed during the EM combination, is of concern for prolonged procedures and needs to be addressed to prevent target-organ damage (Acierno et al 2018). These target-organs include the kidneys, eye, brain, and the heart. Damage to the

kidneys includes decreased renal function, early renal death, and proteinuria. Ocular damage manifests as acute onset blindness, retinal haemorrhage or oedema, retinal detachment, and secondary glaucoma. Left ventricular concentric hypertrophy and encephalopathy are the most common manifestations of damage in the brain and heart (Haskins 2015; Acierno et al 2018). Blindness can predispose the buffalo to injury and are also susceptible to predation. The kidney, cerebral and heart pathology leads to morbidity of the animal and thus succumb to the organ damage or predation during the late recovery period.

Apart from marked cardiovascular effects, clinically relevant respiratory effects were observed with both combinations. In this study, minute ventilation was not determined and so the indicators for ventilation were f_R and $P_E'CO_2$. Minute ventilation is the product of tidal volume and f_R and is defined as the volume of air which moves in and out of the respiratory system per minute (Dugdale et al 2020). Even though the f_R was within expected reference intervals (18-30 breaths minute^{-1}) for both drug combinations, there was significant hypoventilation (decreased alveolar minute ventilation relative to metabolic rate), which could be due to decreased tidal volume (rumen encroaching onto the diaphragm and lungs, decreasing functional residual capacity or a decrease of total lung compliance), resulting in the hypercapnia ($PaCO_2$ or $P_E'CO_2$ > 55 mmHg) (Wilson & Lofstedt 2009; Hattingh et al. 1984; Haskins 2015; Riebold 2015). Zeiler and Meyer (2017) described rigid respiratory muscles in impalas due to effects of potent opioids stimulating the μ opioid receptor in certain areas of the medulla and pons resulting in hypoventilation because of a failure to transition between the inspiratory and expiratory phase of a normal breath cycle. In white rhinoceros, the administration of etorphine increased the metabolic rate, likely related to muscle tremors, and thus an increase in $PaCO_2$ relative to minute ventilation and this etorphine effect could also explain the raised $PaCO_2$ in our buffaloes (Buss et al. 2018). Hypercapnia was observed with both drug combinations but was slightly less with the EM combination, possibly because

midazolam has minimal respiratory depressive effects and may decrease respiratory muscle rigidity (Rankin 2015). An overall negative $P(a\text{-}E')CO_2$ gradient was encountered with both combinations which usually occurs due to higher cardiac output relative to a lower alveolar ventilation. Sampling respiratory gases from the nasopharynx may have also resulted in the negative $P(a\text{-}E')CO_2$ gradient observed since gas samples may be contaminated by CO_2 from the rumen which could have falsely elevated the $PE'CO_2$ (Riebold 2015). An important clinical consequence of hypoventilation, which warrants astute monitoring to prevent mortalities, is the development of hypoxaemia.

Hypoxaemia (PaO_2 < 80 mmHg) occurs by one, or a combination, of the following 1) a decreased PIO_2, 2) hypoventilation, 3) impairment of oxygen diffusion across the alveolocapillary interface, 4) ventilation-perfusion mismatch, 5) reduced venous O_2 content (as seen with shock), 6) high O_2 extraction and 7) right-to-left vascular shunts (Petersson & Glenny 2014; Haskins 2015; Muir 2015a). Clinically relevant hypoxaemia occurs at PaO_2 < 60 mmHg and when diagnosed it should be remedied in a timeously manner (Haskins 2015). During the study, the PIO_2 was not altered and remained at approximately 137 mmHg. Although hypoventilation could have contributed to the severe hypoxaemia (PaO_2 = 30-40 mmHg) observed with both drug combinations, a normal $P(A\text{-}a)O_2$ gradient (< 15 mmHg) would have been present (Haskins 2015; Muir 2015a; Dugdale et al. 2020). However, a widened $P(A\text{-}a)O_2$ gradient was observed and, along with the hypercapnia, suggests that large right-to-left intrapulmonary shunting and impairment of gas diffusion across the alveolocapillary membrane are the primary causes rather than dead-space ventilation or hypoventilation (Muir 2015a; Zeiler & Meyer 2017). A widened $P(A\text{-}a)O_2$ gradient can result from an increased PIO_2 with the administration of supplemental O_2 and caution is advised during interpretation of this gradient because it leads to a limited prediction of a shunt (Armstrong 2007). However, this limitation is not a concern in this study because we did not administer oxygen therapy. The

$P(A\text{-}a)O_2$ gradient incorporates the alveolar gas equation which is only valid in steady-state conditions (Cruickshank & Hirschauer 2004). Three variables affect the PAO_2 which include 1) changes in RQ due to diet, 2) increasing or decreasing the PIO_2, and 3) ease of $PaCO_2$ manipulation in an intubated ventilated animal vs spontaneously breathing animal (Cruickshank & Hirschauer 2004). The RQ is the production of CO_2 divided by the consumption of O_2 and due to the diet, ruminants tend to have a RQ value closer to 1 compared to 0.8 for carnivores (Haskins 2015; Ewart 2020). If the shunt fraction from the right-to-left intrapulmonary shunt is more than 50%, hypercapnia starts to develop (Sarkar et al. 2017). Etorphine has been shown to induce pulmonary hypertension in goats which alongside right-to-left intrapulmonary shunting, contributed to severe hypoxaemia (Meyer et al. 2015). Pulmonary hypertension results in an increase in the hydrostatic pressure which in turn leads to oedema of the interstitium and alveoli. This increase in the diffusion distance restricts the efficiency of less soluble gases like O_2 to transfer across the alveolocapillary membrane (Meyer et al. 2015). Hypoxic pulmonary vasoconstriction is a strong pulmonary reflex in domestic cattle and this may be true in wild bovids as well and could have contributed to ventilation-perfusion mismatching (Ewart 2020). Due to all the possible causes for severe hypoxaemia, the CaO_2 would be lower as well. The closest published reference interval for CaO_2 is 18.6 to 23.5 mL dL^{-1}, however this is for domestic cattle (Burke 1953). To compensate for the decrease in CaO_2, the cardiac output will need to increase to maintain O_2 delivery to the tissues. However, the effects of the drugs on the cardiovascular system, especially etorphine, which causes an increased vagal tone and marked systemic hypertension (baroreceptor reflex) suppresses the HR and no compensatory increase in HR can assist in maintaining O_2 delivery. The cardiac output is usually maintained by an increase in the stroke volume when non-potent opioids are used, but this might not be entirely true for potent opioids (KuKanich & Wiese 2015). Conversely to the maintenance of cardiac output when using non-potent opioid agonists,

the inotropy of the myocardium can be suppressed when fentanyl (μ opioid receptor agonists with little to no effect at the other opioid receptors) is administered (Wu et al. 1997; Kanaya et al. 1998).

Clinical manifestation of hypoxaemia results in production of lactic acid *via* anaerobic glycolysis (Stephenson 2020b). Mild hyperlactataemia (<5.0 mmol L^{-1}) was observed in both arterial and venous blood gases with no significant difference between drug combinations or over time. We speculate the utilisation of the lactate shuttle (transportation of lactate between cells and metabolism thereof) and the Cori cycle (lactate is converted to glucose in the liver and converted back to lactate in the muscle) contributed to the unexpected low lactate concentration despite severe hypoxaemia (Brooks 2018; Dugdale et al. 2020). However, it has been demonstrated that lactate is oxidised within muscles as a source of fuel (Brooks et al. 2018; Lund et al. 2018). The production of lactate is *via* glycolysis and several factors determine the amount produced, which include O_2 availability, oxidative enzyme activity, glycolysis rate and activity of lactate transporters (Lund et al. 2018). Hyperlactataemia is divided into Type A (inadequate tissue O_2 delivery or O_2 demand), Type B1 (disease process), and Type B2 (drug or toxin induced). Etorphine can result in both Type A and B2 hyperlactataemia (Dugdale et al. 2020). Another speculation related to the low lactate concentration is the assumption of greater oxymyoglobin. Buffaloes have darker red coloured muscle compared to domesticated cattle, which indicates greater myoglobin concentration (Zhang et al. 2016). The oxymyoglobin dissociation curve has a hyperbolic equilibrium curve as myoglobin only has one O_2-binding site and demonstrates greater affinity for O_2 and no cooperativity (Hill et al. 2012). The PaO_2 in muscles must decrease to a low enough level to allow the O_2 bound to myoglobin to unload and be utilised (Hill et al. 2012). Myoglobin has also been found to act as an enzyme. During hypoxaemic states, deoxymyoglobin catalyses NO synthesis which in turn inhibits cytochrome oxidase which results in inhibition of O_2 utilisation

and adenosine triphosphate formation by mitochondria (Hill et al. 2012). The compensatory physiological response to clinical hypoxaemia is initially tachycardia and tachypnoea, then during decompensation, bradycardia and tachypnoea occur. In this study, however, no clear indication for the treatment of the hypoxaemia with either O_2 supplementation or intermittent positive pressure ventilation was evident.

The supplementation of O_2 to hypoxemic animals is good veterinary practice, especially during immobilisation and general anaesthesia, as most drugs cause a decrease in alveolar minute ventilation (Fahlman 2014; Haskins 2015; Dugdale et al. 2020). However, if hypoxaemia is due to right-to-left intrapulmonary shunting, O_2 supplementation will be ineffective, particularly if the shunt fraction is > 50% (Sarkar et al. 2017; Dugdale et al 2020). An important clinical concern during O_2 supplementation in animals that are hypoxic and hypercapnic is O_2-induced worsening of the hypercapnia. Petersson and Glenny (2014) reported that O_2-induced hypercapnia can occur either by inhibition of the hypoxic drive to breathe or by an increasing in PAO_2 in low ventilation-perfusion units and inhibiting the pulmonary hypoxic vasoconstriction. This will lead to blood being diverted away from well ventilated units (becoming high ventilation-perfusion units) to low ventilated units resulting in increase in dead space ventilation. The higher $PACO_2$ will diffuse back into the pulmonary circulation. Another proposed explanation for the hypercapnia is the Haldane effect, whereby an increase in PaO_2 decreases binding of H^+ and CO_2 to haemoglobin resulting in a greater level of dissolved CO_2 (Petersson & Glenny 2014).

Differences in venous blood gas variables were noted but not investigated and only speculation on the aetiology of these differences can be made. The K^+ concentration was significantly higher (but within expected reference intervals) in the EM drug combination immobilised buffaloes, possibly due to benzodiazepine agonist effects on decreasing the release of insulin

from the pancreas or the systemic hypertension causing shearing forces on the erythrocytes and thus a release of intraerythrocytic K^+ (Le Quan Sang et al. 1993; Chevassus et al. 2004). Leaking of K^+ from the intracellular compartment of erythrocytes into the serum during long term storage was not a plausible reason because blood gas analysis occurred on site and with immediate analysis (DiBartola & De Morais 2012). The lower Ht of the EA drug combination compared to the EM drug combination is possibly caused by the splenic sequestration that is known to occur when an α_1-adrenoceptor antagonist, like azaperone, is administered (Lamont & Grimm 2014; Rankin 2015). The HCO_3^- and the resultant BE(ecf) and BE(b) will increase due to a rise in $PaCO_2$, mostly because these values are calculated by the blood gas analyser using $PaCO_2$ as a variable within the equations (Dugdale et al. 2020). However, theoretically, an increase in HCO_3^- is due to the diffusion of CO_2 into the erythrocyte which is hydrated to carbonic acid (H_2CO_3) *via* carbonic anhydrase (Arthurs & Sudhakar 2006). The H_2CO_3 dissociates to form H^+ and HCO_3^-ions. The majority of the HCO_3^- diffuses into the plasma, whereas the H^+ remains within the erythrocyte, due to membrane impermeability to cations. Furthermore, electrical neutrality needs to be maintained and as such Cl^- moves into the erythrocyte (the chloride shift) as an exchange for the HCO_3^- moving out. The H^+ binds to deoxyhaemoglobin and is removed from solution contributing to the pH buffering capacity of the body (Arthurs & Sudhakar 2006). Another compensatory mechanism performed by the kidneys, is to reabsorb HCO_3^- from the tubular fluid and excrete H^+ by the collecting ducts (which can also generate HCO_3^-), but this renal compensation is expected to take hours to days to develop and not relevant to our study findings.

4.2 Conclusion

The EM drug combination is a reliable and effective drug combination for immobilisation of boma-confined buffalo bulls and similar in immobilisation characteristics and physiological effects to the routine EA combination. However, systemic hypertension was a clinical concern with the EM combination. Pulmonary hypertension and right-to-left intrapulmonary shunting of blood is speculated to be primarily aetiologies of hypoxaemia and hypercapnia in both drug combinations. Healthy African buffalo bulls demonstrated resilience to the clinically relevant hypoxaemia which did not require rescue interventional therapy. However, this might not be the case in diseased or compromised buffaloes, as the physiological derangements present could exacerbate the effects of the immobilisation drugs. Astute monitoring is advised with both drug combinations, especially if supplemental oxygen is administered.

4.3 Future research

Etorphine-midazolam drug combination is just as effective in inducing reliable immobilisation as the etorphine-azaperone combination. However, further investigation into the mechanism of the marked systemic hypertension during the etorphine-midazolam combination is recommended. Investigation into the substitution of etorphine with thiafentanil, using similar sedative combinations to determine the effectivity and reliability between the two potent opioid drugs is warranted. Furthermore, including medetomidine as a sedative to replace azaperone and midazolam in etorphine and thiafentanil based immobilisations is worth consideration. Importance of continued research in this species will contribute to the scarce literature available and assist in developing safer immobilisation protocols for animals and personnel. Testing these combinations on female African buffalo to ascertain if there might be sex-related physiological or chemical restraint differences is also recommended.

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Addendum

Animal ethics approval certificate V050-18

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

PROJECT TITLE	**Etorphine-azaperone compared to etorphine-midazolam immobilisation in African Buffalo *(Syncerus caffer)***
PROJECT NUMBER	**V050-18**
RESEARCHER/PRINCIPAL INVESTIGATOR	**Dr. JF Grace**

STUDENT NUMBER (where applicable)	**U_10054261**
DISSERTATION/THESIS SUBMITTED FOR	**MSc**

ANIMAL SPESIES/SAMPLES	**African Buffalo *(Syncerus caffer)***
NUMBER OF ANIMALS	**140**
Approval period to use animals for research/testing purposes	**June 2018 - June 2019**
SUPERVISOR	**Prof. G Zeiler**

KINDLY NOTE:

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

APPROVED	Date 10 July 2018
CHAIRMAN: UP Animal Ethics Committee	Signature

S4285-15

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Veterinary Science
Animal Ethics Committee

5 September 2019

Approval Certificate
Annual Renewal
(Extension 1)

AEC Reference No.: V050-18
Title: Comparison of etorphine-azaperone to etorphine-midazolam immobilisation in African buffalo (Syncerus caffer)
Researcher: Dr JF Grace
Student's Supervisor: Prof GE Zeiler

Dear Dr JF Grace,

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

Please note the following about your ethics approval:

1. The use of species is approved:

Species and Samples	Number Available
African Buffalo *(Syncerus caffer)*	140

2. Ethics Approval is valid for 1 year and needs to be renewed annually by 2020-09-05.
3. Please remember to use your protocol number (V050-18) on any documents or correspondence with the AEC regarding your research.
4. Please note that the AEC may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.

Ethics approval is subject to the following:

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

We wish you the best with your research.
Yours sincerely

Prof V Naidoo
CHAIRMAN: UP-Animal Ethics Committee

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Fakulteit Veeartsenykunde
Lefapha la Diseanse tša Bongakadiruiwa

Owner's permission letter

Wildlife Pharmaceuticals (Pty) Ltd

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22/10/2019

Letter of permission (land and animal use)

To whom it may concern,

I, Cobus Raath, hereby give permission to Prof Gareth E. Zeiler (ID) (u21039993) and his student Dr Justin Grace (u10054261) to conduct a MMedVet (Anaes) research project (registered at the University of Pretoria) entitled **"Comparison of etorphine-azaperone to etorphine-midazolam immobilisation in African buffalo (*Syncerus caffer*)"**. During the duration of the project (2019/09/25 to 2019/10/02), I give my full permission to conduct this research on my land (St Pauls' Nature Reserve, Schoemanskloof Valley, Nelspruit) and **TEN** of my privately-owned African Buffalo (*Syncerus caffer*).

I confirm that I have been provided with all the details and methodology of the project stated above and understand the risks associated with such a project.

Please contact me if there are any further concerns:

E-mail: md@wildpharm.co.za

Tel: 082 577 2779

Yours sincerely,

Dr Cobus Raath

Signature

2019-10-22.

Date

Managing Director: Dr JP Raath BVSc – Responsible Pharmacist: M Lines MPharm

Data collection forms

Date		Amb. Temp.		P_B		BCS	
Buffalo ID				Sex (M/F)		Age (months)	

Immobilisation combination	Drug	Dosage (mg)	Route
	Etorphine		
	Azaperone / Midazolam		

Time of dart contact (hh:mm)		Time of first handle (min's'')	
First signs of sedation (min's'')		Time to recumb. (min's")	
Induction Quality Score		Immob. Quality Score	

Comments: _____

_____

Top-up drugs	Drug	Dosage (mg)	Route	Time
	Etorphine			
	Azaperone / Midazolam			

T_0 = time first handle

Parameters	5 min (:)	10 min (:)	15 min (:)	20 min (:)
Heart Rate				
Arrhythmias noted				
Respiratory Rate				
Temperature (°C)				
SpO_2 (%)				
$ETCO_2$ (mmHg)				
SAP (mmHg)				
DAP (mmHg)				
MAP (mmHg)				
Remarks				
Immob. score				
Art + Ven BG (√)		[redacted]		
EDTA (√)				
Serum (√)				
Citrate (√)				
Heparin (√)				

Reversal drugs	Drug	Dosage (mg)	Route	Time
	Naltrexone			
	Flumazenil (if needed)			
Intervention drugs				

1st sign of recovery		Head up (min's'')		Standing (min's'')		Walking (min's'')	

Recovery Quality Score		Tot. time of immob.	

Comments: _____

Presentations and publications arising from the study

Presentations:

EVENT	VENUE	DATE	TITLE	TYPE
SAVA Wildlife Congress	Onderstepoort	07 March 2020	Comparison of etorphine-azaperone to etorphine-midazolam immobilisation in African buffalo	Oral
Onderstepoort Faculty Day 2020	Onderstepoort	20 November 2020	Comparison of etorphine-midazolam and etorphine-azaperone for African buffalo (*Syncerus caffer*) immobilisation	Virtual oral presentation

Publications:

Justin F Grace, Michele A Miller, Jacobus P Raath, Liesel L Laubscher, Peter E Buss & Gareth E Zeiler (Under review) Etorphine-midazolam is as effective as etorphine-azaperone in immobilising free-ranging African buffalo (*Syncerus caffer*). Veterinary Anaesthesia and Analgesia