# Cardiopulmonary effects of ketamine-butorphanol-medetomidine and etorphine-azaperone drug combinations used to immobilise zebra *(Equus zebra*)

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By

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

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# Declaration of originality

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# Abstract

**Cardiopulmonary effects of ketamine-butorphanol-medetomidine and etorphine-azaperone drug combinations used to immobilise zebra *(Equus zebra)***

**Objective** To compare immobilisation efficacy of a non-potent opioid drug combination to the preferred etorphine-azaperone combination in zebras.

**Study design** Prospective, randomised crossover trial.

**Animal population** Ten adult zebras.

**Methods** Each zebra was immobilised, by darting, twice using two different combinations: ketamine-butorphanol-medetomidine (KBM; 7 ml dart) and etorphine-azaperone (EA; 1 ml dart) in random order, three weeks apart. A stopwatch was started once the dart was placed to record times to recumbency and standing during induction and recovery, respectively. Once the zebra were recumbent and instrumented, physiological parameters were measured and recorded, at 5-minute intervals until 20 minutes. The quality of immobilisation was scored subjectively using muscle relaxation, palpebral reflexes and movement as indicators. Antagonist drugs were administered for recovery 5 minutes after the last recordings. Combination KBM was antagonised using atipamezole at 7.5 mg $mg^{-1}$ medetomidine dose (half intravenous then half intramuscular) and intravenous naltrexone at 2 mg $mg^{-1}$ butorphanol dose. Combination EA was antagonised using intravenous naltrexone at 20 mg $mg^{-1}$ etorphine dose. Physiological parameters were compared between combinations using a general linear mixed

model. Data reported as mean (range) unless otherwise stated. The drug and dart cost of immobilisation were compared descriptively.

**Results** The mean dose of ketamine-butorphanol-medetomidine were 3.30 (3.04 to 3.57), 0.40 (0.38 to 0.42) and 0.16 (0.15 to 0.17) mg $kg^{-1}$; and etorphine-azaperone were 0.02 (0.02) and 0.20 (0.18 to 0.22) mg $kg^{-1}$, respectively. KBM and EA induced recumbency in a median (range) of 420 (300 to 600) and 240 (180 to 300) seconds, respectively. Zebras remained recumbent throughout the study procedures. Systolic blood pressures were 225 (136 to 286) and 167 (93 to 268) mmHg and $PaO_2$ were 64 (46 to 90) and 47 (20 to 85) mmHg for KBM and EA, respectively. Median (range) time to standing, after administering antagonists was 90 (18 to 2 220) and 26 (7 to 72) seconds for KBM and EA, respectively. It costed R 5 842.00 to immobilise and antagonise a zebra using KBM which was six-and-a-half times more expensive than EA (R 897.00).

**Conclusions and clinical relevance** The non-potent opioid KBM combination may provide a reliable alternative to the preferred EA combination for the immobilisation of zebras, especially in captive conditions. The superior muscle relaxation and apparent deeper plane of anaesthesia achieved with KBM could make this combination a good alternative to immobilise zebra that require painful procedures. Systemic hypertension and moderate hypoxaemia are clinical concerns when using KBM and EA, respectively. With the currently available preparations, the high costs associated with KBM, and the larger dart required for its administration, may limit its field use. Further evaluation of the KBM combination's efficacy in free-ranging zebras is required.

**Keywords** butorphanol, *Equus zebra*, immobilisation, ketamine, medetomidine, zebra

# List of abbreviations

| | |
|---|---|
| % | percentage |
| °C | degree(s) Celsius |
| BE | base excess |
| boma | temporary enclosure that served as a holding pen, constructed from 2.8-meter-high wooden pole walls |
| bpm | beat(s) per minute |
| cc | cubic centimetre(s) volume used for measuring dart volume (1 cc = 1 ml) |
| $CO_2$ | carbon dioxide |
| DAP | diastolic arterial pressure |
| EA | etorphine-azaperone combination |
| ET-tube | endotracheal tube |
| $HCO_3^-$ | bicarbonate ion |
| HR | heart rate |
| IM | intramuscular |
| IQR | interquartile range |
| IV | intravenous |
| KBM | ketamine-butorphanol-medetomidine combination |
| kg | kilogram(s) |
| kPa | kilo Pascal(s) |
| L | litre(s) |
| m | meter(s) |
| MAP | mean arterial pressure |
| mg $kg^{-1}$ | milligram(s) per kilogram |
| mg | milligram(s) |
| mg $ml^{-1}$ | milligram(s) per millilitre |
| min | minute(s) |
| ml | millilitre(s) |
| ml $min^{-1}$ | millilitre(s) per minute |
| mm | millimetre(s) |
| mmHg | millimetres Mercury |
| mmol | millimole(s) |
| N | newton(s) |
| n | number of animals |
| N $s^{-1}$ | newton(s) per second |
| $PaCO_2$ | arterial carbon dioxide tension |
| $PaO_2$ | arterial oxygen tension |
| $PE´CO_2$ | end-tidal carbon dioxide tension |
| pH | negative log of hydrogen ion concentration |
| RR | respiratory rate |
| SAP | systolic arterial pressure |
| SC | subcutaneous |
| SDS | simple descriptive scale |
| $SpO_2$ | peripheral oxygen haemoglobin saturation |
| T | time of data collection points in minutes (T0, T5, T10, T15, T20) |
| Temp | temperature in degrees Celsius |
| µg | microgram(s) |

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# Introduction

## Literature review

Zebra are frequently immobilised using chemical capture techniques. Immobilisation is achieved by the administration of various classes of drugs, usually including the potent opioid etorphine, delivered intramuscularly via remote darting. Chemical immobilisation of zebra is necessary in several instances such as translocation, or to perform medical or surgical procedures (Senthilkumar et al. 2005; Hoyer et al. 2012), for the collection of biomedical samples and fitting radio-telemetry devices in scientific based conservational efforts (Walzer et al. 2006). There is limited scientific literature describing chemical immobilisation and its physiological effects in zebra, unlike the plethora of readily available information for domestic horses and other wild equids (Quinn 2012; Walzer et al. 2006). Several publications involving various wild equids confirm that chemical immobilisation in equids remains a challenge and despite several proposed drug combinations, opportunities exists, especially in zebra, to make further improvements (Matthews et al. 1995; Walzer et al. 2006; Woolnough et al. 2012).

Opioids, alpha-2 adrenoceptor agonists, phenothiazine derivatives, and benzodiazepine agonists are amongst the most common drug classes used in immobilisation combinations in domestic equines. Studies in the 1980s involving immobilisation of wild equid species suggested that succinylcholine chloride, a neuromuscular blocking agent, is a method of immobilisation despite its variable success rate and high risk of mortalities (Gallagher et al. 1983; Plotka et al. 1987). Concurrently, in 1979, etorphine combined with tranquilizers were investigated by Moore as immobilisation drugs in horses (Plotka et al. 1987), but they did not report dosage details. Subsequently, etorphine in combination with the tranquilizer azaperone or the sedative detomidine was prescribed as the drug combinations of choice to chemically capture zebra (Adin et al. 2007; Allen 1990; Kock et al. 2012; Plotka et al. 1987; Senthilkumar

et al. 2005; Walzer et al. 2006; Young & Penzhorn 1972; Zabek et al. 2015). Ketamine has been added to etorphine drug combinations as an adjunctive drug to improve the overall quality of immobilisation (Adin et al. 2007). Presently, most drug combinations designed to immobilise wild equids have been investigated in feral horses, donkeys and Przewalski's horses, but there is a paucity of information in zebra.

### *Immobilisation drugs of interest*

The commonly used drugs for immobilisation of equine species are reviewed in terms of their class, general clinical and side effects. The drugs will be separated into the primary immobilising drugs and adjuvant drugs. The primary immobilising drugs are considered essential in a drug combination to achieve an adequate state of immobilisation, Whereas, the adjuvant drugs complement the primary immobilising drugs through additive or synergistic interactions which is thought to improve the overall quality of immobilisation.

#### *Primary immobilising drugs*

Etorphine

Etorphine is a highly potent opioid, semi-synthesised from alkaloids found in opium. This drug has become widely used as a primary immobilisation drug in wildlife (Alford et al. 1974; Kock et al. 2012; Zabek et al. 2015). Etorphine is classified as a pure agonist and binds to stereo-specific and saturable receptors in the central nervous system, namely: mu, kappa and delta opioid receptors (Walzer et al. 2006). Intramuscular injection in equids results in rapid immobilisation, sedation and analgesia (Alford et al. 1974). However, animals are still able to respond to various stimuli, such as phlebotomy, when immobilised.

Etorphine is known for an array of side effects in equids, but the most important include: respiratory depression as a result of the direct drug effect on the respiratory centre (mu receptor); prolonged opioid-induced excitement from inadequate dosage of etorphine or failure

to deliver drug intramuscularly with ensuing hyperthermia and hypertension has been reported (Kock et al. 2012; Walzer et al. 2006). Etorphine has also been associated with muscle hypertonicity and catalepsy (Alford et al. 1974; Kock et al. 2012).

The pure opioid antagonist naltrexone and mixed mu-antagonist-kappa-agonist diprenorphine act at the same receptors as etorphine. These drugs act as competitive pharmacological receptor antagonists that displace etorphine from the receptor to "reverse" its pharmacodynamic effects (Kock et al. 2012; Walzer et al. 2006).

Etorphine is seldom used on its own but rather administered in combination with a tranquiliser or sedative to achieve reduced excitement and muscular hypertonicity, which is termed, neuroleptanalgesia.

Ketamine

Ketamine is a frequently used anaesthetic drug that is usually administered in combination with tranquilizers or sedatives. Ketamine induces a short duration of dissociative anaesthesia (Kock et al. 2012; Zabek et al. 2015), characterised by a dissociative loss of consciousness, amnesia and analgesia. Ketamine is frequently combined with medetomidine to immobilise a range of species (Matthews & Myers 1993; Zabek et al. 2015).

When used for immobilisation, hypertonicity of muscles is common, therefore negating its use as a sole agent in most species, especially equids. Thus, tranquilizers and sedatives with muscle relaxation properties are used in combination with ketamine to improve the muscle relaxation during immobilisation. Prolonged recoveries associated with ataxia are thought to be due to the persistence of the active metabolite nor-ketamine (Woolnough et al. 2012). Ketamine is known to have a direct negative cardiac inotropic effect. This direct drug effect is usually not noticed because it stimulated noradrenaline release from the sympathetic branch of

the autonomic nervous system which causes an increase in chronotropic and inotropic effects on the heart. Thus, when ketamine is administered an increase in systemic and pulmonary arterial pressure, heart rate and cardiac output is expected. Despite an apneustic respiratory pattern often seen with the administration of ketamine, minute ventilation is not significantly depressed. However, when administered in combination with other central nervous system depressants then respiratory depression may occur (Muir 2010).

The ketamine dosage required to immobilise larger animals (>100 kg) often exceed the volume capacity of frequently used darts for capture (1, 3 or 5 ml darts). Therefore, it is common practice to use powdered ketamine, whereby a solution of 250 mg $ml^{-1}$ could be made up by dissolving the powder in sterile isotonic saline or water for injection. However, the stability of ketamine formulation concentrations higher than 200 mg $ml^{-1}$ are known to be unstable (Personal communication: Dr Inus Jansen van Rensberg; Chemical Engineer, Koeger Pharmaceuticals; August 2017). If volume remains a restricting factor, anecdotally the ketamine powder could be dissolved with liquid drug formulations such as medetomidine or azaperone.

The effects of ketamine cannot be reversed as there is no antagonist. Clearance and recovery is dependent on initial redistribution followed by hepatic metabolism and renal excretion of ketamine and its metabolites (Kock et al. 2012).

Butorphanol

Butorphanol tartrate is a synthetically derived opioid kappa-agonist-mu-antagonist analgesic of the phenanthrene class (Bush et al. 2012; Kock et al. 2012). Butorphanol has low intrinsic antagonistic (even perhaps mild agonistic) activity at mu opioid receptors and has the potential to antagonise the effects of more potent opioids (Miller et al 2013, Schenellbacher 2010). It is

also an analgesic drug through its agonistic effects at kappa receptors (Bush et al. 2012; Kock et al. 2012).

The interaction with these receptors in the central nervous system mediates its pharmacological effects making butorphanol a good sedative and analgesic drug in horses (Bush et al. 2012). More recently butorphanol has been included in immobilisation combinations for several species such as: rhino, hippo, lions and wild dogs (Kock et al. 2012, Miller et al. 2013). Compared to other opioid drugs, butorphanol generally cause minimal cardiopulmonary depression in equids. Dose-dependent respiratory depression occurs at lower doses, but a ceiling is reached and no further depression occurs (Bush et al. 2012).

As with other opioid drugs, butorphanol can induce unfavourable central nervous system effects such as excitement and ataxia. Equids commonly manifest random head bobbing and twitching of neck and limbs at clinically relevant dose rates. Emesis and nausea has been described in non-equid species and less commonly gastrointestinal effects that lead to hypomotility and constipation (Bush et al. 2012).

Naltrexone can be administered to antagonist the clinical effects of butorphanol when dosed at twice the dose of butorphanol (Kock et al. 2012).

### *Adjuvant drugs*

#### Medetomidine

Medetomidine is a potent selective alpha-2 adrenoceptor agonist (α2-agonist) which acts at both pre- and postsynaptic receptors of neuroeffector junctions. Binding of the agonist, among other actions, inhibits the presynaptic release of: noradrenalin, dopamine and serotonin within

the central nervous system. This causes depression of the central nervous system with effects such as sedation, muscle relaxation and analgesia. The magnitude of effect is dose dependent. Rapid distribution into the central nervous system by medetomidine allows for rapid dose-dependent sedative effects. Medetomidine is known for its potent sedative effect, distinguishing it from less potent detomidine, and other α2-agonists (Scheinin et al. 1989; Virtanen 1989).

The α2-agonists initially cause a peripheral systemic response which encompasses vasoconstriction with accompanying increase in peripheral vascular resistance which results in an initial hypertension. Baroreceptors detect the hypertension and attempt to normalise the blood pressure by increasing vagal tone, causing bradycardia and decreasing the cardiac output and decreased tissue perfusion. Following the initial peripheral effects of the drug, central effects (presynaptic receptors) begin to manifest as a result of decreased noradrenaline release at neuroeffector junctions. The central effects suppress the cardiovascular centre and sympathetic innervation to the myocardium and blood vessels causing a continued bradycardia and variable (normal resting tone or vasodilation) vasotone of the blood vessels. These cardiovascular effects culminate in either a normalisation of the blood pressure (medetomidine), or hypotension (xylazine). Thus, the poor cardiac output persists and therefore metabolising tissues remain poorly perfused (Klein & Klide 1989; Kock et al. 2012; Woolnough et al. 2012). The α2-agonists are known to cause respiratory depression, especially when combined with opioid agonists (Woolnough et al. 2012). Impaired thermoregulation is also a side effect (Klein & Klide 1989). The α2-agonists are poor immobilising agents when used alone, especially in excited animals like zebras and other wild equids, thus they are commonly used in combination with drugs such as opioid agonists (etorphine and butorphanol) or cyclohexylamines (ketamine) (Joubert et al. 1999; Klein & Klide 1989; Walzer et al. 2006; Zabek et al. 2015).

Atipamezole is an α2-adrenoceptor antagonist, generally administrated intramuscularly to antagonist the sedative effects of medetomidine, clinically a dose of 2.5 to 5 times the medetomidine dose is effective, depending on the species (Kock et al. 2012).

Midazolam

Midazolam is a relatively new drug belonging to benzodiazepine agonist group of sedatives. Its current use in wildlife immobilisation is limited, but it may become more widely used in the capture of wild animals as it is more miscible with other drugs within a dart and better absorbed after intramuscular injection when compared to diazepam (Kock et al. 2012). This class of drug has a profound amnesic effect in humans and provides good muscle relaxation in most species. Also, the midazolam appears to be a safe drug to use in cardiovascular compromised patients and when used alone has minimal effects on the respiratory system (Kushiro et al. 2005).

Central sedative effects of midazolam can be antagonised by the administration of flumazenil (Kock et al. 2012).

Azaperone

This tranquilizer is classified as a neuroleptic drug and fails to induce analgesia or hypnosis when used alone, no matter the dose administered. Butyrophenone derivatives, like azaperone, are multi-receptor antagonists with clinically noteworthy anti-dopaminergic effects and mild antagonistic effect on alpha-1 adrenoceptors (Kock et al. 2012).

As a result of the multi-receptor interactions it produces an array of side effects. Some of these unfavourable effects in equids include vasodilatation, bradycardia, respiratory

depression, paraphimosis and priapism (Hillidge et al. 1973). Furthermore, using this drug at high doses can elicit extra-pyramidal effects such as catalepsy, aggression and torticollis (Kock et al. 2012).

There is no antagonist drug for azaperone (Kock et al. 2012). However, administering specific receptor agonists such as anticholinergic drugs (atropine, benztropine, glycopyrrolate) may reverse undesirable clinical effects (Grimm et al. 2015).

### *Immobilising drug combinations used in equids*

Drugs are combined for their additive or synergistic effects. These effects allow for lower primary immobilisation drug dose and decreases the severity or incidence of side effects.

Allen et al. (1994) immobilised twelve Hartmann's mountain zebras (*Equus zebra hartmannae*) using carfentanil alone. This protocol was based on previous work done by the author (Allen 1992) on Mongolian wild horses (*Equus przewalskii przewalskii*). He concluded that short lived sedation of less than ten minutes is achievable when darting this species with carfentanil at an average dose of 0.011 mg $kg^{-1}$ (3.91 mg total dose). Sedation was fully antagonised without renarcotization after intravenous administration of either naltrexone or nalmefene dosed at 50 times the carfentanil dose. Supplementary drugs were administered to half of the zebra in order to improve muscle relaxation and to prolong the immobilisation. The supplementary drugs included ketamine (100-200 mg intravenous), guaifenesin (2-10 g intravenous) or propofol (100-300 mg intravenous). The supplementary drugs reportedly resulted in respiratory depression and hypoxaemia (Allen 1994). Carfentanil is currently not registered in South Africa or even available worldwide anymore. Furthermore, Kock et al. (2012) do not advocate the use of carfentanil and fentanyl (also a potent opioid agonist) in zebra due to doubt in their efficacy.

Etorphine based combinations have been used in zebra (Adin et al. 2007; Senthilkumar et al. 2005; Young & Penzhorn. 1972). Senthilkumar et al. (2005) reported an etorphine-acepromazine drug combination administered to a zoo habituated Grant's zebra (*Equus burchelli boehmi*). The dosage of etorphine reported was similar to what Kock et al. (2012) suggests for immobilising wild zebra. In the study by Senthilkumar et al. (2005), 2.45 mg of etorphine was combined with 10 mg acepromazine maleate. This was a slightly lower dosage than suggested by Kock et al. (2012) who describes a combination of 4-6 mg etorphine and 30 mg acepromazine maleate for wild stallions. Furthermore, a rapid and calm recovery was reported after the administration of 3.26 mg diprenorphine. The higher doses reported by Kock et al. (2012) would most likely be superior in wild animals not accustomed to a zoo environment.

Adin et al. (2007) evaluated the cardiovascular effects of an etorphine-detomidine-azaperone combination to immobilise twenty Grevy's zebras (*Equus grevyi*). The animals were immobilised in order to establish their echocardiac structure and function. Immobilisation was achieved using 4.6 +/-0.6 mg etorphine, 15.2 +/-0.7 mg detomidine and 10 mg acepromazine. To improve muscle relaxation and prolong the period of recumbency, a quarter of the animals received an additional 4 mg of detomidine. A further 20% received 100-150 mg ketamine and others received a 5% infusion of guaifenesin. The drug combinations used in this study are similar to those recommended in the literature (Kock et al. 2012; Senthilkumar et al. 2005; Young & Penzhorn 1972).

After the successful etorphine immobilisation of Burchell's zebras (*Equus burchelli*) was documented by Pienaar in 1969, Young & Penzhorn (1972) investigated the physiological effects of etorphine (2 mg) and azaperone (200 mg) immobilised Cape Mountain Zebras (*Equus zebra zebra*). The zebras moved into a recumbent position 5-10 minutes post darting. These times were similar to what was reported by Senthilkumar et al. (2005), where sternal

recumbency was achieved within 3 minutes post darting and lateral recumbency within in 5 minutes using a similar etorphine dose but a lower azaperone dose (10 mg). Despite conservative etorphine doses being effective in zebras, current recommendations suggest a dose range of 4 to 7 mg should be administered in combination with low azaperone doses to attain effective and predictable immobilisation (Adin et al. 2007; Kock et al. 2012).

### *Cardiopulmonary side effects in anaesthetised equids*

Domestic equids are known to be a species with high numbers of peri-anaesthetic fatalities. Mortality rates as high as 0.9% has been reported in healthy horses. Fatalities are most commonly attributed to cardiovascular complications and injuries sustained during recovery. Hypotension, hypoxaemia and acid base derangements are the major risk factors identified in domestic equids. Prolonged durations of anaesthesia have also been directly associated with fatalities (Nyman & Hedenstierna 1989; Young & Tayler 1993; Johnston et al. 2002; Bidwell et al. 2007; Dugdale et al. 2016).

### *Shortfall in the literature*

Few studies exist that are aimed specifically at evaluating the cardiopulmonary effects of the drug combinations used to chemically immobilise zebras. Furthermore, from the five studies found (Adin et al. 2007; Allen 1994; Hoyer et al. 2012; Senthilkumar et al. 2005; Young & Penzhorn. 1972) only one was in wild zebras (Young & Penzhorn 1972), the remaining were performed in zoo-habituated animals. The largest sample size was 20 zebras (Adin et al. 2007) while one study included a case report of only one animal (Senthilkumar et al. 2005). Although the majority of publications describe combinations containing etorphine it is not always readily available and, in some countries, like the United States of America (Allen 1994), etorphine is not commercially available. Furthermore, in countries, like South Africa, that do have commercial preparations of etorphine has experience stock shortages. These reasons as well as the human drug handling safety issues related to potent opioids, such as etorphine, necessitate

investigations for alternative drug combinations that can be used to effectively immobilise zebras. The ideal immobilisation agent should be highly effective, reliable and provide a rapid onset of action. For remote immobilisation (darting) a small volume for injection is required and the onset of action needs to be rapid, ideally under 15 minutes, and antagonists should exist to allow rapid and calm recoveries (Plotka et al. 1987).

Very little work with regards to immobilisation of zebra has been done. It is, however, evident that alternative immobilisation combinations for zebra are needed. Furthermore, information about the cardiopulmonary effects of drug combinations used to immobilise zebras are scant. Conclusive assessments regarding the efficacy and safety of non-potent opioid based drug combinations used for immobilisation of zebras have not been performed and warrants further investigation.

### *Problem statement*

There is limited literature describing chemical immobilisation and its physiological effects in zebras. Several reports involving other wild equid species confirm that chemical immobilisation remains a challenge and despite several proposed drug combinations, opportunity to improve current combinations exists, especially in zebras (Matthews et al. 1995; Walzer et al. 2006; Woolnough et al. 2012).

The use of etorphine in horses paved the way for its use in zebras and other wild equids (Plotka et al. 1987). Subsequently, etorphine in combination with the tranquilizer azaperone, or the sedative detomidine, have been used commonly to chemically immobilise zebras (Kock et al. 2012; Plotka et al. 1987; Senthilkumar et al. 2005; Young & Penzhorn 1972). Also, ketamine has been added to etorphine combinations to improve the quality and reliability of immobilisation (Adin et al. 2007).

Although etorphine drug combinations have been shown to effectively immobilise wild equids, especially zebras, information on how these combinations affect the cardiopulmonary function of zebras is scant and hence their safety is somewhat undetermined. Potent opioids, like etorphine, are not readily available for clinical use in certain countries, and even in countries where they are available, on occasions shortages have occurred. Apart from issues of availability, the use of etorphine is also a major safety risk to veterinarians. Etorphine is highly toxic in humans. Its accidental administration, either by self-injection, splashing on mucous membranes or absorption through broken skin, can lead to several serious clinical conditions. Dizziness, nausea, severe respiratory depression, hypotension and cyanosis are a few of the effects of this drug in humans. If not treated immediately and appropriately loss of conscious and cardiac arrest can occur (Kock et al. 2012).

Additionally, of the potent opioids available, only etorphine successfully immobilise zebras, whereas thiafentanil, fentanyl and carfentanil appear to only be effective at excessively high doses. Also, etorphine drug combinations are known to cause acidosis, hypercapnia and hypoxaemia, which highlights their potential danger when they are used to immobilise animals (Zeiler & Meyer 2017). Therefore, the need to establish effective and safe alternative non-potent opioid immobilising drug combinations for zebras, and other wild equids, is a necessity.

## Aims and objectives

Due to the frequency at which zebra are chemically immobilised, safety concerns and potential difficulty in acquiring etorphine in sufficient quantities necessitated the primary aim of our study, which was to search for an alternative non-potent opioid drug combination that can be used to safely and effectively immobilise zebras. Additionally, the lack of scientific information pertaining to the cardiopulmonary effects of the most commonly used drug combination in zebra also warranted further investigation.

The aims of this study were accomplished by fulfilling the following research objectives:

1. Investigate a non-potent opioid drug combination and compare its efficacy to immobilise zebras to an established drug combination. The combinations tested were ketamine-butorphanol-medetomidine (KBM) and etorphine-azaperone (EA). Efficacy was established by 1) determining the times to recumbency and quality of induction into recumbency, and 2) if a recumbent state of immobilisation could be maintained for the duration of the study procedures, and 3) if the recovery, after administering antagonists, was rapid and calm.
2. Establish and compare respiratory and cardiovascular parameters to determine the cardiopulmonary safety of the two different immobilising drug combinations by comparing the following:
    a) Heart rate by auscultation
    b) Respiration rate by auscultation and observation
    c) Blood pressure by invasive arterial blood pressure monitoring
    d) Arterial blood gas analysis

The objective of the comparison between the two combinations was to determine if either combination offered greater benefits in terms of immobilisation efficacy and safety.

## Hypotheses

Two hypotheses were proposed for this study as follows:

1. H0: The quality and times of induction into recumbency, immobilisation and recovery will not be different in zebras immobilised with ketamine-butorphanol-medetomidine compared to etorphine-azaperone.

    H1: The quality and times of induction into recumbency, immobilisation and recovery will be significantly different in zebra immobilised with ketamine-butorphanol-medetomidine compared to etorphine-azaperone.

2. H0: The cardiopulmonary effects of ketamine-butorphanol-medetomidine in zebra will not be different to that of etorphine-azaperone.

    H1: The cardiopulmonary effects of ketamine-butorphanol-medetomidine in zebra will be significantly different to etorphine-azaperone

# Materials and Methods

## Pilot study

Prior to commencement of experimental procedures, a pilot study was completed to investigate the efficacy of novel non-opioid drug combinations to immobilise zebras (protocol V095-16). The aim of the pilot study was to determine the effectiveness of two non-opioid drug combinations to achieve a calm induction into recumbency and immobilisation in zebras. The zebras used in this pilot study were scheduled to be translocated to another farm and therefore had to be immobilised to load them into a purpose-built transport trailer.

Both these immobilisation combinations comprised of the same dissociative anaesthetic agent, ketamine, as well as medetomidine to provide sedation, analgesia and muscle relaxation. The two proposed non-opioid drug combinations were:

1. KAM: ketamine (3 mg $kg^{-1}$; Ketonil 200 mg $ml^{-1}$; Wildlife Pharmaceuticals: South Africa), azaperone (0.2 mg $kg^{-1}$ Zapnil 100 mg $ml^{-1}$; Wildlife Pharmaceuticals; South Africa), medetomidine (0.03 mg $kg^{-1}$; Metonil 40 mg $ml^{-1}$; Wildlife Pharmaceuticals; South Africa)

2. KMM: ketamine (3mg $kg^{-1}$; Ketonil 200 mg $ml^{-1}$), midazolam (0.05mg $kg^{-1}$, Midazonil 50 mg $ml^{-1}$; Wildlife Pharmaceuticals; South Africa), medetomidine (0.03mg $kg^{-1}$; Metonil 40 mg $ml^{-1}$)

These two combinations were administered randomly to immobilise a total of three zebras per combination. The zebras were free-ranging on a small farm and habituated to a vehicle and human presence.

Both combinations were unsuccessful at inducing recumbency, even after as long as 30 minutes after dart delivery. Combination KMM was, however, able to induce standing sedation 12-15 minutes post dart delivery. Sedation was characterised by a quiet and calm zebra, showing moderate ataxia and decreased responses to stimuli and manipulation. These zebras

were sedated adequately to allow approach and an intravenous hand-injection of ketamine (300 mg) to induce recumbency.

The zebras darted with KAM only showed signs of sedation such as a pronounced head drop, mild ataxia and isolating themselves from the dazzle. They, however, never reached adequate sedation to allow approach and hand-injection and instead remained skittish and ambulatory. All zebras that could not be approached to induce recumbency were darted again to administer 4 mg of etorphine to induce recumbency.

Following loading into the transport trailer, the medetomidine was antagonised using atipamezole at 5 mg $mg^{-1}$ medetomidine (0.6 mg $kg^{-1}$; Atipamezole 50 mg $ml^{-1}$; Vtech; South Africa) IM. Etorphine was antagonised using naltrexone at 20 mg $mg^{-1}$ etorphine (0.3 mg $kg^{-1}$; Trexonil 10mg $ml^{-1}$; Wildlife Pharmaceuticals) IV. All zebras recovered completely but slowly, taking longer than 20 minutes in some individuals.

From the results of this pilot study it was speculated that KMM could induce recumbency in zebras if the medetomidine dose was increased considerably. The studied dose of the antagonist atipamezole suggested that there was a possible risk of renarcotisation due to the longer than expected recovery. The medetomidine dose was adjusted and the midazolam, which did not appear to contribute meaningfully to the immobilisation, was substituted with butorphanol.

From the outcome of the pilot study, the drug combination KBM was tested. This combination, ketamine (2.5 mg $kg^{-1}$; Ketonil 200 mg $ml^{-1}$; Wildlife Pharmaceuticals: South Africa), butorphanol (0.3 mg $kg^{-1}$; Butonil 50 mg $ml^{-1}$; Wildlife Pharmaceuticals; South Africa), medetomidine (0.12 mg $kg^{-1}$; Metonil 40 mg $ml^{-1}$; Wildlife Pharmaceuticals; South Africa), was administered to a single adult zebra. The zebra successfully went into lateral recumbency without the need to administer intravenous ketamine. Recumbency was achieved

8 minutes after dart delivery, which was comparable to the traditional etorphine-azaperone combination. The animal remained recumbent for 55 minutes, although a single bolus of 200 mg ketamine was administered IV to facilitate loading into the transport trailer. After loading, the medetomidine was antagonised using atipamezole at 7.5 mg $mg^{-1}$ medetomidine (0.9 mg $kg^{-1}$; Atipamezole 50 mg $ml^{-1}$; Vtech; South Africa) IM and the butorphanol was antagonised using naltrexone at 2 mg $mg^{-1}$ butorphanol (0.6 mg $kg^{-1}$; Trexonil 50 mg $ml^{-1}$; Wildlife Pharmaceuticals) IV. Time to standing was under 10 minutes and the transition was calm and coordinated.

The successful immobilisation and induction into recumbency with the combination KBM suggested that a drug combination incorporating butorphanol, a non-potent opioid, may be viable, unlike the non-opioid drug combinations we initially used. This KBM combination was selected as the alternative drug combination and compared to the standard etorphine-azaperone (EA) combination which is most commonly used.

## Main study

The study was approved by the Animal Ethics Committees of the University (Protocol V071-17) and the South African National Parks (#003/17). The study took place from June to July 2017 and was conducted at the South African National Parks (SANParks), Veterinary Wildlife Services facilities Kimberly, South Africa (28º46'22.6" S, 24º44'59.3" E; 1400 meters above sea level).

### *Experimental design*

A prospective, randomised crossover trial was performed.

### *Animals, housing and study design*

Five family groups, comprising of four to six free-ranging zebras (*Equus zebra*), were mass-captured from the Karoo National Park by SANParks as part of a sell-off strategy for population control management. The animals were then transported to the SANParks boma facilities in Kimberly where they were confined in a boma (temporary enclosure that served as a holding pen, constructed from 2.8-meter-high wooden pole walls) for a period of 30 days (habituation period) before commencement of the experimental trials (Image 1). The boma facility consisted of 5 individual adjoining pens, that housed a family group each. The pen consisted of two compartments separated by a metal sliding gate: 1) an outdoor day section (8 x 8 meters, no roof) and 2) an indoor night room (8 x 4 meters, roofed). A series of elevated walkways spanned above the outdoor section and was used for access to the zebras during daily observation and darting procedures. The zebras received Lucerne

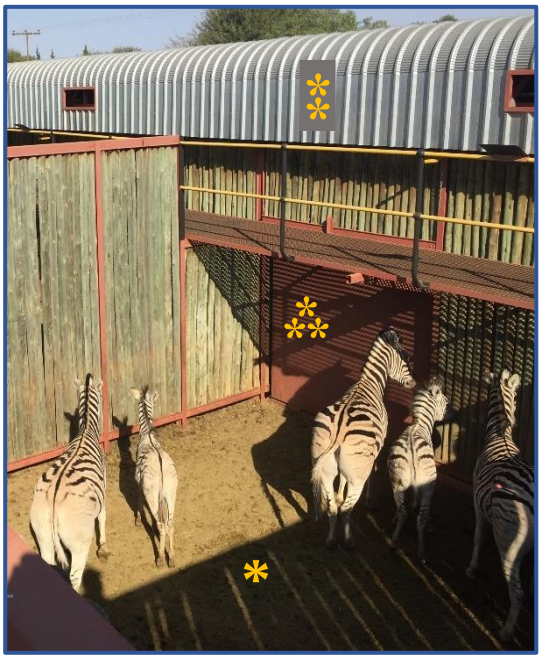

**Image 1** The high-walled enclosure (boma) showing **(⁎)** the outdoor day section and **(⁑)** indoor night room (roofed). **(⁂)** Indicates sliding metal gate separating the day and night sections.

(*Medicago sativa*) and hay (*Eragrostis curvula*) to eat and potable water to drink *ad libitum*, throughout the study period. The pens of the boma were cleaned on a daily basis by shepherding the zebras into the night room and confining them while cleaning the outdoor area and *vice versa* for the cleaning of the indoor night room.

Sample size was estimated by assuming a 3-minute difference between the induction times of the two combinations with a confidence interval at a 95%, normal distribution and an estimated standard deviation of two. Therefore, ten adult zebras (six females; four males) were enrolled in the randomised (ten zebras, two treatments, one block design; www.randomization.com) crossover study. The zebras were sold to a private buyer at the completion of the data collection period of the study.

### *Immobilisation drug combinations*

The two immobilisation combinations that were compared included:

- Combination KBM - ketamine (2.5 mg $kg^{-1}$; Ketonil 200 mg $ml^{-1}$; Wildlife Pharmaceuticals: South Africa), butorphanol (0.3 mg $kg^{-1}$; Butonil 50 mg $ml^{-1}$; Wildlife Pharmaceuticals; South Africa), medetomidine (0.12 mg $kg^{-1}$; Metonil 40 mg $ml^{-1}$; Wildlife Pharmaceuticals; South Africa)
- Combination EA - etorphine (0.015 mg $kg^{-1}$; Captivon 10 mg $ml^{-1}$; Wildlife Pharmaceuticals; South Africa), azaperone (0.17 mg $kg^{-1}$; Zapnil 100 mg $ml^{-1}$; Wildlife Pharmaceuticals; South Africa).

Total drug doses were standardised for a mature stallion zebra weighing 400 kg. The drugs of each combination were drawn up into separate syringes and then injected sequentially into a single dart via a 20-gauge 75 mm spinal needle. Combination KBM was administered using a 7-cc dart (Type U, 13-gauge, 38 mm wire barbed needle, Pneu-Dart, Williamsport, Pennsylvania, USA) and combination EA was administered using a 1-cc dart (Type P, 13-

gauge, 38 mm wire barbed needle, Pneu-Dart, Williamsport, Pennsylvania, USA). Both drug combinations filled the dart to capacity. The zebras were immobilised twice using each combination once with a three-week washout period between immobilisations.

### *Darting and experimental procedures*

All family groups were confined to their indoor night rooms on the data collection days. One family group was released into their outdoor area at a time, prior to darting. The experimental procedures comprised of three phases, as follows: induction (time from darting to recumbency without attempts at standing), immobilisation (recumbency to antagonist administration) and recovery (antagonist administration to standing).

#### *Induction phase*

The zebras were darted over a distance of 3 to 5 meters, into the gluteus muscle group, using a carbon dioxide powered rifle (Dan-Inject $CO_2$ Rifle, model J.M.ST.R., Børkop, Denmark). All darting was performed by the SANParks veterinarians.

Once the dart was placed a stop watch was started to measure the time to recumbency (time from dart placement until being recumbent without attempts to stand). Zebra were observed by the primary investigator and a subjective simple descriptive scale (SDS) was used to score the quality of induction (Table 1).

Once the zebra was sedated enough from the drugs and away from the metal sliding door then the remaining zebras of the family group were shepherded into the night room and confined. If the zebra did not move into sternal or lateral recumbency at 15 minutes, they were either re-darted or approached (if heavily sedated) and a single 300 mg ketamine bolus was hand injected intravenously to induce recumbency.

**Table 1** Simple descriptive scoring system used to categorise the quality of induction into recumbency in boma habituated zebras immobilised using ketamine-butorphanol-medetomidine and etorphine-azaperone by a remote dart delivery system.

| Score | Description | Classifier |
|---|---|---|
| 1 | Slight ataxia observed followed by < 2 attempts to move into recumbency. No signs of CNS excitation. No falling or stumbling. Calm transition from sternal to lateral recumbency. Rapid time to reach sternal recumbency (≤ 4 minutes) and lateral recumbency (≤ 5 minutes). | Excellent |
| 2 | Moderate ataxia observed with > 2 attempts required to move into recumbency. Minimal signs of CNS excitation. Moderate stumbling. Moderate time to reach sternal recumbency (> 4 minutes) and lateral recumbency (> 5 minutes). | Good |
| 3 | Severe ataxia with numerous attempts to move into recumbency. Moderate signs of CNS excitation. Severe stumbling and falling. Moderate risk of injury. Prolonged time to reach sternal (> 8 minutes) and lateral recumbency (> 10 minutes). Zebra may have required re-darting or a hand bolus of ketamine to achieve reliable lateral recumbency. | Fair |
| 4 | Severe ataxia without zebra becoming recumbent. Moderate to severe CNS excitation. Repeated stumbling and falling. High risk of injury or a prolonged time to reach sternal recumbency (> 15 minutes) with lateral recumbency never achieved. Zebra required re-darting or a hand bolus of ketamine to achieve reliable lateral recumbency. | Poor |

Once recumbent the zebra was approached, it was blind folded and cotton wool was inserted into the ears to minimise external sensory stimuli. The zebra was then placed, in lateral recumbency, onto a custom-built metal stretcher and within 5 minutes was instrumented (Image 2).

**Image 2** Zebra placed, in lateral recumbency, onto a custom-built metal stretcher and instrumented.

*Immobilisation phase*

Physiological parameters were monitored continuously, with the exception of temperature, and recorded at 5, 10 and 15 minutes after becoming recumbent.

**Image 3** Cannulated lateral metatarsal artery connected to patient-side pressure monitor.

The lateral metatarsal artery, of the non-dependent pelvic limb, was aseptically cannulated (20-gauge, 25 mm) to allow for continuous measurement of the blood pressure and intermittent blood sampling for gas analysis (Image 3). Invasive systolic (SAP), diastolic (DAP) and mean (MAP) arterial blood pressure and pulse rate were measured using a patient-side pressure monitor (IntraTorr, IntraVitals, United Kingdom) that was connected to an electronic strain-gauge transducer (zeroed to atmospheric air pressure at the level of the right atrium; Deltran II disposable transducer; Utah medical products incorporated; USA).

Arterial blood samples (1 ml each) were collected anaerobically via the arterial cannula into sodium heparinised syringes at 5, 10 and 15 minutes. Once collected, the syringe was sealed using a rubber stopper to maintain an anaerobic environment and placed in a water-tight plastic bag and placed on ice inside a closable container. Once all three samples were collected, they were transported to a laboratory and analysed immediately (within 10 minutes of collecting the final sample) using a daily calibrated bench-top analyser (RapidPoint 500, Siemens, South Africa). Peripheral oxygen haemoglobin saturation ($SpO_2$) was measured by placing a reflectance pulse oximeter probe (Veterinary Pulse Oximeter, Model 9847V; Nonin Medical, USA) onto the mucosa of the cheek (Image 4A). A multi-parameter monitor (SurgVet Advisor V9201, Smiths Medical, USA) was used to measure the respiration rate (RR), end-tidal carbon dioxide ($PE´CO_2$) and non-invasive blood pressure (mmHg) as follows: 1. for RR and $PE´CO_2$

a shortened 10 mm internal diameter cuffed endotracheal tube (machine-end at level of nares Image 4A) was lubricated and placed in the ventral meatus and connected to a side-stream sampling connector and standard sampling line (sampling rate: 200 ml $min^{-1}$); 2. the non-invasive blood pressure cuff (cuff width approximately 40% the circumference of the tail root) was placed at the base of the tail (Image 4 B).

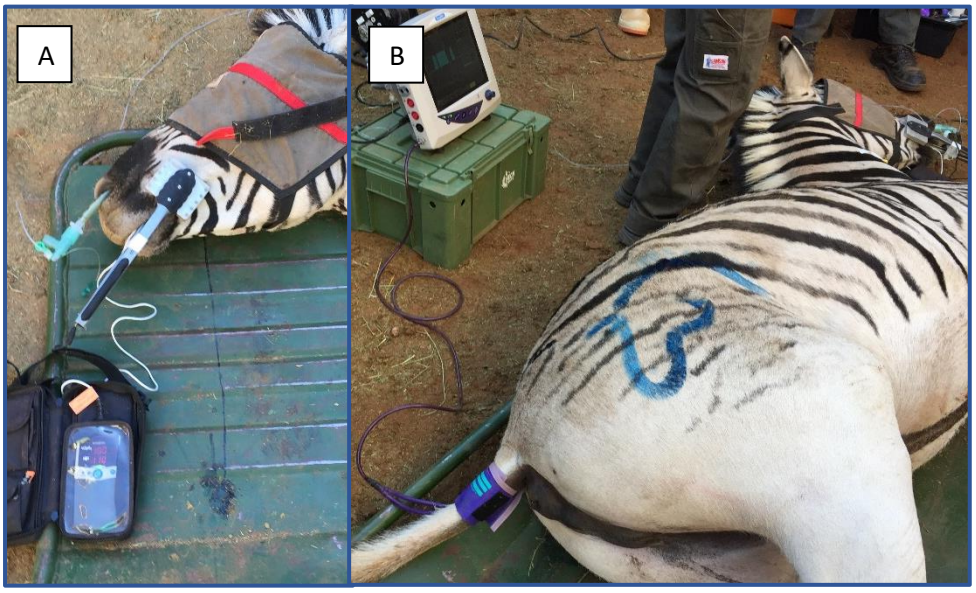


**Image 4 A)** Reflectance pulse oximeter probe placed onto the mucosa of the cheek and a shortened cuffed endotracheal tube placed in the ventral meatus. **B)** Multi-parameter monitor, connected to the non-invasive blood pressure cuff at the base of the tail of the zebra.

Rectal temperature was measured at 5, 10 and 15 minutes, using a quick-responding digital thermometer (HI98509 Checktemp 1; Hanna Instruments, USA). The non-dependent jugular vein was aseptically cannulated (18-gauge, 28 mm) to establish venous access for the administration of any interventional (emergency or ketamine) drugs.

The quality of the immobilisation was scored according to a SDS (Table 2) by the primary investigator at the end of the data collection period (16 minutes). Pain stimulation and response was also evaluated during the mildly painful procedure of transcutaneous cannulation of the blood vessels. Immobilisation quality and response to pain was further evaluated and scored objectively by using an electronic algometer (ProdPro; 3 mm tip rigid probe; Topcat Metrology; UK). This instrument was applied along the coronary band to observe for limb withdrawal reflexes. Increasing application pressure was applied (rate of application guided by

green and red light emitting diodes to apply 2.0 N second$^{-1}$) until a withdrawal response was elicited or the cut-off force of 30 Newton Force was applied.

**Table 2** Simple descriptive scoring system used to categorise the quality of immobilisation in boma habituated zebras immobilised using ketamine-butorphanol-medetomidine and etorphine-azaperone by a remote dart delivery system.

| Score | Description | Classifier |
|---|---|---|
| 1 | Light plane of immobilisation achieved with repeated attempts to stand. Zebra poses a risk to inflict self-induced injury or injury to ground crew. Or, no anaesthetic plane or state of immobilisation achieved. | Limited effect |
| 2 | Zebra remains recumbent without attempts to stand. Continual muscle tremors or rigidity and spontaneous muscle activity may be observed. Mild autonomic and limb withdrawal response to arterial cannulation. Mild limb withdrawal response to an algometer. Palpebral and anal reflex intact and strong. | Deep sedation |
| 3 | Zebra remains recumbent without attempts to stand. Reduced muscle rigidity to complete relaxation. No autonomic and limb withdrawal response to arterial cannulation. No limb withdrawal response to algometer. Palpebral and anal reflex intact, but sluggish. *Moderate level - no involuntary tail movements and tongue easily extracted* . | Light to moderate anaesthetic level |
| 4 | Zebra remains recumbent without attempts to stand. All characteristics of 3 present. Palpebral and anal reflex intact but very sluggish to sometimes absent. | Surgical anaesthesia level |
| 5 | Zebra remains recumbent without attempts to stand. Anaesthetic plane is too deep, all reflexes absent with evidence of life-threatening cardiopulmonary depression. | Excessive level of anaesthesia |

Thereafter, (20 minutes after becoming recumbent) all monitoring equipment was disconnected from the zebra, the dart was removed and the wound treated with a dry cow, intra-mammary anti-microbial preparation (Curaclox DC; Cloxacillin 600 mg, Ampicillin 300 mg; Norbrook Laboratories; South Africa). The zebras were inspected for signs of injury that could have been sustained during the immobilisation. A single vitamin and mineral booster treatment

(20 ml; Kyroligo; Kyron Laboratories; South Africa) was administered intramuscularly in the gluteus muscle (contralateral to the gluteus muscle which was darted).

Lastly, the zebra was weighed by hoisting the metal stretcher using a truck crane and heavy-duty crane scale (Image 5; Crane Scale, max weight 5000 kg; Punit Instruments, India). The weight of the zebra was calculated by subtracting the weight of the metal stretcher and hoisting chains from the total weight recoded.

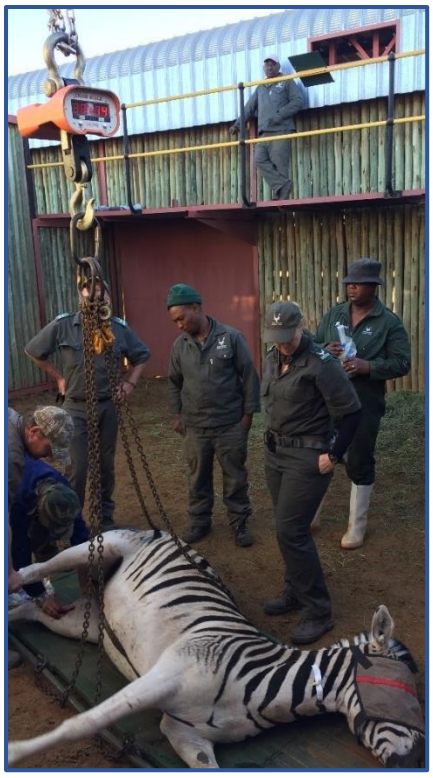

**Image 5** Weighing zebra by hoisting the metal stretcher using a truck crane and heavy-duty crane scale.

*Recovery phase*

After weighing, the zebra was placed in lateral recumbency on the ground and the pen was cleared of all equipment. Then antagonist drugs were administered as follows:

- KBM: medetomidine was antagonised using atipamezole at 7.5 mg $mg^{-1}$ medetomidine (0.9 mg $kg^{-1}$; Atipamezole 50 mg $ml^{-1}$; Vtech; South Africa), half the dosage was administered intramuscularly and the other half intravenously (via jugular cannula). Then butorphanol was antagonised using naltrexone at 2 mg $mg^{-1}$ butorphanol (0.6 mg $kg^{-1}$; Trexonil 50 mg $ml^{-1}$; Wildlife Pharmaceuticals) administered intravenously, via the jugular cannula.
- EA: etorphine was antagonised using naltrexone at 20 mg $mg^{-1}$ etorphine (0.3 mg $kg^{-1}$; Trexonil 10mg $ml^{-1}$; Wildlife Pharmaceuticals) administered intravenously, via the jugular cannula.

A stopwatch was started immediately after administering the antagonist drugs to record the time to standing. The jugular cannula was removed. The quality of recovery was scored

subjectively by the principal investigator using a SDS (Table 3). Once the zebra was standing, without ataxia, it was allowed to re-join its family group.

**Table 3** Simple descriptive scoring system used to categorise the quality of recovery in boma habituated zebras immobilised using ketamine-butorphanol-medetomidine and etorphine-azaperone by a remote dart delivery system.

| Score | Description | Classifier |
|---|---|---|
| 1 | Transition from lateral to sternal occurs with minimal ataxic movements. Stands within 1 to 2 attempts, which are calm and coordinated. Slight ataxia observed during initial ambulation attempt. Recumbency to standing in < 10 minutes post administration of the antagonists. | Excellent |
| 2 | Transition from lateral to sternal occurs with moderate ataxic movements. Standing requires > 2 attempts, which are relatively coordinated. Moderate ataxia observed during initial ambulation attempt. Recumbency to standing in 10-15 minutes post administration of the antagonists. | Good |
| 3 | Frequent and uncoordinated attempts to move from lateral to sternal. Numerous erratic attempts required to stand. Stumbling and falling common. Markedly ataxic gait at initial ambulation attempt. Recumbency to standing in > 20 minutes post administration of the antagonists. | Fair |
| 4 | Zebra remains recumbent for > 30 minutes post administration of the antagonists. No response to stimuli and no attempts to rise. Clinical examination is required and repeat dosing of the antagonists is considered. | Poor |

### *Rescue interventions*

The health and safety of the zebra was of utmost importance and was closely monitored throughout the study. The following potential risks and rescue interventions were identified prior to experimental procedures and were followed in situations where it was deemed necessary to intervene.

#### *1. Failure to immobilise or early recovery*

In the event of a zebra being too lightly immobilised (scoring a 3 in the induction quality score - fair quality) but has clear signs of drug effect and could be approached, then a dose of ketamine (300 mg) was administered intravenously. If the zebra moved into sternal

recumbency or lateral recumbency but demonstrates early attempts to stand prior to the 20-minute data collection point, additional boluses of ketamine (100 mg) were administered intravenously. If the darted zebra scores a 4 in the induction quality score (poor quality) and did not reach a standing sedation, then it was re-darted to administer etorphine (2 to 4 mg) and no further data will be collected from that zebra.

***2. Apnoea***

In the event of a zebra experienced any signs of apnoea (no visible inspiratory effort in 60 seconds) then the etorphine, medetomidine and butorphanol, depending on the combination used, was reversed immediately by administering the appropriate antagonist drug.

***3. Cardiovascular collapse***

In the event of a zebra experienced signs of cardiac arrest (pulseless and apnoea), adrenaline was administered intravenously at a dosage of 0.01 mg $kg^{-1}$, via the jugular cannula, at 3-minute intervals and chest compressions were commence where one of the investigators jumped up-and-down on the highest point of the chest. Also, the antagonist drugs were administered intravenously. If there was no evidence of return of spontaneous circulation within 10 minutes, then all resuscitation efforts were stopped and the zebra was pronounced dead.

### *Data analysis and statistics*

Data from zebras that did not achieve recumbency after the first dart and required intervention (re-darting or a ketamine bolus) were excluded from analysis. Data were assessed for normality by evaluating descriptive statistics, plotting of histograms and performing the Anderson-Darling test for normality. Quantitative serial data with normal distribution (HR, RR, SAP, MAP, DAP, Temp, $SpO_2$, $PE´CO_2$, $PaCO_2$, $PaO_2$, pH, $HCO_3^-$, BE) were compared between the combinations using a general linear mixed model (fixed factors: time, combination; random factor: zebra). Body weights were compared between the two data collection periods using a

two sample T-Test. Times to recumbency and standing were compared between combinations using a Mann-Whitney test. Subjective quality scores were compared between combinations using a Friedman test (response: quality score; treatment: combination; block: zebra). Data were reported as mean (range), unless otherwise stated. Data were analysed using commercially available software (MiniTab 17.1.0; MiniTab Incorporated) and results interpreted at the 5% level of significance ($p < 0.05$). Finally, a total cost of drugs administered to the zebras undergoing KBM and EA immobilisation were calculated to determine if there was a financial difference between the two combinations.

# Results

Of the ten zebras used in the study only nine successfully completed both trails. One of the female zebras died six days after the first immobilisation trial (KBM was used). The post-mortem examination revealed that the zebra was cachexic, likely as a result of maladaptation and chronic stress. A further two zebras immobilised using KBM were excluded from the data analysis because they required an intravenous dose of ketamine to induce recumbency. Therefore, data from seven and nine zebras immobilised using KBM and EA, respectively, were analysed. The weight of the zebras was 300 (278 to 328) kg in the first week and was no different [270 (280 to 312) kg] three weeks later.

For KBM, the final doses of ketamine, butorphanol and medetomidine were 3.30 (3.04 to 3.57), 0.40 (0.38 to 0.42) and 0.16 (0.15 to 0.17) mg $kg^{-1}$, respectively. For EA, the final dose of etorphine and azaperone were 0.02 (0.02) and 0.20 (0.18 to 0.22) mg $kg^{-1}$, respectively. The median (range) time to recumbency for zebras immobilised using KBM was 420 (300 to 600) seconds and was significantly slower [240 (180 to 300) seconds] than EA ($P = 0.0343$). The median (range) induction into recumbency score for zebras was 2 (KBM 1 to 3; EA 2 to 2), regardless of combination used. How the animals moved into recumbency differed between the combinations. The KBM immobilised zebras initially stood stationary and swayed, and just before moving into recumbency they tended to stumble about while trying to stay on their feet before eventually moving tripping or falling into recumbency. Whereas the EA immobilised zebras tended to move in a typical hackney gait, often pushing themselves into the pen wall before eventually losing their balance and falling into recumbency (Figure 6A). All zebras appeared to strongly resist the transition from standing to recumbency and made every effort to maintain a standing position.

During the immobilisation phase, zebras maintained recumbency without attempts to stand until the antagonists were administered, regardless of combination used. The median (range)

immobilisation score for zebras immobilised using KBM was 3 (3 to 3) and 2 (2 to 3) when using EA. Furthermore, KBM provided good muscle relaxation in all zebras unlike EA where frequent marked muscle rigidity and tremors were observed in most zebras (Image 6B). Also, spontaneous motor activity, such as involuntary tail and limb movements was frequently noted when zebra were immobilised with EA. Palpebral and anal reflexes remained intact with both combinations, however, the palpebral reflex was more sluggish when immobilised with KBM compared to EA. A mild limb withdrawal response to the algometer application were elicited in 1 and 3 zebra immobilised with KBM and EA, respectively.

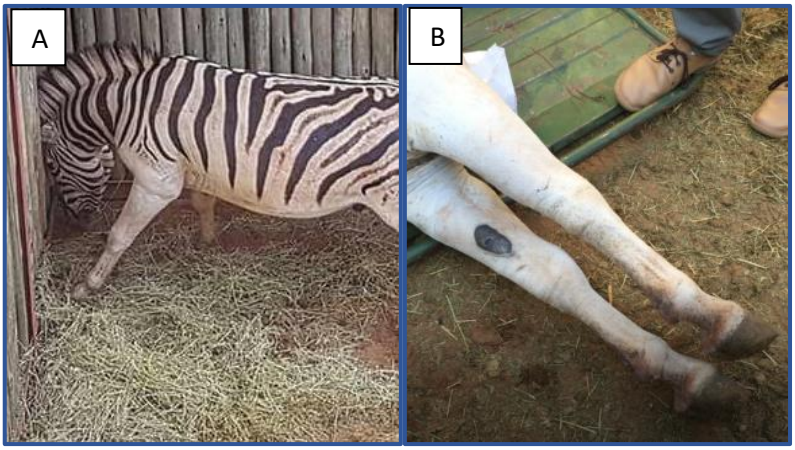


**Image 6 A)** Typical hackney gait, zebra observed pushing themselves into the pen wall before eventually losing their balance and moving into recumbency. **B)** Marked muscle rigidity and tremors were observed in zebra that received combination EA.

The pulse rate of zebra associated with KBM were 45 (32 to 56) beats minute$^{-1}$ which was significantly slower [68 (48 to 104) beats minute$^{-1}$] compared to pulse rates associated with EA ($P < 0.001$). The arterial blood pressures were profoundly elevated in all zebras (Table 4 & Figure 1). The systolic arterial blood pressures of the KBM immobilised zebras was 225 (136 to 286) mmHg, which was significantly greater [167 (93 to 268) mmHg] compared to using EA ($P < 0.001$). The respiratory rates were 17 (8 to 32) breaths minute$^{-1}$ in zebras immobilised using KBM, which was significantly faster than [9 (6 to 16) breaths minute$^{-1}$] using EA ($P < 0.001$). Unexpectedly, arterial blood gas analysis revealed normocapnia but hypoxaemia, and a normal blood pH in zebra when immobilised with either combination. However, zebras were

significantly less hypoxaemic [$PaO_2$: 64 (46 to 90) mmHg] when the KBM combination was used compared to EA [$PaO_2$: 47 (20 to 85) mmHg] ($P < 0.001$; Figure 2).

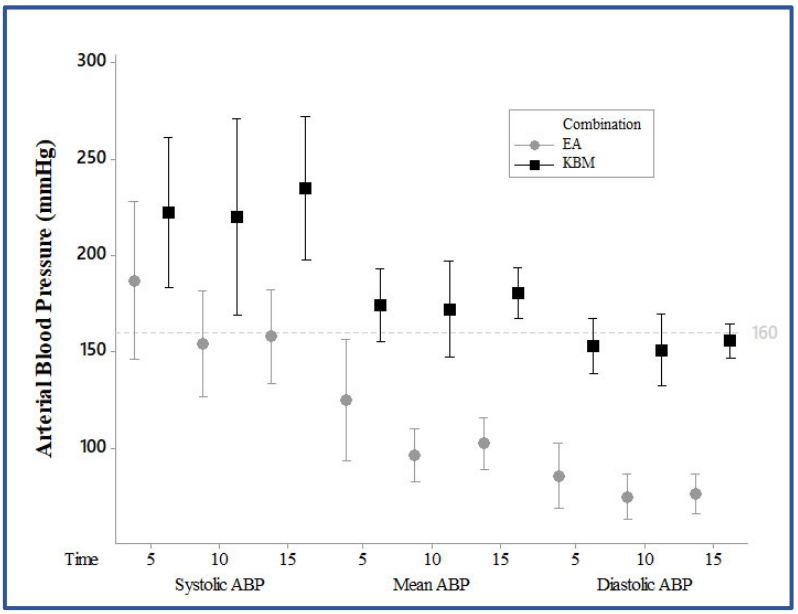


**Figure 1** Systolic, mean and diastolic arterial blood pressure over time in ketamine-butorphanol-medetomidine (KBM) and etorphine-azaperone (EA) immobilised zebras. The plot represents the mean and 95% confidence interval. Systemic hypertension is defined as a systolic arterial blood pressure greater than 160 mmHg, as represented by the reference line.

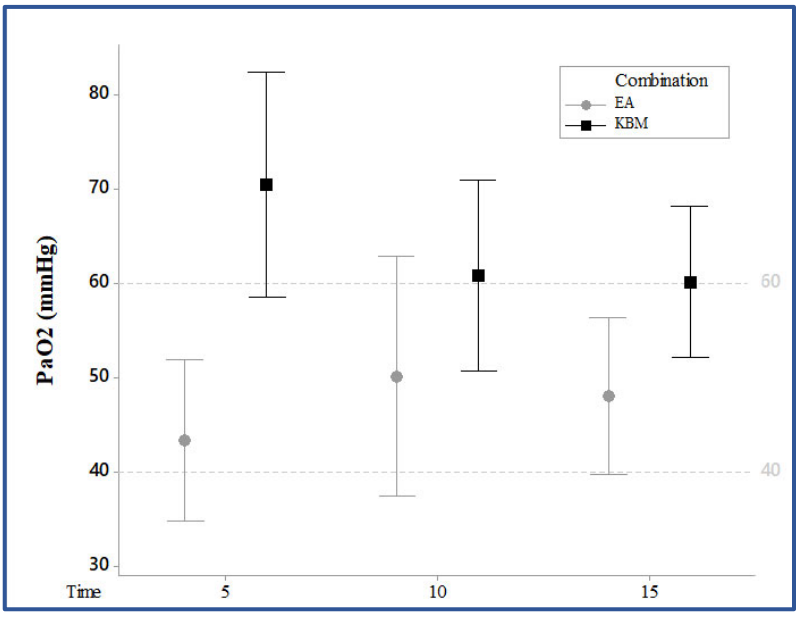


**Figure 2** Partial pressure of oxygen ($PaO_2$) over time in ketamine-butorphanol-medetomidine (KBM) and etorphine-azaperone (EA) immobilised zebras. The plot represents the mean and 95% confidence interval. Moderate hypoxaemia is defined as a $PaO_2$ between 40 and 60 mmHg, as represented by the reference lines

**Table 4** Physiological parameters of ketamine-butorphanol-medetomidine (KBM) and etorphine-azaperone (EA) immobilised zebras (*Equus zebra*) over 15 minutes. Parameters are reported as mean (range) at 5-minute intervals.

| Variable | Unit | Combination | 5 minutes | | 10 minutes | | 15 minutes | | p-value | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Mean | Range | Mean | Range | Mean | Range | between treatments | over time |
| Pulse rate | (beats min$^{-1}$) | KBM | 48 | (40-48) | 48 | (44-51) | 44 | (40-50) | <0.001 | 0.917 |
| | | EA | 67 | (56-81) | 69 | (52-83) | 70 | (54-79) | | |
| Respiration rate | (breaths min$^{-1}$) | KBM | 18 | (16-24) | 12 | (10-28) | 15 | (12-20) | <0.001 | 0.405 |
| | | EA | 8 | (7-11) | 8 | (7-9) | 8 | (7-11) | | |
| SAP | (mmHg) | KBM | 222 | (180-260) | 220 | (136-286) | 235 | (187-274) | <0.001 | 0.534 |
| | | EA | 187 | (93-268) | 154 | (94-227) | 158 | (93-195) | | |
| MAP | (mmHg) | KBM | 176 | (157-188) | 173 | (167-190) | 181 | (169-190) | <0.001 | 0.264 |
| | | EA | 120 | (99-158) | 93 | (83-105) | 105 | (86-115) | | |
| DAP | (mmHg) | KBM | 153 | (136-174) | 151 | (109-172) | 156 | (148-170) | <0.001 | 0.562 |
| | | EA | 86 | (40-112) | 75 | (49-100) | 77 | (55-97) | | |
| $PE'CO_2$ | (mmHg) | KBM | 41 | (37-41) | 32 | (31-45) | 38 | (32-48) | 0.023 | 0.794 |
| | | EA | 44 | (35-49) | 48 | (35-52) | 52 | (33-53) | | |
| pH | - | KBM | 7.42 | (7.41-7.42) | 7.41 | (7.39-7.44) | 7.43 | (7.40-7.45) | 0.001 | 0.375 |
| | | EA | 7.33 | (7.30-7.34) | 7.35 | (7.30-7.38) | 7.35 | (7.30-7.41) | | |
| $HCO_3$ | (mmol $L^{-1}$) | KBM | 20.2 | (18.5-25.3) | 21.3 | (18.2-24.3) | 19.7 | (18.1-23.9) | 0.057 | 0.972 |
| | | EA | 23.9 | (21.1-24.6) | 23.6 | (21.0-24.6) | 25.0 | (23.1-25.4) | | |
| $PaO_2$ | (mmHg) | KBM | 71 | (58-81) | 62 | (50-68) | 60 | (53-70) | <0.001 | 0.816 |
| | | EA | 47 | (36-49) | 50 | (43-54) | 49 | (43-56) | | |
| | (kPa) | KBM | 9 | (8-11) | 8 | (7-9) | 8 | (7-9) | | |
| | | EA | 6 | (5-7) | 7 | (6-7) | 7 | (6-7) | | |
| $PaCO_2$ | (mmHg) | KBM | 38 | (34-39) | 35 | (33-41) | 38 | (32-44) | 0.005 | 0.868 |
| | | EA | 42 | (39-47) | 42 | (39-48) | 38 | (35-45) | | |
| | (kPa) | KBM | 5 | (4-5) | 5 | (4-6) | 5 | (4-5) | | |
| | | EA | 6 | (5-6) | 6 | (5-6) | 5 | (5-6) | | |
| BE | (mmol L$^{-1}$) | KBM | -1.0 | (-4.7-0.4) | -1.8 | (-4-0.3) | 0.6 | (-2.3-1.2) | 0.001 | 0.952 |
| | | EA | -3.9 | (-7.5-2.0) | -4.0 | (-8.0-1.15) | -5.3 | ( -8.6- -0.45) | | |

SAP- systolic arterial pressure, MAP-mean arterial pressure, DAP- diastolic arterial pressure, $PE'CO_2$- end tidal carbon dioxide, $HCO_3$- bicarbonate, $PaO_2$- partial pressure of oxygen, $PaCO_2$- partial pressure of carbon dioxide, BE- base excess

During the recovery phase, the time to standing [median (range)] in zebras recovering from the KBM immobilisation [90 (18 to 2 220) seconds] was significantly longer in duration compared to EA [26 (7 to 72) seconds] (P = 0.0262). However, despite the difference in times to standing, the recovery score [median (range)] of the zebras immobilised using KBM [3 (1 to 4)] was no different to EA [1 (1 to 1)]. Often the standing zebras would tremble and be mildly ataxic. Three of the zebras recovering from KBM immobilisation rolled about during their initial attempts to move into sternal recumbency.

The total current costs to immobilise and antagonise a zebra (400 kg) using KBM was six-and-a-half times more expensive compared to using EA (Table 5).

**Table 5** Drug and dart costs associated with KBM and EA combinations for immobilisation and reversal, as calculated for a single 400 kg zebra stallion (*Equus zebra*), using drug preparations which are currently commercially available in South Africa.

| **KBM** | | **EA** | |
|---|---|---|---|
| | **Immoblisation drugs** | | |
| Ketamine | R 190 | Etorphine | R 640 |
| Butorphanol | R 528 | Azaperone | R 26 |
| Medetomidine | R 40 | | |
| 7-cc Pneudart | R 230 | 1-cc Pneudart | R 90 |
| *Subtotal* | *R 988* | *Subtotal* | *R 756* |
| | **Antagonist drugs** | | |
| Naltrexone | R 282 | Naltrexone | R 141 |
| Atipamezole | R 4 572 | | |
| *Subtotal* | *R 4 854* | *Subtotal* | *R 141* |
| **Grand total** | **R 5 842** | **Grand total** | **R 897** |

# Discussion

Ketamine-butorphanol-medetomidine effectively induced a stable immobilisation in all but two of the zebras and was comparable to the commonly used etorphine-azaperone combination. Systemic hypertension was profound and clinically relevant in KBM compared to EA immobilised zebras. Unexpectedly, zebras were normocapnic and had a normal blood pH balance, regardless of the combination used. However, hypoxaemia was present regardless of the combination administered. Time to recovery was rapid and the recovery phase was mostly uneventful with the exception of three zebras recovering from KBM that rolled about during early attempts to move into sternal recumbency.

It is believed that this is the first report of successful immobilisation of zebras using a drug combination that does not contain a potent-opioid. Immobilisation with ketamine-medetomidine (K-M) combinations have been reported in wild Przewalskii horses (*Equus ferus*; K-M dose range: [(1.8-2.6) – (0.07-0.10) mg $kg^{-1}$]) and feral donkeys (*Equus asinus*; K-M dose: 4.1 – 0.14 mg $kg^{-1}$) using similar doses (Matthews et al. 1995; Woolnough et al. 2012). Despite the use of similar K-M doses in this study, the zebras immobilised using KBM had shorter times to recumbency compared to Przewalski horses [11 minutes] and feral donkeys [nine minutes] (Matthews et al. 1995; Woolnough et al. 2012). This shorter time could be as the result of the addition of butorphanol to the ketamine-medetomidine combination, however, other factors could account for these differences too, such as: 1) species differences in pharmacodynamic and pharmacokinetics, 2) differences in habituation and nutrition levels, and 3) differences in darting equipment. We observed two zebras not attaining recumbency after receiving KBM. Possible causes for the apparent failure could include: 1) poor dart placement, 2) a large volume that was injected could have been administered into fascia or subcutaneous tissue during the injection phase, 3) incomplete injection due to dart failure, or 4) the intervention time of 15 minutes being too quick to allow for full drug effect (Zeiler & Meyer

2017). The times to recumbency in EA immobilised zebras were similar to the 4 minutes reported in Grant's zebras (Senthilkumar et al. 2005), but shorter than the five to nine minutes reported in Cape Mountain zebras (Young & Penzhorn 1972).

All zebras resisted the transition from standing to recumbency. This observation is similar to that in other free-ranging equids (Mathews et al. 1995; Woolnough et al. 2012). Despite the characteristics of the pre-recumbency struggle being different between the two combinations, the greatest risk to injury remains associated with the duration of induction (Kock et al. 2012). Thus, KBM could increase the risk of injury based on its significantly longer induction time compared to EA.

Both combinations induced a reliable state of immobilisation in the zebras for at least 25 minutes after becoming recumbent. Therefore, both combinations could be used for immobilisation during routine management procedures in captive situations. Our finding is unlike ketamine-medetomidine immobilised wild equids, where sudden spontaneous arousal that required additional doses of intravenous ketamine to maintain recumbency was reported (Matthews et al. 1995). Whereas, etorphine based drug combinations do provide an adequate state of immobilisation (Walzer et al. 2006; Plotka et al. 1987). We found that zebras immobilised using EA exhibited muscle rigidity and tremors, similar to previous reports (Walzer et al. 2006; Plotka et al. 1987). Therefore, the superior muscle relaxation and apparent deeper plane of anaesthesia achieved with KBM makes this combination a good alternative to immobilise zebra that require interventions that will induce pain.

Although systemic hypertension is not commonly described in horses it is defined as a systolic arterial pressure of greater than 160 mmHg (Grimm et al. 2015). Systemic hypertension was therefore a consistent finding in the zebras regardless of the combination, but clinically more significant with KBM. Medetomidine was most likely the major contributor to the systemic hypertension, followed by ketamine. Medetomidine interacts with extra synaptic

alpha-2 adrenoceptors to cause profound vasoconstriction which increases the systemic vascular resistance and thus increases the systemic blood pressure (Scheinin et al. 1989; Virtanen 1989). The hypertensive effects of medetomidine was likely augmented by the indirect cardiovascular effects of ketamine; the release of endogenous catecholamines stimulated by ketamine leads to vascular postsynaptic $alpha_1$-adrenoceptors activation, causing vasoconstriction, and release of noradrenaline within the myocardium causes increased chronotropic and inotropic effects. Profound systemic hypertension most likely caused a baroreceptor reflex which resulted in an increased vagal tone and overall slower heart rate compared to what was seen with EA (Scheinin et al. 1989). The systemic hypertension seen with EA was not as profound. We believe that etorphine-induced hypertension was as a result of increased sympathetic tone, similar to that described in horses (LeBlanc et al. 1987), and that azaperone partly reduced this hypertension by decreasing systemic vascular resistance through its alpha-1 adrenoceptor antagonistic properties (Kock et al. 2012). Sustained systemic hypertension can result in end-organ damage which may include renal, ocular or neurologic lesions, therefore currently we cannot recommend KBM for prolonged procedures, without correcting the hypertension (Brown et al. 2007).

Hypoxaemia is defined as a $PaO_2$ of less than 80 mmHg and was a consistent finding regardless of the combination (Grimm et al. 2015). Immobilisation drugs, especially when administered in combination and at high doses will often cause hypoventilation. Minute ventilation was not measured, but respiratory rate, $PaCO_2$ and $PE´CO_2$ were measured as indicators of ventilation. Unexpectedly, these values were within acceptable limits for awake equids (Grimm et al. 2015). Thus, hypoxaemia was more likely to have resulted from other mechanisms than simple drug-induced hypoventilation. Medetomidine and etorphine are known to cause pulmonary hypertension (Kästner 2006; Meyer et al. 2015). This hypertension could contribute to gas diffusion deficits through either pulmonary congestion and oedema, or

decreasing the capillary blood transit time which decreases the opportunity for less soluble gases, like oxygen, to diffuse adequately through the alveolar-capillary membrane (Meyer et al. 2015). Another mechanism, often overlooked, is systemic oxygen extraction. Etorphine, after causing an increase in sympathetic tone, can increase cellular metabolism and thus oxygen consumption resulting in systemic oxygen depletion (Buss et al. 2018). Whereas, medetomidine is known to decrease cellular metabolism and therefore a decrease in systemic oxygen depletion, which could explain why the $PaO_2$ was higher in KBM immobilised zebras (Zeiler & Meyer 2017). Veterinarians are cautioned to heed hypoxaemia and should provide oxygen support, especially in diseased or injured animals where the metabolic rate might be increased, and gas diffusion and transport may be impaired.

The times to standing in the zebras were quick, regardless of the combination used. Ketamine-medetomidine immobilised Przewalski horses [average 13 minutes] and feral donkeys [average 21 minutes] took longer to stand compared to our KBM immobilised zebras (Matthews et al. 1995; Woolnough et al. 2012). This difference could be explained by the different atipamezole-to-medetomidine dose ratios and route of administration used; in the other studies atipamezole was dosed intramuscularly at 2.5 mg $mg^{-1}$ medetomidine dose. The recovery after the KBM immobilisations were mostly calm, however, three zebras rolled about before standing. This effect could result in colon displacement and possibly colic, although these were not seen in our zebras. By comparison, the administration of naltrexone in EA-immobilised zebra resulted in recovery times of less than 60 seconds with no evidence of re-narcotisation. Earlier reports suggest a high incidence of partial re-immobilisation in opioid-immobilised zebras antagonised with diprenorphine (Allen 1990).

The first limitation of the study is that the air-tight arterial blood samples were stored on ice and transported to a laboratory for analysis. The entire process took approximately 30 minutes from the collection of the first sample. Liss and Payne (1993) found that the changes in pH and

gas tensions in adequately stored samples over 30 minutes are not of any clinical relevance, therefore we believe that our reported blood gas results were clinically relevant. The second limitation is that we had to use a large and heavy 7-cc dart to administer the KBM combination. Despite the zebra having well-muscled areas to place a dart, these heavy darts could result in unnecessary soft tissue trauma. Furthermore, it is not recommended to fire these darts at distances that exceed 10 meters due to the kinetic energy required to propel the dart that will unnecessarily increase soft tissue trauma. Therefore, the use of this combination may not be feasible in free-ranging settings where darting from long distances is required. We could also not compare the effect that injection volume had between the two combinations. Ideally, we should have darted the EA zebra using a 7-cc dart to eliminate a potential injection volume effect. However, impala darted with etorphine-medetomidine and thiafentanil-medetomidine using a 3-cc dart (drugs diluted in water for injection to fill the dart; increased volume decreased the drug concentration) had longer than expected induction into recumbency times compared to studies with used 1-cc darts (Zeiler & Meyer 2017). The volume-concentration effect, regardless of the total drug dose administered, has not been well research in wildlife capture and warrants further investigation.

In a clinical setting, several factors are carefully considered when choosing an immobilisation combination for a specific capture operation. The size of the group of animals to be immobilised as well as the cost of immobilisation and antagonism per animal is often weighed up against the pros and cons of different immobilisation combinations. The current total cost of immobilising and antagonising a single zebra, based on drugs available in South Africa, is six-and-a-half times more expensive using KBM compared to EA. While the KBM combination itself is fairly similar in cost compared to EA, the larger dart and amounts of antagonistic drugs required, especially atipamezole, makes the use of KBM much costlier and

less feasible for field work. Further investigation into alternative, cheaper, antagonists, such as yohimbine, would be warranted before KBM could be used cost effectively for field work.

## Conclusion

The non-potent opioid ketamine-butorphanol-medetomidine combination may provide a reliable alternative to the preferred etorphine-azaperone combination for the immobilisation of zebras, especially in captive conditions. Systemic hypertension and moderate hypoxaemia are clinical concerns when using KBM and EA, respectively. The superior muscle relaxation and apparent deeper plane of anaesthesia achieved with KBM could make this combination a good alternative to immobilise zebra that require painful procedures. However, with the currently available preparations, the high costs associated with KBM, and the larger dart required for its administration, may limit its use in clinical cases and routine zebra field management procedures.

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# Addendum

## Data collection forms

South African NATIONAL PARKS

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

**ZEBRA IMMOBILISATION STUDY DATA COLLECTION SHEET**

**DATE:** / /2017

**DATA COLLECTOR DETAILS:**

Dr. GP STEMMET
gideon.stemmet@up.ac.za
082 0722 735

**ANIMAL NR:** ☐

**IMMOBILISATION COMBINATION:**

1. KBM ☐
2. EA ☐

**FAMILY GROUP:**

☐

**SEX:**

☐

**WEIGHT:**

☐

South African NATIONAL PARKS

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**DATE:** / /2017

**DATA COLLECTOR DETAILS:**

Dr. GP STEMMET
gideon.stemmet@up.ac.za
082 0722 735

**ANIMAL NR:**

**VITAL PARAMETERS:**

| | | PARAMETERS | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | HR(bpm) | RR(bpm) | Systolic (mmHg) | MAP (mmHg) | Diastolic (mmHg) | $SpO_2$ | RECTAL TEMP (°C) | $ETCO_2$ |
| TIME (MIN) | 5 | | | | | | | | |
| | 10 | | | | | | | | |
| | 15 | | | | | | | | |

South African NATIONAL PARKS

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

## ZEBRA IMMOBILISATION STUDY DATA COLLECTION SHEET

**DATE:** / /2017

**DATA COLLECTOR DETAILS:**

Dr. GP STEMMET
gideon.stemmet@up.ac.za
082 0722 735

**ANIMAL NR:**

**BLOOD GAS ANALYSIS:**

| | TIME(MIN) | | |
|---|---|---|---|
| PARAMETER | 5 | 10 | 15 |
| $Pa(CO_2)$ | | | |
| $Pa(O_2)$ | | | |

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## ZEBRA IMMOBILISATION STUDY DATA COLLECTION SHEET

**DATE:** / /2017

**DATA COLLECTOR DETAILS:**

Dr. GP STEMMET
gideon.stemmet@up.ac.za
082 0722 735

**ANIMAL NR:**

**ANAETHESIOMETER READING:**

| TIME | NEWTON FORCE AT WHICH A RESPONSE WAS ELICITED |
|---|---|
| 15 MINUTES | |

South African NATIONAL PARKS

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UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

## ZEBRA IMMOBILISATION STUDY DATA COLLECTION SHEET

**DATE:** / /2017

**DATA COLLECTOR DETAILS:**

Dr. GP STEMMET
gideon.stemmet@up.ac.za
082 0722 735

**ANIMAL NR:** ☐

**KNOCK-DOWN SCORE:**

Time from darting to recumbency ☐ min

Notes/ Observations: _____

Knock- down score: ☐

**IMMOBILISATION QUALITY:**

Notes/ Observations: _____

Immobilisation score: ☐

**RECOVERY SCORE:**

Time from reversal to standing ☐ min

Notes/ Observations: _____

Recovery score: ☐

## Presentations and publications arising from the study

**Presentations:**

| EVENT | VENUE | DATE | TITLE | TYPE |
|---|---|---|---|---|
| SAVA Wildlife Group Annual Congress | Misty Hills Country Hotel, Conference Centre | 3 March 2018 | Comparison of chemical capture efficacy of non-potent opioid drug combination to the preferred etorphine based combination in zebra *(Equus zebra)* | Abstract Presentation |

**Publications:**

**Gideon P Stemmet, Leith CR Meyer, Angela Bruns, Peter Buss, David Zimmerman, Katja Koeppel & Gareth E Zeiler**, Ketamine-butorphanol-medetomidine is an effective alternative drug combination to immobilise zebras (*Equus zebra)* compared to etorphine-azaperone. *Veterinary Anaesthesia and Analgesia* (Under review: May 2018).

# Animal ethics approval certificate

UNIVERSITEIT VAN PRETORIA
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## Animal Ethics Committee

| | |
|---|---|
| PROJECT TITLE | **Cardiopulmonary effects of ketamine-butorphanol-medetomidine and etorphine-azaperone drug combinations used to immobilise zebra (*Equus zebra*)** |
| PROJECT NUMBER | **V071-17** |
| RESEARCHER/PRINCIPAL INVESTIGATOR | **Dr. G Stemmet** |

| | |
|---|---|
| STUDENT NUMBER (where applicable) | **U_29022330** |
| 22330DISSERTATION/THESIS SUBMITTED F | **MSc** |

| | | |
|---|---|---|
| ANIMAL SPECIES | **Zebra** | |
| NUMBER OF ANIMALS | **30** | |
| Approval period to use animals for research/testing purposes | | **June 2017-December 2017** |
| 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 15 June 2017 |
| CHAIRMAN: UP Animal Ethics Committee | Signature |

S*4285-15*