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THE PHYSIOLOGICAL IMPACT OF CAPTURE: STRATEGIES FOR IMPROVING IMMOBILIZATION OF WILD EAST AFRICAN MESO- AND MEGA-HERBIVORES

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To the marvellous wildlife of this planet,
From the tallest to the smallest

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ABSTRACT

East African meso- and mega-herbivores have been a key part of the Earth's ecosystem for millions of years, but are now at risk of disappearing. To ensure their conservation, operations that involve veterinary immobilization are becoming essential for wild populations. However, capture morbidity remains high, with both short- and longer-term physiological alterations that can result in acute or delayed death. In large-sized herbivores, the size and unique anatomy and physiology contribute to the high susceptibility to capture stress, drugs adverse effects and alterations due to recumbency. On top of this, the limited knowledge in the species-specific physiological response to immobilization and, as a result, the obliged practice of extrapolating drug doses and protocols from similar species, enhances the risk of complications. Improvement in capture methods and drug protocols are advocated, and as such, in order to develop targeted strategies, it is essential to gain a better understanding of the species-specific physiological impact of capture.

The general objective of this thesis is to advance the knowledge of the physiological mechanism of capture morbidity, and evaluate strategies for the prevention, detection and treatment of complications arising from opioid-based immobilization of selected species of East African megaherbivores, the giraffe (*Giraffa camelopardalis ssp. tippelskirchi and reticulata*) and the black rhinoceros (*Diceros bicornis ssp. michaeli*), and in a large mesoherbivore, the African buffalo (*Syncerus caffer*). A key factor of this study was the collection of data through an opportunistic approach, whereas the research design was shaped for each of the study species based on targeted needs, thus different specific objectives were pursued for each species.

In free-ranging Masai giraffes that were immobilized for a translocation, a combination of etorphine and azaperone was evaluated for physiological and handling safety. Early opioid antagonization – a common procedure performed to reduce etorphine's respiratory depression – was performed at low doses to assess if it would result in smoother restraint and transport. The protocol produced safe inductions, but variable opioid-related excitement occurred and accounted for metabolic derangement. On the other hand, early antagonization with low dose naltrexone allowed calm restraints, a stable physiological function during the recumbency, and enabled smooth recoveries and loading into the chariot with resulting uneventful transport. No delayed complications or resedation were observed during a two-week post-capture *boma* monitoring. Although the protocol allowed safe immobilization and transport, the study highlighted that further research on techniques that reduce induction-induced excitement, which poses severe health risks in giraffe capture, is advocated.

Building up on the study performed in Masai giraffe, the physiological mechanism of capture morbidity occurring in both vehicle and helicopter darted reticulated giraffes, immobilized with an etorphine-azaperone combination, was investigated in order to detect the predisposing factors for homeostatic alterations and to define and guide prevention strategies. Trends over time in blood gases, selected biochemistry variables and cardio-respiratory function were analyzed following early opioid antagonization, and the use of a non-invasive nasal capnometer was investigated. In the helicopter darted giraffes, severe metabolic alterations were observed as a result of an intense startle response,

whereas in vehicle darted giraffes, these were moderate and mainly a result of etorphine-induced excitement. Intense excitement occurred when lower doses of etorphine were administered, whereas higher doses resulted in respiratory depression, severe respiratory acidosis and hypoxemia. Early antagonization produced an improvement over time of gas exchanges, but not of the acid-base status, and resulted in poor immobilization quality. Nasal capnometry proved to be a useful non-invasive monitoring tool for field ventilatory function in giraffes. The severe alterations observed suggest that advances in giraffe immobilization should focus on reducing both opioid-respiratory depression and excitement, and onto providing adequate sedation and analgesia during field immobilizations.

In Eastern black rhinoceroses, two intra-anesthetic treatments, butorphanol and oxygen, or doxapram, butorphanol and oxygen - which are routinely administered to improve gas exchanges, but which efficacy has not been investigated yet in the species - were evaluated. The mechanism of physiological alterations resulting from capture was investigated, and nasal capnometry was evaluated for its accuracy in monitoring carbon dioxide. Hypoxemia and severe lactic acidosis, proportional to more intense pre-dart chase, occurred. After the administration of doxapram and butorphanol, the initial hypoxemia and acidosis improved, presumably as a result of increase in ventilation mediated by doxapram; whereas the same values worsened when butorphanol only was administered. This might suggest that, different to other rhinoceroses, increased oxygen consumption is not the primary mechanism of hypoxemia in black rhinoceros. Nasal capnometry was efficient in monitoring carbon dioxide trends, but not accurate in predicting absolute values. Although intra-anesthetic treatment with doxapram partially improved gas exchanges, and post-capture complications did not occur for at least nine months, the severe metabolic and respiratory alterations observed highlight the need of advances in black rhinoceros capture methods that focus on preventing the origin of physiological alterations.

The physiological safety of two immobilization protocols, etorphine-azaperone and etorphine-medetomidine-azaperone combinations, was compared in free-ranging African buffalos. The aim was to evaluate if medetomidine's sparing effect would have allowed to safely decrease etorphine doses, and its adverse respiratory effects, without increasing the risk of excitement or poor immobilization quality. The addition of a low dose of medetomidine allowed to decrease etorphine dose by 30 %, and resulted in quicker and smoother inductions, and significantly improved immobilization quality. Medetomidine reduced the occurrence of tachycardia and respiratory acidosis, but not of hypoxemia. Etorphine-medetomidine-azaperone combination is recommended for buffalo immobilization as it provides greater physiological and handling safety, and can help to reduce the onset of capture stress.

The new knowledge acquired within the different studies of this thesis has allowed to detect and evaluate species-specific strategies for the prevention (through knowledge of factors influencing capture morbidity, and improved immobilization protocols), detection (through clinical monitoring) or treatment (intra-anesthetic drugs) of capture and drug complications in large-sized herbivores. Species-specific and intra-specific variation of physiological response to capture stress and drugs were individuated, and hence a species-specific approach needs to be endorsed when capturing large-sized herbivores. Furthermore, based on the new information gained in this thesis, further studies can now specifically focus towards targeting solutions for the specific detected physiological alterations. The advances on immobilization methods resulting from this thesis represents a first step towards the improvement of the safety of immobilization of giraffes, black rhinoceroses and buffalos, and by reducing the risk of occurrence of delayed morbidity, it also contributes to the conservation of these East African large-sized herbivores.

LIST OF ABBREVIATIONS

α	alpha
AG	anion gap
BE	base excess
BGA	blood gas analysis
BUN	blood urea nitrogen
°C	degree Celsius
Cl^-	chloride
cm	centimeter
CO_2	carbon dioxide
Crea	creatinine
CTNI	cardiac troponin I
δ	delta
$ETCO_2$	end tidal carbon dioxide
FiO_2	fractional inspired oxygen
Glu	glucose
Hb	hemoglobin
Hct	hematocrit
HCO_3^-	bicarbonate
HR	heart rate
iCa^{++}	ionized calcium
IBP	invasive blood pressure
ID	internal diameter
IM	intramuscular
IV	intravenous
l	liter
Lac	lactate
K^+	potassium
κ	kappa
kg	kilogram
km	kilometer
m	meter
mg	milligram

min	minute
ml	milliliter
mm	millimeter
mmHg	millimeters of mercury
mmol	millimole
n	number
ng	nanogram
Na^+	sodium
O_2	oxygen
$PaCO_2$	arterial partial pressure of carbon dioxide
PAO_2	alveolar partial pressure of oxygen
PaO_2	arterial partial pressure of oxygen
Pb	barometric pressure
P_{H2O}	water vapor pressure
RR	respiratory rate
RQ	respiratory quotient
$P(A\text{-}a)O_2$	alveolar-arterial oxygen partial pressure gradient
SaO_2	arterial hemoglobin oxygen saturation
SpO_2	arterial hemoglobin oxygen saturation measured by pulse oximetry
SD	standard deviation
T	rectal temperature
Tco2	total carbon dioxide
µ	mu
µg	microgram
V/Q ratio	ventilation/perfusion ratio

1 INTRODUCTION

Earth's sixth mass extinction is already underway and the window for effective action to safeguard threatened biodiversity is very short, estimated at two or three decades [1]. Some of the most iconic wild species that have been a key part of the Earth's ecology for millions of years, are now currently at risk of disappearing entirely [2].

Large-sized herbivores face the greatest risk of extinction among all mammals, birds and reptiles owing to their body size and trophic characteristics, which have ecological implications given their important role in controlling ecosystem balance [3]. Being less vulnerable to predators, they shape the habitat structure by opening the habitat for grasses and forbs, and dispersing seeds more homogenously across the landscapes compared to smaller grazers [4–6]. Furthermore, since large-sized herbivores drive ecosystem functions, their loss, as seen in previous historical mass extinctions, is likely to drastically influence the ecology of our planet, and our lives [3,7].

In this thesis I focus on two species of East African megaherbivores, the giraffe (*Giraffa camelopardalis* ssp. *tippelskirchi* and ssp. *reticulata*) and the black rhinoceros (*Diceros bicornis ssp. michaeli*) and a large mesoherbivore, the African buffalo (*Syncerus caffer*). Taxonomic classification of giraffes has recently been debated, concerning the separation of the nine subspecies of giraffes into four species [8–11]. According to these studies, the Masai and reticulated giraffes would be two distinct species. In this study I refer to them as subspecies, since the new hypothetical taxonomic classification has not been recognized yet by the International Union for Conservation of Nature (IUCN), but this hypothesis is taken into account in the interpretation and discussion of the results. The two giraffe subspecies, the Masai and the reticulated, are Endangered and the Eastern black rhinoceros is Critically Endangered, whereas African buffalo populations, although not currently threatened, are declining [12–15]. The thesis focus is on these species because the conservation success of such charismatic species is particularly important as they play a role in catalyzing attention and resources towards

broader biodiversity loss [2]. Since anthropogenic factors, such as illegal hunting for trophies or bushmeat, habitat loss and ecological changes, have drastically reduced their populations to near extinct levels in some species, intensive conservation management, including veterinary work, will be more and more required in the future [15–18]. The Eastern black rhinoceros is one example. Since the remaining populations are enclosed in high protection sanctuaries as an antipoaching measure, to guarantee their meta-population growth, expand their range and genetic diversity, the survival of this species relies on strategic translocations [19,20] during which a safe immobilization is of crucial importance.

Within a multidisciplinary approach, the role of conservation medicine is being increasingly recognized as essential in the arena of wildlife conservation as the extinction of many species is highly probable without veterinary interventions [21,22]. The treatment and rescue of injured individuals from Endangered species are some of the key aspects of this branch of veterinary medicine. Other important activities are the application of GPS units to study wildlife movements which guide conservation policies, disease surveillance within a One Health concept, and translocations to improve the status of targeted population, species or ecosystem, and release rehabilitated animals or solve human-wildlife conflicts [23,24].

Since pharmacological capture is essential to guarantee the veterinary management of highly threatened wild animals, the ability to safely immobilize individuals becomes a priority for the conservation of these species [25,26]. Wildlife medicine is a novel science, thus physiological knowledge, reference values, and response to anesthetic protocols of most wildlife species are still missing. With such data and knowledge gaps, the only available option for veterinarians in the field becomes extrapolation between species belonging to the same genus, or sometimes family. However, since there is significant species-specific responses to stress and drugs, it can result in severe complications for the safety of the animals and that of the capture team [26,27]. Therefore, understanding these response traits in Eastern black rhinoceros, Masai and reticulated giraffe, and African buffalo is the first step to advance the safety of their field immobilization.

In this thesis I address, through an opportunistic research approach, the urgent need to fill some of these gaps, and advance the knowledge on physiological response to stress and capture drugs in selected species of East African large-sized herbivores. This introductive chapter reviews the state of knowledge of wildlife physiology and immobilization, with the aim to highlight the origin for the research questions that I have investigated during my PhD.

1.1 A SAFE CAPTURE

Wild animals have been captured by humans for thousands of years using not only physical restraint, but also a form of chemical restraint using blowpipe darts or arrows prepared with poison to hunt animals. Prior to the development of effective anesthetics and remote delivery systems, veterinary procedures on non-domestic species were either impossible to accomplish or associated with unacceptably high mortality rates. Live capture of wild animals by modern chemical immobilization was introduced in the 1950's [28,29]. Although mortality rates were high during initial immobilization procedures, large numbers of wild animals were captured and rescued using novel drugs and darting techniques. The development of reliable remote drug-delivery systems in conjunction with safer anesthetic drugs over the past four decades has greatly facilitated the capture and handling of many different free-ranging species [26,30,31]. However, given the challenges that are encountered during wildlife capture, it is not surprising that still today morbidity and mortality of animals can be high and injury to people engaged in the capturing procedure not uncommon.

1.1.1 Why chemical immobilization?

Immobilization is the forced restriction of movement of all or part of an animal's body, either by physical or chemical means. In particular, *chemical immobilization* was used in the past to indicate a neuromuscular blockade, obtained with drugs such as succinylcholine. Today this practice is considered obsolete and unacceptable, due to the high levels of stress for the immobilized animal that remains conscious; therefore the term today indicates a field-conditions medium-level anesthetic plane that provides analgesia and muscular relaxation [32]. *General anesthesia* refers to a state of drug-induced unconsciousness that is characterized by controlled but reversible depression of the central nervous system and profound analgesia. In this state, the patient is not arousable by noxious stimulation, and sensory, motor, and autonomic reflex functions are attenuated [33].

Generally, most procedures on free-ranging wildlife, such as sample collection, loading into transport crates or treatment of injuries caused by poaching snares are of short-duration and not, or only marginally, painful and therefore general anesthesia is not necessary; on the other hand, a light plane of sedation to restrain dangerous animals would be too hazardous for the risk of spontaneous recoveries. The goal for most wildlife immobilization is to have adequate unconsciousness, muscle relaxation, analgesia and tranquillization, but avoiding an excessive deepness of the anesthetic plane as ventilatory support is mostly not available in the field. The prevention or limitation of capture stress is fundamental, in order to assure animal welfare, stable immobilization and capture team safety [26,32,34–36].

Chemical immobilization is becoming prevalent in large mammal capture, as it allows to have lower injury rates and mortality than physical restraint methods [37,38], less capture stress as the animals are manipulated while sedated [39,40], and provides the ability to select specific individuals [30]. Capture and immobilization of wild ungulates are likely to be one of the most stressful events in their lives, especially when carried out with snares and nets, as demonstrated by the severe alterations that occur in hematological and biochemical blood constituents [30,37]. Physical restraint in general induces greater stress than chemical restraint, and can be dangerous due to the serious health consequences of stress and risk of injuries [30].

The use of chemical immobilization with drugs that sedate and tranquilize animals is indeed adopted to reduce this arousal and its extreme psychological and physiological response that may result in physiological alterations, trauma, capture myopathy and ultimately death [34]. Furthermore, if the animals are not properly restrained and sedated, it might not only result in manipulation stress and animal welfare issues, but it can increase the risk of injuries for the members of the capture team.

On the other hand, with the use of chemical immobilization, the fright caused to animals during the approach remains a significant factor of risk [26,36,41,42]. In addition, the potent drugs used to capture wildlife have several adverse effects that can compromise the animal's normal physiological function, and add to the already significantly compromised physiologic status of the stressed animal, along with other important complications [32,34,35].

Furthermore, longer-term consequences of capture, although often ignored, can be life-threatening or seriously affect the animal's survival or thriving success [30,34].

1.1.2 Short and longer-term impact of capture and implications for conservation

Chemical immobilization of free-ranging wildlife can be challenging. The nature of the procedure dictates that veterinarians must ignore many of the principals that underlie good anesthetic practice in other settings. It is generally not possible to access the patients for a preanesthetic physical examination or laboratory work. Physical status of the patients cannot be accurately assessed, and therefore the presence of any underlying disease is ignored. Even if physical status and anesthetic risk could be determined, only a few effective drug protocols are available as the use of some drugs is restricted by the volume that can fit into a dart syringe or their suitability for muscular injection [26,32]. The choice of the dosage to administer must be made based on an estimation of body weight that sometimes can be challenging, and on the age, gender, reproductive and nutritional status and temperament before drug administration [32,34,35].

It is well recognized that chemical capture may involve injury, reduction of chances of post-capture survival, and mortality [30,37,43–45]. Mortalities caused by capture and anesthesia of free-ranging wildlife can be caused by direct effects of the stress and immobilizing drug, such as respiratory depression, hyperthermia, bloat and asphyxia due to vomiting or regurgitation, cardiovascular

depression and shock, or by indirect effects, such as injuries during induction or recovery [30,32,34,36]. Secondary effects caused by the capture process, such as various problems with GPS collars or implantable transmitters can also occur. A mortality rate greater than 2% during chemical immobilization is unacceptable, although it is not an uncommon occurrence in some species [36,46].

Mortality rate is generally used as the criterion to establish if an immobilization protocol is safe [26,30]. Unless post-capture follow-up is performed, as in the case of threatened species or in in those in which GPS units have been fitted, it is often challenging to know if delayed mortality occurs, such as in the case of acute or sub-acute capture myopathy [30,36,47]. However, given the significant impact that chemical immobilization still has on an animal's welfare and longer-term complications, more consideration should be placed towards achieving low morbidity and ensuring a stable physiology during the restraint [26,30].

The full impact of capture and manipulation procedures cannot be evaluated without taking into account the physical, physiological and behavioral effects on animals, including in the days or weeks that follow the capture event [26]. Latent effects of both physical and chemical capture include chronic stress, susceptibility to disease, reduction of breeding performances and displacement from the areas surrounding capture sites, altered space and habitat use, depressed movements, and reduction in activity patterns [37,42,48–51]. The advent of these complications can, in addition to affecting the animal's welfare, seriously threaten also the outcome of conservation actions [48,52,53].

In the context of translocations, the capture likely represent the main stressor between capture and transport [54]. When animals are captured for translocation, alterations in their homeostasis triggered by the capture stress can have even more severe consequences, as transport exacerbates the physical stress and anxiety [55]. Many translocations result in failure because the individuals exhibit substantial reproductive delays, or become ill, such as in the case of post-capture anorexia, diarrhea, parasitic diseases and secondary infections, and die [52,56,57]. Even though the direct cause for these mortalities are likely external factors, such as pathogens, the vulnerability to these factors is exacerbated by stress [44,47,52–55]. By reducing the overall exposure and severity of stressors associated with capture and translocation, the effects and duration of stress might decrease, thereby improving translocation success [58]. Since the acute stress triggered during capture can have belated effects, more research is advocated in the effort to successfully prevent it, and to understand its effect on the physiology by monitoring post-capture behaviour and morbidity in large-sized herbivores.

Figure 1. A typical field *boma* used for translocations. The boma temporary confinement is used to acclimatize captured animals before they are transported, or after the transport, before they are released to the new site. The boma represents also an important tool to perform post-capture monitoring. In the photo: Masai giraffes. (Photographer: Francesca Vitali)

To improve veterinary medicine for conservation, it is essential to study new capture techniques and gain a better understanding of the stress and its pharmacological management during immobilization of the different wildlife species. By improving capture protocols and doses with a species-specific approach, and improving our knowledge, and the ability to detect and treat drug adverse effects, it is possible to reduce morbidity and mortality rates [26,30]. Although in this thesis the focus is placed on preventing or treating short-term complications of capture, by doing this a greater outcome is achieved in terms of reducing longer-term morbidity and thus improving conservation success.

1.2 THE PHYSIOLOGICAL EFFECTS OF CAPTURE

Chemical immobilization of wildlife is a type of veterinary anesthesia conducted under difficult circumstances and characterized by an elevated anesthetic risk. Major morbidity and mortality causes are due to the acute stress elicited by the capture, and by drug complications [53,59]. Fear and the chasing associated with the capture event, and the adverse drug effects, are intrinsic to the nature of the restraint itself, and can never be completely eliminated [60]. However, these complications can be mitigated by improving the understanding of their mechanism, investigating the predisposing factors, and advancing techniques for their monitoring, prevention and treatment.

1.2.1 Pathophysiology of capture stress

The stress response is a survival mechanism, particularly sharpened in prey species, that triggers a cascade of events that result in quick adaptations to maximize the chances to address a danger, the so called “fight or flight” reaction [61]. The fight or flight response represent the behavioural and physiological reaction to a threat, resulting from the activation of the autonomic nervous system and hypothalamic-pituitary-adrenal (HPA) axis [61,62].

The stimulation of the sympathetic nervous system, and simultaneous withdrawal of the parasympathetic nervous system results in the release of catecholamines through the stimulation of the adrenal medulla [34]. The catecholamines (e.g. noradrenaline and adrenaline) trigger an immediate response of the body to be prepared for the fight or flight reaction, by mobilizing energy sources to the central nervous system and skeletal muscles. Their release increases arousal, and maximizes the performance of cardio-respiratory function, such as through bronchiole dilatation, increased heart and respiratory rate and blood pressure [63,64]. The HPA axis response follows the catecholamine release, and promotes the release of glucocorticoids (e.g. cortisol) from the adrenal cortex. The major metabolic effect of the increased glucocorticoid secretion is to further mobilize energy by releasing glucose to support the increased physical activity [64].

During capture events, animals are placed under immense physical and psychological stress [65]. These human-induced stressors (e.g. the sound of the helicopter or the proximity to humans) during capture events are usually far greater in duration to any experienced during natural hunting processes. During hunting, a short sprint (for either prey or predator) results in either the death of the prey or in the cessation of the stressor as the animal escapes, or fights, and can then rest and recover quickly. Although the stress response is physiological, when the presence of the stressor is artificially prolonged, it can lead to a derangement of the physiological response and result in chronic stress [61], or if combined with excessive physical activity, such as during a helicopter chase, in overexertion [66].

The increased fast-speed physical activity produces a greater workload on the animal's muscles, and in concomitance with an elevated oxygen consumption, insufficient oxygen is available for the Krebs cycle [67,68]. The catecholamines released contribute to tissue hypoxia through their potent vasoconstriction effect that reduces blood perfusion to muscles [64]. The increased metabolic rate triggered by the stress and overexertion, produces an exhaustion of ATP in muscle cells, altering the delivery of oxygen and nutrients, increasing the production of lactic acid, and causing an inadequate removal of cellular waste products [64,69]. The vast quantity of lactic acid produced overwhelm the capacity of the liver, heart and other tissues to convert lactic acid to useable energy. It dissociates to form lactate and hydrogen ions that in turn causes a rise in hydrogen ion concentration resulting in a state of metabolic acidosis [70]. A low intracellular pH impacts the active transport of sodium and potassium, and results in increased intracellular sodium and chloride, and extracellular potassium levels, and as mitochondrial activity decreases and lysosome ruptures, cellular damage occurs, and enzymes are released. These processes are thought to result in muscular injury and necrosis [71]. The leakage of potassium and intracellular enzymes, such as creatine kinase, into the bloodstream is a result of muscle cell damage and to a lesser degree from increased cellular permeability [59,72–75].

The sudden and continuous rise in plasma potassium, as well as the reduction of calcium ions resulting from an increase in circulating lactate, can induce changes in neuromuscular and heart excitability which can lead to ventricular fibrillation and sudden death [74,75]. Sometimes values of pH as low as 6.5 occur and this can causes acute cardiac arrest especially during massive adrenaline output and sensitization of the heart [76]. When severe acidemia occur, it can cause lethargy, seizures, stupor, coma, and ultimately death [77], and it becomes even more lethal when it is combined with hypoxemia, which is a well-known complication in opioid-based immobilizations.

In case of prolonged catecholamine stimulation, exhaustion of the sympathetic vascular tone occurs and results in vasogenic-neurological shock, and hyper-acute capture myopathy, known as capture shock syndrome [64,69]. Exhaustion of vascular tone results in hypotension and reduced cardiac output, which lead to inadequate delivery of nutrients and oxygen to the tissues and further accumulation of cellular waste products. Tachycardia, tachypnea and hyperthermia are usually followed by death a few hours after capture [47,69]. However signs of capture myopathy more often appear several hours after the capture event, with the acute, sub-acute or delayed forms of capture myopathy, and can lead to mortality within a few days or weeks [47].

Hyperthermia commonly occur as a result of capture, and is believed to be predominantly stress-induced as an anticipatory response of an animal to a stressor, whereas exercise during capture and environmental temperature are not the main contributor to the rise in body temperature [41,72,78–80]. Capture-induced hyperthermia can contribute to the development of capture myopathy and is associated with an increased risk of mortality [37,76,78].

Several studies have highlighted that biochemical and hematological variables change in response within the pathophysiologic alterations of capture stress, and some of them have been used as prognostic factors for mortality or greater morbidity in a few species [30,43,76,80]. Potassium, sodium and creatine kinase are indicative of the degree of stress response and muscle damage associate with capture myopathy [76]. Glucose is often increased due to an acute stressor, or exercise, since

catecholamines and glucocorticoids have an hyperglycemic effect [43,64]. Hematocrit increases as a result of splenic contraction that is caused by either circulating catecholamines or sympathetic nerve activity [83,84]. Its alteration can be triggered by fear or increased exercise, such as in case of delayed induction [80,83]. An increase in BUN is often seen in the acute or sub-acute form of capture myopathy, but early elevations during the restraint have been observed in different wildlife species after capture, and has been linked to strenuous exercise [47,75,76,85]. Cardiac troponin I, that is gaining increasing attention as a biomarker of myocardial injury in wildlife species, is believed to increase in physically restrained ungulates presumably as a result of hypoxic-related myocardial damage, but also after strenuous exercise without indicating cardiac pathology [86,87].

Greater alterations of some of these variables, such as glucose, potassium, calcium, sodium and creatine kinase have been associated with higher mortality and more severe lactic acidosis and hyperthermia [88]. Higher glucose, lactate and hematocrit have also been observed in net-captured ungulates after strenuous exercise [89,90].

Although species-specific values are not available for most species, changes in acid-base and electrolyte status, and biochemical and hematological variables have been observed across a variety of herbivores in response to catecholamines and corticosteroids release during the stress response. More research is advocated on their use and species-specific clinical significance as indicators for capture stress or overexertion [43,72].

1.2.2 Predisposing factors of capture morbidity

Several factors influence the mounting of a stress response when wildlife is captured, and as a result their susceptibility to capture morbidity and mortality. These includes individual and species-specific characteristics and factors related to the capture methods and drugs [76,89].

Species with the highest maximal running speed and large brain mass, such as many ungulates, are considered at higher risk of capture myopathy [69,71]. However, it is well reported that there is not only inter- but also intra-specific variability of reactions to the same stressful stimulus [73,89]. Life-historical extreme stress events, presumably such as previous captures, can also predispose individuals to capture myopathy [71]. Younger and older animals are likely to be more susceptible to the adverse effects of immobilizing drugs, whereas in some species, such as in the buffalo, older age has been associated with the mounting of a greater stress response to immobilization [34,91]. The reproductive status of the females, such as when in estrus or with young, might influence their behavioural response to the capture and drugs, whereas pregnant females usually require higher doses of drugs to achieve recumbency or otherwise might experience prolonged recoveries [89]. Animals in poor health are also likely to be more susceptible to the effects of the immobilizing drugs [34].

Psychological and physical stress are both involved in capture-related morbidity. Commonly the cause for homeostatic imbalance and capture myopathy has been mainly attributed to chase-related overexertion, but recent studies demonstrated the effects of psychological stress in small-sized wild herbivores even when not associated with increased physical activity [41]. Capture-related hyperthermia, and its associated morbidity and mortality, is mainly caused by stress regardless of the amount of exertion [41,78]. Whereas in many species of herbivores, when threatened, psychological stress and behavioural flight response coincide, in large-sized species, that are less susceptible to predation [5,6], psychological stress might rise despite the individual might not flight in response to the stressor.

In general, it is essential to use techniques that invoke lower stress responses such as by decreasing the length, duration and especially intensity of pre-capture exercise in order to limit additional heat production [41]. The development of capture techniques should also consider species-specific ethological traits, especially in prey species, in order to prevent the onset of psychological stress and its disastrous consequences. To lessen the mortality derived from the negative physiological effects of stress, it has been speculated that intensive chasing should be kept to a minimum (less than three minutes), however this is not always possible due to difficult working circumstances [34,36,92].

Figure 2. Helicopter darting can result in an intense flight response. In the photo: A female of Eastern black rhinoceros with her calf are being herded by the helicopter to an open area before being darted, in order to be quickly reached by the ground team once recumbent. (Photographer: Francesca Vitali)

Environmental temperature plays a lesser role on the mechanism of hyperthermia, therefore restricting capture to the cooler hours will not protect animals from developing capture-induced hyperthermia [41]. The amount of stress developed during capture events varies regarding with the type of approach, such by vehicle or helicopter, and severity of chasing. It has been suggested that a

slippery terrain might worsen the fatigue that arises during the capture [93]. Furthermore, habituation decreases the hyperthermic stress-mediated response [41], and might explain the variation in response of individuals of the same species as the habituation to vehicles in certain locations might decrease the response to the darting vehicle.

Rapid times to recumbency are usually believed to reduce capture morbidity by decreasing physical exertion [34,41]. However, the shorter induction times have been observed in association with higher cortisol concentrations, and drugs with the best knock down times have the greatest dose-dependent respiratory depressant effects [94]. In a species of wild perissodactyl, the khulan, it was observed that the longer the animals kept moving after darting and without being chased any longer, the lower the lactate level and acidosis [95]. In human athletes it has been shown that active recovery after strenuous exercise clears accumulated blood lactate faster than passive recovery in an intensity-dependent manner [96]. However, a prolonged induction is dangerous as usually leads to a partially sedated animal in an "excitatory phase" running around, facing higher risks of injuries [92]. Prolonged inductions are more often due to under-dosed opioids administration, although the administration of drugs in a poorly vascularized area is likely to have a role due to a delayed absorption. Dart placement can affect induction times, with the quickest absorption occurring after injection in large muscle masses, such as the neck, shoulder, or hindquarters. Animals that are excited or stressed can have induction times that are considerably longer than calm animals [34].

Prevention is often much more efficient than treating capture myopathy and other capture-related complications [97]. Other than minimizing the pre-darting stress, the choice of an optimal drug combination can decrease the stress in the animal. One example is the addition of drugs with anxiolytic and muscle relaxant properties that have been used for treatment and prevention of capture myopathy [79,97,98].

It is important to further understand the causes of morbidity in each species and how these relate to the capture procedure, so that changes in capture techniques can be made in order to prevent complication [88]. Most of the variables playing a role on stress response during capture have not been properly considered or studied at a species-specific level, partially due to the lack of a systematic collection of data and information on free-ranging individuals. The correlation between possible predisposing factors and alterations in blood variables indicating stress and morbidity, can be used to detect which ones account for more severe physiological derangement [95], and might provide guidance on how to prevent capture morbidity.

1.3 THE PHYSIOLOGICAL EFFECTS OF OPIOID-BASED IMMOBILIZATION

Opioids are the most common class of drugs used to immobilize large-sized herbivores. Among their advantages there are quick inductions, potent sedation, catatonia, analgesia, the availability of antagonists and high concentrations that allow them to fit them in the small volume of the dart syringes [34,35]. However, potent opioids, such as etorphine, thiafentanil and carfentanyl have significant adverse effects.

Four major opioid receptor classes (μ-, κ- and δ- and nociception- receptors) exist and are known to regulate, together with endogenous ligands, various physiological processes by binding the different receptors, such as stress response, respiratory, pain and appetite control, and thermoregulation [99]. Exogenous opioids and opioid antagonists bind the receptors differently, and this mechanism accounts for their different specific pharmacological effects.

Etorphine is a synthetic morphine-like drug with strong agonism for μ, κ and δ and is 5,000 – 10,000 times more potent as an analgesic than morphine [100]. It is believed that the μ-receptor is responsible for most of the clinically relevant effects such as sedation, catatonia and analgesia, but on the other hand it can result in excitation in some species with increased locomotor activity [35,101]. It is also well described that etorphine, mainly through its μ-agonism, produces a significant dose-dependent respiratory depression, which results in severe hypoxemia, hypercapnia and acidemia in a variety of wildlife species [34,35].

The activation of μ-receptors, that are distributed in respiratory control centers of the brain stem, in the lungs, in carotid bodies and vagus nerve, impact the regulation of the respiratory rhythm, and reduces the sensitivity of chemoreceptors to carbon dioxide and pH alterations thus impacting breathing frequency and tidal volume [99,102]. The resulting hypoventilation, and lack of carbon dioxide elimination is particularly problematic in wildlife capture, as it inhibits the respiratory response essential to compensate for metabolic acidosis resulting from the exertion and can lead to respiratory or mixed acidosis. The respiratory depressant effects are even more enhanced by an increase in upper airway resistance and a decrease in chest and abdominal wall compliance [103,104].

Hypoxemia and hypercapnia resulting from opioid immobilizations are associated with an increased alveolar-arterial gradient, that reflects the occurrence of intrapulmonary mechanism such as ventilation perfusion mismatching, oxygen diffusion impairment and right-to-left shunting of blood [104–106]; and with an elevation in oxygen consumption and carbon dioxide production [26,107].

Etorphine stimulates catecholamine release which results in an hypermetabolic state associated with, depending on the species, tremors, tachycardia, increase in cardiac output, and pulmonary hypertension [25,107,108]. Although hypoventilation is involved in the development of hypoxemia,

the predominant mechanism seems indeed to be caused by etorphine-mediated effects on pulmonary blood pressure [109]. Etorphine-induced pulmonary hypertension can alter the pulmonary blood flow and cause congestion or interstitial oedema at the alveolar-capillary membrane, or decrease the time of blood passage through pulmonary vasculature, thus hindering gas exchange across the membrane and causing oxygen diffusion impairment and ventilation-perfusion mismatch [107,109]. However, etorphine's effect on pulmonary vascular resistance and pressure is independent from its effect on systemic blood pressure [92,109,110]. In domestic goats, the systemic blood pressure, heart rate and cardiac output decrease after intramuscular etorphine administration, whereas in boma-habituated white rhinoceroses, heart rate and cardiac output increase in alignment with pulmonary arterial pressure after etorphine is administered intravenously [108,109].

If the severe opioid-mediated hypoxemia, hypercapnia and acidosis are not treated, this can result in cell and organ damage, cardiac arrhythmias and increase the risk of capture myopathy, which can lead to acute or delayed death. When insufficient oxygen delivery and inadequate oxygen levels in the body occur, hypoxemia can result in hypoxia. Tissue hypoxia rapidly leads to cell damage and can result in brain cell death and multi organ damage, including impaired myocardial contractility [26].

Normally in case of hypercapnia, the increase in $PaCO_2$ stimulates the respiratory centers to regulate its levels in the body. Indeed the occurrence of ventilation perfusion mismatching should not result in hypercapnia physiologically, but the hypoventilation resulting from reduced central respiratory drive and decrease in the sensitivity of chemoreceptors mediated by the opioids can lead to a dangerous increase in carbon dioxide [104,107]. Hypercapnia might lead to respiratory acidosis, or complicate the metabolic acidosis turning it into a mixed acidosis, and result in consequent acidemia, and hemodynamic instability, tachyarrhythmia and impaired diaphragmatic contractility [26,77].

Thermoregulation is also impacted by opioids, which can further affect this severe homeostatic derangement [35]. Indeed since the mechanism of ventilation and gas exchanges is closely connected with acid-base and electrolyte status, the alteration of the metabolic demand, respiratory function and body temperature can alter this balance and result in further myocardial dysfunction, multi-organ failure or capture myopathy [111].

As a result of the complex physiological alterations resulting from the use of potent opioids, and that further complicate the significant physiological alterations resulting from the capture-related stress, it is not surprising that mortality and severe morbidity has been associated with etorphine-based immobilizations.

1.4 ADDITIONAL CONSIDERATIONS FOR THE PHYSIOLOGICAL EFFECTS OF IMMOBILIZATION IN LARGE-SIZED HERBIVORES

Large-sized herbivores have unique anatomical, physiological and behavioural characteristics that can further expose them to morbidity when chemically immobilized.

Megaherbivores, and large mesoherbivores like the African buffalo, are less vulnerable to predation compared to smaller ungulates. This might expose them to a higher risk of capture myopathy, since they might be less adapted to intense exercise due to not being regularly chased by predators, as reported with other herbivores located in predator-free areas [76,88].

To maximize food absorption, large-sized herbivores have evolved with a massive digestive system, which is represented by the large colon in the monogastric species (e.g. black rhinoceros) or by the rumen apparatus in ruminants (e.g. giraffe and buffalo). Change in posture from standing to lateral or sternal recumbency causes their digestive system to push forward onto the diaphragm. Furthermore, the expansion of the thorax during inspiration is also reduced on the dependent side. As a result, lung volumes are reduced, impacting the alveolar ventilation [26,34], furthermore worsening the already small tidal volumes observed during etorphine immobilizations [104].

In large-sized herbivores, changes in posture also significantly affects the dynamic between blood flow and ventilation in the lungs, resulting in dependent areas of the lungs being well perfused but less well ventilated, and vice versa. When ventilation-perfusion mismatch occur, it impacts gas-exchange and results in hypoxemia and hypercapnia [34].

A number of studies have been conducted on the physiologic response to immobilization in white rhinoceroses compared to black rhinoceroses or any other large-sized herbivore. In this species it was discovered that several alterations occur because of a combination of drug effects and alterations due to recumbency. In white rhinoceroses immobilized in lateral recumbency, it has been observed that the dependent lung is minimally ventilated and perfused, although it remains aerated with minimal detectable lung collapse. Due to the decrease in PaO_2 in the dependent lung, the hypoxic pulmonary vasoconstriction reflex produces a shift of blood to the hilum of the non-dependent lung, thus forcing the blood volume through a diminished vascular bed. The resulting increase in the pulmonary pressure, furthermore significantly influenced by a greater cardiac output, and also worsened by the initial etorphine-induced vasoconstriction that decreased the perfusion in the peripheral parts of the non-dependent lung, results in the recruitment of intrapulmonary arteriovenous anastomoses, thus leading to an increase in venous admixture. As such, unless the perfusion ventilation ratio is improved, gas exchange will be heavily affected in this recumbency [105].

It is also well-documented that shunting occurs especially in lateral recumbency in large mammals like the horses, and has been observed in rhinoceros [112–114]. When severe shunts occur, such as in a fraction of shunting above 50%, it represents a significant physiological complication, since even the supplementation of oxygen is not effective in ameliorating the hypoxemia [26,112,115].

In a study conducted on black rhinoceroses, a greater dead space ventilation, linked with an increased alveolar dead space, and a decrease in PaO_2 has been observed in lateral recumbency compared to sternal, and assumed to be the cause of greater hypoxemia in this position [104]. An increase in alveolar dead space can be caused by a decrease in overall pulmonary blood flow such as in case of decreased cardiac output, redistribution of pulmonary blood flow away from ventilated alveoli, and regional changes in chest wall movement, which all can be enhanced by both etorphine and lateral recumbency. Although the rhinoceroses seem to ventilate better in this position, dead space ventilation occurs and, since it does not contribute to gas exchanges, it is a wasted effort and results in hypoxemia [104].

In ruminants and in the rhinoceros, the sternal recumbency seems to guarantee less ventilatory and perfusion alterations, and it is therefore recommended when possible [34,104,106,116]. On the other hand, it seems that the lateral recumbency facilitates lactate clearance in black rhinoceroses, and the alveolar dead ventilation might play a role in heat dissipation [104].

Sternal decubitus is also recommended to decrease ruminal bloating, and when possible the head must be positioned with the nose sloping downwards to decrease the risk of inhalation in case regurgitation occurs, as it might lead to fatal aspiration [34]. However, not only the difference between sternal and lateral recumbency may be important in the respiratory and perfusion dynamic [104–106,116], but in those ruminants like giraffes where the sternal recumbency is not possible, the difference between right and left decubitus during immobilization might have important effects on cardiorespiratory physiology, as well as on the occurrence of bloat and regurgitation.

Further consideration must be made for giraffes. Due to the distance between their heart and their head, giraffes have developed a higher blood pressure compared to other mammals, with normal levels at the heart level of 200 mmHg. The arterial pressure near the head is about 100 mmHg, similar to other mammals, whereas in dependent areas, such as the legs, is might exceed 400 mmHg, suggesting that the high mean arterial pressure is a necessity to perfuse the brain [117–119]. Various anatomic adaptations have evolved in giraffes to adapt to the extreme blood pressure [119–124]. In particular, the giraffe kidney has developed a thick capsule and an extreme interstitial pressure [121]. Since recumbency, through a change in gravity forces, can significantly affect giraffe blood pressure regulation, when under anesthesia a minimal MAP of 120 mmHg at heart level is required in order to allow renal function [119].

When giraffes are fully anesthetized, the head is generally elevated on a board, in order to reduce aspiration risk, but also as this might help the regulation of blood pressure [125]. Indeed mean blood pressure and cardiac output are significantly influenced by the position of the head in giraffes, and when the head is raised, blood returns from the jugular to the circulation restoring the cardiac output [123]. In the field, where giraffes are often antagonized as soon as they reach the ground, and then only

manually restrained, the neck and head are pushed on the ground to prevent them from standing up [125,126]. This process might further affect their blood pressure and their delicate cardio-respiratory physiology, which is already compromised by the intense physical activity that occur before the recumbency, even if they are awake. Indeed, because of their unique cardio-vascular adaptations, including a thick left ventricular wall to generate high blood pressure, the giraffe heart might be prone to injury from oxygen debt during hypoxemia [127].

Figure 3. A reticulated giraffe being roped to reach recumbency. Giraffe's capture is characterized by an abrupt passage from intense physical activity to recumbency, which can significantly alter giraffe's physiology. (Photographer: Francesca Vitali)

Furthermore, giraffes have small lungs characterized by low compliance, and a long and narrow trachea that might impose a high respiratory resistance during or after physical exercise [128]. Investigation on the physiological response of giraffes to the practice of early antagonization has not been performed, and elucidations are urgently needed to understand the potential benefits, or risks, of these techniques regarding, other than their welfare, the physiological stability.

The unique physiological mechanism that has evolved as result of their immense size requires particular attention when immobilizing large-sized herbivores. On top of these physiological challenges, large-size herbivores can be particularly dangerous due to their fractious temperament, and the development of a protocol that guarantees safe inductions, handling and recoveries is even more required in these species.

1.5 CLINICAL MONITORING OF THE PHYSIOLOGICAL EFFECTS OF IMMOBILIZATION

The physiological changes that result from capture stress and overexertion, anesthetic drugs adverse effects, and recumbency position, compromise the animal homeostasis. Hypoxemia, hypercapnia, acidosis and hyperthermia, together with cardio-respiratory and metabolic alterations commonly occur and can lead to severe short- or longer-term consequences. These clinical conditions are not always treated or even detected, as appropriate diagnostic systems such as blood gas analysis, and clinical monitoring devices might not be always available in the field [26].

Anesthetic crises characterized by a rapid onset can easily occur and can be devastating. The use of continuous monitoring devices is a standard practice during human and domestic animal anesthesia, where multiparametric monitors provide real time data on physiological function. The early detection of alterations allows anticipation of crises and intervention to prevent or correct them. In the field, where supportive ventilatory devices are not available in most cases, and rescue maneuvers are limited by the size of the patients, the maintenance of a stable cardio-respiratory function and the early detection and prevention of these crises is even more important.

Although in recent years the availability of portable monitoring tools has increased, their use is rare in the field, or is based on the extrapolation of techniques and range values from other species, since these have not been evaluated in most species. More frequently, the clinical monitoring is only restricted to the empirical observation of the animal physiological function, which can be misleading. For example in large animals an apparent adequate thorax excursion might not reflect optimal gas exchange, as dead space ventilation might occur [104].

Clinical continuous monitoring during chemical immobilization must be improved for many wildlife species in order to be able to recognize potentially harmful cardio-respiratory changes and to proactively intervene in time [26]. A deep investigation on gas-exchanges, acid-base and electrolyte status through point of care analyzers must be undertaken to understand the mechanism of complications and allow us to develop an understanding of how to modify drug protocols to reduce the risk of morbidity and mortality. Furthermore, ideally the use of blood gas analyses should be adopted as a clinical monitoring tool when enough personnel is available.

1.5.1 Continuous monitoring

Basic monitoring of chemical restraint should include the monitoring of anesthesia depth, assessment of circulation and temperature, and adequacy of ventilation and oxygenation [129].

Depth of anesthesia should be closely monitored throughout the procedure in order to assess if the plane of immobilization gets too light or excessively deep. Factors that increase the risk of sudden arousal include loud noises, movement of the animal or changes in the body position, or painful stimuli.

To reduce the visual and noise stimulation, the eyes should be covered and plugs inserted in the ears [34]. The assessment of the level of anesthesia depth can be obtained by observing the pattern of ventilation, eyeball position, reflexes, muscle tone and response to surgical stimulation [129]. Muscle relaxation is poor during potent opioid immobilization, and since it is associated with tremors, higher metabolism, and oxygen consumption, its monitoring and the addition of drugs with muscle relaxant properties should be adopted. Scores have been used in a variety of species to assess the immobilization level, and can be useful to quantify the observation and make them comparable between different individuals and treatments [130–133].

The role of hyperthermia in the genesis of capture stress and in the risk of developing capture myopathy is well described [41,78,82,88,134]. Furthermore, several capture drugs, including opioids can impair thermoregulation [34,35]. If hyperthermia occurs, measures have to be undertaken in order to limit its effects, and when values in ungulates reach 41 °C, it should raise immediate concern [32]. In larger herbivores it is important to increase measurement accuracy, to collect the temperature from a deeper reach within the rectal ampulla since the presence of feces or relaxation of the sphincter might create a bias due to the external temperature. Modification of food thermometers characterized by a longer probe has been used and validated in rhinoceroses (Leith Meyer, pers. Comm., 2019).

Cardiovascular monitoring includes heart rate, pulse rhythm, blood pressure and tissue perfusion. Monitoring of the cardiovascular system is vital in preventing complications since capture stress and capture drugs can cause both bradycardia and tachycardia, and the occurrence of tachycardia can be a symptom of cardiovascular shock and hyperacute capture myopathy [47,69,94,110]. The heart rate is determined by auscultation of the heart using a stethoscope, which can also facilitate the identification of irregular heartbeats and murmurs. The peripheral pulse rate, rhythm and strength may be counted by palpation of a peripheral arterial pulse, or measured with a pulse oximeter [129,135].

Opioids, as well as other drugs used for chemical immobilization, can significantly influence the blood pressure and results in either hyper- or hypotension. Excessive blood pressure can result in lung and brain oedema and retinal damage, and is associated with an increased metabolism. Low blood pressure will instead lead to inadequate tissue perfusion, hypoxia and anaerobic metabolism. Non-invasive methods to measure blood pressure are considered inaccurate in some large herbivores, although have been widely used [119,125,136,137]. Invasive blood pressure is the gold standard and can be obtained through the use of an intra-arterial catheter and an anesthetic monitor. As an alternative, an aneroid manometer is an unexpensive and field-friendly method to continuously measure mean blood pressure [129].

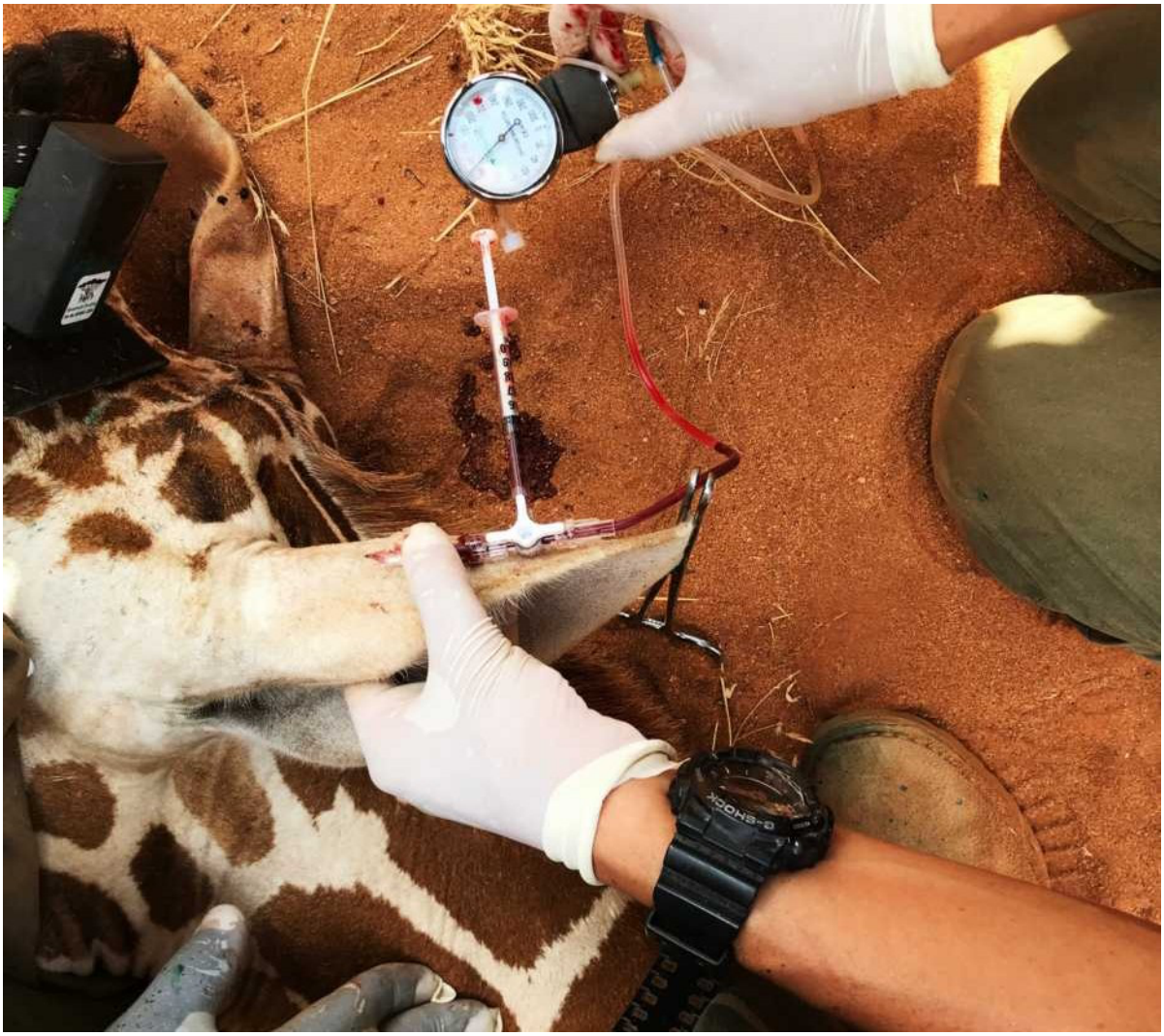

Figure 4. An aneroid manometer used to monitor invasive blood pressure in a reticulated giraffe. Through the integration of a three-way stopcock it is possible to simultaneously withdraw arterial samples for BGA. (Photographer: Francesca Vitali)

Adequacy of ventilation can be monitored through visual observation of respiratory rate and depth of breathing, and observation of chest movement. Clinical signs of hypoxemia are unspecific and include dyspnea, cyanosis, tachycardia, and arrhythmias. The color of mucous membranes can be evaluated to asses tissue oxygenation and the occurrence of cyanosis [26]. Cyanosis in an important sign of hypoxemia, but can be misleading since it can be altered by the use of drugs such as α2- agonists that cause peripheral vasoconstriction, or might be absent in anemic but hypoxemic individuals [26,135]. Furthermore, a normal respiratory rate does not reflect adequate ventilation, and even an increase in respiratory rate might not compensate for a decrease in tidal volume, resulting in hypercapnia. The fact that hypoxemia and hypercapnia, and acidosis, are clinically silent, and as a result undetected and untreated, demands for the use of specific monitoring equipment [26].

Pulse oximetry is a non-invasive method for continuous measurement of hemoglobin oxygen saturation (SpO_2). The principles of measurement are based on the different light absorption spectra of

oxyhemoglobin and reduced hemoglobin, and the detection of a pulsatile signal. A pulse oximeter detects inadequate blood oxygenation, which should be taken as an indication to supplement the animal's inspired oxygen concentration and to search for the cause [135]. The SpO_2 should be higher than 95% for an animal to be considered within normal limits. A SpO_2 value of less than 90% (which corresponds to a PaO_2 of 60 mmHg at sea level according to the hemoglobin oxygen saturation curve) indicates severe hypoxemia and could indicate hypoventilation, intrapulmonary alterations such as diffusion impairment of oxygen and V/Q mismatch, or increased use of oxygen due to an increase in metabolism [26,112].

Although pulse-oximeters have been largely used in wildlife immobilizations, these devices have different limitations. Indeed pulse oximeters are calibrated for use in humans and, as a result, absolute accuracy of any given saturation value cannot be guaranteed [135]. Limitations often affecting the use of pulse oximetry in large animals are due to the thickness of tissue placed within the sensor, dark skin pigmentation, the presence of hair, and movement of the patient [138]. These factors can be responsible for the oximeter failing to measure oxygen saturation as the light penetration is more difficult [135,139]. Ambient light may also interfere with probe function. It may be challenging to obtain a reading from a pulse oximeter in case of hypotension, peripheral vasoconstriction (for example after administration of α2-agonists) or in case of circulatory shock. The pulse oximeter will not accurately reflect blood oxygen content in patients with carbon monoxide or methemoglobin poisoning, or in case of hypoxic anemic individuals with low hemoglobin concentration, the pulse oximeter may falsely display normal values for SpO_2 [135]. Accuracy of pulse oximeters seems greater when oxygen hemoglobin saturations are high (>90%), and only in precise sampling sites, therefore its readings must be interpreted with caution, and used in conjunction with additional monitoring equipment such as capnometers and blood gas analysis [139].

Capnometry is based on monitoring equipment routinely used, in human and veterinary anesthesia, to evaluate continuously end tidal carbon dioxide ($ETCO_2$) and respiratory rate. $ETCO_2$ indicates if ventilation, and thus the gas exchange in the lungs, is adequate. Elevated levels indicate hypercapnia due to hypoventilation or ventilation-perfusion mismatching, whereas lower values indicates hyperventilation, dead space ventilation or a cardio-vascular depression [104,129,138].

The difference between PaO_2 and $ETCO_2$ is physiologically considered to be 2 – 5 mmHg [140,141]. In human medicine, a reduced accuracy is usually due to the capnometer underestimating the CO_2 level, especially in conditions such as metabolic and respiratory acidosis, or due to dilution with ambient air [142]. The difference between $ETCO_2$ and the arterial value depends on patient size, posture, and dead space [119,140]. Reversal of the normally positive $PaCO_2$-$ETCO_2$ gradient has been reported in human and domestic animals [140,141]. These are normally attributed to conditions where dead space and ventilation-perfusion mismatch are minimal such as in children, or to late emptying of well-perfused alveoli with higher carbon dioxide tensions, or to reduced functional residual capacity as in pregnant or obese patients [141,143].

Although capnometers are usually attached to endotracheal tubes, the use of nasal capnometry has been spreading in human medicine as has the advantage of being less invasive. Nasal capnometry

is routinely used in human medicine to measure carbon dioxide of sedated, non-intubated patients [144]. In veterinary medicine capnometry is routinely used associated with endotracheal intubation, whereas the use of nasal capnometry, especially in wildlife practice, has been reported only in few studies and its accuracy has not been evaluated [104,145]. Although in large animals such as horses and giraffes with a large dead space, accuracy of capnometry associated to endotracheal intubation is normally poor in predicting the absolute values of arterial carbon dioxide [119,140], the use of nasal capnometry as a clinical monitoring tool is yet to be evaluated in large-sized herbivores. Nasal capnometry might be a non-invasive and simple way to monitor trends of $ETCO_2$ in the field, and might be useful and more sensitive compared to pulse oximeter use in detecting ventilatory alterations.

Figure 5. A mainstream capnometer (Masimo EMMA Capnometer 9632, Masimo Corporation, California, United States) attached to a nasal tube in a young Eastern black rhinoceros (left) and in an African buffalo (right). (Photographer: Francesca Vitali)

1.5.2 Point of care field analyzers

Arterial blood gas analysis is considered the gold standard for the assessment of the respiratory and metabolic status [138]. Point of care blood gas analyzers have become widely used in the study of wildlife immobilization since these provide, in just a few minutes, measures of pH, partial pressure of oxygen (PaO_2) and of carbon dioxide ($PaCO_2$). PaO_2 and $PaCO_2$ are more sensitive indicators of hypoxemia and hypercapnia compared to pulse oximetry and capnometry [146]. Other variables such

as bicarbonate (HCO_3^-), total carbon dioxide (TCO_2), base excess (BE) and oxygen saturation (SaO_2) are calculated by the analyzer. Many analyzers also provide measures of lactates, electrolytes and selected biochemistry and hematology, and calculation of the alveolar-arterial gradient (P(A-a)O2).

The measurement of PaO_2 and $PaCO_2$ are key factors to evaluate the gas exchanges in the lungs, whereas pH, lactate, bicarbonate, base excess and electrolytes are essential for the interpretation of the acid-base status [26,138]. Lactate is a marker of tissue oxygenation and perfusion, and of anaerobic metabolism and stress induced glycolysis [68,147]. Blood gases and acid-base status are essential for evaluation of the physiological effects that different capture methods and drugs have on wild animals [26]. $P(A\text{-}a)O_2$ indicates the integrity of alveolocapillary membrane and the effectiveness of gas exchange. In case of alteration of the membrane, such as in case of ventilation perfusion mismatch, diffusion limitation and shunt, the gradient is increased, whereas it is normal in case of hypoventilation. The gradient is therefore helpful in the evaluation of the mechanism of hypoxemia [115].

Similar values of blood gases are usually described across mammals, although caution is advised as some difference may exist [146]. For example, white rhinoceroses have range values of carbon dioxide higher than most mammals, between 44 and 53 mmHg [148]. Furthermore, the values of SaO_2 might not be accurate since they are calculated from an algorithm based on a human oxygen dissociation curve (ODC). In white rhinoceros , since the oxygen dissociation curve is left shifted, an algorithm was created to correct the SaO_2 in this species [149,150], whereas the same evaluation has not been performed in other large-sized herbivores. Similarly, since the ODC is influenced by the body temperature, but the algorithms to correct pH, PaO_2 and $PaCO_2$ are based on human physiology, the accuracy of these corrections in wildlife species has not been evaluated, therefore temperature-corrected values should be interpreted with caution.

Pre-analytic errors are not uncommon with blood gas analyzers, and are mainly due to inaccurate sample collection, such as the presence of air bubbles in the syringe that can affect the values of gases. Furthermore if the sample is not analyzed immediately, it should be stored in ice and analyzed within 30 minutes to maintain accurate measurements of the blood gases, or within one hour for the biochemical analytes [138].

The advent of portable blood gas analyzers has allowed us to greatly advance the monitoring of chemical immobilization and its safety. Even though sometimes in the field it is not possible to immediately analyze the blood gases due to quick procedures and lack of personnel, this analysis still represents an important tool to advance research for safer immobilization.

Changes in the levels of blood gases and acid-base values, electrolytes and biochemical analytes, when retrospectively correlated to capture techniques, can help in understanding the mechanism of physiological alterations occurring as a result of capture stress and drugs [43,72,151]. When these same values are correlated with alterations in basic monitoring of physiological parameters, they can help to determine cut-off in basic clinical values in order to recognize clinically important alterations [119].

1.6 CLINICAL PHARMACOLOGY

Although wildlife immobilization has an elevated intrinsic risk of morbidity and mortality, the continuous research for improved species-specific protocols has allowed us to significantly reduce this risk [30]. Wildlife immobilization has indeed progressed a great deal in recent years, and new techniques continue to develop, especially in particularly sensitive species such as the white rhinoceros, but many gaps are yet to be covered in most species.

Research for improved immobilization in wildlife medicine has focused on two main objectives. Attention has been placed on the improvement of darting combinations that produce smoother and shorter inductions, and reduce cardio-respiratory side effects at the same time, with the aim to prevent the complications. Research has also been performed on the use of intra-anesthetic drugs to treat complications when the animal is already recumbent. This has included the use of partial opioid antagonists, or non-opioid respiratory stimulants, often combined with oxygen administration.

1.6.1 Synergistic drug combinations to prevent the onset of physiological alterations

Despite a recurring hazard of severe side effects, the use of very potent drugs such as opioids is essential when immobilizing large and dangerous wildlife. The first ten minutes after opioids administration, has been observed to be the time with higher mortality as a result of severe physiological alterations, therefore the prevention of complications can be more effective than their post-induction treatment [92,109]. However, if doses of opioids are lowered in order to avoid respiratory depression, this can result in excitement, longer inductions and overexertion [80].

The choice is controversial since a longer time to recumbency is related to severe stress (higher cortisol and catecholamines), tachycardia and lactic acidosis [80]. For these reasons, it is essential to conduct further research on safer protocols that are able to reduce etorphine doses, thus impacting less on the animal's physiological function from the beginning of the immobilization when higher chances of mortality occur, and without resulting in greater excitement and prolonged inductions.

To decrease the severity of complications such as respiratory depression, excitement and hypertension, it has been established that the use of "drug cocktails" can be advantageous in counteracting opioid side effects by providing opposite actions on same receptors [92]. Indeed, the combination of different anesthetic drugs decreases the required dose of each agent, thus increasing the therapeutic index of the combination. A safe therapeutic index is desirable in field capture as it allows a margin for error in estimating body weight and for individual variations in physiological response. The use of multi-drug protocols, including sedatives and tranquillizers in the dart mixture,

can be beneficial in terms of taking advantage of synergism between drugs, that allows a decrease in the overall doses, and consequently in their side effects. On the other hand, thanks to drugs synergism, the same anesthetic result can be obtained, or sometimes improved, by providing additional analgesia, anxiolysis or desirable physiological effects. Clinical studies are needed since their use could be beneficial in preventing major complications related to capture in different mammalian free-ranging species [92].

1.6.1.1 Azaperone

Azaperone is a butyrophenone derivative that binds to autonomic (adrenergic and muscarinic), serotonin and histamine receptors [152]. Azaperone is a tranquilizer and has sedative, anti-anxiety, and mild muscle relaxant, but not analgesic, effects. Through antagonism at α1-receptors in peripheral arterioles, it causes peripheral vasodilatation and reduces mean arterial blood pressure [153].

Thanks to this property, azaperone is routinely used in combination with etorphine for chemical immobilization of wildlife, since it can counterbalance opioid-mediated hypertension [43,70,154,155]. Furthermore, the synergism between azaperone and etorphine seems to be able to reduce induction times and improve the immobilization quality through additional anxiolysis and muscle relaxation [70,103,133,154,155]. Although there are reports of adverse effects in wild ungulates, such as severe hypotension and extrapyramidal effects, these are rare and thought to result from high intravenous doses in stressed animals [154,156]. Although azaperone can improve the safety of etorphine immobilization by stabilizing the physiological function, severe physiological alterations including muscle rigidity, respiratory impairment and acid-base imbalance persist with this combination in white and black rhinoceroses, where it has been largely used [104,106,116,147,157–159].

Despite etorphine-azaperone being recommended combination for most herbivore species, the study of its effects and safety has been restricted to only a few species only [34]. Therefore, the evaluation of its physiological effects on gas-exchanges, acid-base status and cardio-respiratory function is needed for the other species where it is routinely used.

1.6.1.2 A2-agonists

A2-agonists exert their central effects by stimulating presynaptic α2-receptors, thus preventing the release of norepinephrine and damping or preventing sympathetic drive from the central nervous system [160,161]. A2-agonists are among the most used drugs in veterinary medicine, as they produce reliable dose-dependent sedation, analgesia, and muscle relaxation, which can be fully reversed with selective antagonists [35,160,161]. Furthermore, thanks to their potent anesthetic sparing effect, they allow a severe reduction in the amount of other anesthetics, including opioids, required to induce and maintain anesthesia and analgesia [160–164]. These agents have been successfully used in wild herbivores, and have been shown to improve muscle relaxation, decrease the amount of opioid required

and ease induction and recoveries [136,165,166]. Thanks to their anxiolytic effect, α2-agonists might also be useful in attenuating the stress response, by influencing the endocrine system [160].

These drugs exhibit agonism also at postsynaptic α2-adrenoceptors, often exerting a stimulating action similar to that exerted by α1-adrenoceptors at the same site. Peripheral postsynaptic α2-receptors are spread widely in many tissues explaining many of the actions of α2-agonists, including cardio-respiratory effects.

The use of a α2-agonists is indeed not universally supported because of concerns relating to the respiratory depression and potent cardiovascular effects of α2-agonist drugs, such as the significant bradycardia [160,161,167]. Hypoxemia is commonly observed in response to administration of α2-agonists in mostly ruminants, and is believed to occur mainly through an inflammatory reaction mediated by pulmonary intravascular macrophages specific for this suborder [167]. These adverse effects are however typically dose related, and mostly associated with less selective α2-agonist drugs (e.g. xylazine), and the intravenous route [160,161,167].

Medetomidine is highly selective for the α2-receptor, with an α2:α1 binding ratio of 1620:1, compared to the 160:1 of xylazine. Higher selectivity results in the occurrence of lesser side effects, which are mostly mediated by the side action of α2-agonists on α1-receptor [35,160].

The addition of medetomidine to the drug mixture, might provide benefits in terms of additional sedation, muscle relaxation and analgesia, limiting anxiety and its pathophysiological alteration, and perhaps also preventing post-capture chronic stress. Furthermore, medetomidine might allow a decreased dose of opioids, and in turn, their adverse effects, without impacting the speed of inductions or safety of the restraints. Medetomidine can be safely reversed with atipamezole, which is the antagonist with the highest α2-receptor selectivity, and is the preferred antagonist for reversal of medetomidine [160].

However, medetomidine in association with etorphine and azaperone has been used only in one study among large-sized herbivore species [168], and a throughout evaluation of its physiologic effects including gas-exchanges, safety, and potential benefits remains to be investigated.

1.6.2 Intra-anesthetic treatment to reduce the impact of physiological alterations

If hypoxemia and hypercapnia resulting from opioid-mediated respiratory depression are not successfully prevented, strategies to reduce their intensity must be adopted during the immobilization, in order to reduce morbidity and mortality risk.

Mechanical ventilation combined with oxygen insufflation might be able to solve the respiratory depression in most cases, however ventilatory support is often not available in the field [135]. On the other hand, intranasal oxygen administration is routinely administered to immobilized wildlife, but its efficacy depends on several factors including the type of hypoxemia [115,135]. Some drugs have

pharmacological properties that can improve gas-exchange. These drugs are opioid partial antagonists, such as butorphanol, that reduce opioid adverse effects by partially antagonizing their actions, or respiratory stimulants, like doxapram, which act on non-opioid receptor systems [27,34,92].

Even if it is commonly believed that reducing the length of immobilization might reduce the risk of morbidity and mortality, this is not true. Indeed hyperacute mortality or alterations that lead to a delayed death can still occur [169]. Therefore, the prompt treatment of hypoxemia and hypercapnia is also essential in short field immobilization.

1.6.2.1 Oxygen

Oxygen supplementation has been recommended during immobilization of wildlife for several large species [26,103,106,170]. Intranasal oxygen administration increases arterial blood oxygenation and can correct or limit hypoxia, and might also be helpful at reducing metabolic acidosis in hyperthermic and stressed animals. In these animals, oxygen supplementation can meet the increased oxygen requirements and protect the brain against hyperthermic damage [171].

Oxygen therapy is usually effective in case of hypoventilation, ventilation-perfusion mismatch and oxygen diffusion impairment, but not in the case of shunts [115]. However, administration of oxygen is not able to correct respiratory acidosis, but might reduce hypoxic respiratory drive, resulting in hypoventilation and worse respiratory acidosis [69].

Indeed in white rhinoceros it has been observed that blood carbon dioxide rises when high inspired oxygen is administered, whereas oxygenation does not improve, but it worsens the already compromised animal's respiratory status [112]. Proposed explanatory mechanism are, other than hypoventilation, a worsening of ventilation-perfusion mismatch caused by absorption atelectasis, hypoxic vasoconstriction that results in an increased alveolar dead space, or displacement of carbon dioxide from hemoglobin into the plasma (Haldane effect) as a result of increased arterial oxygenation [107,112,157,172]. In white rhinoceroses it has instead been observed that when oxygen is combined with a partial opioid antagonist, hypoxemia is treated or improved, and carbon dioxide decreases slightly [107,112,157,172].

Since intranasal oxygen is commonly administered to immobilized wildlife, more research should be performed also in other species to evaluate if its use is beneficial, and to investigate which flows of oxygen are adequate.

1.6.2.2 Butorphanol

Butorphanol is a mixed action opioid drug that displays antagonism at μ-opioid receptor, and agonism at k-receptor. Butorphanol provides sedation, mild analgesia and has minimal effects of cardiopulmonary function [35].

Butorphanol has been widely use in wildlife immobilization, other than its use as a sedative, for partial antagonism of potent opioids, as it improves hypoxemia in white rhinoceroses [112,133,157,172,173]. It was recently demonstrated that improvement in arterial oxygenation is not

caused in this species by an increase in respiratory minute ventilation, as previously thought, but is primarily due to a decrease of oxygen consumption associated with decreased muscle tremors [107]. Tremors can significantly increase metabolism and oxygen consumption, which can be deleterious during the anesthesia of animals already compromised due to over-exertion. In white rhinoceroses tremors are associated with increased plasma catecholamines concentrations [25], and it seems that butorphanol reduces tremors by antagonizing etorphine-induced sympathetic nervous system activity [107]. In black rhinoceros and other ungulate species, butorphanol is often administered, combined with oxygen, to improve hypoxemia, but there is no scientific evidence of its benefits in these species.

When butorphanol is given at higher doses, it partially antagonizes the μ-opioid receptor effects of the etorphine, creating a partial antagonization, which can result in undesired arousal. This principle is used to produce a state of sedation and "walk" white rhinoceros into transport crates [54,174]. It is likely that etorphine cardio-respiratory adverse effects are displaced along the μ-mediated sedative and analgesic effects when butorphanol is given at higher doses.

1.6.2.3 Doxapram

Doxapram is a potassium channel blocker used to stimulate ventilation through its action on the carotid bodies [175]. Doxapram has been largely used in veterinary medicine to transiently increase respiratory rate and tidal volume, as it diminishes the magnitude of opioid-induced hypoventilation across a range of species [35,103,158].

In the field of wildlife medicine only a few studies have assessed the effect of doxapram on gas exchanges, and most evaluations are based on empirical observations, such as in the black rhinoceros where transient respiratory effects of doxapram have been observed to last for 10-15 minutes [176]. In white rhinoceroses, improvement in arterial oxygenation has been recorded when used in association with oxygen insufflation and a partial opioid antagonist, nalorphine, but no improvement in gas-exchange has been observed when doxapram is given alone [158,177]. On the other hand, when used in constant rate infusion (CRI), doxapram produces a decrease in $PaCO_2$ and an increase in pH in anesthetized humans and ponies [178,179]. In anesthetized dogs, low dose of CRI doxapram improved PaO_2, whereas $PaCO_2$ was decreased by higher doses [180].

Besides its activity on ventilation, it is not clear if doxapram is able to act on intrapulmonary factors such as on the ventilation perfusion ratio. Doxapram increases cardiac output in hypotensive individuals [181], but only marginally in normotensive individuals. The mechanism whereby doxapram increases blood pressure and cardiac output is unknown but may be related to increased circulating catecholamines and not direct vasoconstriction [181], whereas the ability of doxapram to restore hypoxic pulmonary vasoconstriction, a reflex to re-direct blood to more ventilated lung areas and that reduces ventilation perfusion mismatch, is controversial [182].

Side effects have been linked with the use of high doses of doxapram, such as the insurgence of muscle tremor in rhinoceroses with doses of 100 – 200 mg, or rapid arousals and recoveries, which might result from doxapram being a central nervous system stimulant [109,159].

1.6.2.4 Opioid antagonists

A unique immobilization technique, characterized by early opioid antagonism, has been routinely used in the last decades to restrain giraffes.

Giraffes are particularly susceptible to developing opioid-induced excitement, thus in the attempt to reduce the risk of overexertion, high doses of opioids are usually administered to knock them down quickly [34,125,126,183]. On the other hand, giraffes are also particularly sensitive to opioid respiratory depression, therefore as soon as they reach the ground, an opioid antagonist is administered to reverse etorphine adverse effects. Although this practice reduce the risk of gas exchange impairment, it leaves them fully awake, and exposed to manipulation stress, which can turn into excitement and risk of injuries [125,126].

The agents used to antagonize etorphine are naltrexone, a long-acting pure μ-antagonist or diprenorphine, a partial agonist-antagonist [34,35]. Diprenorphine is often preferred over naltrexone for giraffe restraint since the pure antagonistic effect of naltrexone induce complete reversal of the opioid immobilization and, as such, giraffes have been considered too excited to be safely manipulated such as in case of translocations. On the other hand, diprenorphine poses a greater risk of renarcotization [184].

Doses of naltrexone up to 100 mg/mg etorphine have been traditionally used in wildlife immobilization, while, more recently, between 5 and 25 mg naltrexone per milligram of etorphine have been reported to be effective without apparent renarcotization [34,126,184]. A low dose of naltrexone might therefore be effective in antagonizing etorphine adverse effects without resulting in excessive excitement, or increasing the renarcotization risk, and its use needs to be evaluated in this species.

1.7 ADVANCING IMMOBILIZATION SAFETY THROUGH A RESEARCH OPPORTUNISTIC APPROACH

An increasing number of captures are being performed on wildlife for conservation-related purposes, such as translocations, application of GPS units, or for epidemiological studies [21]. These capture events represent a unique occasion to opportunistically collect samples and precious information on endangered species to understand how to improve the safety of their immobilization. Furthermore, these can provide an opportunity to compare different protocols with marginal bias, since the animals are often captured in the same location and period. Indeed protocol variation often occurs within a capture operation, dictated by clinical needs, or in the attempt to ameliorate the immobilization safety after the use of advanced monitoring devices highlights the presence of underlying complications that have gone unnoticed until that moment [36].

Another advantage of performing anesthetic studies in an opportunistic manner is that it would not be ethical to capture wildlife, often endangered, if not within a bigger conservation mission.

On the other hand, this approach poses limitations regarding the logistics, the duration of the immobilization that are often very quick, and does not control for a variety of unpredictable factors that occur in field conditions. Even though controlled conditions obtained in studies conducted in captivity have allowed to perform high-end studies that elucidated unique physiological mechanisms of large-sized herbivores [105,123], field research provides the opportunity to tackle complications usually faced in the fields, and is therefore equally important for the advancement of wildlife immobilization techniques.

Opportunistic approaches are commonly embraced in wildlife immobilization studies [25,54,55], and are a valid tool also for other disciplines of wildlife research [185,186].

The opportunistic observation of the alterations that occur during the immobilization of large-sized herbivores can thus help elucidate the pathophysiological mechanisms that threaten the safety of their capture. The retrospective analysis of the incidence of acute and delayed complications and mortality in relation to physiological alterations, capture methods and environmental factors can be a valuable method to understand species-specific factors of risk that have to be considered to prevent complications when capturing these species, and might allow to identify potential improvements resulting from different drug doses and protocols used.

Furthermore, through the opportunistic collection of samples and clinical monitoring, the gap of knowledge on species-specific physiological reference values, such as blood-tested parameters and cardiorespiratory dynamics, can be finally refined.

2 AIM OF THE THESIS

The general objective of this thesis is to advance the knowledge of the physiological mechanism of capture morbidity in East African large-sized herbivores, with the aim of developing new strategies to improve the safety of their capture. A species-specific approach was used to investigate on predisposing factors for severe physiological derangement and its prevention using innovative anesthetic protocols, and on improving techniques for intra-anesthetic monitoring of the physiological function and the pharmacological treatment of physiological alterations.

This thesis focuses on selected species of large-sized herbivores; the megaherbivores giraffe (*Giraffa camelopardalis ssp. tippelskirchi and reticulata*) and black rhinoceros (*Diceros bicornis ssp. michaeli*), and the mesoherbivore African buffalo (*Syncerus caffer*). These species share common characteristics, such as their susceptibility to capture stress and myopathy, opioid adverse effects, and alterations due to changes in recumbency, in addition to the challenges faced in the field as a result of their size and fractious temperament.

The selection of species was made according to results of my preliminary studies conducted during my PhD and field observations on a wide variety of wildlife species, which showed major capture morbidity in these three species. Furthermore, limited research had been previously conducted on pathophysiological response to capture of these species. This lack of data prevented the establishment of targeted strategies to improve immobilization safety in these species. The research design in my thesis was shaped for each of the species based on their specific need, and within an opportunistic approach for collection of data, but a common objective to all studies was to unveil the major, species-specific complications that occur when immobilized with etorphine-azaperone combination, which is a protocol commonly used in the field to capture these species.

The following specific aims were investigated:

1. In Masai giraffes, the early antagonization of etorphine-azaperone combination with a low naltrexone dose was evaluated for physiological and handling safety during a translocation. In addition, the role of startle response and induction-induced excitement in affecting the physiological derangement was investigated in order to improve the understanding of the mechanism of capture morbidity in giraffes.
2. Building up on the results of the first preliminary study performed in Masai giraffe, the physiological mechanism of capture morbidity occurring in both vehicle and helicopter darted immobilized reticulated giraffe was researched in order to detect the predisposing factors for homeostatic alterations and to define and guide prevention strategies. The evaluation of the effects of early antagonization on the physiological function was also pursued through the evaluation of trends over time in blood gases, selected biochemistry variables and cardio-respiratory function, and the use of a non-invasive nasal capnometer to allow early detection of complications.
3. In Eastern black rhinoceroses immobilized with a combination of etorphine and azaperone, the respiratory gas exchange and acid-base status were evaluated, and the efficacy between two post-induction treatments, butorphanol and oxygen, or butorphanol, doxapram and oxygen, in improving physiologic alterations was compared. In addition, the mechanism of pathophysiological response to capture stress in Eastern black rhinoceroses, and the accuracy of a nasal capnometer were investigated to help identify potential ways to reduce and allow early detection of complications.
4. Etorphine-medetomidine-azaperone and etorphine-azaperone immobilization protocols were compared in free-ranging African buffalos with the aim to evaluate if the addition of ultralow doses of medetomidine could decrease capture morbidity and improve physiological and handling safety. Furthermore, since this was the first study to evaluate gas-exchange and acid-base function in this species, a secondary aim was to provide further advancement on the understanding of the mechanism of capture morbidity.

The ultimate aim of this thesis is that the new detailed species-specific knowledge, and the resulting strategies to reduce capture morbidity, will not only improve the short-term safety during immobilization, but also indirectly maximize the longer-term outcome of conservation actions, therefor contributing to the advancement of the field of conservation medicine and biodiversity conservation.

3 RESEARCH STUDIES

RESEARCH STUDY I

ETORPHINE-AZAPERONE IMMOBILIZATION FOR TRANSLOCATION OF FREE-RANGING MASAI GIRAFFES (*GIRAFFA CAMELOPARDALIS TIPPELSKIRCHI*): A PILOT STUDY

This chapter has been published as a research paper with the journal Animals. The research paper has been adjusted to this thesis format.

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Vitali, F.; Kariuki, E.K.; Mijele, D.; Kaitho, T.; Faustini, M.; Preziosi, R.; Gakuya, F.; Ravasio, G. Etorphine-Azaperone Immobilization for Translocation of Free-Ranging Masai Giraffes (*Giraffa Camelopardalis Tippelskirchi*): A Pilot Study. *Animals* **2020**, *10*, 322.

Simple Summary

Due to their peculiar anatomy and sensitivity to drugs, giraffes are among the most challenging mammals to immobilize. Masai giraffes have recently been listed as endangered. Hence, their conservation needs actions that require veterinary capture such as translocations. In this study, we evaluated a new protocol of immobilization for translocation of free-ranging Masai giraffes. The hypothesis is that, by combining a potent opioid with a tranquilizer, it is possible to mitigate the capture stress, which is a major cause of disastrous homeostatic consequences, including capture myopathy and death. The combination produced, in all individuals, smooth and quick inductions and reliable immobilizations. Although hypoxemia in a few individuals and acidosis were seen, the overall cardiorespiratory function was adequate. Whereas the initial stress to the capture was limited in the individuals, likely due to tourism-related habituation, the opioid-related excitement and resulting increased exertion was responsible for worse immobilization and physiological derangement. A low dose of an antagonist was used and evaluated and, in the two-week boma follow-up, it proved to be efficient in providing safe recoveries and transport. At the investigated doses, the combination provided partially reversed immobilization that allowed uneventful translocation in free-ranging Masai giraffes.

Abstract

Etorphine-azaperone immobilization was evaluated for translocation of Masai giraffes. Nine giraffes were darted with 0.012 ± 0.001 mg/kg etorphine and 0.07 ± 0.01 mg/kg azaperone. Once ataxic, giraffes were roped for recumbency and restrained manually. Naltrexone (3 mg/mg etorphine) was immediately given intravenously to reverse etorphine-related side effects. Protocol evaluation included physiological monitoring, blood-gas analyses, anesthetic times, and quality scores (1 = excellent, 4 = poor). Sedation onset and recumbency were achieved in 2.6 ± 0.8 and 5.6 ± 1.4 min. Cardio-respiratory function (HR = 70 ± 16, RR = 32 ± 8, MAP = 132 ± 16) and temperature (37.8 ± 0.5) were stable. Arterial gas analysis showed hypoxemia in some individuals (PaO_2 = 67 ± 8 mmHg) and metabolic acidosis (pH = 7.23 ± 0.05, $PaCO_2$ = 34 ± 4 mmHg, HCO_3^- = 12.9 ± 1.2 mmol/l). Minor startle response occurred, while higher induction-induced excitement correlated to longer inductions, worse restraint, and decreased HCO_3^-. After 19 ± 3.5 min of restraint, giraffes were allowed to stand and were loaded onto a chariot. Immobilizations were good and scored 2 (1–3). Inductions and recoveries were smooth and scored 1 (1–2). Translocations were uneventful and no complications occurred in 14-days boma follow-up.

1. Introduction

Giraffe populations are declining and they are undergoing a silent extinction. The Masai giraffe (*Giraffa camelopardalis ssp. tippelskirchi*), which is found only in East Africa, might be listed soon as a separate species as debated in recent genetic studies [8–11]. Masai giraffes have been declared endangered mainly due to poaching and habitat loss, and their status calls for urgent conservation actions [12].

Translocations are increasingly important tools for endangered species conservation to increase genetic exchange between isolated populations or to assist the recovery of declining populations [58]. Translocating wild giraffes is challenging mainly due to the potential dangers posed by their size, the harsh environmental conditions, and the lack of proper anesthetic equipment in the field [34,125]. Chemical capture is usually needed in order to attach the ropes necessary to lead the giraffes onto a chariot (i.e., a trailer modified to transport giraffes). The common procedure for long distance translocations requires a boma confinement for two to three weeks before a long journey is faced [183].

Giraffe captures have been defined as the “art and science of giraffe immobilization” due to the numerous challenges faced because of their unique anatomy and physiology [126]. Dramatic consequences are not rare and can result in unacceptable morbidity and mortality [34,125,126]. Anatomical features of giraffes make their handling very complicated. They are heavy and are prone to develop stress and injure their long neck. Additionally, aggressive movement of their legs can pose a danger to the capture team [34,125,126,187].

Giraffes are among the most susceptible African ungulate species to capture myopathy [47]. Because of their unique cardiovascular physiology, the giraffe heart may easily get damaged from

oxygen debt, complicated by their smaller lung volumes and low compliance [188]. Their delicate physiological balance can easily be altered by potent opioids used for captures because giraffes are particularly sensitive and are prone to side effects [125]. Opioid-related respiratory depression and alterations in the thermoregulatory response commonly occur and worsen the oxygen debt and the acid-base derangement, and, in case of stress-driven hyperthermia, further increase the metabolic demand [125]. Furthermore, not only the immense physical activity, but also the psychological stress developed during the capture event produce a severe homeostatic imbalance that can lead to capture myopathy and death [47,189,190]. The high morbidity and mortality rates (sometimes >10%) encountered with previously reported opioid-based protocols has resulted in a hesitancy to anesthetize this species [46]. In the field, to keep induction times and physical exertion to a minimum, giraffes are usually knocked down by overdosing with a potent opioid, which is immediately reversed when the giraffe is recumbent. This is followed by manual restraint. Naltrexone, which is a long-acting pure opioid antagonist that is given at dose rates between 5–100 mg per mg of etorphine, is often used, and is able to fully antagonize etorphine action and side effects, even though there are some controversial opinions on the effective doses [34,125,184]. This anesthetic technique greatly limits what can safely be done to giraffes while they are recumbent, as they are completely awake [34,125,126,183].

Azaperone is a butyrophenone tranquilizer that has been widely used as an adjunct to an opioid-induced chemical restraint. Azaperone has sedative, anti-anxiety, and mild muscle relaxant effects, and, due to its α1-antagonistic effect, induces vasodilatation that can counterbalance opioid hypertension [43,70,154,155]. Reports of adverse effects in wild ungulates, such as severe hypotension and extrapyramidal effects, are rare and thought to result from high intravenous doses in stressed animals [154,156]. Guideline advice is that giraffes should be fully reversed before loading and that neuroleptics should not be given while being transported due to the danger of disorientation, unsteadiness on their feet, and collapse [183]. However, from previous experiences of the authors, it emerged that azaperone at low doses might provide useful tranquilization during the physical restraint phase, when the giraffes are recumbent and reversed, which decreases the insurgence of manipulation stress without affecting the physiological function. Having calmer individuals might also benefit the safety of the loading and transport operations for both the animals and the capture team.

The aim of this preliminary study was to investigate the physiological and handling safety of etorphine combined with azaperone for the immobilization and translocation of free-ranging Masai giraffes. The evaluation of low doses of naltrexone in its efficacy for etorphine antagonisation was also performed by taking advantage of a two-weeks boma confinement follow-up.

We assumed that both the initial stress associated with the darting procedure and the physical exertion resulting from a mix of opioid-related excitement and anxiety influence the insurgence of acid-base and cardio-respiratory function derangements and their severe complications. Since they have different pathways and different prevention strategies, we aimed to investigate their role in affecting the physiological imbalance and the immobilization outcome.

2. Materials and Methods

2.1. Animals, Drugs, and Procedures

This study was performed in the Rift Valley Region in Kenya, in the area surrounding Lake Naivasha, which is a forest region situated at 1884 m of altitude. It was performed on giraffes that were part of a translocation carried out for management and conservation purposes, organized by the Kenya Wildlife Service, which is the authority for wildlife and parks management in the country. The project required the capture of free-ranging individuals of Masai giraffe from the Naivasha area, in Nakuru County, and the subsequent translocation to a wildlife sanctuary in the Mombasa County.

The Kenya Wildlife Service (KWS) Department of Veterinary Services and the Biodiversity Research and Monitoring Office (KWS/BRM/5001) approved the project, which complied with the KWS guidelines to conduct research on wild mammalian species.

Nine free-ranging Masai giraffes, five females and four males, both subadults and young adults were considered for this study. The animals were captured over three consecutive mornings during which the environmental temperature was always between 18 and 25 °C. The capture was planned during the dry season in order to have a favorable terrain, even though the area is characterized by spots with thick vegetation and rough terrain. This requires particular care during the induction phase. Individuals suitable for the translocation were first spotted from a vehicle, and, after estimating the age and body size, and checking their apparent health status, a 3-mL dart (Dan-Inject 3 mL, 2.0 x 60 mm needle, S300 Syringe Dart, Dan-inject International, Skukuza, South Africa) containing the drug mixture was prepared.

The immobilization protocol included a combination of 8 or 9 mg of Etorphine (Captivon 9.8 mg/mL, Wildlife Pharmaceuticals, White River, South Africa) and 50 mg of Azaperone (100 mg/mL, Kyron Laboratories, Johannesburg, South Africa) depending on the estimated size of the giraffe. The individuals were cautiously approached in a vehicle, and when a proper distance was reached (15 – 20 m), a CO_2 pressurized dart gun (Model JM; Dan-inject International, Skukuza, South Africa) was used to deliver the dart syringe intramuscularly in the upper hind leg or shoulder area. After being darted, the individuals were observed from a distance until the first signs of sedation were observed.

When the giraffes appeared severely ataxic, and, in order to facilitate their recumbency and prevent them from both overexertion and falling in undesired areas, they were casted with ropes from an experienced capture team. A heavy rope, at chest height, was placed in the path of the giraffes, and, as the giraffes encountered the rope, the ends were crossed behind the hindquarters, which stopped the forward motion of the giraffes and led to recumbency [187]. Once the individuals were recumbent, naltrexone (3 mg/mg etorphine, 40 mg/mL Kyron Laboratories, Johannesburg, South Africa) was administered intravenously to reverse etorphine, in order to antagonize the opioid-related side effects. After the reversal, in order to maintain the lateral recumbency, the giraffes were manually restrained by holding the neck against the ground, and a blindfold was applied to minimize stress.

During the recumbency phase, the capture team positioned the ropes needed to subsequently load the individuals onto the chariot. During this time, blood and tissue samples were collected, age was estimated, and body measurements were recorded. The body weight of each giraffe was estimated from

the length and girth measurements following the method described by Hall-Martin [191]. When the giraffe was ready to be loaded, the neck restraint was lifted, and the giraffe was allowed to stand up. Once standing, the blindfolded giraffe was guided through the use of the ropes, onto the chariot, and then transported to a boma 5–10 km away. The nine giraffes were housed in the boma for 14 days after which they have been translocated to a new site with a hard release [183].

2.2. Monitoring

Anesthetic times and appositely descriptive scores were used in order to standardize the evaluation of the immobilization procedure quality. The time from the darting (referred as time 0) to the first signs of sedation ("sedation time"), the successful application of ropes ("roping time"), when recumbency was reached ("recumbency time"), were recorded. The distance that the giraffes walked while chased by the darting vehicle before they were successfully darted, and the distance from the darting spot to the recumbency site were estimated.

Ad-hoc qualitative scores were created and used for each individual in order to differentiate between the initial behavioural reaction of the giraffe to the stress of the darting procedure, the "startle response", from the excitatory behaviour displayed after the drugs showed the first effects, the "induction induced excitement", which is characterized by intense physical activity and is likely due to both opioid-induced central excitation and anxiety.

The "startle response score" (Table 1) took into account the individual's reaction to the darting vehicle, and the resulting duration and velocity of the chasing phase.

Startle Response Score	Description
1	No reaction when approached by the darting vehicle, keep on with the previous activity or stop to observe the vehicle
2	Suspicion over the darting vehicle. Intermittent walking away for maximum 2 min only when the vehicle is close
3	Keep distance from the darting vehicle, walking away for up to 5 min before being successfully darted
4	Sustained chasing involving a high velocity gallop before being successfully darted

Table 1. Startle response score.

Table 2 referred to the individual's behaviour after the first signs of sedation occurred. It was created to describe solely the degree of excitement and the consequent physical exertion displayed by the giraffes, which results from the excitatory effects of the opioid during the induction phase. Although this score mainly focused on the physical component of the capture stress, it is not possible to exclude that the excitatory behaviour is not only influenced by the drugs, but also by the anxious response of the giraffes to the perception of sedation signs. The behaviors considered for the

excitement score were star gazing, high gait stepping, and ataxic accelerated ambling or galloping with no regard to the surrounding environment.

Excitement Score	Description
1	Excitement shorter than 1 min, mainly slow, high gaited step ambling
2	Excitement phase of maximum 2 min, involving low velocity ambling or galloping
3	Excitement phase between 2 and 4 min, including a high velocity gallop
4	Sustained high velocity gallop, characterized by unsafe and repetitive unsuccessful roping attempts

Table 2. Induction-induced excitement score.

In order to evaluate the quality of induction, a descriptive score was used to define the safety of rope-assisted recumbency for the giraffes and the capture team (Table 3).

Induction Score	Description
1	Excellent induction. Motionless star gazing or slow ataxic gait and no reaction to the capture team's approach. When roped, the recumbency is quickly achieved without struggling. Minimum risk for the safety of the giraffe and the capture team.
2	Good induction. The individual shows a severe ataxia and an accelerated high gait ambling when approached by the capture team. When roped, shows no fight response and reaches recumbency easily.
3	Fair induction. The individual shows a severe ataxia but gallops. While being entangled by the capture team's ropes, it is still strong and keeps on galloping for meters before falling.
4	Poor Induction. The giraffe shows a severe excitement and has been galloping for minutes. Failure of roping attempts due to a fight reaction. Dangerous to rope, it would require a second dosing.

Table 3. Induction score.

Once giraffes were recumbent, the time of administration of naltrexone was recorded. The physiological function and behaviour of the giraffes were monitored continuously throughout the recumbency. Since etorphine was early antagonized, the recumbency was maintained thanks to a combination of manual restraint and tranquilization with azaperone. Occurrence of regurgitation and

of other complications was recorded. The first record (T1) was made 2 min after naltrexone was administered IV, and the following records were made every 5 min (T2, T3).

The respiratory rate (RR) was monitored through chest movement observation and heart rate (HR) by auscultation of the heart with a stethoscope. Body temperature (T) was measured with a digital thermometer inserted in the rectum (Veterinary rectal thermometer, 25588 Gima S.p.a., Gessate, Italy). A pulse oximeter with a transmission probe (MD 300 Handheld Pulse Oximeter, ChoiceMMed Co., Tianjin, China) was attached to the rectal or vulvar mucosa to measure hemoglobin oxygen saturation (SpO_2). Non-invasive blood pressure (NIBP) was measured oscillometrically using a cuff placed on the tail (Omron Digital Blood Pressure Monitor Model HEM-400 C, Omron Corporation, Kyoto, Japan).

At time T2, arterial blood was collected anaerobically from an ear artery by using a 1-mL heparinized syringe and analyzed within 15 min using a portable blood gas analyzer (VetStat Electrolyte and Blood Gas Analyzer and Respiratory/Blood Gases cassettes, IDEXX Laboratories Italia Srl, Milano, Italy). The analysis included measured values for pH, partial pressure of arterial carbon dioxide ($PaCO_2$), partial pressure of arterial oxygen (PaO_2), sodium (Na), potassium (K), and chloride (Cl). Base excess (BE), bicarbonate (HCO_3^-), anion gap (AG), arterial hemoglobin oxygen saturation (SaO_2), and hemoglobin (Hb) were calculated by the blood gas analyzer from measured variables. The alveolar-to-arterial oxygen tension gradient [$P(A\text{-}a)O_2$] was calculated by subtracting PaO2 measured by the blood gas analysis (BGA) from the alveolar oxygen tension (PAO_2). PAO_2 was calculated with the following equation.

$$PAO_2 = FiO_2\,(Pb\text{-}P_{H2O}) - PaCO_2/RQ$$

where FiO_2 is the fraction of inspired oxygen (21% for room air), Pb is the barometric pressure measured during the study by the blood gas analyzer, P_{H2O} is the partial pressure of vapor in the alveoli (47 mmHg), $PaCO_2$ is the partial pressure of carbon dioxide measured by the BGA, and RQ is the respiratory quotient, dependent on metabolic activity and diet, and is considered to be 1.0 for ruminants [94]. Since the capture site was not at sea level, we calculated the PaO_2 expected for the average altitude we worked at (i.e., 1884 m) in order to use it as the cut-off value for defining hypoxemia, using the alveolar-to-arterial oxygen tension formula. Assuming a normal alveolar-oxygen tension difference of 15 mmHg, the expected PaO_2 was calculated by subtracting 15 mmHg from the calculated PAO_2 value (as described above, but assuming a barometric pressure of 604 mmHg and $PaCO_2$ of 35 mmHg for this estimation, which is considered the physiological reference value at this altitude) [170,192]. The resulting expected PaO_2 at 1884 m of altitude was 66 mmHg, and animals with PaO_2 values lower than this were considered hypoxemic.

Restraint quality was assessed using an ad hoc descriptive qualitative score (Table 4), which considered the reactive behaviour of the individuals to human manipulation and painful stimuli like arterial punctures.

Restraint Score	Description
1	No attempts to stand or fight. The giraffe is alert but quiet. Safe handling.
2	Weak attempts to stand. The giraffe is quiet, but manual restraint is essential. Safe handling.
3	Repetitive attempts to stand. The giraffe is strong and sometimes kicks. Caution required.
4	Strong attempts to stand, excitation, and aggressive behaviour. Extremely dangerous situation.

Table 4. Restraint score.

The total time of the duration of the recumbency phase ("restraint length"), the time that occurred from when the manual restraint was lifted to standing ("standing time"), the time that occurred for loading the giraffes on the chariot ("loading time"), and the duration of the transport to the boma were recorded. Descriptive scores were used to define the quality and safety of the recovery ("recovery score") and of the loading procedures ("loading score") (Tables 5 and 6).

Recovery Score	Description
1	Excellent recovery. Standing at the first attempt as soon as manual restraint is lifted, immediate balance, and coordination.
2	Good recovery. Standing at the first attempt once manual restraint is lifted and balance is gained after a few steps.
3	Fair recovery. Few attempts before standing. Weakness and poor balance in the first several seconds. Risk of injuries.
4	Poor recovery. Struggle to stand with one or more attempts and poor balance once standing. High risk of injuries.

Table 5. Recovery score.

Loading Score	Description
1	The giraffe has good coordination and easily follows the rope guidance to load into the chariot at the first attempt.
2	The giraffe has a good balance but is not loaded at the first attempt as it does not follow correctly the rope guidance. The giraffe is calm and the situation is not dangerous.
3	The giraffe has a poor balance and falls during the loading procedure. Repetitive attempts before a successful loading. Unsafe procedure.
4	The giraffe has a good balance, but makes attempt to fight the ropes, i.e., kicking or running away. Loading is difficult and dangerous for the team and the giraffe.

Table 6. Loading score.

Giraffes' behaviour was observed during the chariot transport from the capture site to the boma and adverse reactions were recorded. During the boma confinement, giraffes were monitored in order to assess their health status, welfare, and possible signs of renarcotization and capture myopathy. Giraffes' apparent health status was recorded every hour for the first 12 h, and at least every 6 h for the remaining 14 days. Personnel from the capture team were always available on site and were instructed to record the occurrence of possible complications.

2.3. Data Analysis

Statistical analyses were performed using JMP, version 7.0 for Windows (SAS Institute, Cary, United States). Numeric data are presented as mean values ± standard deviation, with ranges where relevant. Scores are presented as median values with ranges. A non-parametric Spearman correlation coefficient was used to evaluate the correlations between the scores and the measured variables. The correlation coefficients and significances have been calculated for all pairs of variables. Only the most relevant statistical results are reported. A Student's t-test was used to evaluate the difference between SpO_2 and SaO_2 values. The statistical method was chosen on the basis of the nature of data (mixed measured/score data) and of the sample size. A *p*-value below 0.05 was considered significant.

3. Results

Data collected from all nine Masai giraffes were included in this study. In all the individuals, etorphine-azaperone combination provided a reliable immobilization and no additional doses were needed. According to estimations made during restraint, the individuals weighed 722 ± 97 kg and were 3.6 ± 0.7 years old. Administered doses of etorphine were 0.012 ± 0.001 mg/kg (range 0.011 – 0.013 mg/kg) and azaperone 0.07 ± 0.01 mg/kg (range 0.063 – 0.083 mg/kg). Etorphine and azaperone doses in mg/kg administered to individuals of different sizes showed little variations, and there was no correlation between the variations in the doses in mg/kg and times for the first signs of sedation and casting nor induction time and score. Naltrexone was given intravenously at 0.036 ± 0.003 mg/kg (range 0.034 – 0.04 mg/kg), which reflects a 3:1 ratio with an etorphine dose. No dart failures were experienced and no individuals required a second dart. The dart impact sites were the muscles of the shoulder or the hindquarters, and all were considered excellent sites. The terrain was dry for all nine immobilizations, but, in six immobilizations, the area was characterized by a thick vegetation and rough terrain that required particular care during the induction.

The median startle response score was 2 (range 1 – 3) and showed some variability. Three giraffes appeared completely undisturbed by the presence of the darting vehicle. Four giraffes calmly walked away only when the vehicle was closer than 10 m. Two giraffes seemed to be uncomfortable and kept a distance from the vehicle. However, they always kept a relaxed gait and never broke into a gallop in this phase. Most giraffes were darted while they were eating and mostly within the herd. After being darted, they stayed on the spot or moved less than 20 m. All the giraffes quickly became calm and returned to the previous activity until the first signs of sedation occurred. The first signs of initial drug

effects were observed within 2.6 ± 0.8 min (range 1.5 – 3.4 min) and included signs of ataractic tranquilization defined as a decreased interest for external stimuli, increased motor activity with an ataxic gait, and isolation from the herd. In all giraffes, an excitatory phase was observed 1 – 3 min after darting, which was characterized by a sudden gallop or accelerated amble usually following the rising of ataraxia. The induction-induced excitement score also showed high variability among individuals. The median value was 2 and ranged between 1 and 4. Most of the giraffes showed a mild excitement. In three giraffes, the excitement lasted less than one minute until they were successfully roped down and was characterized by a low velocity amble, and three other giraffes displayed a mixed speedy amble/slow gallop for a couple of minutes. In three giraffes, a severe excitement was observed: a high velocity gallop was observed and maintained for up to 4 min and, in one case, roping was considered dangerous. According to the Spearman correlation test, the startle response developed during the darting procedure and the successive opioid-related excitement were not correlated. The startle response score also did not show correlations with any other score, anesthetic time, or physiological variable. A higher induction-induced excitement score (i.e., greater excitement) was positively correlated with a longer time to successful roping ($r = 0.76$, $p = 0.01$) and recumbency ($r = 0.94$, $p = 0.002$), and worse (higher) induction score ($r = 0.74$, $p = 0.03$) and restraint score ($r = 0.78$, $p = 0.01$), but was not correlated to drug doses used.

Giraffes were approached to be cast down with ropes within 4.9 ± 1.3 min (range 3 – 6.6 min) when signs of adequate sedation were apparent, such as high gaited and accelerated ambling or galloping, star gazing, and no reaction to external stimuli. Recumbency was achieved 5.6 ± 1.4 min (range 3.5 – 7.6 min) after darting, and, in all the giraffes, it was reached in less than a minute from the beginning of the roping procedure. In all individuals except for two, the induction was rated as excellent (median induction score: 1, range 1 – 3), characterized by severely ataxic giraffes that were quickly roped down by the capture team, and safely reached recumbency. Naltrexone was administered in the jugular vein within 6.3 ± 1.4 min (range 5.1 – 8.2 min) and in less than a minute from when the individuals reached lateral recumbency. Five giraffes fell in the right lateral decubitus, whereas four giraffes fell in the left decubitus. None of them showed regurgitation at any stage. The duration of the recumbency phase under manual restraint was 13.3 ± 3 min (range 10.5 – 18 min). The median restraint score was 1 (range 1 – 3) and was rated excellent in six individuals out of nine. The immobilizations were safe except in one individual that required caution due to occasional but potent leg kicks. Most of the giraffes showed mild attempts to lift the neck at the beginning of the recumbency and occasionally moved the legs, but, within a few minutes, they accepted the manual restraint. There was no specific reaction to painful stimuli like arterial punctures. There were no correlations between differences in etorphine or azaperone doses in mg/kg and restraint score, which was also not correlated with induction time and score.

RR remained stable throughout the restraint (T1: 32.2 ± 7.3, T2: 31.8 ± 8.5, T3: 31.9 ± 8 breaths/minute), whereas HR slightly increased over time (T1: 68 ± 21, T2: 70 ± 16, T3: 73 ± 15 beats/minute). Rectal temperature ranged in different individuals from 37.0 to 38.5 °C (mean values in T1: 37.8 ± 0.4, in T2: 38.0 ± 0.5). NIBP was recorded at T2 only, and the mean values were 154 ± 22

mmHg for the systolic pressure, 122 ± 16 mmHg for the diastolic pressure, and 132 ± 16 mmHg for the mean arterial pressure (MAP). SpO_2 values showed hypoxemia in all individuals, which ranged from 72% to 80% (T1: 78 ± 3, T2 77 ± 1, T3: 77 ± 2). These values, however, did not correlate with SaO_2 obtained from the blood gas analyses, as the difference between the two values in each individual was statistically significant (t = 3.6; p = 0.0018). There were no correlations between differences in etorphine or azaperone doses in mg/kg and HR, RR, T, SpO_2, and NIBP. No significant differences were seen in these variables over time.

Arterial blood was drawn at T2 from seven individuals only, and the results of the arterial blood gas analysis are summarized in Table 7. The results from each individual are also shown in Table 8 with other variables such as RR, SpO_2, and P(A-a) in order to better highlight the pathophysiological mechanism that occurred in the giraffes and the compensatory response. Furthermore, since negative correlation was demonstrated by the Spearman correlation test between excitement and HCO_3^- (r = -0.83; p = 0.005), details on each individual's induction-induced excitement score (1 = low, 4 = high) were also included in Table 8.

Variable	Mean	Standard Deviation
pH	7.23	0.05
HCO_3^- (mmol/l)	12.9	1.2
$PaCO_2$ (mmHg)	34	4
Anion Gap (mmol/l)	28	3
tCO_2 (mmol/l)	13.8	1.3
BE (mmol/l)	−12.4	1.8
PaO_2 (mmHg)	67	8
tHb (g/dl)	12.5	1.1
SO_2 (%)	87	5
Na (mmol/l)	142	1.2
K (mmol/l)	4.6	0.8
Cl (mmol/l)	105	4.6

Table 7. Arterial blood gas analyses analyzed from samples collected at T2.

Excitement Score	HCO_{3-}	pH	$PaCO_2$	BE	RR	PaO2	AG	Cl_-	SaO_2	SpO_2	P(A-a) O_2	PAO_2	Pb
1	14.5	7.2	41	−12.2	32	58	25.9	108	81	77	19.2	77.2	609.8
1	13.5	7.22	36	−12.2	30	55	26.7	108	80	78	27.4	82.4	611.1
2	13.4	7.26	32	−11.4	32	67	26.1	108	91	80	19.7	86.6	612.1
2	13.3	7.28	31	−10.7	32	68	33.4	97	91	79	18.9	86.9	608.4
3	12.7	7.26	31	−11.6	50	78	27.9	109	92	74	9.5	87.6	611.7
3	11.9	7.25	29	−12.5	36	75	28.5	109	92	80	14.6	89.5	611.6
4	10.8	7.14	35	−16.2	32	66	33.7	102	84	84	17.6	83.6	612.1

Table 8. Most relevant BGA values and physiological variables organized according to the individual's induction-induced excitement score.

The BGA showed a slight to moderate metabolic acidosis in all individuals, characterized by bicarbonates variously decreased in comparison to ungulate reference values. In four individuals out of seven, $PaCO_2$ was lower to what would normally be expected at this altitude [193]. Hypoxemia, defined as PaO_2 values lower than 66 mmHg, which is the expected PaO_2 at the capture site altitude, was present in three individuals only, whereas the calculated SaO2 from the BGA were higher than 90% in four individuals out of seven. In all the individuals, the electrolytes were within ranges previously reported in giraffes [194]. The mean alveolar-to-arterial oxygen tension gradient $P(A\text{-}a)O_2$ was 18.2 ± 5.4 mmHg, with values <20 mmHg in six individuals out of seven. As highlighted by the Spearman correlation test, individuals with higher induction-induced excitement scores had lower bicarbonates, whereas there were no statistical correlations for the other variables with the degree of excitement. No variables measured by BGA were correlated with the differences in etorphine or azaperone doses in mg/kg.

When the manual restraint was lifted, all the giraffes were able to stand up immediately, on average 18.8 ± 2.9 min from the darting. The median recovery score was 1 (range 1–2), with seven individuals rated excellent, and they stood up in a coordinated manner at the first attempt. The loading procedures on the chariot were rated good (median value 2, range 1–2), and it took 3.9 ± 2.4 min to safely load the giraffes. There were no correlations between differences in etorphine or azaperone doses in mg/kg and recovery and loading times and scores, which were not correlated to induction times and score, startle response, excitement, and restraint score.

The giraffes were transported in the chariot for 10 – 25 min and no complications occurred during the transport or the unloading procedure in the boma. During the 14 days in the boma, the nine giraffes appeared to be healthy and their vital functions were maintained. No complications such as renarcotization or signs of capture myopathy were observed during the follow-up period.

4. Discussion

This is the first study to evaluate an immobilization protocol specifically in Masai giraffes. Since taxonomic classifications might be quickly changing, and Masai giraffes could emerge as a species itself, it is important to begin to have a species-specific approach from a veterinary point of view. Masai giraffes diverged 1.25 to 2 million years ago [8], so it would not be surprising to find differences, for example, in drug sensitivity as it is anecdotally reported from field veterinarians for Masai giraffes, and as is recognized for other similar species [8,34,103]. Etorphine-azaperone combination and its early reversal with naltrexone provided safe and reliable immobilizations and translocations in the nine free-ranging Masai giraffes. The doses used in this study provided quick and smooth inductions, which allowed all the individuals to reach recumbency safely in a challenging area characterized by thick vegetation and rough terrain. The purpose of the knock-down phase was to have an early ataxic state in order to cast the giraffes with ropes as soon as possible and lead them to reach recumbency in suitable spots to avoid overexertion on one side, and injuries on the other side [126].

All the individuals showed the first signs of drug effects between 1.5 and 3.4 min, and they were evaluated as sedated enough to be safely casted 3 to 6.6 min after they were darted. All the individuals fell recumbent in less than a minute with a minimum effort from the capture team, on average 5.6 ± 1.4 min after the dart injection. Seven out of nine inductions were rated as excellent, whereas only one individual posed a danger, as it was still galloping when approached by the capture team. Compared to other protocols used in ground darted free-ranging giraffes, the mean time for the first signs of drug effects and time to recumbency in this study, were similar to those achieved with the protocol thiafentanil-medetomidine-ketamine [195], but quicker than in the protocols butorphanol-azaperone-medetomidine [196] and carfentanyl-xylazine [46] or quicker than in protocols used for captive giraffes, such as medetomidine-ketamine [197], etorphine-medetomidine-ketamine, thiafentanil-medetomidine-ketamine [195,197], and etorphine-acepromazine [198]. Times of etorphine-azaperone combination for the first signs of sedation and recumbency also resulted quicker than in combinations such as medetomidine-ketamine [11] or thiafentanil-medetomidine-ketamine [195] used in helicopter darted free-ranging giraffes. However, the chase with the helicopter might have elicited a greater stress response that influenced not only the physiological balance of the individuals but also the drug effects.

Even though there were no correlations between the etorphine and azaperone doses and the times to first drug effects and induction, we have seen some individual variability in the rapidity and depth of the drug effects. A possible explanation is that, because the body weight was estimated, errors in the given dosage per kg might have occurred. The mg/kg doses are displayed only in order to make an easier comparison with other studies and must be taken with caution.

Another possible explanation is that it has been demonstrated, in humans, that there is different genetic-mediated individual sensitivity to opioids. A common single nucleotide polymorphism, A118G, in the μ-opioid receptor gene (OPRM1), can affect opioid function and, consequently, has been suggested to contribute to individual variability in drug response to pain and drug addiction, through a regulation of the quantity and distribution of opioid receptors [199–202]. Although a similar allele and mechanism has been found in other species like mice and dogs [200,202], this field has not yet been investigated in wildlife, where it could provide an explanation for the many differences in drug response seen at individual and species' level. In addition, the higher opioid-related excitement seen in some individuals, which was significantly correlated to longer induction times, might be caused by individual differences at the OPRM1 locus [200].

The doses of etorphine, azaperone, and naltrexone used in this study were based on previous experiences of the authors. The etorphine dose was higher compared to other opioid-based protocols described in giraffes [34,195,197,198], while azaperone was lower than previously reported [125,196]. The use of high doses of opioids for the knock down of capture myopathy-sensitive herbivores is common practice in order to obtain a fast recumbency and decrease the risk of overexertion and resulting homeostatic consequences such as acidosis and hypoxemia [34,125]. Giraffes are particularly sensitive to both overexertion as well as to opioid side effects. For this reason, when capturing them for short field procedures, full antagonism is usually provided as soon as the giraffe reaches the ground

[34,125,126,183]. They are then manually restrained, but this greatly limits what can be done to them and might also cause psychological stress with deleterious physiological consequences [47,125,126].

The addition of azaperone to the combination might add a few benefits. The synergism between etorphine and azaperone could have reduced induction time and improved immobilization quality, thanks to the additional tranquilization and anxiolytic effect provided, which could decrease the stress of the individuals and provide greater safety for the capture team [47,103,133]. Our results confirm that, at the doses used, a combination of 8 – 9 mg of etorphine and 50 mg of azaperone were adequate in knocking down subadults and young adult Masai giraffes quickly and with limited excitement, as shown by the limited homeostatic alterations that occurred and the safety of the restraint. In order to further decrease the opioid-related excitement shown in some individuals, future studies should evaluate multimodal drug protocols that include the addition of sedatives, since, by working in synergism with opioids, they might decrease induction times and excitement.

Naltrexone is a long-acting pure μ antagonist used for the antagonism of opioid immobilization in wildlife [184]. Despite naltrexone's half-life being longer than that of etorphine, renarcotization has been observed between 2 and 72 h after immobilization, even though the precise mechanism of renarcotization is not fully known [43,184]. In the early days, doses of naltrexone up to 100 mg/mg etorphine were used, while, more recently, between 5 and 25 mg naltrexone per milligram of etorphine have been reported to be effective without apparent renarcotization [34,125,184]. In a study performed in goats, the minimum effective naltrexone dose was 20 mg/mg etorphine, whereas renarcotization signs showed up between 20 and 133 min after antagonisation and lasted for 2 to 8 h when lower doses were given [184]. In our study, naltrexone at 3 mg/mg etorphine was administered intravenously as soon as the giraffes were recumbent. Even though it was challenging to record the exact time of recovery as it overlapped with the induction phase movements, the giraffes were clearly awake during the physical restraint. The mild hypoxemia and the adequate cardiorespiratory function seen in the individuals also suggest that etorphine was reversed, as those would have likely been seriously compromised before naltrexone was given. However, the quick times and quality of recoveries and the 14 days boma follow-up are the proof that naltrexone at 3 mg/mg etorphine was effective in reversing etorphine in all the nine Masai giraffes. The small number of individuals in our study, and the fact that our results are innovative and in contradiction with previous reports in other species [184,203] suggest that further research with a larger sample size is needed, and that this dose of naltrexone should be used with caution until further work has been done on its use in giraffe to confirm its safety.

However, novel research on polymorphism at OPMR1 could provide an explanation for species and individual variation in sensitivity not only to opioids, but also for their antagonists, i.e., by coding for different opioid receptor stability and brain expression, which can alter the binding of the antagonist [204]. The effect of naltrexone, which is used for treating alcoholism in human medicine, also seems to be moderated by the Asn40Asp single-nucleotide polymorphism of the μ-opioid receptor gene, and, moreover, to vary substantially as a function of ethnic background [205]. Although this is an emerging research topic and is currently under further development as it is being used in the novel field of human precision medicine, it is promising and could find a unique application for the advancement of wildlife immobilization research.

After naltrexone was administered, the giraffes were manually restrained by the capture team for 10–18 min, which was the time that was needed to position the halters needed for the chariot loading. All the individuals were calm and occasionally made weak attempts to stand up at the beginning of the restraint, which generally decreased over time. To evaluate the restraint quality, an ad hoc descriptive score that considered the peculiarity of the protocol in which the knockdown drug was antagonized was created. The descriptive score scale differed from classic anesthetic scales that consider the depth of anesthesia and the presence of reflexes, but considered the overall giraffe fight reaction and safety working conditions for the capture team (Table 4). Six giraffes out of nine displayed an excellent level of restraint during which, even if they were awake, minimum fight reactions occurred and the safety conditions were appropriate, whereas only one giraffe required caution for occasional but potent kicks.

Although no reactions to painful stimuli like arterial punctures were seen, this combination might not be appropriate for more painful procedures, as the early reversal with naltrexone also displace the analgesia provided by opioids. In some species, manual restraint has more controversial effects than the use of potent drugs for chemical restraint in the development of capture stress and its consequences such as catecholamine release, hyperthermia, reactive oxygen species (ROS) production, and cell damage [41,47]. Since, in our study, naltrexone was administered at the beginning of the immobilization, giraffes were technically awake, and potentially more prone to psychological stress.

The administration of tranquilizers and sedatives during capture procedures are recommended practices that appear to decrease the incidence of capture myopathy and improves survival rates [47]. The addition of azaperone might have provided additional tranquilization and anxiolytic action that helped to keep the stress to a minimum during manual restraint, while not compromising the physiological compensation needed after the post-capture homeostatic derangement. A better restraint score was not correlated to etorphine, azaperone, or naltrexone doses, but was correlated to a lesser degree of excitement. We speculate that excitation-related poorer immobilization could have been a consequence of catecholamines release by etorphine, which continued to display some excitation even when etorphine was reversed. However, since, in the same giraffes that had poorer restraint quality, their recovery and chariot loading were smooth, it showed that the low dose of naltrexone administered was efficient and was not correlated to the restraint quality level.

Few studies report physiological variables and blood gas analyses in detail in free-ranging restrained giraffes. Heart rate in our study slightly increased over time from 68 to 73 bpm, but remained within resting giraffe heart rates measured by telemetry or in trained unanesthetized giraffes [117,206]. It was higher than in α2-agonist-based protocols, as these are commonly reported to cause bradycardia [160]. Etorphine stimulates catecholamines release, which, in many species, results in tachycardia [41,70], whereas, in the only study described in giraffes where etorphine was not immediately reversed, heart rate did not increase [198]. In our study, etorphine was immediately reversed with naltrexone, and, as such, the physiological variables reported in this study were technically collected under the effect of solely azaperone, which does not directly influence cardiac frequency [70]. The mean respiratory rate was 32 bpm and did not show variation over time. This value was lower than in other protocols described in immobilized giraffes with combinations such as medetomidine-ketamine [187] and butorphanol-azaperone-medetomidine [196], but was higher than in non-reversed opioid-based

protocols such as etorphine-acepromazine [198] and etorphine or thiafentanil-medetomidine-ketamine [197]. It was also higher than the giraffe resting respiratory rate, which is 8–15 bpm depending on the age and size [188]. A slight increased respiratory rate is advocated in free-ranging giraffe opioid captures as it means that depression of the bulbar respiratory network does not occur and that a proper compensative response to balance the capture-related acidosis is maintained. In our study, since a low dose of naltrexone was administered, the presence of an adequate respiratory function is particularly important, as it highlights that the reversal dose was effective in the short term. A slightly increased respiratory rate, compared to giraffe resting rates, might have been beneficial in order to compensate metabolic acidosis, as demonstrated by the lowered $PaCO_2$ levels, when compared to normal ranges and measured by the BGA. Furthermore, since the animals were technically awake, the absence of severe tachypnoea, which could be a consequence of manipulation stress, could be explained by the adjunct of azaperone in the dart, which seems to have been effective in tranquilizing the giraffes.

Rectal temperature slightly increased throughout time, but it never exceeded 38.5 °C in any individuals. In a study conducted in impala, hyperthermia was not primary related to the effects of drugs such as metabolic and vascular effects of catecholamine release, environmental conditions, or physical activity but rather appeared to be strongly related to the level of initial stress (startle response) in response to capture [41]. Capture-induced hyperthermia may contribute to the development of capture myopathy [47,82]. Hence, its monitoring and prevention requires particular care, especially when using opioids that alter the thermoregulation mechanism [34]. In our study, the rise in rectal temperature was not correlated to startle response or excitement scores nor to longer times for induction. The small sample size might have limited our ability to detect this, but the fact that no severe hyperthermia was evident might find an explanation with the fact that the giraffes were captured in a highly touristic area where most animals are habituated to vehicles.

Due to the distance between their heart and their head, giraffes have developed a higher blood pressure compared to other mammals, and, when under anesthesia, a minimal MAP of 120 mmHg is required in order to allow renal function [119]. In our study, in all individuals, the MAP recorded were above 120 mmHg, but since NIBP in giraffes is considered imprecise and inaccurate in estimating the true value, a further investigation deploying invasive blood pressure monitoring is needed [119]. Azaperone can reduce the hypertensive effects of the opioids thanks to its affinity to α1- receptors, which produces peripheral vasodilation and reduces mean arterial blood pressure. Since, in our study, etorphine and its side effects were immediately reversed with naltrexone, more research on the possible occurrence of hypotension in the awake giraffe would be needed [70,103].

Pulse-oximetry in our study showed moderate to severe hypoxemic values between 72% and 80%, which is similar to that reported with medetomidine-ketamine combination [187]. In accordance with the results reported by Bertelsen et al. [119], these values were statistically different from the values obtained from blood gas analyses, where calculated oxygen saturation was corrected according to the rectal temperature with values above 90% in four individuals. Furthermore, Bertelsen et al. [119] found the pulse-oximetry tended to overestimate hemoglobin oxygen saturation, but, in our data, the pulse-oximetry gave lower values when compared to SaO_2. Accuracy of pulse oximeters and failure to produce a reading can vary widely between different models of pulse oximeters as well as between different species and skin pigmentation. Therefore, the readings should be interpreted with care [207].

Nonetheless, the values of SaO_2 might not be accurate since they are calculated from an algorithm based on a human oxygen dissociation curve (ODC), which does not reflect the actual SaO_2 in giraffes, as it is likely that the ODC is left shifted in this species. This is similar to other megaherbivores [149]. Although the calculated SaO_2 values are not representative of the actual value of hemoglobin saturation, and, as such, cannot be used to validate the pulse-oximeter sensitivity, the fact that, in our study, the measured levels of PaO_2 are mostly within physiologic ranges suggests that the SpO_2 values recorded with the pulse-oximeter are not reliable.

Blood gas analyses revealed a slight to moderate metabolic acidosis with decreased values of bicarbonates in all individuals [193], whereas non-increased values of $PaCO_2$ likely shows that there was no respiratory component for the acidosis. Although values of blood lactate were not available, it is likely that the acidosis was caused by an increase in lactic acid as commonly occurring in free-ranging herbivore capture. This is also supported by an overall increase in the anion gap [147].

Nonetheless, giraffes that had a greater excitement score, as well as those that, as a result, had longer times to casting and recumbency and have run for longer times or distances, had a significantly greater decrease in bicarbonates. The giraffes with worse excitement scores and lower bicarbonates did not show more severe acidosis (Table 8). Indeed, initial respiratory compensation occurred in some individuals seen as decreased $PaCO_2$ values compared to reference values. PaO_2 and SaO_2 were also higher in these individuals whereas $P(A\text{-}a)O_2$ was lower, which is likely a consequence of an increased respiratory rate within the compensatory response. The occurrence of respiratory compensation is particularly important in opioid-based protocols, as these depress the respiratory driven response to carbon dioxide, which leads to a potentially uncompensated acidosis and homeostatic catastrophe [34]. A similar or worse metabolic acidosis was reported in captive giraffe immobilized with medetomidine-ketamine-etorphine/thiafentanil and medetomidine-ketamine combinations, and, in a study of helicopter darted free-ranging giraffes, it showed improvement within 30 min [187,197]. A limit of our study is that we took arterial samples only once, at the beginning of the restraint. Therefore, it is not possible to know if an improvement of acidosis occurred as well as in our individuals.

Considering the PaO_2 that would be expected in a normal awake animal at the altitude of the capture area, only three giraffes could be considered mildly hypoxemic with values of PaO_2 lower than 66 mmHg. $P(A\text{-}a)O_2$ was not elevated (normal < 20 mmHg) except in one individual (27.4 mmHg). These results highlight that cardiorespiratory function was maintained after the early administration of naltrexone, and, also in those individuals with a mild hypoxemia, there was no oxygen diffusion impairment or physiological right-to-left intrapulmonary shunting of blood. Even though in our study oxygen was not administered and minor hypoxemia was recorded in few individuals only, hypoxemia is a common eventuality in giraffe immobilizations [126,187,195,197], thus oxygen insufflation should always be provided even in field situations to prevent poor tissue oxygenation [197,207]. In case of oxygen demand, giraffes have evolved a mechanism to increase the respiratory rate and oxygen diffusing capacity, rather than increasing the tidal volume due to anatomical constraints such as small lung volume and low lung compliance [188]. This inability to increase the tidal volume indicates the need for focusing attention on preventing acidosis and hypoxemia in giraffes, as they might struggle

more than other mammals to compensate a large homeostatic derangement [188]. Due to their long trachea, it is unclear whether an increased and shallow respiration would be able to overcome the dead space in the upper portion of the respiratory tract, as an increased respiratory rate would exacerbate the hypoxemia [187]. Recent studies, however, have demonstrated that, since the trachea is narrow, giraffes have a low dead space-tidal volume ratio [208,209], but this might impose a high respiratory resistance during physical exercise, which is partially compensated by the longer inspiratory time and due to no pause between inspiration and expiration [188]. In our study, an increased respiratory rate accounted for an improvement of gas exchanges, as demonstrated by an increase in oxygenation and a decrease in carbon dioxide. This shows that the oxygen demand-driven mechanism was functionally maintained despite the drugs used.

Sodium and chloride were within free-ranging giraffes' ranges, whereas potassium was similar to values reported in captive giraffes, but lower than in free-ranging captured giraffes [194]. Potassium elevations are due to high levels of capture stress since it results from muscle damage and is a major component in the pathogenesis of capture stress and myopathy [43]. Non-increased values of potassium recorded in this study supports that etorphine-azaperone combination kept the stress to a minimum, and, even though some excitement occurred, it had little consequences. Sodium in this study was lower than in other studies in free-ranging and captive giraffe [194], and the differences seen may be because of the small sample size and narrow range of values.

The excitement score evaluated the increased motor activity, which was likely due to both opioid excitation and anxiety. The startle response score was focused on the behavioural stress reaction of the individual when approached by the darting vehicle. In the giraffes of this study, the startle response score ranged between 1 and 3, and it did not involve any increased physical activity (i.e., full speed chase). The reaction after the dart impacted the animals was not taken into consideration as all the giraffes showed little reaction to it, such as a galloping for a few seconds, after which all the giraffes returned to their previous activity. Most capture events are naturally likely to induce an acute stress response, which can be defined as psychological stress [82]. This level of stress has higher influence on the development of hyperthermia and related homeostasis disturbance, than the physical activity itself or drug effects [41]. Although acute or chronic stress responses are usually evaluated respectively through the measurement of catecholamines or cortisol and metabolites, the effects of habituation can also be evident in the animal's behaviour [41,65].

In our study, we used an ad hoc descriptive score based on behavioural reactions such as startle response to darting to define the presence of psychological stress of the giraffes. If alarmed, a giraffe can go quickly from a walk to a fast gallop of up to 56 km/hour and can sustain this for many kilometers [183]. In our study, the giraffes reacted differently to the sight of the car. A few stood still and others walked away to keep some distance. However, compared to helicopter captures among giraffes, the stress reaction displayed was minimal. Since we did not observe any correlation between higher startle response developed during the darting operation and alterations of the acid-base or cardiorespiratory function, it is likely that vehicle darted captures in giraffes in highly touristic areas have a minimum impact on their stress response. Further investigations involving helicopter-based capture would be

required in order to understand how a greater psychological stress influences the homeostatic balance and how the use of capture techniques that consider the species-specific susceptibility to different stressors can prevent it.

In our study, the startle response during the darting operation and the drug-related excitement were not correlated. Regardless of the initial stress, our results show that the induction-induced excitement caused a higher degree of bicarbonate loss and, indirectly, metabolic acidosis. It is likely that it was due only to the increased physical activity resulting from the excitation, which likely results from the development of hypoxemia and lactic acidosis. Psychological and physical stress are the two sides of the same question, which is capture stress. Our opinion is that it is fundamental to differentiate and deeply investigate their roles in the development of acid-base disturbance, as they have different prevention strategies.

The homeostatic derangements seen in this study resulting from a higher excitement were not severe. Usually, it will take time for the heart rate, blood pressure, and respiration to normalize once giraffes are restrained and reversed [183]. Thus, it is likely that the cardiorespiratory function we observed had further improved rapidly beyond our short-term monitoring. It is likely that the practice of roping the giraffes not only prevents injuries, but also decreases the physical activity during induction, which limits the overexertion effects.

Recoveries in all the giraffes were safe and smooth. When the neck restraint was lifted, they immediately stood up in a coordinated manner at the first attempt between 16 and 27 min after the initial darting. The recovery score was excellent in all individuals, except for two that we rated as good. Diprenorphine is often preferred over naltrexone for giraffe translocations for its partial agonist/antagonist effect, even though it has a greater renarcotization risk [184]. Pure antagonists such as naltrexone induce complete reversal of the opioid immobilization and, as such, giraffes have been considered too excited to be safely managed and loaded. In our study, the combination of naltrexone, which fully reversed etorphine even if administered at low doses, and the addition of the tranquilizer action provided by azaperone allowed coordinated recoveries and loading procedures, as the giraffes were conscious but calm. The use of sedatives or tranquilizers are usually not advised for giraffe translocations, as they are reported to decrease the coordination and balance [34,183]. In our study, all the giraffes regained coordination from the beginning and maintained it during the loading procedure and during the 10 – 25 min drive to the boma. Azaperone is not reversible, but it did not prevent satisfactory recoveries, as giraffes were loaded in the chariot quickly and effectively in less than 4 min, and no signs of excessive tranquilization were evident. Tranquilization has likely made the transport to the boma smoother and uneventful, and it might have reduced the giraffe stress and their fight reactions that can be deleterious during the loading procedures. Recovery and loading times as well as scores were not correlated to drug doses nor to alterations seen in the cardiorespiratory function and blood gases, which confirms that no serious physiological compromise occurred from the capture.

The boma period represented a valuable tool that allowed to closely monitor the giraffes and evaluate possible tardive complications. During the 14 days of a follow-up, particular attention was taken toward signs of re-sedation and capture myopathy. Since renarcotization has been reported to

mostly occur within the first hours and lasts 2–8 h, our monitoring protocol was likely to be adequate in spotting possible signs of renarcotization such as fear loss, aimless wandering, ataxia, and depression [184]. The giraffes maintained their vital functions and remained healthy during the monitoring period.

5. Conclusions

The results in this preliminary study showed that the etorphine-azaperone combination provided reliable immobilizations in ground darted Masai giraffe with adequate physiological and handling safety. Early reversal with a low naltrexone dose (3 mg/mg etorphine) was successfully obtained and the two weeks boma follow-up excluded the occurrence of renarcotization or capture myopathy. Although a control group was not available, the addition of azaperone provided tranquilization that likely increased the restraint quality and facilitated a smooth loading and safe chariot transport. Casting giraffes with ropes is a valuable tool to minimize the overexertion, especially when due to the combination of high opioid doses and tranquilizers, the giraffes move with a slow high stepping gate, which minimizes the injury risks for the capture team.

Startle reactions and induction-induced excitement both occur during the capture of giraffes, and, since they have different pathways and origins, it is important to analyze them separately whenever possible. Although this is only a pilot study on a small number of individuals, the encouraging results obtained can provide an initial insight into the causes of the stress induced in the giraffe. Opioid-related excitement accounted for greater physiological derangement, and further research on the use of multimodal anesthetic drug mixture might provide a solution to mitigate it.

The use of ad hoc descriptive scores seems to be a useful tool for evaluating the predisposing factors in influencing the morbidity in chemical and physical restraint, and might give important information if used in a larger study. Systematic monitoring that includes the analysis of arterial blood gases should always be adopted to be able to detect physiological changes that might otherwise go unnoticed. A good knowledge of the physiological responses of the animals is important to ensure the success of a capture operation by preventing possible complications.

RESEARCH STUDY II

MECHANISM OF CAPTURE MORBIDITY IN ETORPHINE-AZAPERONE IMMOBILIZED FREE-RANGING RETICULATED GIRAFFE (*GIRAFFA CAMELOPARDALIS RETICULATA*) AND EFFECTS OF EARLY OPIOID ANTAGONIZATION ON THE PHYSIOLOGICAL FUNCTION

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

Abstract

Free-ranging giraffe immobilization is characterized by life-threatening physiological alterations resulting from capture stress and drug adverse effects. In this study, the mechanism of capture morbidity was investigated in reticulated giraffes immobilized with an etorphine-azaperone combination and antagonized immediately after recumbency. The accuracy of nasal capnometry was investigated for its efficacy in monitoring continuously $ETCO_2$. Eighteen giraffes were darted by helicopter (n = 4) or vehicle (n = 14) with etorphine (13 – 17 mg) and azaperone (70 – 100 mg). Scores were used to evaluate the startle response to darting and the induction-induced excitement. Naltrexone and/or diprenorphine were administered as soon as giraffes reached recumbency; the effect of early antagonization was meticulously evaluated through serial blood gas analyses, physiological monitoring, and immobilization scores. Severe acid-base and metabolic alterations occurred, especially in helicopter darted giraffes as a result of intense chase, with respiratory compromise (hypoxemia and hypercapnia) in some individuals. In ground darted giraffes, where acid-base alterations were less severe being the flight response marginal, severe respiratory acidosis occurred in case of higher etorphine dose, due to respiratory depression, whereas lower doses resulted in greater excitement and slight/moderate metabolic acidosis. Early antagonization resulted in an improvement over time of $PaCO_2$ (BGA1: 41 ± 11 mmHg; BGA2 34 ±7 mmHg; p=0.02), but not of the acid-base status (pH, BGA1: 7.201 ± 0.1, BGA2: 7.202 ± 0.1) whereas cardio-respiratory function and PaO_2 (BGA1: 84.1 ± 39 mmHg, BGA2: 84.5 ± 33 mmHg) were stable. On the other hand, the immobilization quality was poor, which poses giraffe welfare and capture team safety concerns. Nasal capnometry was shown to be a useful non-invasive monitoring tool for field assessment of ventilation function, even though was not accurate in predicting absolute values of $PaCO_2$. Despite the severe alteration observed, all giraffes recovered and no mortality occurred for at least the four months of GPS follow-up monitoring period.

1. Introduction

Giraffes are vulnerable to extinction as their populations are decreasing in numbers across Africa [17]. reticulated giraffe is a unique subspecies found mainly in Kenya, with a current population number estimated around 11,000 mature individuals and is classified as Endangered [13,210]. According to recent genetic studies, it has been debated that instead of one, there might be four distinct giraffe species, and that reticulated giraffe might be classified as a species on its own [8–11]. Over the last 30 years, reticulated giraffes faced a 50% decline, mainly due to habitat loss and deterioration, and illegal killing [13]. In order to support their conservation, and similarly to other giraffe subspecies facing extinction, operations that require veterinary interventions and chemical capture, including the application of GPS units, are often required [211,212]. To prevent the avoidable loss of rare individuals, clinical treatment of injured reticulated giraffes is routinely performed, even more justified by the fact that most injuries are of anthropogenic origin, such as those caused by poaching snares.

Chemical capture of free-ranging giraffes remains challenging and involves a high risk of morbidity and mortality [34,81,125,126,187]. Major complications include not only physical injuries and regurgitation that can lead to fatal asphyxia, but severe pathophysiological alterations resulting from the psychological and physical stress, and adverse effects of the drugs used [46,81,126,187,195,197,198,213,214]. Giraffes are sensitive to capture stress and are indeed among the most susceptible species to capture myopathy [47]. Giraffes that are captured in touristic areas display little behavioural stress response, and as a result moderate homeostatic alterations when they are darted from vehicles [81], whereas when giraffes are darted from helicopters, the fight-flight response can be extreme and results in severe morbidity [187]. They easily develop severe homeostatic imbalance, and due to their unique cardio-respiratory anatomy and physiology, are prone to develop oxygen debt, which combined with small lungs and low lung compliance, results in poor compensation ability [188]. Giraffes are also particularly sensitive to opioid adverse effects. This is not only the deleterious cardio-respiratory depression that is seen in most species, but also an excitatory phase, especially when opioids are underdosed, that can lead to extreme over-exertion and acid-base imbalance [46,125]. The degree of this excitement has been linked with prolonged and dangerous inductions characterized by high-velocity galloping, poor immobilization quality, and higher physiological derangement linked with low bicarbonates [81].

In order to reverse etorphine adverse effects on cardio-respiratory depression, giraffes are usually antagonized immediately when they become recumbent and are then manually restrained [34,125,126,183]. This technique results in smooth restraint and limited physiological alterations when Masai giraffes are immobilized for non-painful intervention with a combination of etorphine and azaperone [81]. Azaperone, thanks to its anti-anxiety and vasodilatory properties, might provide tranquilization during the physical restraint, once etorphine is antagonized [43,70,154,155]. However, azaperone does not have analgesic effects, therefore the same protocol might not be indicated for surgical procedures. Furthermore, when manually restrained, giraffes can be dangerous for the capture team and the treatment of limb injuries might be impossible due to continuous kicking. When early antagonization is performed, a clinical improvement is observed, and it is commonly assumed that no major complication occurs because giraffes are awake. No studies have investigated if giraffes, when

awake and manually restrained, actually have an improving trend for their acid-base and gas exchange function, or if other factors occur. Indeed the recumbency might easily impair the giraffe delicate blood pressure regulatory system or the ventilatory dynamic, as seen in other megaherbivore species [104,105,120,122,123,215,216]. Furthermore, it is well documented that physical restraint can trigger a stress cascade that can be more deleterious than the adverse effects of the drugs used for chemical immobilization [41,87].

In order to decrease giraffe immobilization morbidity and mortality, a better understanding of giraffe pathophysiological response to capture, drug use and manipulation stress, is required to detect predicting factors and prevention strategies. Especially when interventions involve painful stimuli and require precise surgical interventions, the availability of a smooth chemical immobilization that guarantees steadiness and reduce manipulation stress is essential, both for the welfare of the giraffes and the safety of the capture team. Advances in giraffe immobilization protocols are needed, such as through the use of multi-drug protocols that might be able to reduce complications. A systematic assessment of the morbidity mechanism involved is therefore needed, in order to detect which drug combinations, and through which pharmacological properties, morbidity can be reduced.

In addition, advancing the monitoring of the physiological function in order to detect the pathophysiological changes that occur as a consequence of exertion, drugs adverse effects, and manipulation stress, is essential in these delicate animals. Nasal capnometry is routinely used in human medicine to measure carbon dioxide of sedated non-intubated patients, as this technique offers the advantage of being less invasive compared to the classic endotracheal intubation [144]. In veterinary medicine, capnometry is routinely used associated with endotracheal intubation, whereas the use of nasal capnometry, especially in wildlife practice, has been reported only in few studies and its accuracy in predicting arterial carbon dioxide has not been evaluated in giraffe [104,145]. If accurate, the adoption of nasal capnometry might be extremely useful to detect complications early, such as the insurgence of respiratory depression, especially in species like giraffes that are prone to immobilization fatalities and where other real-time monitoring devices such as pulse-oximeters have shown to have poor accuracy [81].

The principal aims of this study were to advance the understanding of the physiological alterations occurring in vehicle and helicopter darted reticulated giraffe immobilized with an etorphine-azaperone combination, and to evaluate the effects of early antagonization on the physiological function during clinical procedures. The final objective was to elucidate the mechanism of capture morbidity in order to pave the way towards the identification of potential drug combinations that improve giraffe capture in the field. In addition, nasal capnometry was used and its accuracy investigated in order to validate its use for field based, real time monitoring purposes and early prediction of complications. Furthermore, building on the empirical observations of different sensitivity between giraffe subspecies to capture drug adverse effects, this was the first study to evaluate the safety of etorphine-azaperone immobilization protocol specifically in the reticulated giraffe subspecies.

2. Materials and Methods

2.1 Animals, drugs and procedures

Eighteen free-ranging reticulated giraffes that were captured in Kenya for conservation-related purposes were included in this study. The Kenya Wildlife Service (KWS) Department of Veterinary Services and the Biodiversity Research and Monitoring Office (KWS/BRM/5001) approved the project, which complied with the KWS guidelines to conduct research on wild mammalian species. The study was carried out in an opportunistic manner, with no interference with procedure durations or protocols. Giraffes were either immobilized for clinical purposes involving a limb lameness that required clinical treatment (n=3) or for fitting of GPS units attached to the ossicones (n=15). Captures took place in the Northern Kenya region, in several ecosystems including semi-arid desert (Marsabit, Samburu, Isiolo areas) and savannah (Laikipia area), at environmental temperatures between 24 and 30 °C and at altitudes between 850 and 1750 meters (barometric pressure 619 – 689 mmHg).

Fourteen giraffes were darted from a vehicle, whereas four giraffes that were located in a remote area (Marsabit) were darted from a helicopter. A combination of 13 – 17 mg of etorphine hydrochloride (Captivon 9.8 mg/mL, Wildlife Pharmaceuticals, White River, South Africa) and 70 – 100 mg of Azaperone (100 mg/mL, Kyron Laboratories, Johannesburg, South Africa), which dose varied according to the clinical needs, were administered intramuscularly in the upper hind leg or shoulder area in a 3-mL dart syringe (Dan-Inject 3 mL, 2.0 × 60 mm needle, S300 Syringe Dart, Dan-inject International, Skukuza, South Africa) delivered through a CO_2 pressurized dart gun (Model JM; Dan-inject International, Skukuza, South Africa). After being darted, the individuals, both ground and helicopter darted, were monitored from a distance until the first signs of sedation were observed. When the giraffes appeared severely ataxic, and, in order to facilitate their recumbency and prevent them from both overexertion and falling in undesired areas, they were casted with ropes [81,187]. Once the individuals reached recumbency, a combination of diprenorphine (12 mg/ml; Wildlife Pharmaceuticals, White River, South Africa) and naltrexone (40 mg/ml; mL Kyron Laboratories, Johannesburg, South Africa), with dose variation depending on the veterinarian in charge, were administered intravenously in order to antagonize the etorphine-related cardio-pulmonary adverse effects, and the giraffes were successively manually restrained through neck pressure on the ground [81].

During the recumbency, body measurements were taken in order to estimate body weight according to the method described by Hall Martin [191]. When the clinical procedures or the GPS unit attachment were accomplished, the neck restraint was lifted and the giraffes were able to stand up.

2.2 Monitoring

In order to standardize the evaluation of the quality of the immobilizations, anesthetic times and scores were adopted. These included the time of darting (referred as time 0), the time of first signs of sedation (Sedation time), and the time when recumbency was reached (Recumbency time). Videos of darting and inductions were recorded, and two qualitative scores that used behavioural descriptive scales were selected among those described by Vitali et al., based on significance criteria [81]. The scores were used to tentatively differentiate between the psychological stress measured as the initial

behavioural reaction to the darting procedure, either performed by vehicle or helicopter, the “startle response score”, from the physical stress due to the drug-mediated excitement, the “induction-induced excitement score”. Once giraffes reached recumbency, the time of administration of diprenorphine and naltrexone were recorded.

The monitoring of physiological function was performed continuously throughout the recumbency phase. The respiratory rate was measured through chest movement observations, and heart rate by auscultation of the hearth with a stethoscope. Rectal temperature was measured with a thermometer inserted in the rectum (Veterinary rectal thermometer, 25588 Gima S.p.a., Gessate, Italy). Mean invasive blood pressure (IBP) was measured through an aneroid manometer connected to a catheter inserted in the auricular artery. A mainstream capnometer (Masimo EMMA Capnometer 9632, Masimo Corporation, California, United States) was used to continuously monitor end tidal carbon dioxide ($ETCO_2$), and respiratory rate. The device was attached to a cut 20 cm long endotracheal tube (10 mm ID) inserted in one nostril. Two arterial blood samples were withdrawn from the auricular artery in pre-heparinized syringes immediately after the antagonist was administered (BGA1), and at the end of the procedure (BGA2), and the time of sampling was recorded. If there were any air bubbles, these were immediately removed and the syringe sealed with a tip cap and put in ice inside a cooler box. The whole blood was analyzed within 20 minutes from collection with an i-ISTAT 1 handheld blood analyzer using CG4+ cartridges, and within 60 minutes for CHEM8 and CTNI cartridges (Abbott Laboratories, Abbott Park, Illinois, USA). Measured values included pH, partial pressure of carbon dioxide ($PaCO_2$) and oxygen (PaO_2), and lactate, whereas bicarbonate (HCO_3^-), base excess (BE), total carbon dioxide (TCO_2), and arterial saturation of oxygen (SaO_2) were calculated. Electrolytes (Na, K, Cl, iCa), Glucose (GLU), BUN, creatinine (Crea), hematocrit (Hct), hemoglobin (Hb), Anion Gap (AG) and cardiac troponin I (CTNI) were also analyzed.

The alveolar-to-arterial oxygen tension gradient [$P(A\text{-}a)O_2$] was calculated for BGA1 and BGA2 by subtracting PaO_2 measured by the blood gas analysis (BGA) from the alveolar oxygen tension (PAO_2). PAO_2 was calculated with the following equation:

$$PAO_2 = FiO_2\,(Pb\text{-}P_{H2O}) - PaCO_2/RQ$$

where FiO_2 is the fraction of inspired oxygen (21% for room air), Pb is the barometric pressure measured during the study for each giraffe by the blood gas analyzer, P_{H2O} is the partial pressure of vapor in the alveoli at 37 °C (47 mmHg), $PaCO_2$ is the partial pressure of carbon dioxide of each giraffe. RQ is the respiratory quotient, which depends on metabolic activity and diet, and for carbohydrate diet it is considered to be 1 [81,106]. Since the capture site was not at sea level, the PaO_2 expected for the average altitude of the capture site (range 863 – 1780 m) was calculated for each giraffe in order to use it as the cut-off value for defining hypoxemia, using the alveolar-to-arterial oxygen tension formula. Assuming a normal alveolar-oxygen tension difference of 15 mmHg, the expected PaO_2 was calculated by subtracting 15 mmHg from the calculated PAO_2 value (as described above, but using the measured barometric pressure at each capture site and $PaCO_2$ of 35 mmHg for this estimation, which is considered the physiological reference value at this range of altitudes)

[170,192].The expected PaO_2 at the altitude of each giraffe capture site was subtracted from the measured PaO_2 values of each individual with the following equation:

$$\Delta PaO_2 = PaO_{2\ measured} - PaO_{2\ expected}$$

Individuals with negative values were considered hypoxemic, and the ones with values lower than -20 mmHg severely hypoxemic.

The restraint quality was assessed using a descriptive qualitative score (Restraint score [81]), which considered the reactive behaviour of the individuals to human manipulation and painful stimuli like arterial punctures, GPS fitting and wound treatment. The side on which each giraffe fell was recorded, as well as the occurrence of complications, including regurgitation. The duration of the recumbency phase (Restraint length), the time that taken for the giraffes to stand up (Standing time), and the quality of recovery (Recovery score [81]) were recorded.

2.3 Data Analysis

In order to improve the understanding of the mechanism of physiological alterations occurring in etorphine-azaperone immobilized giraffes, a non-parametric Spearman correlation coefficient was used. In particular, correlations between variation in anesthetic doses, anesthetic times and scores (startle response, excitement, restraint) and physiological and blood tested variables were investigated. When different conditions emerged between helicopter and ground darted giraffes, these were treated as separate groups, either in correlation or descriptive analyses. A Student's t-test was used to evaluate the difference over time between mean values of blood gases and selected biochemistry, and physiological variables analyzed at BGA1 and BGA2. To evaluate the trend of blood sample variables after antagonization, the delta for each variable was calculated subtracting the value analyzed at BGA1 from the one analyzed at BGA2. These deltas were tested for correlations with drug doses, scores and times to investigate possible relationships between these factors and the trend of physiological alterations. A Bland-Altmann plot was used to evaluate the concordance between $ETCO_2$ and $PaCO_2$ values for each sampling time point.

The statistical methods were chosen on the basis of the nature of data (mixed measured/score data) and the sample size. Numeric data are presented as mean values ± standard deviations, with ranges where relevant. Scores are presented as median values with ranges. Only the most relevant statistical results are reported; a *p*-value below 0.05 was considered significant.

3. Results

In the present study, the data collected from all the eighteen reticulated giraffes were included. The giraffes, twelve females and six males, were both young adults and adults. According to the estimations made from the measurements, individuals weighed 838 ± 142 kg. Administered doses per kilo of etorphine were 0.017 ± 0.002 mg/kg and 0.101 ± 0.02 mg/kg of azaperone. The doses varied

among individuals. Indeed, in some of the giraffes, prolonged induction and high excitement that are usually attributed to etorphine underdosing [46] occurred, as well as a rough restraints that can be linked to a higher induction-induced excitement [81]. To tentatively reduce this undesired phase, the doses were increased to 15 mg of etorphine and 100 mg of azaperone in eight giraffes (captured in Samburu and Marsabit). In two old male giraffes immobilized for limb injuries, a dose of 17 mg of etorphine was administered. A higher total dose of etorphine was inversely correlated to a lower excitement score ($r = -0.54$, $p = 0.02$), and shorter induction time ($r = -0.48$, $p=0.05$), but was not correlated with the restraint quality. A higher dose of etorphine per-kg was not correlated to excitement, but was correlated to lower levels of potassium ($r = -0.53$, $p = 0.02$). Higher doses of azaperone per-kg were correlated to higher sodium ($r = 0.56$, $p = 0.02$), chloride ($r = 0.64$, $p = 0.007$) and to a lower pH ($r = -0.59$, $p = 0.02$). However, azaperone doses were correlated also to the area of the capture site ($r = 0.69$, $p = 0.004$), as higher azaperone was given in two capture sites (Samburu and Marsabit), in one of which helicopters were used for darting and severe exertion occurred. Most individuals were darted once, except for two individuals that required a second darting since the first dart did not discharge.

The startle response of the giraffes was extremely varied (median score 2, range 1 – 4). Vehicle darted giraffes in general showed little startle response (score 1 or 2), except for those that were darted two times (because the first dart did not discharge, $n = 2$). In these two individuals, after the first darting attempt that resulted in a quick sprint, the giraffes were more alert, and kept distance from the darting vehicle with an accelerated pace, and thus a score 3 was given. Two of the three giraffes with a limb lameness had an increased score (2 and 3) as they kept on moving away from the darting vehicle for a few minutes, although this did not involve ambling or galloping. The helicopter darted giraffes were chased at a fast galloping gait for several minutes (5 – 7 minutes), in order to be herded to open areas, and as such a score of 4 was given. Startle response was correlated with the area of capture ($r = 0.579$, $p = 0.01$), which was divided in 4 categories based on the geographic region, touristic flow and ecosystem similarity (reflecting Laikipia, Samburu, Marsabit and Isiolo counties). After darting, all giraffes including the helicopter darted individuals, sprinted for a maximum of 30 seconds, and then returned to their previous activity. The first signs of initial drug effects were observed within 4.0 ± 1.6 min and included signs of ataractic tranquilization defined as a decreased interest for external stimuli, increased motor activity with an ataxic gait, isolation from the herd, and beginning of excitement, characterized by a sudden gallop or accelerated amble usually following the rising of ataraxia. Median induction-induced excitement was 3 (range 1 – 4). Extreme excitement with prolonged and dangerous attempts to rope were seen in four giraffes that were administered lower doses of etorphine and azaperone (15 and 70 mg respectively). Excitement was not higher in helicopter darted giraffes, and two of them went recumbent alone without roping. Also four ground darted giraffes reached recumbency spontaneously, three of which were immobilized for limb injuries. A higher excitement score was correlated to longer induction times ($r = 0.809$, $p < 0.001$). Inductions were achieved on average 8.2 ± 7.6 minutes after darting. Eleven giraffes fell recumbent in left recumbency, whereas seven in right recumbency, and none of them regurgitated at any stage. No difference was observed in the physiological variables according to the side of recumbency.

Antagonism was administered on average 1.3 ± 1.0 minutes after recumbency was reached. Different doses of diprenorphine and naltrexone were given, according to the veterinarian in charge. These were, given as a ratio with the mg of etorphine administered, either diprenorphine in a ratio 2 – 2.5:1 to etorphine, diprenorphine 2:1 combined with naltrexone 3:1, or naltrexone 6:1. The different antagonist doses did not account for correlations with any of the physiological or blood gas analysis values or restraint and recovery score, and no clinical difference was observed. Immobilization quality (median 3, range 2 – 4) was however varied. Some giraffes kicked occasionally (n = 7), and others showed repetitive attempts to stand up and kick (n = 7), often in coincidence with painful stimuli, such as during ossicone drilling or wound curettage but also non concurrently to it. Four giraffes tried to lift their neck aggressively, making the restraint dangerous. The restraint score was also not correlated to the initial etorphine or azaperone doses.

In order to evaluate the trends of the different physiological variables, the values recorded were divided in four time periods. These were: t0, within one minute after recumbency was reached and before the administration of the antagonist; t1, between 1 and 3 minutes after recumbency and after the administration of the antagonist; t2, between 4 and 8 minutes after recumbency; t3, between 9 and 15 minutes after recumbency.

Respiratory rate, recorded in breaths/minute did not change between t1 (19.2 ± 3.2), t2 (20.3 ± 5.4) and t3 (21.1 ± 4.2), whereas heart rate, recorded in beats/minute slightly decreased over time, although not significantly ($p > 0.05$) from the first recording at t1 (68.2 ± 22.6) to the second at t3 (63.9 ± 16.2). Respiratory rate did not show any correlation ($p > 0.05$) with anesthetic doses, times or scores. An initially higher HR was correlated with a higher excitement score (r = 0.51, $p = 0.03$), with the area of capture (r = 0.5, $p = 0.03$), and with azaperone per-kg (r = 0.56, $p = 0.01$). Furthermore, a higher initial HR was correlated with a lower pH (r = -0.72, $p < 0.001$), HCO_3^- (r = -0.61, $p = 0.008$) and base excess (r = -0.70, $p = 0.002$), and with higher lactates (r = 0.59, $p = 0.01$), sodium (r = 0.67, $p = 0.002$), chloride (r = 0.76, $p < 0.001$), glucose (r = 0.60, $p = 0.008$). Heart rate, both at the beginning and at the end of the restraint, was correlated with a higher (worse) restraint score (r = 0.67, $p = 0.008$ and r = 0.86, $p < 0.001$). The invasive mean blood pressure was recorded in 10 giraffes only, and the mean value was 122 ± 32.9 mmHg (range 90 – 200 mmHg). The IBP was correlated with worse excitement score (r = 0.80, $p = 0.005$), longer time of induction (r = 0.81, $p = 0.08$), but not with startle score, drug doses and antagonists. Rectal temperature was 38.3 ±1.1 °C at t1 and 38.4 ± 1.0 °C at t3, and did not change significantly. Rectal temperature at t1 ant t3 was associated with a lower initial $PaCO_2$ (r = -0.69, $p = 0.001$; r = -0.64, $p = 0.004$), and $ETCO_2$ (r = -0.79, $p = 0.01$; r = -0.84, $p = 0.004$), and with higher creatinine (r = 0.64, p = 0.004 and r = 0.73, $p < 0.001$). In ground darted giraffes the temperature at t1 and t3 was associated with higher startle score (r = 0.69, $p = 0.006$ and r = 0.81, $p < 0.001$), and at t3 only with higher glucose (r = 0.52, $p = 0.02$) and anion gap (r = 0.58, $p = 0.01$). The rectal temperature was not higher in case of environmental temperature.

Two arterial samples were obtained respectively at t1 (BGA1, 1.7 ± 1.3 minutes after antagonist administration) and at t3 (BGA2, 7 ± 2.4 minutes after antagonist administration). BGA1 was obtained from all giraffes, whereas BGA2 in only 10 giraffes. The results of BGA1 and of selected biochemical

variables from all the individuals are shown in Table 1, together with other variables such as RR, HR, $ETCO_2$ recorded at the time of BGA1, in order to better highlight the pathophysiological mechanism that occurred in the giraffes and their compensatory response. The results in the table are ordered according to the startle response score and the excitement score, as the two scores had several correlations. Information regarding the type of darting (helicopter Vs. vehicle) and the area of capture are included since these influenced some of the variables. Indeed helicopter and ground darting accounted not only for different behavioural response, but also in remarkable differences in the physiological responses and alterations, and for this reason their results are presented and discussed separately, through descriptive comparisons. The mean values and standard deviation of BGA1 and BGA2 of the 10 individuals in which two blood samples were successfully obtained are represented in Table 2. In the table both corrected and non-corrected by the rectal temperature are represented, whereas in the text, unless specified, pH, $PaCO_2$ and PaO_2 refers to values non-corrected for the rectal temperature.

The initial pH was acidic in all giraffes except three, whereas overall mean $PaCO_2$ and PaO_2 were within normal ranges for mammals. However, hypercapnia (> 45 mmHg) was detected in five individuals, and hypocapnia (< 35mmHg) in five individuals. In particular the increased levels of $PaCO_2$ were recorded in ground darted giraffes that were immobilized with higher doses of etorphine and azaperone (15 and 100 mg respectively), in which extreme low pH values (mean pH 7.138) were also observed. Arterial oxygenation was also various, with ranges between 48 and 162 mmHg. Although no significant correlations were observed, increases of PaO_2 were more common in individuals that underwent higher excitement, supported by the fact that initial PaO_2 was correlated to lower bicarbonates ($r = -0.65$, $p=0.006$). The alveolar-arterial gradient (mean 8.1 ± 29.2 mmHg) was increased (> 20 mmHg) in seven giraffes, and interestingly the gradient was negative in four individuals that underwent extreme post-induction excitement and had high arterial oxygenation levels. Bicarbonates and base excess were variously decreased in most individuals. The initial low levels of pH were inversely and significantly correlated with higher lactate ($r = -0.72$, $p = 0.002$), but not with increased $PaCO_2$. Lactates were increased to various extents in all individuals, and were beyond the maximum values detected by the analyzer (20 mmol/l) in two individuals that were helicopter darted. Higher lactates were also correlated with higher sodium ($r = 0.75$, $p < 0.001$), potassium ($r = 0.57$, $p = 0.01$), chloride ($r = 0.62$, $p = 0.008$), anion gap ($r = 0.66$, $p = 0.005$), glucose ($r = 0.57$, $p = 0.01$), hematocrit ($r = 0.57$, $p = 0.01$), hemoglobin ($r = 0.57$, $p = 0.01$), and BUN ($r = 0.48$, $p = 0.04$). Mean values recorded for sodium, potassium, calcium (1.2 ± 0.1 mmol/l), and for anion gap (21.9 ± 3.4 mmol/l) were within ranges reported for wild giraffes, although in some individuals values were severely altered (Table 1) [194,217]. Mean chloride was higher compared to giraffe range values [194]. Glucose, hematocrit and hemoglobin levels were variously increased. Creatinine was within normal ranges, whereas mean BUN was elevated [194,217]. Cardiac troponin I was detected in 11 individuals, most of which was below 0.03 ng/ml, except for two giraffes that had values of 0.04 and 0.07 ng/ml, and that were immobilized because of a limb injury. In these giraffes the blood sample was collected later compared to the other giraffes, respectively 30 and 60 minutes after the giraffes were darted.

Area	Capture mean	Startle score	Excitement score	RR (bpm)	HR (bpm)	T (°C)	pH	$PaCO_2$ (mmHg)	PaO_2 (mmHg)	BE (mmol/l)	HCO3- (mmHg)	Lactate (mmol/l)	$P(A\text{-}a)O_2$ (mmHg)	Na (mmol/l)	K (mmol/l)	Cl (mmol/l)	Glu (mmol/l)	BUN (mg/dl)	Creatinine (mg/dl)	PCV (%)	Hb (g/dl)	CTNI (ng/ml)
1	V	1	1	16	40	36.8	7.283	45.9	74	-5	21.7	2.89	3.16	138	4.8	104	98	15	1.4	18	6.1	0.00
1	V	1	1	16	48	38.1	7.37	38.9	NA	-3	22.5	1.96	NA	144	5	108	85	28	1.3	31	10.5	0.01
2	V	1	2	16	80	36.5	7.090	55.3	68	-13	16.8	5.76	11.1	143	5.2	116	101	19	1.4	23	7.8	0.03
2	V	1	3	26	80	36.8	7.119	49.7	132	-13	16.1	11.51	-48.3	153	4.4	119	144	21	1.6	32	10.9	0.02
2	V	1	3	16	64	37.5	7.059	45.4	54	-17	12.8	8.42	35	148	4.2	120	99	20	1.5	23	7.8	0.00
1	V	1	4	20	60	37.4	7.135	50.1	47	-12	16.9	11.65	23.4	149	5.1	115	125	32	1.5	41	13.9	0.01
1	V	1	4	20	40	38.3	7.345	37.1	77.0	-11	14.8	6.05	19.2	143	5.1	109	74	20	1.6	34	11.6	0.00
1	V	1	4	16	76	39.4	7.241	37.4	54	-11	16.1	10.53	29.4	145	4.1	110	214	10	1.7	37	14.6	0.01
1	V	2	1	16	44	39.8	7.23	31.6	60	-14	13.2	13.29	30.8	140	5.7	109	86	36	1.7	41	13.9	0.07
1	V	2	1	20	60	39	7.334	41.7	57	-4	22.2	NA	23.1	142	4.7	108	99	26	1.6	38	12.9	NA
2	V	2	3	20	54	38.5	7.11	40	116	NA	NA	14.16	-21.1	153	5.2	115	138	30	1.8	32	10.9	0.03
4	V	3	1	22	40	40.6	7.316	34.7	48	-8	18	10.1	43.9	148	4.6	111	200	19	2	39	13.3	0.04
1	V	3	3	20	80	38.2	7.305	32.6	53	-10	16.2	8.62	37.6	145	4.2	110	164	32	2	40	13.6	0.01
1	V	3	4	24	68	39.8	7.293	17.6	162	-18	8.5	13.14	-59.3	145	4.7	110	214	25	2.3	43	14.6	0.01
3	H	4	4	22	100	38.1	6.826	37.9	96	-28	6.3	> 20	3.2	155	7	123	186	27	2	37	12.6	0.00
3	H	4	3	22	100	37.5	6.99	42.5	85	-21	10.2	> 20	9.6	153	6.2	118	191	32	1.6	46	15.6	0.01
3	H	4	3	20	100	38.4	7.022	42.3	82	-20	11	17.15	12.8	153	5.3	122	203	27	1.5	30	10.2	0.01
3	H	4	3	14	90	38.2	6.953	36.5	116	-24	8.1	14.69	-15.3	155	5.1	127	173	27	1.5	43	14.6	0.00
Mean				**19.2**	**68**	**38.2**	**7.168**	**39.2**	**81.2**	**-13.6**	**14.77**	**11.2**	**8.15**	**147**	**5.0**	**114.2**	**145**	**24.8**	**1.7**	**34.9**	**11.9**	**0.015**
Standard Deviation				**3.2**	**21.3**	**1.0**	**0.157**	**8.9**	**33.1**	**6.9**	**4.8**	**5.2**	**29.2**	**5.3**	**0.7**	**6.3**	**50.6**	**6.8**	**0.3**	**7.7**	**2.6**	**0.019**

Table 1. Most relevant BGA1 values and selected biochemistry and physiological variables of each giraffes, organized according to the startle and excitement score. The areas of capture are 1 = Laikipia, 2 = Samburu, 3 = Marsabit, 4 = Isiolo. Capture means are V = vehicle, H = helicopter. Mean and standard deviation are reported, NA = non analyzed value.

A more intense startle response was correlated to higher levels of lactate, (r = 0.78, $p < 0.01$), sodium (r = 0.54, $p = 0.02$), glucose (r = 0.58, $p = 0.01$), hematocrit (r = 0.54, $p = 0.01$), hemoglobin (r = 0.54, $p = 0.01$), anion gap (r = 0.64, $p = 0.005$), and the respiratory rate measured by the capnometer (r = 0.61, $p = 0.04$), and negatively with lower bicarbonates (r = -0.60, $p = 0.01$), base excess (r = -0.58, $p = 0.01$) and $ETCO_2$ (r = -0.60, $p = 0.05$). However, since the helicopter darted giraffes underwent severe exertion which might have confounded the results, the same correlation was tested considering ground darted giraffes only, and, as expected, the correlations changed. The startle response was still positively correlated to hematocrit and hemoglobin (both r = 0.64, p=0.01), anion gap (r = 0.59, $p = 0.03$), and $ETCO_2$ RR (r = 0.61, $p = 0.04$), and inversely correlated to $ETCO_2$ (r = -0.60, $p = 0.05$); whereas it was not correlated to lactates, sodium, glucose or bicarbonates in the ground darted individuals. Furthermore, a higher startle response in ground darted giraffes was correlated to a higher rectal temperature (r = 0.69, $p = 0.006$), and to a lower initial $PaCO_2$ (r = -0.59, $p = 0.02$).

Excitement score was correlated in all giraffes to more elevated glucose (r = 0.51, $p = 0.03$) and heart rate (r = 0.51, $p = 0.03$), and negatively to lower bicarbonates (r = -0.59, $p = 0.001$), and $PaCO_2$ corrected by the temperature (r = -0.51, $p = 0.04$).

Variable	BGA1	BGA2
pH	7.201 ± 0.10	7.202 ± 0.10
$PaCO_2$ (mmHg)	41.0 ± 11.3*	33.6 ± 6.7*
PaO_2 (mmHg)	84.1 ± 39.0	84.5 ± 33.6
BE (mmol/l)	-11.5 ± 4.6	-13.6 ± 5.6
HCO3- (mmol/l)	16.2 ± 4.1	14.1 ± 4.4
SaO_2 (%)	86.6 ± 10.8	90.1 ± 7.4
Lactates (mmol/l)	9.3 ± 3.7	9.0 ± 3.8
$P(A\text{-}a)O_2$ (mmHg)	1.5 ± 43.2	8.6 ± 32.4
T (°C)	38.1 ± 1.3	38.2 ± 1.2
pH (mmHg; corrected for T)	7.185 ± 0.1	7.197 ± 0.08
$PaCO_2$ (mmHg; corrected for T)	42.0 ± 11.0*	35.3 ± 6.0*
PaO_2 (mmHg; corrected for T)	87.2 ± 41.5	91.4 ± 37.5

Table 2. Mean ± standard deviations of BGA1 and BGA2 values of the giraffes in which two arterial samples were obtained (n=10). *= difference between BGA1 and BGA2 is statistically significant (p = or < 0.05).

The mean pH did not change between the two sampling points. When pH corrected by rectal temperature was considered, pH slightly increased between the two sampling points in six giraffes, even though all giraffes except for one remained in acidosis in BGA2 with a pH lower than 7.35; it remained stable in two giraffes and worsened in two. When non corrected pH was used, acidosis improved only in four giraffes and worsened in six. Both $PaCO_2$ non-corrected and corrected by temperature decreased significantly from BGA1 and BGA2 ($p = 0.02$ and $p = 0.05$ respectively). All five of the initially increased values of $PaCO_2$ (> than 45 mmHg) seen in BGA1 decreased below 45 mmHg in BGA2. $PaCO_2$ decreased below the physiological levels of 35 mmHg in six giraffes in BGA2. The mean PaO_2 did not show significant increase in BGA2, even though when the values were corrected by the rectal temperature there was a slight improvement over time. In order to evaluate the degree of hypoxemia according to the altitude, the delta PaO_2 between the measured PaO_2 and the

expected PaO_2 for each giraffe capture site altitude was calculated in the ten giraffes with both blood gas values. This value was 13.4 ± 34.2 mmHg at BGA1 and 13.8 ± 36.8 mmHg at BGA2, and when values were corrected by the rectal temperature, respectively 19.5 ± 37.2 mmHg in BGA1 and 20.7 ± 39.2 mmHg in BGA2. In BGA1 the gradient was negative in five giraffes, whereas in BGA2 it was negative in three giraffes only, and only in one giraffe the hypoxemia was considered severe as the values were below -20 mmHg. In all those giraffes that had an improvement in pH, there was a decrease in $PaCO_2$, and in all but one an improvement in PaO_2. The two giraffes that underwent a worsening for pH, $PaCO_2$ and PaO_2, were characterized by a hyperoxygenation and hypocapnia at the beginning of the immobilization, and included two giraffes with a prolonged excitement phase. A higher (worse) restraint score might have also influenced the physiological response during the restraint, or vice versa, since it was correlated to a higher initial $PaCO_2$ ($r = 0.54$, $p = 0.03$) and to a lower pH ($r = -0.61$, $p = 0.02$) at BGA2. The alveolar-arterial gradient overall increased, with some individual differences, reflecting the heterogeneity of the trend of PaO_2 and $PaCO_2$ between BGA1 and BGA2. In four giraffes the gradient became pathological in BGA2, with values higher than 20 mmHg, whereas in all three individuals that had an initially increased gradient, it improved in BGA2. Lactate values did not change in most individuals, whereas both bicarbonates and base excess decreased at BGA2.

In order to evaluate the trend of the blood gas analysis values over time, a delta for each value was calculated as the difference between each value at BGA2 and BGA1. A higher delta represented an increase of the values in BGA2, whereas a lower delta represented a decrease of the value. A higher PaO_2 delta was correlated to lower initial PaO_2 ($r = -0.69$, $p = 0.03$), and a higher delta $PaCO_2$ was correlated to lower initial $PaCO_2$ ($r = -0.85$, $p = 0.006$). The delta bicarbonate was also correlated to lower initial $PaCO_2$ ($r = -0.82$, $p = 0.006$), and positively to the trend of $PaCO_2$ ($r = 0.97$, $p < 0.01$).

Nasal capnometry was recorded in 14 individuals, and at four time points during the monitoring period. The values of $ETCO_2$ and respiratory rate measured by the capnometer were, at t0 53.3 ± 12.2 mmHg and 16.8 ± 3 breaths/minutes, at t1 43.3 ± 9.6 mmHg and 17.8 ± 3.8 breaths/minute, at t2 37.1 ± 6.7 mmHg and 18.8 ± 3.9, and at t3 33.4 ± 7.2 mmHg and 16.5 ± 4.6 breaths/minute. $ETCO_2$ values changed significantly between t0 and t1 ($p = 0.003$), and then between t1 and t2 ($p < 0.001$), and t2 and t3 ($p < 0.001$). $ETCO_2$ significantly decreased from t1 (time of BGA1) and t3 (time of BGA2) ($p = 0.006$), and the greater decrease was correlated to higher initial $PaCO_2$ ($r = 0.64$, $p = 0.04$), but not with the trend of $PaCO_2$. Although $PaCO_2$ at BGA1 was not correlated with $ETCO_2$ at BGA1, the same variables were correlated at BGA2 ($r = 0.828$, $p = 0.006$). The respiratory rate measured by the capnometer correlated at BGA1 and BGA2 with respiratory rate at t2 ($r = 0.77$, $p = 0.005$) and at t3 ($r = 0.96$, $p < 0.001$).

The difference between $PaCO_2$ and $ETCO_2$ corrected by the temperature were -1.1 mmHg at BGA1 and 0.56 mmHg at BGA2, whereas those non corrected by the temperature were -3.7 mmHg at bga1 and -1.4 mmHg at BGA2. The agreement at all time sampling points for $ETCO_2$ and $PaCO_2$, corrected by rectal temperature, are represented in Bland-Altman plots, divided by BGA1 and BGA2. At BGA2 (Figure 2) the level of agreement was more narrow (-8.0 the lower level of agreement, 6.7 the upper, bias -0.62) than BGA1 (lower level of agreement -16.6, upper 18.8, bias 1.08; Figure 1). The gap between PaO_2-$ETCO_2$ at t1 was correlated with lower times of induction ($r = -0.65$, $p = 0.04$)

and PaO_2 (r = -0.78, p = 0.001), where for BGA2 there were no correlations. In BGA1 the biggest differences between PaO_2-$ETCO_2$ were observed in giraffes with initial PaO_2 higher than 100 mmHg, whereas in all other giraffes the difference was within a difference of ± 3 mmHg.

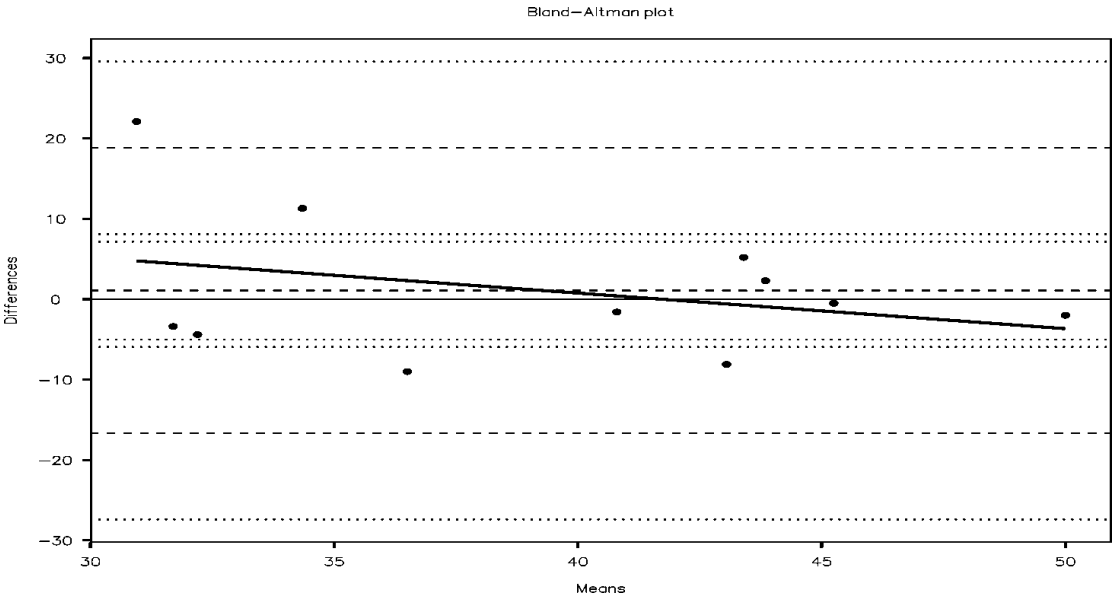

Figure 1. Bland-Altman plot representing the difference of $ETCO_2$ and $PaCO_2$ corrected by temperature at BGA1, in relation to the mean values of $PaCO_2$ at BGA1.

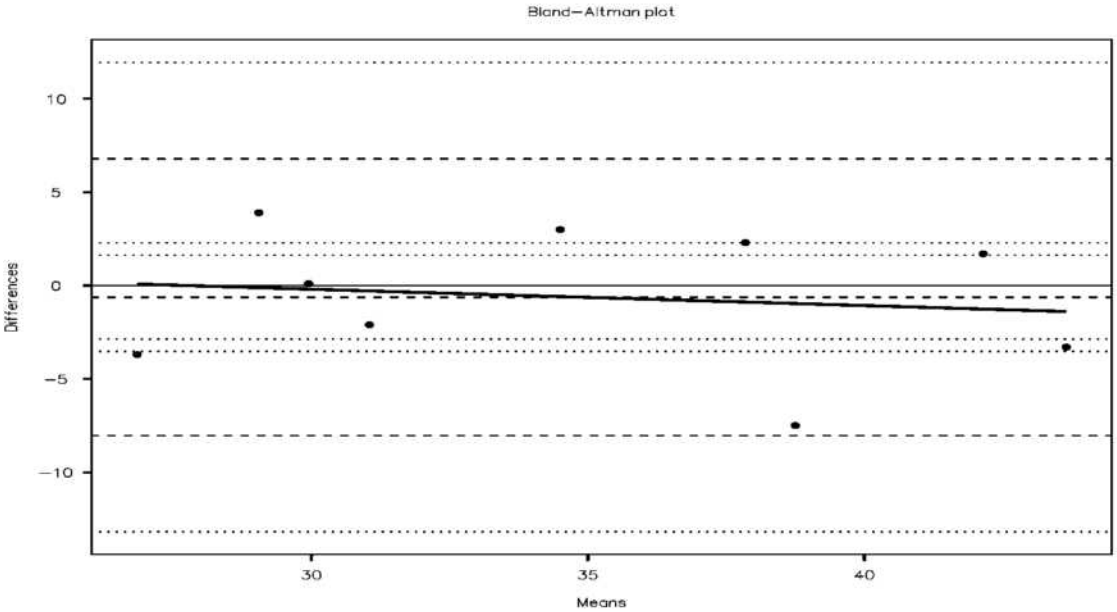

Figure 2. Bland-Altman plot representing the difference of $ETCO_2$ and $PaCO_2$ corrected by temperature at BGA2, in relation to the mean values of $PaCO_2$ at BGA2.

When the manual restrained was lifted, all the giraffes woke up immediately, and the recovery score was rated excellent in all giraffes, except for two individuals that took around 20 seconds and

were scored 2. One of these giraffes had a limb injury, and the other one had gone severe exertion as was darted from the helicopter. The two other giraffes that were immobilized because of limb injuries were humanely euthanized due to the severity of the injuries, therefore data on their recoveries are missing.

Through the monitoring of the movement registered by the GPS units it was possible to detect eventual mortality. Some of the GPS units had technical issues such as voltage drop, and stopped transmitting data earlier than expected. In three ground darted giraffes the ossicone units stopped transmitting data 17, 43 and 68 days after capture, but it was confirmed by the monitoring team that it was not due to mortality but technical problems or due to ossicone-units that fell off. All the other giraffes were reported alive at least four months after the capture.

4. Discussion

Most of the free-ranging reticulated giraffes included in this study, immobilized with an etorphine and azaperone combination, experienced various degrees of homeostatic imbalance. This included moderate to severe acid-base and metabolic alterations, combined in some individuals with respiratory compromise.

The main factors of risk for giraffe immobilization are believed to be their susceptibility to capture stress which can easily lead to overexertion and capture myopathy, the sensitivity to opioid's adverse effects, and capture-related injuries [81,119,126,187,195,197,198,214]. Etorphine-azaperone combination is commonly used for free-ranging herbivore immobilization, as etorphine provides quick and reversible restraint that is advantageous in the field, whereas azaperone is believed to shorten induction times, counteract etorphine pulmonary hypertension and add tranquilization during the physical restraint [34,70,81,154]. This combination is usually preferred in free-ranging giraffes as it is characterized by short induction times, which are important to keep excitement to a minimum and reduce the risk of injuries. Indeed the occurrence of overexertion is peculiar in giraffes since it can result from the eventual exertion triggered during the darting process (especially when helicopter darting is involved) and from an induction-induced excitement that has been linked to opioid underdosing and prolonged inductions, and whose intensity is far more extreme in giraffes than in other species [46,81]. However, although etorphine-azaperone combination provides some logistic benefits for field capture, in this study resulted in moderate to severe acid-base and gas exchange alterations, similarly to other combinations that have been used in giraffes including opioid- and ketamine-based [81,187,197] protocols. In particular, moderate to extremely severe metabolic acidosis, with pH values in some giraffes below 7, and characterized by increased lactates, decreased HCO_3^- and BE, were observed in most individuals. Lactates were the main cause for acidosis in our study, as showed by a stronger correlation of pH with lactates, compared to the respiratory component. Concomitant, or predominant, respiratory compromise was indeed observed only in a few giraffes, in which elevated $PaCO_2$ resulted in severe mixed acidosis. Only three giraffes maintained a physiological acid-base balance, either because lactates were not elevated or respiratory compensation occurred. Severe acidosis is of particular concern since it can be life-threating, especially in opioid-based immobilization where respiratory depression might limit the ventilatory compensation, and it is linked with major

alterations such as electrolyte imbalance and muscle cell breakage that increase mortality in wildlife capture [47,64,76]. In our study this is supported by the lower pH associated not only to higher lactates, but also to alterations in other indicators of severe physiological derangement. Indeed, lactates increased together with potassium, chloride, sodium, anion gap, glucose, hematocrit, hemoglobin and BUN. In particular, the high levels of potassium observed in helicopter darted giraffes were of concern as hyperkaliemia can induce changes in neuromuscular and heart excitability which can lead to ventricular fibrillation and death [74,75]. Mean chloride, which elevations are not uncommon in captured wildlife species [218,219], was higher compared to ranges reported for wild captured giraffes [194,217], and its increases were correlated also with hyperglycemia. Indeed, similar to other studies in captured free-ranging giraffes [194], glucose levels were elevated, likely as a result of the hyperglycemic effect of the catecholamines and glucocorticoids released after an acute stressor or exercise [43,64]. An increase in BUN has been observed in different wildlife species after capture, and has been linked to strenuous exercise [75,76,85], whereas hematocrit and hemoglobin can increase as a consequence of both fear-mediated splenic contraction or strenuous exercise [83]. Although cardiac troponin I can be elevated not only after myocardial injury, but also after strenuous exercise without indicating cardiac pathology, in this study no significantly increased levels were detected [86]. However the fact that the two highest value recorded (0.04 and 0.07 ng/ml) were from those samples collected more far (30 and 60 minutes) from when the stressor occurred, and that peak elevation usually occur 2 – 6 hours after exercise [87,220], it might suggest that even a small early increase might be significant and should be taken into account, and that further investigations are needed to understand the clinical value of increases in cardiac troponin I in giraffes.

The acidosis seen in most of the giraffes, was not directly correlated to possible predisposing factors such as a higher startle response to darting or the intensity of induction-induced excitement. However, other indicators of acid-base imbalance, such as low bicarbonates, were instead correlated to a greater excitement, similarly to a study performed in Masai giraffes where the same scoring system was used [81]. In contrast with the previous study, in this study of reticulated giraffes we observed that also a higher startle score was linked to acid-base alterations, specifically to lower bicarbonates and base excess. Furthermore, the two scores were differentially associated with other homeostatic alterations commonly associated with capture stress, thus suggesting that the stress response resulting from the darting operation, and the exertion induced by opioid excitement, triggered different pathophysiological mechanisms. Startle response score is a score built in order to tentatively evaluate the stress response of giraffes during the darting procedure, based on the observed behaviour, and does not reflect increased physical activity or exertion until its higher level, which in this study was seen only in the four helicopter darted giraffes. On the contrary, induction-induced excitement always involves a degree of exertion as it reflects the etorphine-mediated excitement that results in increased motor activity until recumbency is reached by roping, or spontaneously in a few cases. In this study, a higher startle response accounted for increases in metabolites commonly associated with capture stress, such as lactates, sodium, chloride, glucose, hematocrit and hemoglobin, with some of these values substantially increased [43,47,64,75,76,85]. However, increased physical activity linked to the startle response occurred only in the four helicopter darted giraffes, whereas in the ground darted giraffes no

or limited activity preceded darting, which highlights that these alterations might have been mainly triggered by the extreme overexertion due to minutes of fast gallop that occurred during the helicopter chase, and not by the psychological stress itself. This is supported by the fact that helicopter darted giraffes had extremely low pH and high lactate levels, and the maximum alterations in potassium, glucose, sodium, chloride and BUN levels, which were far from all the values observed in ground darted giraffes. Thus, when the startle response was considered in ground darted giraffes only, a higher score was not associated with acid-base alterations, but it had an effect on hyperthermia, hematocrit and hemoglobin. This result is in agreement with the concept that it is also elevated psychological stress, and not only the intense physical activity, that can trigger a homeostatic cascade with pathological consequences. Indeed, hyperthermia is an indicator of psychological stress, and can indicate other physiological alterations, such as elevations in glucose and creatinine, that in our study were associated with hyperthermia and that can both increase as a result of catecholamine release in fright responses [41,85]. The fact that hyperthermia was not associated with higher excitement or environmental temperature, is in agreement with a study conducted in another herbivore, the impala, where it was not primarily related to the effects of drugs such as metabolic and vascular effects of catecholamine release, environmental conditions, or physical activity but rather appeared to be strongly related to the level of initial psychological stress in response to capture [41]. Since capture-induced hyperthermia can contribute to the development of capture myopathy [41,47,81,82], it is important to consider the startle response when darting free-ranging giraffes, and its prevention requires particular care, especially when using opioids that alter the thermoregulation mechanism [34,41,134]. A greater induction-induced excitement instead accounted, other than decreased bicarbonates, for hyperglycemia, and for an increase in heart rate. A greater excitement was correlated with lower etorphine doses in mg, however not to the doses calculated per-kg. The doses per kg are calculated on the estimated weight based on body measurements, therefore might not be completely accurate and are showed only for comparison. However, lower potassium, which is usually elevated after strenuous activity as a result of muscle damage [74,75], was associated with higher etorphine doses, and a difference in terms of less excitement was clinically appreciable when the doses of etorphine and azaperone were increased. The correlations observed between higher doses of azaperone with sodium, chloride and lower pH might be instead caused by confounding factors, since higher azaperone was given to the helicopter darted giraffes where severe exertion occurred. In the light of these results and the interaction observed, elevations of lactates, electrolytes, glucose and BUN seems associated with increased physical activity. Hematocrit and hemoglobin instead seemed to be more associated to psychological stress in ground darted giraffes regardless of physical activity, probably as a consequence of fear-mediated splenic contraction [83]. Creatinine, which also has been linked with capture stress [76,85], in our study increased more in hyperthermic individuals which were those with greater startle response, but also in those subjected to intense exertion. This finding might support the idea that elevation in creatinine might happen as a consequence of both psychological and physical stress; however further investigations on a larger sample size are needed to validate these hypotheses.

Although excitement was correlated with some severe metabolic alterations, acidosis was not constantly observed in giraffes that underwent more excitement. This is supported by the fact that a

higher excitement score was correlated with hypocapnia, presumably resulting from the respiratory compensatory system that was activated to limit the metabolic acidosis that occurred [221]. Ground darted giraffes that were darted with higher doses of etorphine and azaperone were instead hypercapnic, and had extremely decreased pH. It would be speculative to say if hypercapnia in these giraffes was due to etorphine-mediated respiratory depression or to less respiratory compensation since they had run less, as the excitement phase was less intense [222]. However, in these ground darted giraffes, the acidosis was mixed and was particularly severe, with lactates moderately high, but no major alterations in electrolytes, glucose and hematocrit were observed. In contrast, in helicopter darted giraffes that were also darted with high doses of etorphine and azaperone, the carbon dioxide was below physiological levels, probably as a consequence of the hyperventilation triggered pre-darting by the strenuous run while chased by the helicopter [223]. Even though the occurrence of a respiratory response seen with initial hypocapnia might be beneficial in partially compensating metabolic acidosis [221], the lack of a second BGA sample in the helicopter darted giraffes prevented to establish if improvement over time actually occurred. Not only initial hypo- and hypercapnia were observed in this study, but also initial arterial oxygenation showed some variability, with both hypo- and hyperoxemic giraffes. According to the PaO_2 gradient, created in order to evaluate the degree of hypoxemia in relation to the altitude of each capture site, eight giraffes were hypoxemic, and of these three were severely hypoxemic, and four giraffes had values higher than 100 mmHg of PaO_2. Giraffes are particularly susceptible to hypoxemia when immobilized [126,187,195,197], and the fact that initial severe hypoxemia was found only in a few giraffes, might be mainly attributed to the early etorphine antagonization. The initial degree of arterial oxygenation was not correlated to startle or excitement scores, nor to etorphine or antagonists doses administered. However, we observed more elevated PaO_2 in individuals with higher excitement or that underwent a strenuous exertion in helicopter darted giraffes. This initial heterogenous scenario of hypo- and hypercapnia and oxygenation is in agreement with reports from other large herbivore species sensitive to capture stress and that commonly undergo both exertion and opioid adverse effects [95,106]. Although giraffes have a small lung volume and low lung compliance that prevent them from increasing tidal volume, when their oxygen demand is increased such as during exercise, they have evolved a mechanism to increase oxygen diffusion capacity together with respiratory rate [188]. This mechanism is efficient in responding to changes in oxygen demand, and physiologically supports giraffe athletic performances, as they can run up to 65 km/h for 5 minutes [222], and might explain the initial hyperoxygenation and the low alveolar-arterial gradients seen in many giraffes that had run for long.

However, in order to better understand the mechanism of the physiological alterations resulting from the capture, and the changes occurring over time following the early antagonization, it was fundamental to consider the trends of the BGA values in the ground darted giraffes [224]. In giraffes, opioid antagonists are administered as soon as the giraffe reach the ground, to limit the adverse effect of etorphine on cardio-respiratory function [34,125]. When early antagonization is not provided, mortality has been experienced and anecdotally reported by field veterinarians, and was attributed to hypoxemia and respiratory depression. In this study the different antagonist ratios used, including a full dose of diprenorphine, a combination of diprenorphine and naltrexone, or naltrexone alone,

resulted in similar effects. Although the dose of naltrexone, when it was administered alone, might be considered low compared to those recommended [34], it produced adequate effects in this study, and was also higher than that reported in Masai giraffes, where resedation was excluded by a meticulous 2 weeks follow-up [81]. In this study it was not possible to obtain blood gas analyses before the antagonist was administered, but the fact that the arterial oxygenation recorded at the beginning of the immobilization was not severely compromised, supports the idea that this practice provides some benefits. In addition, the fact that, although only a small time passed between the two sampling points, there was an improvement in gas-exchange in most giraffes is encouraging. However, acid-base balance was more affected and severe metabolic alterations remained. Carbon dioxide is the only variable that significantly changed over time. However, if the trends are evaluated individually, pH slightly increased in 6 giraffes, along with improvement in carbon dioxide and arterial oxygenation in most of the same individuals. Instead, the values of giraffes with prolonged pre-induction excitement and that were characterized by a hyperoxygenation and hypocapnia at the beginning of the immobilization, worsened over time. In most individuals the early antagonization resulted in stabilization towards more physiological levels for arterial oxygen and carbon dioxide levels, but this did not occur for pH. In particular, in those giraffes that underwent a predominantly respiratory acidosis, presumably as a result of opioid overdosing [225,226] and limited pre-induction exertion, the pH remained extremely low ($pH<7.16$) despite the fact that ventilation appeared restored after antagonist administration. A slower compensation mechanism in case of respiratory acidosis might be explained by the fact that BGA2 was analyzed only few minutes after ventilation, which had been severely impacted when etorphine kicked in [92,225,226], was restored following the antagonization. In contrast compensation might have been more successful in case of metabolic acidosis because the respiratory compensation started immediately when the giraffe began to run [223] and lasted several minutes, until the opioid respiratory depression overcame. After etorphine antagonization, arterial carbon dioxide levels decreased to hypocapnic levels in several giraffes, and bicarbonates slightly decreased in the same manner, likely as a mechanism of physiological compensation to prevent respiratory alkalosis [221]. Lactates however did not change over time. In human athletes and in other herbivores that underwent extensive chasing, it has been suggested that an active recovery phase, characterized by slow movement, clears accumulated blood lactate faster than in passive recovery [95,96]. In our study, the fact that all giraffes ran until they either fell recumbent or were roped down, and as such no active recovery phase was present, might explain the lack of lactate clearance, in comparison to other species where it occurs faster.

The restraint quality and the physiological function were also monitored meticulously over time with an objective of evaluating if physical restraint in concomitance with painful and stressful procedures might have triggered a stress cascade and severe cardio-respiratory alterations. Indeed, an early antagonization means that giraffes are completely awake, and susceptible to a stress cascade that can be more deleterious than the effects of drugs, including catecholamine release, hyperthermia, reactive oxygen species production, and cell damage, [41,47,87,227]. Furthermore, lateral recumbency is not a physiologic position for giraffes, especially considered they have an already low pulmonary compliance, that can further be affected by compression due to the recumbency, and a delicate blood

pressure regulatory system that includes a jugular venous pooling that is affected by the gravity when the head is lowered [123,188,197]. Unlike a study performed in Masai giraffes that were not exposed to any painful procedures, and where the restrain quality was excellent in most individuals [81], in this study none of the giraffes had an excellent restraint quality, as all of them showed some attempt to stand and fight against the restraint. In particular, in four giraffes the restraint became dangerous as the giraffes repetitively kicked and tried to lift their neck. Although the administration of azaperone might have provided additional tranquilization and anxiolytic action that helped to reduce the stress, azaperone does not have analgesic or sedative properties [34]. In our study the level of restraint was not correlated to doses of etorphine and azaperone, nor to the antagonists doses, or startle and excitement score. However, a smoother restraint was associated with lower initial $PaCO_2$ values, and appeared less rough in helicopter darted giraffes. A possible explanation would be that individuals with lower $PaCO_2$, that was associated with more strenuous activity might have been exhausted and less prompted to react. A worse restraint was correlated instead with a lower pH at BGA2, and higher heart rate at both the beginning and end of the restraint. This finding might find an explanation with the fact that higher stress, or perceived pain, increased the heart rate both acting on direct sympathetic system stimulation, and through the increased physical activity, and in turn by increasing the metabolism led to a decrease in pH [223].

Although the physiological function was monitored only after antagonization was performed, the values recorded, especially at the beginning of the immobilization, are also the result of a combination of factors such as startle response, induction-induced excitement and opioid adverse effects [95,106,224]. The physiologic function was surprisingly not severely compromised despite the stressful captures, and only marginal variations over time occurred for most values, likely also as a result of the short monitoring time. Heart rates were within giraffe resting rates in most giraffes [117,206], and slightly decreased from 68 to 63 bpm, although the trend varied among individuals. Heart rate was significantly elevated only in the four helicopter darted giraffes (90 – 100 bpm), and also decreased over time. An initial higher heart rate was correlated with higher excitement score, and in turn, presumably as a consequence of the exertion and catecholamine release, with homeostatic alterations such as higher metabolic acidosis, lactates, sodium, chloride and hyperglycemia [43,64,75,76,85]. According to our results, an initial heart rate of 80 bpm or above was linked to greater homeostatic alterations, in particular acidosis and glycemia. This value might represent a cut-off for increased heart rates and be used as an alarm bell when other monitoring devices such as blood gas analyzers are not available in the field. Higher IBP was also correlated to higher excitement, and it reached a peak of 200 mmHg in one individual that underwent extreme excitement. Accurate measurement of blood pressure is particularly important in giraffes since, due to their peculiar anatomy, they are sensitive to blood pressure changes and require a minimum mean blood pressure of 120 mmHg when recumbent under anesthesia in order to allow renal function [119]. In our study IBP was on average 122 mmHg, and therefore was considered adequate, although it was under this threshold in four individuals, with values between 90 to 110 mmHg. Although in our study IBP was not correlated with the different doses of azaperone, or with the antagonist doses, the use of azaperone, which due to its affinity to α1-receptors can reduce the hypertensive effects of opioids [35], might explain the non-

elevated IBP. Compared to giraffe resting respiratory rate, which is between 8 and 15 bpm depending on the age and size [188], the mean respiratory rate in our study, on average 20 bpm along all the immobilization, was slightly elevated. These values were however lower compared to combinations such as medetomidine-ketamine, and butorphanol-azaperone-medetomidine, but higher than in non-reversed opioid-based protocols such as etorphine-acepromazine and etorphine or thiafentanil-medetomidine-ketamine [187,196–198]. The respiratory rate was also lower compared to the same etorphine-azaperone combination in Masai giraffes, which is unexpected since Masai giraffes were administered a lower dose of both etorphine and antagonists, and underwent less excitement compared to giraffes of this study [81]. Giraffes have evolved with a mechanism to increase both respiratory rate and oxygen diffusion capacity [188], and the fact that in this study overall PaO_2 was adequate and higher compared to values recorded in the Masai giraffes, might explain the non-elevated respiratory rates. Rectal temperature also did not change over time, and was within physiological giraffe temperature ranges in most individuals [228]. Being hyperthermia an indicator for psychological stress [41], it is promising that it did not increase during the immobilization as a result of stressful manipulation under solely physical restraint [34,41,134]. Hyperthermia, tachypnea, tachycardia and weak pulse are early symptoms of capture myopathy, and usually are associated with severe acidosis and electrolyte imbalances such as hyperkaliemia [47,76,229]. In the giraffes of this study, especially those helicopter-darted, acidosis was extreme and severe hyperkaliemia was measured. However, tachycardia, tachypnea, hyperthermia and hypotension were not experienced, and giraffes survived for at least four months after capture, ruling out the occurrence of a sub-acute form of capture myopathy which usually occurs and lead to death from few days to few weeks [47].

In addition, the fact that heart rate and IBP did not overall increase, as well as respiratory rate and rectal temperature, after antagonization was performed, and during the stressful manual restraint, is promising. The adjunct of azaperone in the dart might have prevented the occurrence of severe stress-mediated tachypnea and tachycardia, by providing tranquilization in giraffes [81]. However, a rough manual restrain was experienced, which is not only a concern for giraffe welfare when individuals are awake during stressful and sometimes painful procedures, but it is dangerous for the capture team and limits the precision of surgical treatments that are sometimes required even in the field. The results of our study highlighted that a protocol that provides sedation and analgesia during the restraint of giraffes, without increasing the risk of mortality is a priority and needs to be evaluated. The use of partial antagonism, such as through the administration of post-induction butorphanol instead of early full antagonists, might be able to partially reverse etorphine μ-mediated effects [54], such as respiratory depression, whereas through its agonism at the k-opioid receptor, it would provide additional sedation and mild analgesia, but with fewer cardio-respiratory consequences.

The quick passage from fast gallop to recumbency can be seen as a homeostatic shock, as it requires quick physiological adaptation [117,123], and being furthermore impaired by the knock down drug's adverse effects [35], this is thus one of the most challenging components of giraffe capture. Higher doses of opioids are associated with a reduction of excitement [34], and in turn might reduce the complications due to the intensity of this unexpected change from strenuous activity to

recumbency. The administration of higher doses of etorphine can also be beneficial as, by reducing the time for induction, they can reduce the risk of injuries [34,125]. However according to the results of this study, no other major benefits were observed. Although giraffes that displayed less excitement, presumably due to higher etorphine doses, had less severe decrease in bicarbonates and hyperglycemia, other biochemistry alterations such as lactates increased and electrolyte changes still occurred. Furthermore, our results show that the acidosis presumably resulting from the etorphine-mediated respiratory depression was more serious compared to the metabolic acidosis triggered by higher excitement, and in addition showed less improvement over time despite gas-exchanges being rapidly restored with antagonization. Indeed compensatory mechanisms for metabolic acidosis are impaired by opioids, which can cause a respiratory depression, including hypoventilation through a reduction of central respiratory drive, an increase in chest wall rigidity and upper way resistance [34,104], or hindering of gas exchanges through the alveolar-capillary membrane, caused by congestion or decreased time of blood passage through pulmonary vasculature caused by etorphine hypertension [109]. In giraffes that underwent exercise as a result of excitement or fear, the proper functionality of the mechanism of oxygen and carbon dioxide exchanges across the alveolar-capillary membrane is essential to compensate for metabolic acidosis. Higher doses of opioid might thus explain the hypercapnia that occurred in some giraffes in this study, which resulted in worrisome acid-base complications. These findings suggest that overdosing etorphine in order to produce a shorter excitement does not improve the physiological safety of giraffe immobilization. The use of alternative combinations of drugs that provides the same short inductions and limited excitement, but reduces etorphine doses and its resulting respiratory depression, might improve giraffe immobilization safety and needs to be evaluated.

According to the results of this study, nasal capnometry is a valid non-invasive clinical tool to monitor changes in expired carbon dioxide. It showed to be more accurate in predicting arterial carbon dioxide compared to a technique that utilized intratracheal monitoring through a percutaneous needle in immobilized giraffes [187]. The accuracy of the nasal capnometry values, in relation to the $PaCO_2$ measured by the blood gas analyzer, was lower at the beginning of the immobilization compared to the second sample. This finding may find an explanation with the fact that at the beginning of the recumbency, greater and more dynamic ventilation alterations happen due to a combination of exertion and initial drug effects, which might affect more the reading of the capnometer. This is supported by the fact that a bigger depth of breath is associated with a smaller gap in the $PaCO_2$-$ETCO_2$ difference [230]. The fact that in our giraffes a smaller breath depth might have occurred at the beginning of the restraint, since the first recordings at t0 and t1 were made before and just after the antagonist was administered, might justify the initially bigger gap observed. Furthermore, the difference between $ETCO_2$ and the arterial value depends on patient size, posture, and dead space, and in large animals such as horse and giraffes with a large dead space, accuracy even in intubated animals is poor [119,140]. According to the Bland Altman plot, which represented the difference between $PaCO_2$ and $ETCO_2$ according to the mean values of $PaCO_2$, accuracy at BGA2 was significantly improved with a narrower range of agreements. In our study, giraffes were awake when the second samples were obtained, and therefore less respiratory dynamic alterations that might increase capnometer inaccuracy

(e.g. dead space ventilation and ventilation perfusion mismatch) might have occurred. A correlation between higher arterial oxygenation and overestimation of $ETCO_2$ was also observed, in particular overestimations occurred only in three giraffes with PaO_2 initially higher than 100 mmHg, and it decreased in the second sample when arterial oxygenation also decreased. This finding might suggest that respiratory dynamics were altered in these giraffes, which might have influenced the accuracy of the capnometer.

The difference between $PaCO_2$ and $ETCO_2$ were -1.1 mmHg at BGA1 and 0.56 mmHg at BGA2. The difference between PaO_2-$ETCO_2$ is physiologically considered to be 2 – 5 mmHg, but reversal of the normally positive $PaCO_2$-$ETCO_2$ gradient has been reported in human and domestic animals [140,141]. These are normally attributed to conditions where dead space and ventilation-perfusion mismatch are minimal, to late emptying of well-perfused alveoli with higher carbon dioxide tensions or to reduced functional residual capacity as in pregnant or obese patients [141,143]. Other possible explanations are rebreathing of carbon dioxide from relatively under-ventilated compartments [143]. Even if the trachea in giraffes is narrow and they are considered to have a low dead space-tidal volume ratio, its length might impose a high respiratory resistance during physical exercise [208,209]. Increased and shallow ventilation might lead to the creation of a dead space in the upper portion of the respiratory tract, which might enhance carbon dioxide rebreathing [230]. An alternative interpretation is that rebreathing of carbon dioxide could be happening within the tube inserted in the nostril because it might accumulate carbon dioxide. More accurate results have been observed with smaller diameter cannulas [144], therefore more research on the optimal nasal capnometry sampling technique in giraffes is needed. In human medicine, a reduced accuracy is usually due to the capnometer underestimating the CO_2 level, especially in conditions such as metabolic and respiratory acidosis [142], or due to dilution with ambient air. In this study, the nasal capnometer rarely underestimated the level of arterial carbon dioxide, which is particularly important considering the high risk of hypercapnia resulting from the opioids and recumbency alterations. Further studies that consider greater numbers of individuals should be undertake in order to validate if this device could also be indicated for the study of gas-exchange dynamics as a substitute of endotracheal capnometry.

The results of this study confirmed the anecdotal difference observed in the field between physiological response to capture for different giraffe subspecies. Compared to a previous study that evaluated the same protocol in Masai giraffes [81], reticulated giraffes seems to be less sensitive to opioid effects as inductions were prolonged and induction-induced excitement more intense, despite the higher doses administered. Also differences in the pathophysiological response were observed, including more severe acidosis, and better arterial oxygenation despite lower respiratory rate. A possible explanation for the difference between the two studies would be in the genetic divergence between Masai and reticulated giraffes [8], so it would not be surprising to find differences, for example, in drug sensitivity and as it is recognized for other similar species [11,74], perhaps due to genetic-mediated sensitivity to opioids [199–201]. Since taxonomic classifications might be quickly changing, and Masai giraffes and reticulated giraffes could emerge as different species [8–11], it is important to begin to have a species-specific approach from a veterinary point of view, and account for the clinical differences observed.

5. Conclusion

The immobilization of free-ranging reticulated giraffes with an etorphine-azaperone combination resulted in severe acid-base and metabolic alteration, with respiratory compromise in some individuals.

In both helicopter and ground darted giraffes the stress that resulted from the darting approach triggered physiological alterations, suggesting the need for capture techniques that allow to keep the stress to a minimum according to the animal's behaviour. However, these were extremely severe when helicopter darting is performed, since it triggered an intense chase that resulted in overexertion and severe metabolic alterations. In ground darted giraffes the major predisposing factors for morbidity was either the metabolic acidosis due to drug-induced excitement – enhanced by etorphine underdosing – or the respiratory depression and resulting hypercapnia and severe respiratory acidosis, resulting from etorphine higher doses. In the light of these results, a combination of synergistic drugs might improve giraffe immobilization by preventing the development of alterations. By including drugs like sedatives in the dart combination, it might result in limited excitement, but on the other hand might allow reduced etorphine doses and its associated adverse cardio-respiratory effects. Early antagonization can be beneficial as it improves gas-exchanges, but on the other hand, the fact that manual restraint was rough, and poses giraffe welfare and capture team safety concerns, suggests the need for researching on protocols that provide sedation without impacting the physiological function. Early partial antagonization with a mixed-action opioid such as butorphanol might improve gas-exchange similarly to full antagonization, but would provide adequate sedation and analgesia, thus decreasing the stress resulting from giraffes being awake during the restraint and improving the safety of the immobilization.

Nasal capnometry was shown to be an accurate non-invasive monitoring tool for field assessment of ventilation function in giraffes, even though values recorded immediately after recumbency should be interpreted with caution as the fast ventilation changes occurring might affect accuracy.

Different sensitivity to drugs occurs between giraffe subspecies, suggesting that dedicated capture protocols should be implemented that account for the evolutionary divergence.

RESEARCH STUDY III

PHYSIOLOGICAL ALTERATIONS IN FREE-RANGING EASTERN BLACK RHINOCEROSES (*DICEROS BICORNIS MICHAELI*) IMMOBILIZED WITH ETORPHINE-AZAPERONE COMBINATION, AND EVALUATION OF THE EFFECTS OF BUTORPHANOL WITH OR WITHOUT DOXAPRAM ON GAS EXCHANGES AND ACID-BASE STATUS

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

Simple Summary

Eastern black rhinoceroses are a critically endangered subspecies of black rhinoceros. To enhance their conservation, veterinary interventions that require immobilization are commonly performed. Morbidity and mortality associated with immobilization remain high, with severe respiratory and metabolic alterations occurring due to overexertion and drug adverse effects. This study aimed to better understand the mechanism of physiological alterations resulting from capture, and to evaluate two intra-anesthetic treatments for their ability to improve these alterations: butorphanol and oxygen, or butorphanol, doxapram and oxygen. The rhinoceroses were severely affected by low arterial blood oxygenation, and by lactic acidosis in a manner dependent to the amount of time rhinoceroses were being chased by the helicopter prior to darting. The treatment with butorphanol and doxapram slightly improved oxygen and pH, whereas butorphanol resulted in worsened blood oxygenation. Nasal capnometry was evaluated for its accuracy in monitoring trends of carbon dioxide, and resulted to be a useful non-invasive monitoring device. The results of the study highlight the partial improvements obtained by administering butorphanol, doxapram and oxygen to immobilized black rhinoceros. However further research is advised into understanding physiological alterations resulting from capture, and to further advance drug treatment options to improve the safety of black rhinoceros immobilization.

Abstract

Black rhinoceros immobilization is characterized by severe physiological alterations. After induction, butorphanol and/or doxapram, combined with oxygen, are routinely administered to improve gas exchanges, but their efficacy has not been investigated yet. Twenty-seven rhinoceroses were helicopter darted with etorphine (2.5 – 4.5 mg) and azaperone (40 – 80 mg). Once recumbent, they were administered oxygen, plus butorphanol (10 mg; group B) or doxapram (20 – 30 mg) and butorphanol (5 mg) (Group BD). The physiological function was monitored pre- and post-treatment.

Hypoxemia (PaO_2 68.5 ± 33 mmHg BD, 52.5 ± 14.7 mmHg B), and severe lactic acidosis (pH 7.112 ± 0.09 BD and 7.168 ± 0.11 B), proportional to more intense pre-dart chase, occurred. After treatments, in BD PaO_2 (79.0 ± 37 mmHg) and pH (7.146 ± 0.12) slightly improved, whereas worsened in B (PaO_2 42.2 ± 7.0 mmHg, pH 7.155 ± 7.12). Nasal capnometry was not accurate in predicting $PaCO_2$ but was proved efficient in monitoring $ETCO_2$ trends. Butorphanol alone did not improve gas exchanges, presumably being increased oxygen consumption not the primary hypoxemia mechanism in black rhinoceroses, different to other rhinoceroses. Doxapram-mediated ventilation effects might have instead improved ventilation and intrapulmonary gas exchanges. Despite the severe alterations, no complications occurred in the nine-months post-capture monitoring.

1. Introduction

Black rhinoceroses are listed as Critically Endangered, and their fragmented populations are severely threatened by poaching [16]. Eastern black rhinoceroses (*Diceros bicornis ssp. michaeli*) are a subspecies of black rhinoceroses originally found in East Africa, and to avoid their extinction, conservation management procedures that require veterinary chemical immobilization are becoming essential [14,19,231]. Eastern black rhinoceroses are acutely susceptible to stress and anxiety [48,232]. The immense physiological and psychological stress developed during capture can produce severe homeostatic imbalance, leading to massive lactic acid production, metabolic acidosis and hyperthermia that can result in capture myopathy [41,47,147]. In addition, etorphine, a potent opioid commonly used to perform free-ranging rhinoceros capture, has a wide range of severe adverse effects such as respiratory depression, and catecholamine release due to sympathetic stimulation that further increases metabolism, and worsens the oxygen debt, and acid-base imbalance [99,103,104,106]. The respiratory depression mediated by etorphine results in hypoventilation and chest rigidity, and the occurrence of other intrapulmonary factors such as ventilation perfusion mismatch, shunting and decreased oxygen diffusion, enhanced by the etorphine-mediated pulmonary hypertension, impair the physiological mechanism of compensation that normally activates after exertion, causing severe hypoxemia and hypercapnia [104–106]. If hypoxemia and acidosis are not treated, this can result in cell and organ damage, which can lead to hyperacute or delayed death [47,104,106,116]. Although the addition of azaperone, a butyrophenone tranquillizer, can reduce opioid-related hypertension, and improve muscle relaxation and induction times [70,103,154], severe hypoxemia and acidosis are documented in black rhinoceroses immobilized with this combination [104,106,116,159].

In order to support cardio-respiratory and acid-base function, oxygen supplementation is considered good practice during immobilization [106]. However, this is controversial as there is evidence, in other rhinoceroses species, that a high fraction of inspired oxygen (FiO_2), when not combined with other post-induction drugs such as partial opioid antagonists or analeptic drugs, has detrimental effects on $PaCO_2$, pH and respiratory rate and does not improve arterial oxygenation [112]. Instead, when combined with the administration of intravenous butorphanol (a mixed opioid agonist-antagonist), administration of oxygen is able to mitigate etorphine cardiorespiratory effects in white rhinoceroses through a decrease in oxygen consumption [107]. Doxapram – an analeptic drug that stimulates ventilation through the central nervous system – is also routinely used in black and white

rhinoceroses immobilization for its properties of increasing transient respiratory rates and tidal volumes [103,177]. When doxapram is given alone, no improvement in gas-exchanges has been observed in white rhinoceroses [177], whereas improvement in arterial oxygenation has been recorded when used in association with oxygen insufflation and a partial opioid antagonist [158].

To date there are no studies on the effectiveness of oxygen insufflation and post-induction butorphanol alone or combined with doxapram in terms of improving pulmonary gas exchanges and acid-base in black rhinoceroses. This is primarily because treatment protocols and physiological knowledge are extrapolated from studies conducted on white rhinoceroses. The mechanism of physiological alteration in response to capture stress and drugs have been poorly investigated in Eastern black rhinoceros, and as such, a better understanding of the pathophysiological mechanism of capture morbidity is urgently needed. To further advance the safety of black rhinoceros immobilization in the field, the use of non-invasive clinical monitoring devices such as nasal capnometry, which has been recently spreading in human medicine [144,230], might be beneficial. Its accuracy in early detecting ventilatory complications in field immobilizations needs to be evaluated yet in rhinoceroses.

The aims of this study were to (1) evaluate the physiological alterations occurring as a result of capture in Eastern black rhinoceroses immobilized with a combination of etorphine and azaperone, and (2) compare the efficacy in ameliorating the physiological function between two post-induction treatments: butorphanol and oxygen (group B) and butorphanol, doxapram and oxygen (group BD); (3) the use of nasal capnometry as a clinical monitoring device and its accuracy in predicting $PaCO_2$ were also investigated.

2. Materials and Methods

2.1 Animals, Drugs, and Procedures

The study was performed on free-ranging Eastern black rhinoceroses that were chemically immobilized for conservation measures unrelated to this study, such as ear notching and implanting of horn transmitters. The study took place in Kenya, specifically in Ngulia Rhino Sanctuary and the surrounding intensive protection zone (IPZ) in Tsavo West National Park (TWNP) (altitude 670 – 920 m), and in Lewa and Borana Conservancies (altitude 1740 – 2020 m) in the Northern Kenya region. The Kenya Wildlife Service (KWS) Department of Veterinary Services and the Biodiversity Research and Monitoring Office (KWS/BRM/5001) approved the project, which complied with the KWS guidelines to conduct research on wild mammalian species.

Twenty-seven Eastern black rhinoceroses (15 females, 12 males) were considered for the study. The animals were individually identified from a fixed-wing aircraft by rhinoceros monitoring personnel before the capture. Total doses of etorphine (Captivon 9.8 mg/mL, Wildlife Pharmaceuticals, White River, South Africa) and azaperone (100 mg/mL, Kyron Laboratories, Johannesburg, South Africa) were chosen according to the age class of the rhinoceroses. These were, respectively for etorphine and azaperone, 2.5 mg and 40 mg (2 – 2.5 years old) or 3 mg and 60 mg (3 – 4 years old) for calves, 3.5 mg and 60 mg for sub-adults (5 – 7 years old), and 4.5 mg and 80 mg for adults (> 7 years

old). All rhinoceroses were successively darted (3 ml S300 Syringe Dart, 2.2 × 60 mm needle, Model JM; Dan-inject International, Skukuza, South Africa) from a helicopter.

Once recumbent, rhinoceroses were allocated to one of the post-induction treatment groups and administered either butorphanol (10 mg, group B) or butorphanol (5 mg) and doxapram (20 mg in calves and subadults, 30 mg in adults) (group BD) into the auricular vein (IV), combined with oxygen administration through a cannula inserted in one nostril at 10 l/min flow rate.

When the procedures were terminated, the rhinoceroses were rolled into sternal recumbency. All animals were given IV diprenorphine (3 times etorphine doses; 12mg/ml, Wildlife Pharmaceuticals, White River, South Africa), plus naltrexone (5 times etorphine; 40 mg/mL Kyron Laboratories, Johannesburg, South Africa) in rhinoceroses showing signs of respiratory depression, to antagonize etorphine.

2.2 Monitoring

The amount of time a rhinoceros was chased with the helicopter before darting, i.e. to be pushed away from thick areas, and the intensity of activity during this period was recorded by a veterinarian with the support of videos. The information was summarized into categories in Table 1 with a "pre-dart chasing intensity score" to evaluate the physiological alterations in relation to the intensity of chasing and resulting exertion, and if this represented a confounding factor for the evaluation of the two intra-anesthetic treatments. The behaviour of each rhinoceros after being darted and the amount of time in minutes from darting to recumbency (Induction time) were also recorded.

Pre-darting Score	Description
1	The rhinoceros was darted in 2 minutes or less from the beginning of the startle response that started when the helicopter was at a darting distance. The chase included both trot and gallop gait. The rhinoceros might have been spotted from the helicopter earlier from a distance without having caused any startle response in the individual.
2	The rhinoceros was darted between 2 and 5 minutes from the begin of the startle response due to the proximity of the helicopter, which included both trot and gallop gait. It might have been spotted from the helicopter earlier from a distance without having caused any startle response in the individual.
3	The rhinoceros was darted between 5 and 10 minutes from the begin of the startle response. As it was guided from the helicopter from a thick to an open area, it was not an extensive chasing. It was mostly characterized by trot gait, with some gallop when the helicopter got closer to dart.
4	The rhinoceros was darted between 10 and 20 minutes from the begin of the startle response. The rhinoceros was guided towards an open area for darting, but being resilient to leave the thickness and often aggressive, it was an extensive chasing characterized by alternation of fast trot and gallop gait.

Table 1. Pre-dart chasing intensity score. Categorization of the amount and intensity of helicopter chasing performed before the rhinoceroses were successfully darted.

Since rhinoceroses included in the study are part of an intensive rhinoceros monitoring program mandated by the Kenya Black Rhino Action Plan [19], they are individually known and historic data on previous immobilization were recorded.

As soon as the animal reached recumbency, physiological function was monitored, and if the rectal temperature was higher than 38 °C the animal was sprayed with water. Environmental temperature was also recorded with a thermometer placed in the shade close to each immobilized rhino. Ear plugs and blindfold were applied, and if the animals were found in sternal position, they were rolled immediately into lateral in order to facilitate the drilling of the horn to fit the transmitter.

Respiratory rate (RR) was monitored through chest movements observation and heart rate (HR) by auscultation of the hearth with a stethoscope. In order to monitor the rectal temperature more accurately, an industrial thermometer (HI-98509 Chektemp1 Pocket Thermometer, Hanna Instruments, Rhode Island, United States), characterized by a 10 cm long probe which was modified by covering its sharp tip with a protective sheath using epoxy glue and an artificial insemination pipette sleeve (L Meyer, pers comm, 2019), was inserted deep (15 – 20 cm including the handle) within the rectum. At ambient conditions of -30C to 50C this thermometer measures temperature over a large range (-50 to 150C) at a resolution of 0.1 °C and an accuracy of 0.2 °C. A mainstream capnometer (Masimo EMMA Capnometer 9632, Masimo Corporation, California, United States) was used to monitor end tidal carbon dioxide (ETCO2). The device was attached to a shortened 20 cm long endotracheal tube (11 mm ID) inserted in the opposite nostril to the oxygen tube. A pulse oximeter with a transmission probe (PM-60, Mindray Medical, Shenzen, China) was attached to the rectal mucosa to measure hemoglobin oxygen saturation (SpO2). When possible, the mean invasive blood pressure was measured through an aneroid manometer connected to a catheter inserted in the auricular artery [233].

Arterial blood samples were collected from the auricular artery into 1 ml heparinized syringes 3 minutes after the animal became recumbent (BG1). After either butorphanol (group B) or butorphanol-doxapram (group BD) were administered IV, and nasal oxygen administration was started, a second and third samples were collected respectively after 5 minutes (BG2) and 10 minutes (BG3). The time of administration of the treatment (Treatment time), and the time when oxygen insufflation started (Oxygen time) were recorded. Variation in the time of sample collection was recorded. Eventual air bubbles were immediately removed from samples, and the syringe was sealed with a tip cap and put on ice inside a cooler box. The whole blood was analyzed within 20 minutes of collection with an i-ISTAT 1 handheld blood analyzer using CG4+ cartridges, and within 60 minutes for CHEM8 and CTNI cartridges (Abbott Laboratories, Abbott Park, Illinois, USA). pH, partial pressure of carbon dioxide ($PaCO_{2)}$ and oxygen (PaO_2), and lactate were measured directly, while HCO_3^-, BE, TCO_2, and SO_2, were calculated by the analyzer. Electrolytes (Na, K, Cl, iCa), Glucose (GLU), BUN, creatinine (Crea), hematocrit (Hct), hemoglobin (Hb), Anion Gap (AG) and cardiac troponin I (CTNI) were also measured from the BG2 sample.

The alveolar-to-arterial oxygen tension gradient [$P(A\text{-}a)O_2$] was calculated for BG1 and BG2 by subtracting PaO2 measured by the blood gas analysis (BGA) from the alveolar oxygen tension (PAO_2). PAO_2 was calculated with the following equation:

$$PAO_2 = FiO_2(Pb\text{-}P_{H2O}) - PaCO_2/RQ$$

where FiO_2 is the fraction of inspired oxygen (21% for room air), Pb is the barometric pressure measured using the blood gas analyzer, P_{H2O} is the partial pressure of vapor in the alveoli at 37 °C (47 mmHg), and $PaCO_2$ is the partial pressure of carbon dioxide for each rhinoceros. RQ is the respiratory quotient, which depends on metabolic activity and diet, and is considered to be 1 for a carbohydrate diet [106]. Since the capture site was not at sea level, we calculated the PaO_2 expected for the average altitude we worked at for each rhinoceros (range 670 – 2020 m) in order to use it as the cut-off value for defining hypoxemia, using the alveolar-to-arterial oxygen tension formula. Assuming a normal alveolar-oxygen tension difference of 15 mmHg, the expected PaO_2 was calculated by subtracting 15 mmHg from the calculated PAO_2 value (as described above, but using the measure barometric pressure at each capture site and $PaCO_2$ of 35 mmHg for this estimation, which is considered the physiological reference value at this range of altitudes) [170,192]. The expected PaO_2 at the altitude of each rhinoceros capture site was subtracted from the measured PaO_2 values of each individual using the following equation:

$$\Delta PaO_2 = PaO_{2\ measured} - PaO_{2\ expected}$$

the individuals with negative values were considered hypoxemic, and the ones with values lower than -20mmHg severely hypoxemic.

Descriptive quality scores were used to evaluate the quality of immobilization before (t1) and after the treatment (t-b1), represented in Table 2. The presence of tremors was recorded throughout the immobilization.

Immobilization Score	Description
1	Poor immobilization with attempts to stand. The situation is dangerous for handlers, re-dosing is required.
2	Light immobilization with spontaneous motor activities such as head or limb movements and continuous ear twitching, especially in reaction to ear notching or horn drilling.
3	Moderate immobilization with good muscle relaxation, and occasional ear twitching, no spontaneous motor activity.
4	Excellent immobilization, complete muscle relaxation and stable cardio-respiratory function.
5	Excessive immobilization, complete muscle relaxation and respiratory depression or apneas.

Table 2. Immobilization score. Clinical description of the quality of immobilization in the black rhinoceros.

The time in seconds between administration of the antagonists and full recovery was recorded (Recovery time). Videos were recorded for each recovery and a behavioural descriptive score scale was used to compare the quality of the recovery between the treatments (Recovery score; Table 3).

Recovery Score	Description
1	The rhinoceros wakes up within 3 minutes. The individual appears calm and stay around cars for a few minutes or shy and disappear immediately in the bush.
2	The rhinoceros is fully awake within 3 minutes from antagonist administration. It is aggressive and charges vehicles although no incident happens.
3	The rhinoceros wakes up within 3 minutes, but shows signs of moderate ataxia. It takes a few minutes to gain normal ambulation.
4	The recovery takes longer than 3 minutes, an additional antagonization is required.

Table 3. Recovery score. Behavioural description of the quality of recovery following antagonisation based on video.

2.3 Data Analysis

In order to elucidate underlying mechanism for physiological derangement and to evaluate the interaction for each individual between physiological and blood tested variables and factors such as drug doses, descriptive scores and anesthetic times, previous immobilization and anagraphic information, a Spearman correlation test was used. A Student's t-test was used to assess differences in the distribution in the B and BD groups of elements such as drug doses, age, gender, induction times, altitude and barometric pressure, in order to detect confounding factors for evaluation of the two intra-anesthetic treatments. The difference over time of physiologic and blood tested variables in the treatment groups was evaluated with one- and two-way analysis of variance (ANOVA). A multivariate analysis of variance (MANOVA) test was used to establish if the time of administration of oxygen in relation to BG1 arterial sample collection acted as a confounding factor on blood gases with any of the treatments. The test considered both the treatment group and the occurrence of oxygen before (in a range of 10-50 seconds) and after BG1; the Hotelling trace multivariate test was adopted to evaluate the differences between groups. Bland-Altmann plot was used to evaluate the concordance between $ETCO_2$ and $PaCO_2$ values for each time point. The results are shown as mean and standard deviations, or median and ranges for non-parametric results. Statistical significance was considered at $p < 0.05$.

Finally, exclusion criteria included individuals that had an induction time longer than 10 minutes, those where physiological monitoring began and first BG1 were drawn more than 10 minutes after recumbency, and individuals that required multiple darting.

3. Results

Of the twenty-seven rhinoceroses included in this study, ten (7 females, 3 males) and sixteen (8 females, 8 males) were included respectively in the B and BD group. One rhinoceros belonging to BD

group was excluded from the study as it was darted twice. The two areas where the study was performed had different ecological characteristics. Capture sites within TWNP were located at an altitude of 670 – 920 meters above sea level, and the recorded environmental temperatures were between 27 and 31 °C. Capture sites in Lewa and Borana Conservancies were located at a higher altitude, between 1740 and 2020 meters, where the climate was cooler – between 21 and 27°C. In our study, capture sites were not equally distributed between the two treatment groups, since all rhinoceroses in BD group were immobilized in TWNP, whereas in group B, two individuals were immobilized in TWNP and eight in Lewa and Borana Conservancies. For this reason, the barometric pressure (693 ±5.2 mmHg in BD, 635 ± 37.9 mmHg in B; $p < 0.001$) and environmental temperature (29 ± 1.9 °C in BD, 25.3 ± 3.5 °C in B; $p = 0.003$) between the two groups were significantly different.

The age and the estimated body weight of rhinoceroses based on the age class were similar between the two treatment groups (group BD: age 9.6 ± 7.6 years; estimated body weight 1064 ± 179 kg; group B: age 6,5 ± 6,3 years; estimated body weight 985 ± 137 kg; $p > 0.05$). The amount of etorphine and azaperone administered through the dart were converted in µg/kg according to the estimated body weight of each individual to facilitate group comparison. The resulting estimated per-kilo dose of etorphine were similar (3.6 ± 0.4 µg/kg in BD and 3.5 ± 0.6 µg/kg in B group; $p > 0.05$), whereas azaperone was given at lower dose ($p < 0.001$) in BD (62 ± 7 µg/kg) compared to B group (77 ± 12 µg/kg). However, according to the Spearman correlation test, estimated per-kilo azaperone doses did not show any correlation with induction times ($p > 0.05$). The intensity and duration of the chasing with the helicopter prior to successful darting, represented by the median pre-darting score, was 3 in both the groups (range 2 – 4 in BD; 3 – 4 in B). However, regardless of the group, in rhinoceroses that had a higher pre-darting score, which represented a more intense chase due to logistic reasons, it was correlated with more severe physiological derangement. These included lower initial pH (r = -0.451, $p = 0.04$), bicarbonates (r = -0.596, $p = 0.006$), $PaCO_2$ (r = -0.537, $p = 0.01$) and higher lactate (r = 0.598, $p = 0.005$). The time from darting to recumbency did not show any significant difference between the two groups (5.4 ± 2.2 minutes in group BD and 5.3 ± 1.8 in B), and most rhinoceroses displayed a similar behaviour after darting, such as a moderate trotting until the first signs of sedation appeared, followed by ataxic ambulation and recumbency. The induction time was not correlated with any significant change in gas-exchange or acid-base values. No severe post-darting excitement was observed, although it was not possible to continuously monitor the rhinoceroses during the induction phase due to thick vegetation. Potential predisposing factors for greater physiological alterations were investigated in the rhinoceroses of both the treatment groups. Ten rhinoceroses were immobilized in the past, but according to Spearman correlation test, history of capture did not result in a longer chase, longer induction time or variation in blood gases and acid-base values. Two females were heavily pregnant, but did not undergone longer induction times or higher physiological alterations. An older age was correlated with slightly longer induction times (r = 0.47, $p = 0.01$), whereas the gender resulted in no correlations.

The mean duration of immobilization was slightly different between the BD and B groups (13 minutes in BD, 9.5 minutes in B), and this resulted in an earlier treatment administration in group B in relation to the time of recumbency (5 ± 2.8 minutes in BD and 2 ± 1.1 minutes in B; $p = 0.001$). When

calculated as a ratio with etorphine, the administration of 5 mg and 10 mg of butorphanol in the BD and B group resulted respectively in a mean dose of 1.35 ± 0.2 mg and 3 ± 0.6 mg of butorphanol per mg of etorphine in BD and B group. Post-induction doxapram was administered in the BD group only, at a ratio of 7 ± 1.6 times the etorphine. Physiological data and blood variables collected during immobilization were organized by sampling periods to facilitate data interpretation and comparison between different groups by statistical analysis. Pre- and post-treatment sampling periods are shown in relation to when each rhinoceros reached recumbency to reduce the risk of variation in sampling times acting as a confounding factor on the physiological response to treatments. These were t0 (0 – 1 minutes after recumbency), t1 (2 – 5 minutes after recumbency), tb-1(2 – 5 minutes after treatment), and tb-2 (6 – 10 minutes after treatment).

Respiratory rate (RR) decreased over time in both groups ($F = 19.83$; $p < 0.001$), but no difference was found between the trend in the two groups in a two-way ANOVA ($p > 0.05$). In particular, RR (reported as breaths per minute) decreased between t1 (12.8 ± 2.7 BD; 12 ± 5 B) and t2 (9.9 ± 2.2 BD; 10 ± 4.7 B) ($p = 0.02$), and from t2 to tb1 (7.5 ±1.5 BD; 6.3 ± 1.7 B) ($p = 0.03$) but did not change between tb1 and tb2 (7.6 ± 2.2 BD; 5.6 ± 2.7 in B) ($p > 0.05$). Episodes of hypopnea occurred in two rhinoceroses of group BD, and all rhinoceroses but two of B group, in which apnea also occurred towards the end of the immobilization. Heart rate (in beats per minute) slightly decreased over time in both the groups. Heart rate before the treatment, at t1, was 92.6 ± 18 in BD and 92.4 ± 15 in B, and after the treatment, at tb1, was 87.4 ± 12 in BD and 84.7 ± 17 in B, although no statistical difference was found over time or according to the treatment group ($p > 0.05$). Due to the challenge of inserting an intra-arterial cannula when ear notches were being performed IBP was only recorded in 8 rhinoceroses belonging to group BD, and 2 rhinoceroses in group B. The mean arterial pressure in group BD was 91 ± 21 mmHg pre-treatment and 87.1 ± 12 post-treatment, and 115 ± 7 pre-treatment and 100 ± 14.1 post-treatment in group B, although these differences were not statistically significant.

Rectal temperature (in °C) was significantly higher in BD group compared to B group at all time points ($F = 37.9$, $p < 0.001$), and slightly increased in both groups over time although no statistical difference was found ($p > 0.05$) (t1: 39.0 ± 0.8 in BD, 38.0 ± 1.0 in B; t2: 39.2 v 0.7 in BD and 38.1 ± 0.9 in B; tb1: 39.3 ± 0.7 in BD and 38.1 ± 0.8 in B; tb2: 39.1 ± 0.7 in BD and 38.2 ± 0.9 in B). Rectal temperature was significantly correlated to the site of capture ($p < 0.001$ at all time points), but not to higher pre-darting score or longer induction times ($p > 0.05$).

Nasal end-tidal carbon dioxide ($ETCO_2$) did not differ either over time or in the two groups, although a peak after butorphanol treatment in group B was recorded. The mean values, in BD and B groups respectively, were in t2 before the treatment 69.6 ± 11.5 and 60.3 ± 12.4 mmHg, and, after the treatment, 59.8 ± 10.7 and 60.4 ± 9.5 mmHg in tb-1, 59.0 ± 11.9 and 72.0 ± 13.2 mmHg in tb-2 and 57.9 ±9.2 and 55.7 ± 10.6 mmHg in tb-3. In those rhinoceroses with signs of respiratory depression such as apneas, shallow breath or breath holding, $ETCO_2$ values were quickly changing within few seconds. In these individuals, the values are calculated as the mean of three close following values. According to Spearman correlation test, values of $ETCO_2$ sampled at the same time of BG1 were not correlated with levels of $PaCO_2$, either corrected or not for the temperature, but values of $ETCO_2$ collected at BG2 were positively correlated with $PaCO_2$ ($R = 0.750$, $p < 0.001$) and negatively with

PaO_2 (r = -0.563, p = 0.001) measured at BG2. The agreement at all time points for $ETCO_2$ and $PaCO_2$, corrected by rectal temperature, of all rhinoceroses are represented in Bland-Altman plots, divided by BGA1 (figure 1a; lower level of agreement -27.08, upper level of agreement 14.1, bias -6.8) and BGA2 (figure 1b; lower level of agreement -24.7, upper level of agreement 14.2, bias -5.2).

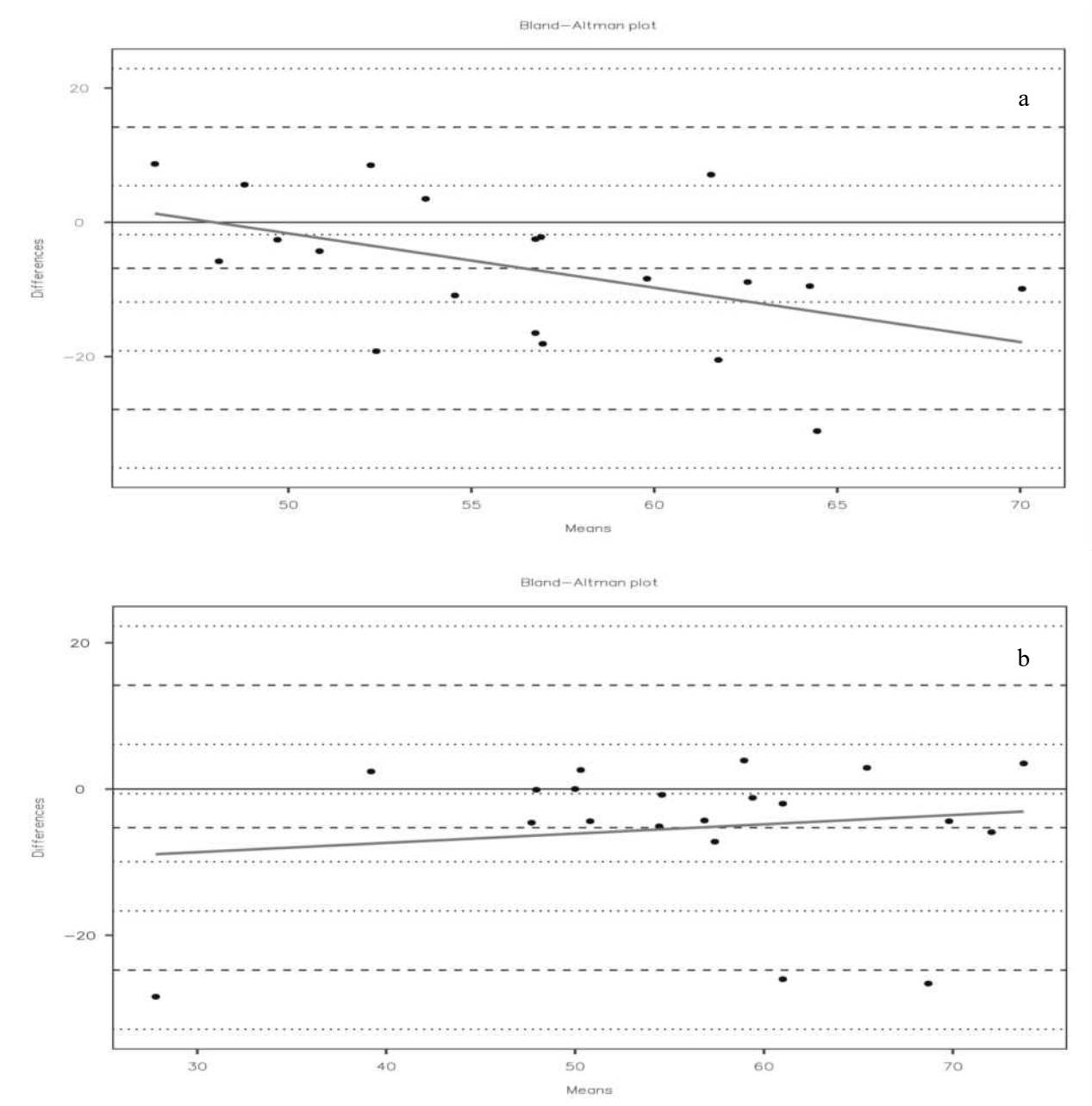

Figure 1. Bland-Altman plot representing the difference of $ETCO_2$ and $PaCO_2$ corrected by temperature of all rhinoceroses (group B and BD) at BGA1 (a) and BGA2 (b), in relation to the respective mean values of $PaCO_2$ at BGA1 and BGA2.

Pulse-oximetry was measured in 10 rhinoceroses only due to a technical problem with the device. Values before treatment at t2 were 87.5 ± 10 %, and after treatment were 88.4 v 7.1 % at tb-1 and 94.2 ± 5.4 at tb-2. When SpO_2 values were compared to the SaO_2 measured by the blood gas analyzer, no

correlation was found, as the pulse-oximeter tended to both over and underestimate the hemoglobin saturation of oxygen.

Although three arterial blood samples were collected (BG1 3 minutes after recumbency and before the treatment; and BG2 and BG3 respectively 5 and 10 minutes after treatment), it was not possible to collect the third sample from most rhinoceroses as the duration of the immobilization was often quicker than initially planned due to logistics challenges. For this reason, we have only considered BG1 and BG2 in statistical analyses. Furthermore, it was not possible to obtain arterial blood samples in all individuals both pre- and post-treatment. As such, in order to have comparable data, the trend of blood gases was evaluated in 12 rhinoceroses in group BD and 8 in B. The time of the first arterial blood sample differed between the two groups ($p = 0.015$), as in group BD blood was withdrawn later, 4 ± 2.4 minutes after recumbency, compared to 1.9 ± 0.9 minutes in B, however this did not interfere with any of the blood gas values ($p > 0.05$). In 10 individuals (four in BD and six in B) oxygen insufflation started just before the collection of BG1 sample, in a range of 10 to 50 seconds before, whereas in all the other rhinoceroses it was administered with the intravenous treatment. The different time of oxygen administration accounted for a significant difference in PaO_2 levels at BG1 (HL = 1.88, $p = 0.001$). This difference was due to both the treatment group ($p = 0.002$) and to the occurrence of oxygen administration before the sample BG1 ($p = 0.0008$). The difference in PaO_2 seen at BG2 was also statistically significant (HL = 0.93, $p = 0.02$), but in relation to the treatment group only ($p = 0.008$) and not to the occurrence of oxygen before BG1 ($p < 0.05$). Carbon dioxide level was not influenced at either BG1 or BG2 by the early administration of oxygen ($p > 0.05$). Arterial blood gases values are represented in Table 4 and uncorrected and corrected values for the rectal temperature, pH, $PaCO_2$, and PaO_2 are reported. Unless specified, values reported within the text are uncorrected.

Variable	**BG1**		**BG2**	
	Group BD	**Group B**	**Group BD**	**Group B**
pH	7.112 ± 0.09	7.168 ± 0.11	7.146 ± 0.12	7.155 ± 7.12
$PaCO_2$ (mmHg)	50.0 ± 6.1	47.3 ± 4.5	48.2 ± 15.4	51.4 ± 6.4
PaO_2 (mmHg)	68.5 ± 33.7	52.5 ± 14.7	79.0 ± 37*	42.2 ± 7.0*
BE (mmol/l)	-10.3 ± 10.2	-10.8 ± 6.7	-12.8 ± 5.6	-10.4 ± 6.5
HCO3- (mmol/l)	16.3 ± 4.3	17.8 ± 5.0	16.2 ± 5.1	18.6 ± 4.8
SaO_2 (%)	75.3 ± 22.7	73.4 ± 11.7	82.3 ± 21.4*	62.5 ± 7.1*
Lactates (mmol/l)	12.4 ± 3.9	10.2 ± 4.8	10.3 ± 4.4	8.7 ± 4.6
$P(A\text{-}a)O_2$ (mmHg)	17.1 ± 36.0	23.8 ± 23.9	7.5 ± 32.0	29.9 ± 10.5
T (°C)	39.1 ± 0.7	38.2 ± 0.8	39.3 ± 0.7*	38.3 ± 0.6*
pH (mmHg; corrected for T)	7.085 ± 0.08	7.149 ± 0.11	7.116 ± 0.11	7.135 ± .113
$PaCO_2$ (mmHg; corrected for T)	54.8 ± 6.2	50.3 ± 4.7	53.0 ± 16.2	54.8 ± 6.2
PaO_2 (mmHg; corrected for T)	77.8 ± 36.2	57.4 ± 14.8	91.0 ±39.9*	49.9 ± 6.7*

Table 4. Blood gases of the black rhinoceroses divided by group (BD and B) and by sampling time (BG1 and BG2). The results shown represent the mean ± the standard deviation. * = Statistical difference with $p < 0.05$ between group BD and group B at the same sampling time.

Pre-treatment arterial blood gases (BG1) were similar in the two groups, as no statistical difference was found for any of the variables. At BG1, all rhinoceroses were acidemic, characterized by a similar decrease in BE and HCO_3^- in both groups. At BG1, rhinoceroses of both groups had $PaCO_2$ between 40 mmHg and 60 mmHg, whereas all but three individuals were hypoxemic in group BD and all but one was hypoxemic in group B. Low levels of PaO_2 were not correlated to increased levels of $PaCO_2$. Although not statistically significant, PaO_2 at BG1 was higher in the BD group. However, the different altitudes of the areas of the study must be considered, since these could have influenced oxygenation given that all rhinoceros in group BD were from TWNP. The cut-off for hypoxemia in TWNP was 85 mmHg and in Lewa-Borana Conservancies was 69 mmHg. Taking into consideration the expected PaO_2 for each rhinoceros given the altitude of the capture site, the delta PaO_2 was -17.2 ± 33.6 mmHg in group BD and -21.2 ± 15.3 mmHg in group B. Seven rhinoceroses in BD and four in B were severely hypoxemic, with values of delta PaO_2 lower than -20 mmHg. In group BD, four rhinoceroses had initial high levels of PaO_2 (> 80 mmHg) associated with normal $PaCO_2$, whereas in B, all PaO_2 where below 80 mmHg. All the rhinoceroses of both groups (except one in group B) that suffered from hypoxemia at the beginning of the immobilization (n = 7 in BD, n = 6 in B) had initial increased alveolar to arterial gradients (> 20 mmHg, range 21 – 62). Spearman correlation test showed, considering rhinoceroses of both groups together, a positive correlation between the initial PaO_2 and the lactates (r = 0.51, p = 0.02), which were correlated also to a higher pre-dart score (r = 0.59, p = 0.005). Lactates were in addition correlated to a lower pH (r = -0.88, $p < 0.001$) and HCO_3^- (r = -0.92, $p < 0.001$)). Initial elevated $P(A\text{-}a)O_2$ was correlated with lower PaO_2 (r = -0.87, $p < 0.001$), lactates (r = -0.51, p = 0.02), a higher pH (r = 0.56, p = 0.01), but not with $PaCO_2$.

The time of post-treatment arterial blood gases (BG2) from the administration of the treatment did not differ between the two groups (4 ± 2 minutes in BD; 3.5 ± 1.5 minutes in B). Although not statistically significant, mean PaO_2 increased over time after treatment with butorphanol and doxapram, whereas in B group, where only butorphanol was given, PaO_2 decreased significantly compared to initial levels (p = 0.05). The delta PaO_2 post-treatment improved in group BD (-5.7 ± 36.7 mmHg), whereas it worsened in B group (-31.3 ± 11.6 mmHg). In group BD, hypoxemia was treated in 50% of the individuals, or 75 % if considering the PaO_2 corrected for the temperature, three individuals showed no or little improvement of hypoxemia, and one worsened. All the rhinoceroses remained hypoxemic (six rhinoceroses severely hypoxemic) compared to the expected PaO_2 for the altitude in group B. Although not statistically significant, $PaCO_2$ increased in B group and slightly decreased in BD group towards more physiological values. Initial alveolar-arterial gradient was increased (> 20 mmHg) in all rhinoceros in group BD except five, and in all in group B except three. Post- treatment gradient was overall decreased in group BD and increased in B, however this result is not accurate since a higher FiO_2 was administered, but the formula was not corrected according to it since the FiO_2 administered was not measured. Lactates, that were initially elevated with values above 10 mmol/l in 12 individuals of both groups, and over 20 mmol/l in one rhinoceros, decreased in similarly both groups in BG2. On the other hand, pH slightly increased in BD, but slightly decreased in B.

Electrolytes, selected biochemical variables and cardiac troponin I measured on arterial heparinized blood collected at BG2 are represented in Table 5. No statistical difference was found between the two groups for any of the variables, although levels of potassium were slightly higher in BD group (> 5.5 ng/ml in 2 rhinoceroses in BD). Cardiac troponin was measured in 19 individuals only. It was not detected in most individuals, except two individuals in group BD where it was 0.01, and 0.02 and 0.03 ng/ml in other two rhinoceroses. Higher levels of glucose were positively correlated to higher pre-dart score ($r = 0.615$, $p = 0.001$), lactate ($r = 0.669$, $p < 0.001$), rectal temperature ($r = 0.4$, $p = 0.04$), and negatively to a lower pH ($r = -0.061$, $p = 0.004$), and $PaCO_2$ ($r = -0.46$, $p = 0.03$). A low level of blood glucose (< 60 mg/dl) was observed in only one individual in group B.

Variable	Group BD	Group B
Na (mmol/l)	130.0 ± 1.9	130.3 ± 2.0
K (mmol/l)	5.0 ± 0.4	4.5 ± 0.6
Cl (mmol/l)	98.4 ± 2.9	97.1 ± 2.6
iCa (mmol/l)	1.58 ± 0.11	1.53 ± 0.07
Glu (mg/dl)	116.2 ± 29.3	105.0 ± 30.0
BUN (mg/dl)	16.4 ± 6.0	13.2 ± 5.4
Crea (mg/dl)	0.81 ± 0.15	0.63 ± 0.23
Hct (%)	42.1 ± 12.8	40.6 ± 6.8
Hb (g/dl)	14.3 ± 4.3	13.8 ± 2.3
Anion Gap (mmol/l)	19.1 ± 2.8	19.6 ± 4.5
Cardiac Troponin I (ng/ml)	0.004 ± 0.009	0 ± 0

Table 5. Electrolytes, selected biochemistry variables and cardiac troponin I. Samples were collected from arterial blood after the treatment (BG2). The results are shown as mean values ± standard deviation.

The median pre-treatment immobilization quality was 4 in both the groups. It was considered moderate or excellent (score 3 or 4) in all rhinoceroses with the exception of one animal in B group (which was light, score 2), and one animal in BD that received an excessive level of immobilization (score 5). After the treatment in BD group, most of the rhinoceroses maintained the same level of immobilization (median score 4, range 2 – 4), except for one rhinoceros where the level of immobilization became too light (score 2) and head movements were experienced, and another rhinoceros which shifted from score 5 to 4. In B group, the median post-treatment score was 5 (range 2 – 5), with most rhinoceroses experiencing an excessive level of immobilization with signs of respiratory depression such as apnea and shallow breathing towards the end of the immobilization. Moderate tremors were experienced in two rhinoceroses from the BD group, which decreased after the administration of butorphanol and doxapram. The rhinoceroses that experienced tremors were the individuals that received higher etorphine doses per-kg (4.5 and 4.1 μg/kg).

Diprenorphine at three times the dose of etorphine was administered IV in all rhinoceroses. Additional naltrexone, which was administered in case of signs of respiratory depression, was given to 6 rhinoceroses of B group and 1 from BD group. The time from administration of the antagonists to recovery was equal in both groups (161 ± 80 seconds in BD; 140 ± 55 seconds in B; $p>0.05$). In B, median recovery score was 2 (range 1 – 2) and most of the rhinoceroses displayed an aggressive behaviour and attempted to charge the vehicles, whereas in BD – where the median recovery score was

1 (range 1 – 4) – individuals were mostly calm and often stayed around the vehicles for a couple of minutes to sniff the ground before disappearing into the bush. Except from one recovery in BD group, which was prolonged and required additional antagonist administration, all recoveries were uneventful. No signs of resedation or post-capture morbidity and mortality have been observed in the rhinoceroses since the immobilization event (9 – 12 months) during the daily anti-poaching patrols performed by the rhinoceros monitoring teams.

4. Discussion

Immobilization of helicopter darted free-ranging Eastern black rhinoceroses with an etorphine-azaperone combination produced a condition of severe hypoxemia and acidosis. Nasal oxygen insufflation and post-induction treatment with butorphanol and doxapram slightly improved the arterial oxygenation and acidosis, whereas the administration of oxygen and butorphanol alone resulted in a more severe acidosis and hypoxemia.

4.1 Physiological effects of etorphine-azaperone combination

Although etorphine-azaperone combination is the preferred protocol for the immobilization of free-ranging black rhinoceroses, high morbidity has been reported [104,116,159,170]. The strong opioids administered to immobilize the rhinoceroses produce μ- and δ-mediated respiratory depression, experienced as hypoventilation and chest rigidity, and pulmonary hypertension that contributes to ventilation perfusion mismatch, thus heavily affecting the physiological mechanism of compensation needed after the exertion-related homeostatic imbalance resulting from helicopter darting [70,104,106]. Azaperone is often used in combination with etorphine, as thanks to its sedative, anti-anxiety, mild muscle relaxant properties and α1-antagonistic-mediated vasodilatation, it can counterbalance opioid hypertension and improve induction times and muscle relaxation [43,70,81,154,155]. Even though in other rhinoceros species the addition of azaperone to the dart mixture was proven to reduce etorphine side effects, exertion-driven homeostatic derailment remains severe [70], similar to the blood gases and acid-base results found in this study, which were far from physiological values.

The severe hypoxemia that was observed in our study, which was in line with other reports of helicopter darted free-ranging black rhinoceroses with combinations of opioids and azaperone, with and without an α2-agonist, could be explained by the occurrence of intrapulmonary factors and hypoventilation [104,106,116]. Indeed, all the rhinoceroses of both groups (except one) that suffered from hypoxemia at the beginning of the immobilization had initial increased alveolar to arterial gradients (> 20 mmHg), which is indicative of the occurrence of intrapulmonary alterations, such as ventilation-perfusion mismatching, right to left shunting or diffusion limitation of gases across the alveolar-capillary membrane, as a result of combinations of etorphine adverse effect, over-exertion and recumbency [104,106,116]. Range values of arterial carbon dioxide for resting non-anesthetized black rhinoceroses are not known, but in white rhinoceroses these have been reported to be higher than in most mammals, being between 44 and 53 mmHg [148]. If black rhinoceros carbon dioxide values are similar to those of white rhinoceros, most rhinoceroses in our study would have been normocapnic.

The fact that low PaO_2 was generally not accompanied by an increase in $PaCO_2$ could support the occurrence, in particular, of oxygen diffusion impairment, which might have developed due to congestion at the alveolar-capillary membrane, or decreased time of blood passage through pulmonary vasculature caused by etorphine-induced pulmonary hypertension, which as a result hindered gas exchanges across the membrane [107,109]. Indeed, since CO_2 is twenty times more soluble in water than oxygen, it is less likely to be affected by diffusion limitation and as such hypercapnia is uncommon in this condition [115].

Similar to other studies performed in helicopter darted black rhinoceroses, the initial cardio-respiratory function was also altered, presumably as a consequence of the capture stress and drugs [104,106,116]. The decreased respiratory rates recorded in this study were similar to those reported in other studies in etorphine-azaperone immobilized field black rhinoceroses [104,116], and to black rhinoceroses immobilized with combinations of opioids and xylazine [234,235]. Considering the resting range values reported for heart rates in black and white rhinoceroses (30 – 40 beats/minute), the rhinoceroses in our study were tachycardic [236]. However, despite the strenuous exertion that our rhinoceroses underwent due the helicopter chasing, severe tachycardia (with values over 120 beats/minute) reported in other studies [176], occurred only in one individual of BD group. Compared to black rhinoceroses immobilized with combinations of opioids and xylazine, where α2-agonist mediated bradycardia reduced heart rates almost to physiological levels [234,235], the mean heart rates in our study were higher [234,235]. There are no reports of blood pressure values in black rhinoceroses for comparison; instead the blood pressure was lower compared to white rhinoceroses immobilized with either etorphine or etorphine-azaperone, and compared to resting values in white rhinoceroses [70,195,237].

On the other hand, the degree of acidosis and the increase of lactate levels were more severe in our study compared to other studies in black rhinoceroses, even in those individuals chased only briefly [104,106,238]. Since Eastern black rhinoceroses are considered more aggressive than their southern counterpart, they might also be more susceptible to psychological stress developed during capture, which can exacerbate the homeostatic imbalance caused by the immense physical activity [47,190,239]. A more intense chase with the helicopter, which reflected both psychological stress and strenuous physical activity, was, not surprisingly, the major predisposing factor for physiological derangement in the study, in particular for severe lactic acidosis. In most cases, helicopter darting produces an intense sympathetic response that results in hyperthermia, cardiovascular and respiratory alterations, increased metabolism and oxygen consumption, and lactic production and metabolic acidosis, and exacerbate the respiratory depression caused by opioids [104,106,166,172]. However, helicopters are essential for black rhinoceros darting due to the thickness and harshness of areas where these elusive animals are found [116,240]. In our study indeed it was necessary to guide the rhinoceroses for several minutes to open areas before darting them, so that the ground team could quickly reach the rhinoceros when they became recumbent to intervene immediately in case of complications. A more intense chase accounted also for hypocapnia and initial hyperoxygenation in some individuals. Although the mean values of PaO_2 showed an overall hypoxemia, there was indeed variability in the levels of initial PaO_2 between individuals, and variability in the trend of improving or worsening, as some rhinoceroses had high initial levels of PaO_2, which tended to decrease at BG2.

This finding is similar to studies in other perissodactyls [95,106] where both hypercapnia as a direct consequence of exercise and hyperventilation-induced hypocapnia as a compensation mechanism for the severe metabolic acidemia [67,68] have been reported. However, initial individual high values of PaO_2 have scarce clinical significance, since initial values reflecting hyperoxemia and hypocapnia, invert in trend after minutes and slowly stabilize after 15 minutes from the anesthetic induction [224] – similarly to our observations.

Although severe acidosis and hypoxemia occurred, other severe alterations in possible indicators of capture metabolic derangement did not occur in this study. Lactates were initially significantly increased in many rhinoceroses, in a way correlated with the duration of the chasing, but these rapidly decreased in BG2. Since it happened in both treatment groups in similar ways, most likely it was due to the lactate clearance that physiologically occurs after a recovery from exercise [95], and in which the lateral posture has been reported to facilitate in comparison to sternal recumbency, maybe due to better perfusion in this posture [116]. Although lactates are used as prognostic factor for severity of illness including muscular damage and exertion [95,241], in this study no adverse complication were associated with high lactate (and low pH levels). Electrolytes shift, and in particular potassium elevation may be a result of muscle damage due to excessive exertion, and is a major component in the pathogenesis of capture myopathy [43]. Potassium was more elevated in some individuals of BD group, although it did not account for any post-capture sign of morbidity. The rhinoceroses in our study did not display alterations in other electrolytes levels, nor in BUN and creatinine, and these were similar to other both free-ranging or captive black rhinoceroses [84,236,242]. Hematocrit was slightly increased in respect to values reported in captive black rhinoceroses, whereas hemoglobin was not [236]. Hematocrit can be increased in case of stress [84], however in our results hematocrit was not correlated with other variables such as lactates or pH. Glucose, which was increased compared to captive black rhinoceroses but similar to free-ranging ones [84,232,242], was associated with higher pre-dart stress and indicators of physiological derangement such as lactates, rectal temperature, and lower pH and $PaCO_2$. Hyperglycemia can be increased due to an acute stressor, or after exercise, since epinephrine release increases gluconeogenesis [43]. Hypoglycemia which can be secondary to exertion during the lag period before compensation occur [238], was observed in one individual only, and no adverse complications have been observed. Cardiac troponin I can increase as a consequence of myocardial injury, or after strenuous exercise without indicating cardiac pathology [86]. In rhinoceroses the normal ranges for cardiac troponin I are not known, whereas in horses values higher than 0.03 ng/ml are considered pathological [243] and a cut-off of 0.08 ng/ml is suggested for a variety of zoo mammals [86]. Of the four individuals where cardiac troponin I was detected, three had high pre-darting scores and high lactate and low pH. Peak elevation of cardiac troponin I are usually occurring 2 – 6 hours after the myocardial injury or the activity [87,220]. In this study, the samples were collected on average 12 minutes after the begin of the exertion, and thus also a small increase might be significant. However, further investigations are needed to understand the clinical value of increases in cardiac troponin in black rhinoceroses.

The higher rectal temperature observed in BD might have been caused by higher environmental temperature recorded in the capture site of those rhinoceroses. Psychological stress is the major factor contributing to the rise of hyperthermia [41], thus even if we did not observe a different reaction to

helicopter chasing, it is not possible to exclude that rhinoceroses in BD had a higher mounting stress response. Although the water sprayed to hyperthermic rhinoceroses did not help to decrease it during the monitoring period, the fact that no complications were seen after the capture suggest that rhinoceroses were able to restore their normal temperature once antagonized, as a severe or prolonged hyperthermia might result in mortality or exacerbate capture-related pathologies like capture myopathy [41,72].

The availability of a long-term follow-up resulted to be an important method to exclude major delayed effects of capture on the behaviour, health and survival of the captured animals [30,48]. The correlations between pre-darting score and physiological derangement highlight however that there is need to rethink strategies that reduce time spent chasing rhinoceros during immobilization procedures, in order to avoid life- threatening metabolic alterations. On the other hand, the severity of the initial homeostatic derangement appeared not to be correlated to time of inductions, and not dependent on opioid dosages, as we did not find any correlation to variation in etorphine pro-kg doses which were similar in all rhinoceroses. Azaperone per-kilo doses instead varied between individuals, but no difference in the physiological response or induction times were observed according to this, suggesting that azaperone in this species might not shorten induction times in a dose-dependent manner. However, per-kg doses were calculated based on weight estimations based on the age and measurements of the rhinoceroses, and therefore errors in the dose per-kg might have occurred. Surprisingly, the history of previous immobilization did not influence greater pathophysiological mechanisms or any different behavioural reaction. This finding is important since in case of a stressful experience earlier in life, the chances of developing capture myopathy are greater [47,71].

4.2 Post-induction treatments

As a measure to improve the severe homeostatic imbalance that commonly results from rhinoceros capture, postinduction butorphanol and nasal or tracheal oxygen are routinely administered in both black and white rhinoceroses [103]. Butorphanol and other partial antagonist have been used in free-ranging black rhinoceroses [106,116], although no dedicated analysis of their effect on gas exchanges has been performed yet in this species. On the other hand, since white rhinoceroses are extremely sensitive to etorphine side effects and develop severe hypoxemia, more studies have been carried out in this species to identify ways to reduce capture-related complications. Post-induction butorphanol combined with oxygen insufflation has shown to improve and sometimes treat hypoxemia and hypercapnia in white rhinoceroses [107,112,133,157,172]. In our study, different to white rhinoceroses, the administration of 10 mg of butorphanol combined with nasal oxygen insufflation led to a decrease in arterial oxygenation. Overall arterial PaO_2 and pH decreased, and $PaCO_2$ slightly increased in respect to the initial values. On the other hand, the administration of 20 – 30 mg of doxapram and 5 mg of butorphanol combined with nasal insufflation of oxygen resulted in an improvement in gas exchanges and acid-base status in most individuals. Overall PaO_2 and pH increased and $PaCO_2$ slightly decreased. This finding was in agreement with a study performed in white rhinoceroses, where doxapram produced an improvement in PaO_2 if used in conjunction with supplemental oxygen and the partial opioid antagonist nalorphine [158]. It is also consistent with the

observation of a decrease in $PaCO_2$ and an increase in pH in anesthetized humans and ponies [178,179], and improvement of PaO_2 in dogs [180].

Respiratory and heart rate had instead a similar decreasing trend after both treatments, with no relevant difference between groups. Resting respiratory rate ranges of black rhinoceroses are 6-12 breaths per minute [236], therefore hypopnea occurred in 2 rhinoceroses of BD group towards the end of the immobilization (5 breaths/minute) and in all rhinoceroses of B group but two, with rates down to 3 breaths/minute and apnea towards the end of the immobilization. Studies in both field and boma white rhinoceroses show that the administration of post-induction butorphanol produced an immediate transient increase of respiratory rate [172], which instead did not happen in our rhinoceroses. The respiratory rates in our study were similar to boma-habituated or vehicle darted white rhinoceroses [107,133,157,172], but lower than in free-ranging helicopter darted white rhinoceroses, both after being treated with postinduction butorphanol and oxygen [157,172]. After butorphanol and oxygen are administered to white rhinoceroses, a drop of almost half of the initial values of heart rates was observed, whereas the small decrease seen in our results might be explained with the fact that initial heart rates were less increased. Indeed heart rates in our study are lower than the initial heart rates in boma and field vehicle darted white rhinoceroses immobilized with etorphine or etorphine-azaperone [70,133,157], or etorphine-midazolam [244], before the butorphanol and oxygen were administered, and similar [133,157], or higher [70,173,244] after the treatment in the same rhinoceroses. Invasive blood pressure, which was only recorded in a few individuals, was stable in BD group and decreased in group B, although it was not possible to perform statistical analysis in the two groups due to the small numerosity of these records, or to discern the effects of butorphanol from those of azaperone. The use of azaperone in combination to etorphine creates a bigger drop in mean blood pressure compared to physiological values, whereas blood pressure remains unchanged when postinduction butorphanol is administered in white rhinoceroses [70]. In our study, interestingly azaperone per-kg doses were significantly higher in B group where blood was pressure more elevated. Since the first recording of physiologic variables were obtained immediately after the rhinoceroses reached recumbency, the trends of physiological monitoring should be interpreted with caution. Changes in some of these variables occur more slowly than changes in blood gases, therefore it is challenging to distinguish the potential effect of both treatments from the physiological changes that happen when physical activity ends [224]. However, the fact that in black rhinoceroses seems that there is less cardiovascular stimulation compared to values reported in etorphine-azaperone immobilized white rhinoceroses, highlights that the predominant mechanism of hypoxemia, potentially involving perfusion alterations, might be different in the two species.

The existence of different mechanism of hypoxemia might also explain the different effects of butorphanol and oxygen on the arterial oxygenation in the two species. Indeed in white rhinoceroses, the improvement in arterial oxygenation is not caused by an increase in respiratory minute ventilation as previously believed [107,112,133,147,173], but is primarily due to a decrease of oxygen consumption associated with decreased muscle tremors, through the antagonism of butorphanol on etorphine-induced sympathetic nervous system activity [107]. Tremors, that are associated with increased plasma catecholamines [25] can significantly increase metabolism and oxygen consumption. Although tremors in black rhinoceroses have previously been reported [106], and this was experienced

in two individuals in this study that received slightly higher etorphine doses, their occurrence is less common and of lesser intensity compared to white rhinoceroses. Therefore, being higher oxygen consumption due to increased metabolism, such as for tremors, a lesser concern in black rhinoceroses, it presumably doesn't contribute greatly to hypoxemia of this species, explaining the absence of an improvement following butorphanol and oxygen treatment in B group. The greater hypoventilation that occurred in this group, manifested as shallow breathing, hypopnea and apnea, compared to group BD where doxapram might have instead supported ventilation [103], might provide a possible explanation, or concomitant factor, for the worsening of gas exchanges following the treatment in group B. As a combination of etorphine-related hypoventilation (more severe when doxapram is not given), and lateral recumbency that compress the dependent lung and alters ventilation and perfusion dynamics, the percentage of non-ventilated and perfused alveoli might have increased between BG1 and BG2 in group B. The alterations occurring in the dependent lung can indeed lead to severe alterations, such as dead space ventilation and increase in venous admixture due to an hypoxic pulmonary vasoconstriction triggered by a decrease in PaO_2 in the dependent lung [105]. It is also well-documented that shunting occurs in other perissodactyls like horses, and in rhinoceroses [105,112,113], especially in lateral recumbency, and when the fraction of shunting is over 50% oxygen therapy does not improve PaO_2 [157]. However, since the flow rates of oxygen administered in our study were far lower than those given to white rhinoceroses, it is unlikely that, if atelectasis occurred, it was a consequence of absorption atelectasis, which usually occur as a consequence of high FiO_2 (> 0.8) [112,245]. Whichever mechanism applies, if atelectasis happened increasing the shunt fraction, this could explain why oxygen insufflation was non effective in B group. Furthermore, the fact that butorphanol didn't improve alveolar-arterial gradient and dead space ventilation in white rhinoceroses [107], is consistent with our results where the alveolar-arterial gradient increased after butorphanol and oxygen treatment. Carbon dioxide also increased in group B in BG2, probably as a consequence of both hypoventilation and intrapulmonary alterations, and consequently, even though lactates decreased, acidosis worsened due to its respiratory component.

On the other side, one could speculate that by increasing the tidal volume [103], doxapram might have prevented an increase of the portion of non-ventilated alveoli in the dependent lung, reducing the hypoxic pulmonary vasoconstriction reflex and resulting pulmonary hypertension, and thus reducing the mechanism that lead to the venous admixture observed in white rhinoceroses [105]. However, shunt probably still occurred although in a lower percentage as the level of oxygenation was far from normal in most rhinoceroses of this group. Indeed the low PaO_2, might find an explanation in the fact that lateral recumbency, that was needed in our study to safely drill the horn to place transmitters, results in greater dead space ventilation in black rhinoceroses, and in lower PaO_2 compared to sternal recumbency [104], which is further supported by the higher initial alveolar-arterial gradients found in this study compared to other etorphine-immobilized black rhinoceroses in sternal recumbency [104,106]. The gradients however decreased in this group after the treatment, together with an improvement of PaO_2, a decrease in $PaCO_2$ and increased pH, as a consequence of both lactate clearance and elimination of carbon dioxide, since elimination is less impacted by lateral recumbency compared to PaO_2 exchange [104]. In addition, if doxapram increases the cardiac output in rhinoceroses, as demonstrated in other species [181], it might have been able to increase perfusion in

areas where ventilation occurred but perfusion was reduced, thus reducing the mismatch. Although the exact mechanism by which ventilation-perfusion mismatch in free-ranging black rhinoceroses occurs is unclear, it is well-demonstrated that this pathological mechanism occurs during immobilization and a decrease in cardiac output might be one of the causes [104]. In contrast, in boma-habituated immobilized white rhinoceroses, a high cardiac output significantly influenced the amount of venous admixture, although differently from our study, these individuals did not undergo exertion prior to the immobilization [105]. In humans a lower cardiac output leads to increased extraction of oxygen in the tissues, thereby decreasing partial pressure of oxygen in the venous blood. This, in the presence of conditions that decrease the ratios of ventilation to blood flow, amplifies the effects of the ventilation perfusion mismatch, leading to a decrease in arterial oxygen pressure [246].

Pharmacological properties of azaperone and butorphanol might have had a role on ventilation-perfusion mismatching mechanism in this study. Azaperone reduces mean blood pressure through a reduction in systemic vascular resistance, whereas cardiac output is slightly increased after azaperone administration in horses [153]. Butorphanol seems to be able to antagonize etorphine-mediated catecholamine release, as when given intravenously it is associated with a decrease in heart rate and blood pressure in both boma and field white rhinoceroses [70,133], and with a decrease of systemic vascular resistance in alpacas [247]. Therefore, it is not possible to exclude that butorphanol, especially in group B where it was given at higher doses, decreased cardiac output, and in a scenario where blood pressure was not elevated such as in this study, might have increased the mismatch. This could be supported by the fact that in group B blood pressure dropped more quickly. Although the monitoring of the blood pressure was performed in two individuals only in this group, the bigger decrease in blood pressure seen without increase in heart rate might have led to a decrease of cardiac output in this group, which would explain a bigger V/Q mismatch. Furthermore, differently from most of the studies performed on white rhinoceroses where there was no or little chasing prior to the immobilization, the fact that the rhinoceroses in our study were subjected to a massive exertion might have further altered the hemodynamic scenario and the pathophysiological mechanisms, including through catecholamine depletion [248].

The mechanism of hypoxemia and interaction between exertion, drugs and recumbency would need further dedicated research in black rhinoceroses to confirm these speculative assumptions. Indeed, as demonstrated by the different main source of hypoxemia in the black rhinoceros, and the different response to butorphanol, other pathophysiological mechanisms that lead to severe gas exchange and acid-base alteration might happen in black rhinoceroses compared to the white rhinoceroses. The choice over the doses used in this study might have also played a role. Doses of butorphanol that were able to fully treat or improve hypoxemia in white rhinoceroses, 10 – 20 times the mg used of etorphine, are higher than those recommended for black rhinoceroses [107,147,173]. Indeed black rhinoceroses appear to be particularly sensitive to the partial antagonist effect of butorphanol, and since sudden arousal are not uncommon, it is normally used at a ratio of 0.5 – 1 times etorphine [34]. Doses of butorphanol used in this study are thus high for the species, considering that higher doses provoke awakenings and are used to guide semi-sedated rhinoceroses into crates during translocations [54,174]. Doxapram is instead used at 100 – 200 mg in bolus in black rhinoceroses, based on empirical observation of increasing depth of ventilation [137]. Since both drugs are reported to cause

awakenings, a lower dose of doxapram was used in combination with butorphanol in group BD in this study, and based on previous experience of the authors. Other side effects that have been linked with the use of high doses of doxapram being a central nervous system stimulant, such as the insurgence of muscle tremor in rhinoceroses with doses of 100 – 200 mg [92,159] did not occur in this study. In addition, the fact that immobilization plane remained stable throughout the immobilization in most individuals, and doxapram did not account for more abrupt or quicker recoveries might be a result of the lower doses of doxapram used. The findings of this study, and in particular that the administration of butorphanol and oxygen was detrimental on gas exchanges, but became beneficial when it was combined with low dose doxapram, needs further investigation to elucidate the underlying mechanism and further improve the techniques of postinduction treatment in this species.

4.3 Capnometry

Capnometry measured in non-intubated patients with a nasal cannula is commonly used in human medicine in order to monitor respiration in sedated patients [144]. In our study, according to the Bland Altman plot, the nasal $ETCO_2$ did not appear accurate in predicting the values of $PaCO_2$. On the other hand, the higher correlation observed between $ETCO_2$ and $PaCO_2$ at BG2, compared to BG1, may find an explanation with the fact that at the beginning of the recumbency, greater and more dynamic ventilation alterations happen due to a combination of exertion and initial drug effects, which might have affected more the reading by the capnometer. Indeed in human medicine, it has been observed that the depth of breathing can influence the $PaCO_2$-$ETCO_2$ difference, which gap decreases when the breath is more deep [230].

In our study $ETCO_2$ values where overall higher than $PaCO_2$ values in both treatment and sample time points. Although the difference between PaO_2-$ETCO_2$ is physiologically considered to be 2 – 5 mmHg, reversal of the normally positive $PaCO_2$-$ETCO_2$ gradient negative has been reported in human and domestic animals, in cases of late emptying of well-perfused alveoli with higher CO_2 tensions or reduced functional residual capacity (FRC), rebreathing of carbon dioxide from under ventilated compartments, and Haldane effect [141,249]. Since according to our results, the V/Q matching was far from being optimal, the more likely options are a reduction in FRC resulting from the increased pressure on diaphragm of abdominal organs in lateral recumbency, similarly to the mechanism in pregnant and obese human patients, or rebreathing of carbon dioxide due to increased anatomical dead space. When the respiratory cycle is altered and the inspiration time is prolonged compared to the expiration time, the chances of accumulation of carbon dioxide in the upper respiratory ways, or even in the capnometry sampling tube, are increased. Indeed a prolonged inspiratory time, which has the benefit of increasing alveolar recruitment, and improving oxygenation, on the other hand decreases the clearance of carbon dioxide by decreasing the time available for passive expiration, leading to the rebreathing of carbon dioxide [250]. In white rhinoceroses and horses, an inspiratory breath holding reflex has been observed and interpreted as an effort of "auto-recruitment" and influence distribution of ventilation, and was associated with increased $PaCO_2$ levels [105,251]. In our study breath holding was observed in some rhinoceroses, even though it was not quantified; however this mechanism could explain a lower carbon dioxide clearance and thus the higher $ETCO_2$ found in this study. In alternative, rebreathing of carbon dioxide could be due to a technical problem due to the tube inserted in the nostril

that accumulated carbon dioxide. Indeed, in human medicine the smaller diameter of the cannula lead to more accurate results, and in black rhinoceroses, where a small diameter cannula was used, negative values of $PaCO_2$-$ETCO_2$ were not reported [104], therefore more research on the optimal nasal capnometry sampling technique needed.

Our results suggest that it may be difficult to predict $PaCO_2$ from the $ETCO_2$ due to the wide limits of agreement, but that nasal capnometry can be used as a clinical tool to early detect ventilation-related complications, and in our study seemed more reliable than pulse-oximetry to detect respiratory flaws. Indeed the pulse-oximeter tended to both over- and under-estimate the hemoglobin oxygen saturation, in line with previous studies [106]. Furthermore, based on our descriptive observations, most of the capnometer overestimations were occurring in case of hypoxemia and when quick variation of values was observed and were concomitant with hypopnea and apnea, and as such it could play as an alarm for hypopnea and apnea. On the other hand, underestimations that commonly occur with nasal capnometry due to dilution with ambient air [142], did not happen in our study, which is particularly important considered the high risk of hypercapnia resulting from the opioids and recumbency alterations. More research is needed in order to improve the sampling techniques for nasal capnometry in black rhinoceroses, within a more experimental design study with limited cofounding factor.

4.4 Limitations

In this study, some limitations occurred and affected the possibility to draw definitive conclusions. These included the short monitoring period and the lack of monitoring of more advanced physiologic techniques such as tidal volume, cardiac output, and pulmonary pressures. Furthermore, since the study was conducted opportunistically and in the field, interpretation of the treatment effects was confounded by a number of variables that could not be controlled for, such as chase-related exertion. The different distribution of the individual's locations in the two groups might have also affected the results. The use of a delta PaO_2 to calculate the difference between the expected PaO_2 for the specific altitude and the measured one, allowed to exclude that PaO_2 was higher in BD in respect to B group as a result of the higher altitude of capture sites for individuals of group B. On the other hand, a genetic-mediated different sensitivity to etorphine's side effects might exist in separated populations. The role of single nucleotide polymorphism in the μ-opioid receptor gene (OPRM1) has been elucidated in some veterinary species [35,37] and further investigation in rhinoceroses is auspicated, as it could provide an explanation for the differences in drug response seen at individual and species' level [81,200–202]. Another possible confounding factor for the evaluation of the two treatments, is that in some individuals oxygen was administered at the time of the first sample. The administration ranged from 10 to 50 seconds before the sample was collected, due to a delay in getting samples in the field. Considering the limited and sometimes detrimental effects of oxygen in improving the arterial oxygenation in white rhinoceroses, it is unexpected to have such an effect so shortly after administration [172]. A MANOVA test confirmed otherwise that the initial PaO_2 levels were influenced by the occurrence of oxygen insufflation that preceded BG1 sample in both the groups. Instead, the fact that PaO_2 measured at BG2 was not influenced by it in neither of the groups supports that the evaluation of the effects of the two different treatments (at BG2) was not biased by the oxygen administered before BG1, and as such can be considered reliable.

5. Conclusions

We observed severe homeostatic imbalance during the capture of free-ranging Eastern black rhinoceroses, in a manner correlated to the intensity of pre-darting helicopter chase. The administration of doxapram and butorphanol, combined with oxygen insufflation, produced a partial improvement in arterial oxygenation, pH and carbon dioxide. This was likely caused by an increase in ventilation mediated by doxapram, that might have diminished the severity of intrapulmonary alterations such as ventilation-perfusion mismatch and oxygen diffusion impairment. In contrast with results obtained from white rhinoceroses, the administration of butorphanol and oxygen alone did not result in improvement of physiological function in study subjects. Our results suggest black and white rhinoceroses share different mechanisms of physiological alterations and more research is therefore needed to understand pathophysiological mechanisms in response to the administration of capture drugs and presence of stress in this species. New drugs combinations that enable a reduction in etorphine doses and therefore its adverse effects need to be evaluated in this species, as they might further improve the safety of field capture. Although more research is needed to evaluate the accuracy in predicting arterial PaO_2 levels, nasal capnometry is a useful tool for clinical monitoring of ventilation trends in wildlife in the field. A long-term post-capture follow-up allowed to assess that regardless of the severe capture-related morbidity, no delayed complications occurred. Future studies may be considering to include non-invasive methods to quantify and assess the occurrence of chronic physiological alterations in order to further advice on how to improve capture protocols to increase not only short- but also long-term safety and success.

RESEARCH STUDY IV

COMPARISON OF ETORPHINE-AZAPERONE AND ETORPHINE-MEDETOMIDINE-AZAPERONE COMBINATIONS FOR THE IMMOBILIZATION OF FREE-RANGING AFRICAN BUFFALOS (*SYNCERUS CAFFER*)

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

Abstract

The physiological safety of two immobilization combinations, etorphine-azaperone (EA) and etorphine-medetomidine-azaperone (EMA) was evaluated in free-ranging African buffalos. The hypothesis was that the α2-agonist sparing effect would have allowed to decrease etorphine doses, and its adverse respiratory effects, without increasing the risk of excitement or poor immobilization quality. Buffalos were darted intramuscularly with etorphine (0.010 mg/kg) and azaperone (0.11 mg/kg) (n=34) or medetomidine (0.003 mg/kg), etorphine (0.007 mg/kg) and azaperone (0.11 mg/kg) (n=14). Protocol evaluation included physiological monitoring, blood-gas analyses (BGA), anesthetic times and *ad-hoc* quality scores (1 = excellent; 4 = poor). Immobilization was then reversed with diprenorphine, plus atipamezole in EMA. Inductions were faster in EMA (6.9 ± 1.5 minutes Vs. 12.0 ± 2.7 minutes) and smoother, scored 1 (1 – 3) Vs. 2 (1 – 4). Immobilization quality was improved in EMA, where median score was 1 (1 – 4) Vs. 3 (1 – 5) in EA ($p < 0.001$). Heart rate was significantly lower in EMA (69 ± 12 Vs. 110 ± 34; $p < 0.001$), whereas respiratory rate and rectal temperature were similar in the groups. SpO_2 was higher in EMA (90.8 ± 6.9 % Vs. 85 ± 5.8%), whereas nasal end-tidal CO_2 was lower (respectively 43.8 ± 7.0 mmHg Vs. 47.6 ± 11.0 mmHg). The results of BGA revealed slight acidosis, mainly of respiratory origin, in EA and hypoxemia in both groups. After reversal, recoveries were quick in both combinations (1.2 ± 0.4 minutes in EMA, 1.8 ± 0.9 in EA) and qualitative, scored respectively 1 (1 – 2) Vs. 2 (1 – 3). Medetomidine's sparing effect allowed to decrease etorphine doses, decreasing its respiratory adverse effect, but nonetheless providing smoother inductions and immobilizations. EMA combination resulted in physiological and handling safety and is recommended for free-ranging buffalo immobilization.

1. Introduction

African buffalos (*Syncerus caffer*) have been recently declared as near threatened, as their populations are decreasing as a result of anthropogenic factors [15]. Being a favored target of illegal bush meat hunters, buffalos are often victims of poaching snares, which removal requires chemical immobilization. In addition, African buffalos are routinely immobilized across Africa for epidemiological studies since they are important reservoir of livestock diseases [252,253], and for translocations within the game ranching industry in several African countries [254].

Chemical immobilization of wild buffalos is challenging since they have a fractious temperament that make them particularly dangerous, and yet they are commonly known for their susceptibility to capture stress and morbidity [91,136,165]. Their physiology and size expose them to metabolic derangement, respiratory and circulatory compromise, hyperthermia, tympany, regurgitation and capture myopathy [165]. Indeed, during field capture operations, if chased or frightened, they can sustain high speed run for minutes, developing a high risk of overexertion [34].

Ultrapotent opioids like etorphine are the most common group of drugs for the restraint of wild bovids, as they provide quick and reversible immobilization. However, their use is associated with significant adverse effects that further increase the risk of capture morbidity, such as respiratory depression, poor muscle relaxation and sympathetic stimulation that results in catecholamine release [34,35,136,165]. Etorphine-azaperone combination is the protocol of choice in African buffalos, since the addition of azaperone, a butyrophenone tranquillizer, can reduce opioid-related hypertension, and improve muscle relaxation and induction times [70,103,154,155]. However, even if no research study has been conducted to investigate the effects of etorphine-azaperone combination on gas exchanges and acid-base function in this species, severe morbidity including hypoventilation, hyperthermia and regurgitation has still been observed by field veterinarians [255]. For this reason, in the field, where proper monitoring equipment and anesthetic support machines are often unavailable, doses are kept low to limit severe cardio-respiratory adverse effects. However this can result in excitement and longer inductions that can lead to severe physiological derangement due to overexertion, and poor immobilization depth that can sometimes result in dangerous restraints [34,136].

A2-agonists are among the most used drugs in veterinary medicine, as they offer several advantages including potent sedation, analgesia, muscle relaxation and reversibility with selective antagonist [35,160,161]. Furthermore, thanks to their potent anesthetic sparing effect, they allow a severe reduction in the amount of other anesthetics, including opioids, required to induce and maintain anesthesia and analgesia [160–164]. These agents have been successfully used in other wild bovids, and showed to improve muscle relaxation, decrease the amount of opioid required and ease induction and recoveries [136,165]. Furthermore since buffalos mount a stress response to immobilization that might have a longer-term impact on their health [91], α2-agonists might be useful since they are anxiolytic and attenuate the stress response influencing the endocrine system [160].

However, in spite of the several benefits and the results of clinical studies indicating their efficacy and safety in healthy animals, the use of a α2-agonists is not universally supported because of concerns relating to the respiratory depression and potent cardiovascular effects of α2-agonist drugs

[160,161,167]. Hypoxemia is commonly observed in response to administration of α2-agonists in most ruminants, and is believed to occur mainly through an inflammatory reaction mediated by pulmonary intravascular macrophages specific for this suborder [167]. These adverse effects are however typically dose related, and mostly associated to less selective α2-agonist drugs (e.g. more with xylazine compared to medetomidine), and the intravenous route [160,161,167]. Furthermore, sensitivity to different anesthetics among nondomestic bovids tend to be species-specific [165], therefore in African buffalos the effects of α2-agonists, and in particular of those highly selective like medetomidine, needs to be evaluated.

The aim of this study was to compare etorphine-medetomidine-azaperone and etorphine-azaperone immobilization in free-ranging African buffalos to evaluate if the addition of ultralow doses of medetomidine could decrease capture morbidity and improve physiological and handling safety. Furthermore, since this was the first study to evaluate gas-exchanges and acid-base function in this species, a secondary aim was to provide further advancement on the understanding of capture morbidity in this sensitive species.

2. Materials and Methods

2.1 Animals, drugs and procedures

This study was conducted opportunistically within the implementation of an international epidemiological project aimed to investigate the role of African buffalos as reservoir for livestock pathogens in East Africa. The study took place in Masai Mara National Reserve and Mara Triangle (altitude 1600 – 1700 meters). The Kenya Wildlife Service (KWS) Department of Veterinary Services and the Biodiversity Research and Monitoring Office (KWS/BRM/5001) approved the present study, which complied with KWS guidelines to conduct research on wild mammalian species.

Forty-eight free-ranging African buffalos were included in the study. The individuals were divided in two groups based on the chemical immobilization protocol: a combination of etorphine and azaperone (group EA) or etorphine, medetomidine and azaperone (group EMA). Etorphine (Captivon 9.8 mg/mL, Wildlife Pharmaceuticals, White River, South Africa) was administered at ranges of 3 – 5 mg in EA and 2 – 5 mg in EMA group, whereas 50 mg of azaperone (100 mg/mL, Kyron Laboratories, Johannesburg, South Africa) were administered in both groups. Doses of etorphine were chosen based on the estimated size and age of the individuals. In EA doses of 3 and 3.5 mg of etorphine were chosen for subadults (< 400 kg) and 5 mg for average-sized adult females and males. In EMA doses of 2 – 3 mg were chosen for subadults (< 400 kg), 3.5 mg for average-sized females and males, and 5 mg for the only three older bulls of the study that were estimated over 700 kg. In EMA group, 1 – 1.5 mg of medetomidine (Domitor 1 mg/ml, Orion Pharma, Espoo, Finland) were added to the dart mixture for all individuals, whereas the dose was increased to 2.5 mg for the three bulls. The drugs were delivered intramuscularly in the upper hind leg or neck or shoulder area in a 3-mL dart syringe (Dan-Inject 3 mL, 2.0 × 60 mm needle, S300 Syringe Dart, Dan-inject International, Skukuza, South Africa) through

a CO_2 pressurized dart gun (Model JM; Dan-inject International, Skukuza, South Africa). All the buffalos were darted from a vehicle, in groups of two to four animals at a time.

During the recumbency, the age was estimated by the teeth [256], and the weight was successively estimated based on age categories and growth curve [257].

Once the sample collection was terminated, the buffalos were administered in both groups intravenous diprenorphine (12mg/ml, Wildlife Pharmaceuticals, White River, South Africa), combined with atipamezole in EMA group (Antisedan, 5 mg/ml, Orion Pharma, Espoo, Finland) to reverse medetomidine.

2.2 Monitoring

In order to evaluate the quality of the inductions and compare it between EA and EMA groups, pre-darting and induction scores were created (Table 1 and 2). The time from the dart injection to the recumbency was recorded (Induction time).

Darting Stress Score	Description
1	Calm reaction when approached by the darting vehicle, keep on with the previous activity or stop to observe the vehicle. Herd bulls on alert.
2	Suspicion over the darting vehicle. Intermittent walking away within the herd for maximum 2 minutes only when the vehicle is close
3	Keep distance from the darting vehicle, intermittent trot/gallop for up to 5 minutes before successfully darted
4	Sustained chasing involving high velocity gallop before successfully darted

Table 1. Darting stress score in free-ranging African buffalos

Induction Score	Description
1	Excellent induction after a short sprint; slight ataxia with little signs of excitement or falling over during the process. Smooth recumbency quickly achieved.
2	Good induction after a short sprint; moderate ataxia and little signs of excitement, the buffalo may stumble.
3	Fair induction; flighty reaction leads to minutes of gallop before achieving ataxia. Severe ataxia and excitation.
4	Rough induction; severe excitatory reaction leading to over-exertion. The animal might require a second dose of drugs before becoming recumbent.

Table 2. Induction score in free-ranging African buffalos

Once the buffalos were recumbent, they were placed in sternal decubitus. The physiological function was monitored, and the occurrence of regurgitation was recorded. Respiratory rate (RR) was monitored through the observation of chest movements, and the heart rate (HR) by auscultation of the

hearth with a stethoscope. Rectal temperature (T) was measured with a thermometer inserted in the rectum (Veterinary rectal thermometer, 25588 Gima S.p.a., Gessate, Italy). A mainstream capnometer (Masimo EMMA Capnometer 9632, Masimo Corporation, California, United States) was used to monitor end tidal carbon dioxide ($ETCO_2$). The device was attached to a cut 20 cm long endotracheal tube (11 mm ID) inserted in one nostril. A pulse oximeter with a transmission probe (PM-60, Mindray Medical, Shenzen, China) was attached to the vulvar or preputial mucosa to measure hemoglobin oxygen saturation (SpO_2). Since more than one individual was recumbent at the same time, often it was not possible to monitor the physiological function overtime, therefore for consistency of data only the first recording of each animal is reported.

Arterial blood samples were withdrawn from the auricular artery in pre-heparinized syringes. If there were any air bubble, these were immediately removed and the syringe sealed with a tip cap and put in ice inside a cooler box. The blood was analyzed within 30 minutes from collection with an i-ISTAT 1 handheld blood analyzer using CG4+ cartridges, and within 60 minutes for CHEM8 and CTNI cartridges (Abbott Laboratories, Abbott Park, Illinois, USA). Measured values included pH, partial pressure of carbon dioxide ($PaCO_2$) and oxygen (PaO_2), and lactate, whereas bicarbonate (HCO_3^-), base excess (BE), total carbon dioxide (TCO_2), and arterial saturation of oxygen (SO_2) were calculated. Electrolytes (Na, K, Cl, iCa), Glucose (GLU), BUN, creatinine (Crea), hematocrit (Hct), hemoglobin (Hb), Anion Gap (AG) and cardiac troponin I (CTNI) were also analyzed. Values of pH, $PaCO_2$ and PaO_2 are shown both as corrected and non-corrected for rectal temperature.

The alveolar-to-arterial oxygen tension gradient [$P(A\text{-}a)O_2$] was calculated by subtracting PaO_2 measured by the blood gas analysis (BGA) from the alveolar oxygen tension (PAO_2). PAO_2 was calculated with the following equation:

$$PAO_2 = FiO_2\ (Pb\text{-}P_{H2O}) - PaCO_2/RQ$$

where FiO2 is the fraction of inspired oxygen (21% for room air), Pb is the barometric pressure measured during the study (620 mmHg), P_{H2O} is the partial pressure of vapor in the alveoli at 37 °C (47 mmHg), $PaCO_2$ is the partial pressure of carbon dioxide of each buffalo. RQ is the respiratory quotient, which depends on metabolic activity and diet, and is considered to be 1 for ruminants [81].

Since the capture site was not at sea level, we calculated the PaO_2 expected for the average altitude of the capture sites (range 1600 – 1700 m) in order to use it as the cut-off value for defining hypoxemia, using the alveolar-to-arterial oxygen tension formula. Assuming a normal alveolar-oxygen tension difference of 15 mmHg, the expected PaO_2 was calculated by subtracting 15 mmHg from the calculated PAO_2 value (as described above, but using the measured barometric pressure of the capture site (620 mmHg) and $PaCO_2$ of 35 mmHg for this estimation, which is considered the physiological reference value at this range of altitudes) [170,192]. The expected PaO_2 at the altitude of the capture site was subtracted from the measured PaO_2 values of each individual with the following equation:

$$\Delta PaO_2 = PaO_{2\ measured} - PaO_{2\ expected}$$

the individuals with negative values were considered hypoxemic, and the ones with values lower than -20 mmHg severely hypoxemic.

The quality of the immobilization was monitored using the appositely created descriptive score (Table 3).

Immobilization Score	Description
1	Surgical level. Deep sedation and myorelaxation, no reaction to pain. Safe handling.
2	Good sedation and myorelaxation. Involuntary tail/ear movements. Safe handling.
3	Voluntary movements, reaction to manipulation and muscle tone, but the immobilization is safe.
4	Voluntary movements, reaction to manipulation and muscle tone. Caution required, manual restrained is essential for sample collection.
5	Strong attempts to stand, excitation and aggressive behaviour. Extremely dangerous situation.

Table 3. Immobilization score in free-ranging African buffalos

The time of the administration of the antagonists, and the time that took for the buffalo to stood up (Recovery time) were recorded. The quality of the recovery was assessed through the recovery score (Table 4).

Recovery Score	Description
1	Excellent recovery; standing at the first attempt with adequate balance and coordination.
2	Good recovery; standing at the first attempt, balance is gained after few steps.
3	Fair recovery; few attempts before standing, weakness and poor balance in the first seconds. Risk of injuries.
4	Poor recovery; struggle to stand with one or more spills and poor balance once standing. Risk of injuries.

Table 4. Recovery Score in free-ranging African buffalos

2.3 Data Analysis

A Student's t-test was used to compare doses, anesthetic times, physiological function and blood variables between EA and MEA groups; a Mann-Whitney U test was used to compare the non-parametric data (scores). A non-parametric Spearman correlation coefficient was used to evaluate the

correlations between the anesthetic doses, times and scores and the measured physiological variables and blood analytes in each of the two groups. Only the most relevant statistical results are reported. Numeric data are presented as mean values ± standard deviation, with ranges where relevant. A *p*-Value equal or below 0.05 was considered significant. Scores are presented as median values with ranges.

3. Results

Thirty-four buffalos were immobilized with EA combination and four-teen buffalos with EMA combination. The distribution of the age and gender within the two groups was similar. In EA group the mean age was 5.8 ± 3.3 years old and individuals were 16 females and 18 males, whereas in EMA group the mean age was 5.3 ± 2.5 years old and individuals were 8 females and 6 males.

The estimated body weight was 463 ± 110 kg in EA and 489 ± 133 kg in EMA. Doses of etorphine per-kg estimated on the body weight were significantly higher ($p < 0.001$) in EA, 0.010 ± 0.002 mg/kg Vs. 0.007 ± 0.002 mg/kg in EMA. Azaperone doses per-kg were instead not different between the two groups, 0.116 ± 0.03 mg/kg in EA and 0.110 ± 0.03 mg/kg in EMA. Medetomidine was administered only in EMA group, and doses per-kg were 0.003 ± 0.001 mg/kg. Three individuals were excluded from EA group and one from EMA group since they had prolonged inductions (over 20 minutes) that required a second darting. In two of these individuals the darting site was not optimal (tail), and might have accounted for longer inductions, whereas in all the other individuals the darting site included muscles of the neck, shoulder or hindquarters.

The pre-darting stress was overall minimal, and was similar between the two groups (median score 1, range 1 – 3 in EA, median score 1, range 1 – 4 in EMA). Induction times were significantly faster in buffalos given EMA combination ($p < 0.001$), on average 6.9 ± 1.5 minutes Vs. 12 ± 2.7 minutes in EA, and a better induction quality was also observed in EMA combination (median score 1, range 1 – 3, $p = 0.05$) compared to EA (median score 2, range 1 – 4). In EMA, higher doses of medetomidine accounted for shorter induction times ($r = -0.65$, $p = 0.04$) and better induction scores ($r = -0.53$, $p = 0.04$), whereas in both groups etorphine and azaperone doses per-kg did not account for any difference ($p > 0.05$).

The immobilization quality was significantly better ($p < 0.001$) in EMA (median score 1, range 1 – 4) compared to EA (median score 3, range 1 – 5). In EA group, most buffalos were not adequately sedated, reacted aggressively to sample collection trying to hit personnel with horns and managed to stood up in some occasions. Additional manual restraint with ropes was needed in most cases and the restraint was dangerous for the capture team, and stressful for the animal. In EMA group, the buffalos had a satisfactory plane of anesthesia and level of myorelaxation. No reaction to sample collection was observed, and additional manual restraint was not required. Even though immobilization score was not correlated to the doses of the darting drugs in neither of the groups ($p > 0.05$), worse immobilization quality seemed to occur when mistakes on weight estimation were made. In particular, in EMA, immobilization quality was excellent in all individuals except two, which received lower doses of medetomidine (0.002 mg/kg). However, in the same individuals, a higher startle response to the darting

vehicle also occurred. In EA, the immobilization quality was unaffected by the doses administered and the startle response, as no correlations were found ($p > 0.05$).

Mean heart rate was significantly lower in EMA (69 ± 12 bpm), compared to EA ($p < 0.001$) where HR was increased (110 ± 34 bpm) with rates up to 180 bpm. Respiratory rate was slightly higher in EMA, although non significantly (25.0 ± 12.9 bpm Vs. 19.8 ± 7.9 bpm), whereas rectal temperature (39.1 ± 0.9 °C Vs. 39.2 ±1.0 °C) was similar in the two groups. SpO_2 was measured only in 15 individuals in EA and 9 in EMA due to technical issues, and it showed higher values in EMA (89.0 ± 7.9 % Vs. 85 ± 5.8%). Even though, according to paired sample t-test no significant difference was observed between the values of SpO_2 and arterial SaO_2 ($p > 0.05$), the pulse-oximeter tented to both over- and under-estimate the arterial saturation values. Although not significantly different, nasal $ETCO_2$ was overall lower in EMA (43.8 ± 7.0 mmHg Vs. 47.6 ± 11.0 mmHg). The distribution of HR, RR, rectal temperature and $ETCO_2$ in the EA and EMA groups are represented in the histogram in Figure 1. The darting doses did not influence any of the physiological variables in EMA, whereas in EA a higher etorphine dose per-kg was correlated to lower rectal temperatures (r = -0.36, $p = 0.04$).

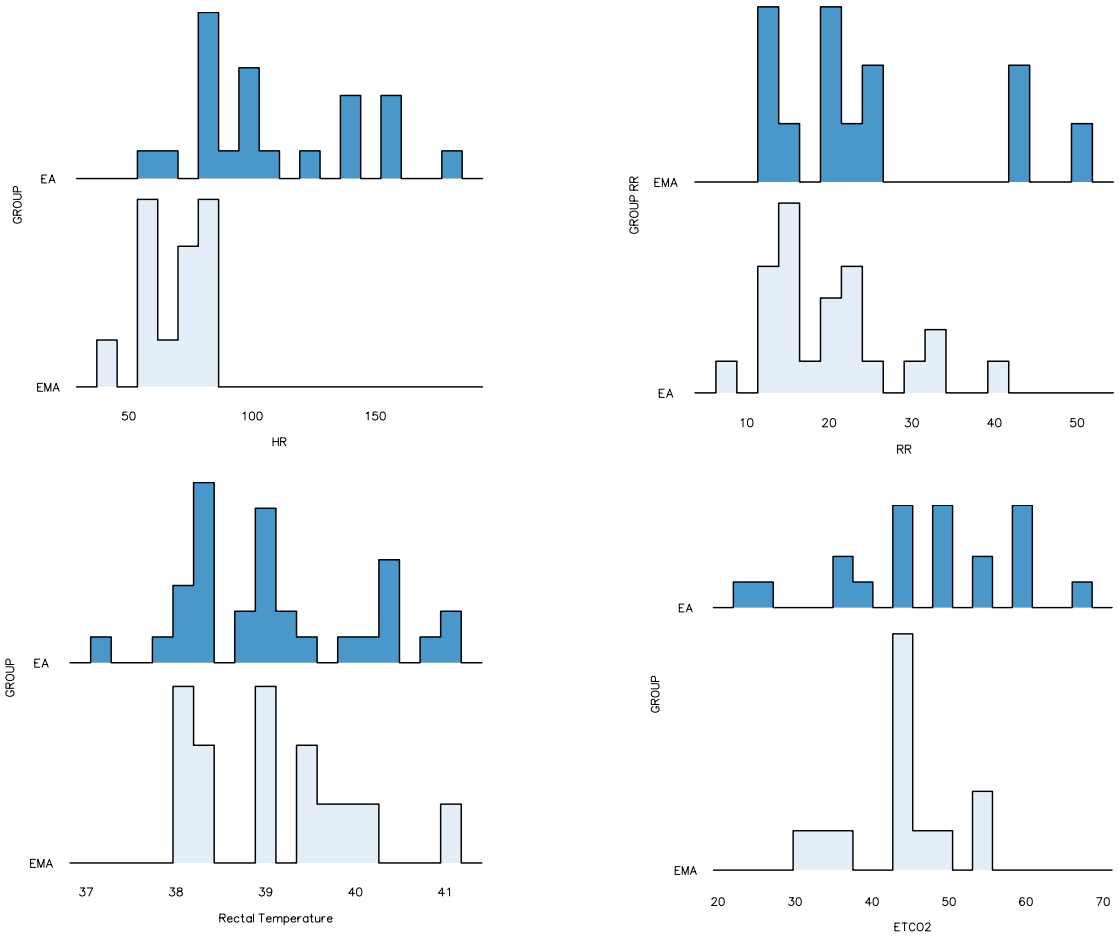

Figure 1. Representation of the distribution of the values of HR, RR, T and $ETCO_2$ across the EA (dark blue) and EMA (light blue) groups. Each histogram represents the number (in proportion) of animals within each range of recorded value.

Arterial samples were successfully obtained in 27 individuals in EA group and 13 in EMA group. The results of the BGA are shown in Table 5. Since more than one buffalo was darted simultaneously, it was not possible to standardize the time of sample collection in regard to the start of the recumbency. However, in all buffalos this was obtained within 10 minutes from the start of the recumbency.

Variable	EMA		EA	
	Mean	**SD**	**Mean**	**SD**
pH	7.389	0.08	7.352	0.09
$PaCO_2$ (mmHg)	37.7	7.4	42.6	11.3
PaO_2 (mmHg)	51.4	10.2	55.4	18.6
BE (mmol/l)	-2.4	4.1	-2.6	3.3
HCO3- (mmol/l)	22.6	3.6	22.9	3.2
TCO_2 (mmHg)	23.6	6.7	24.2	3.5
SaO_2 (%)	83.0	9.8	81.9	13.3
Lactates (mmol/l)	4.7	3.9	4.1	2.8
T (°C)	39.2	0.8	39.0	0.9
pH (mmHg; corrected for T)*	7.356 *	0.08	7.311*	0.07
$PaCO_2$ (mmHg; corrected for T)*	41.7*	8.1	47.3*	10.1
PaO_2 (mmHg; corrected for T)	60.0	12.3	64.2	21.3
$P(A\text{-}a)O_2$*	31.1*	6.8	21.3*	16.9
$PaO_{2\ measured\text{-}expected}$	-18.0	8.5	-14.8	18.6

Table 5. BGA values in EMA and EA groups, showed as mean values and standard deviation (SD) (CG4 cartridges, I-Stat1, Abbott Laboratories). * = statistically significant difference between EMA and EA group ($p < 0.05$).

Overall pH was within physiological ranges. A slight acidosis occurred in EA in 9 individuals, and a moderate acidosis with values < 7.25 in three individuals, whereas in EMA a slight acidosis occurred in three buffalos only. Moderate alkalosis (pH > 7.5) occurred in two buffalos in EA, and in one in EMA [193]. When the pH was corrected for the rectal temperature, the mean pH became slightly acid in EA group, with significantly lower values ($p = 0.04$) compared to EMA. Arterial carbon dioxide was also significantly higher ($p = 0.04$), when corrected by the rectal temperature in EA group. It was beyond physiological levels (> 45 mmHg) in five individuals in EA (16 when T corrected values are used), with peaks of 67 mmHg, and it was decreased (< 35 mmHg) in six individuals, down to 19 mmHg. In EMA, the $PaCO_2$ was slightly decreased in four individuals and increased in two, but the range of variations was more narrow (26 – 47 mmHg). Arterial oxygenation was similar in the two groups. When the delta between measured and expected PaO_2 (the cut-off of hypoxemia calculated for the altitude was 70 mmHg) for the capture site's altitude was calculated, it was negative in both groups. In particular, severe hypoxemia (delta PaO_2 < -20 mmHg) occurred in eleven individuals in EA, and five individuals in EMA.

The mean alveolar-arterial gradient was more elevated in EMA ($p = 0.02$), in which it was beyond physiological levels (> 20 mmHg) in all individuals but two. It was instead within normal levels in 14 individuals out of 25 in EA. Interestingly, negative gradients were observed in three buffalos in EA, that had increased levels of arterial oxygen (> 85 mmHg). The difference between $PaCO_2$ and $ETCO_2$ was -0.2 ± 9.9 mmHg in EA group and -2.1 ± 10.6 mmHg in EMA. However due to field condition

challenges, it was not possible to record $ETCO_2$ values and sample arterial blood in a precise simultaneous manner in all animals.

Mean bicarbonates and base excess were within normal ruminant ranges and were similar in the two groups. Bicarbonates were decreased under the threshold of 20 mmol/l in four buffalos in EA and one in EMA, and no buffalos had elevated levels. Lactates were slightly increased in both groups, however, were beyond 10 mmol/l in two individuals only, one in each group. Increases in lactates were correlated in both groups with worse induction scores ($r = 0.69$, $p < 0.001$ in EA, $r = 0.6$, $p = 0.02$ in EMA), but not with induction times ($p > 0.05$).

Based on the evaluation of bicarbonates and $PaCO_2$, in EA the acidosis was of respiratory origin in seven buffalos, metabolic in one and mixed in five, whereas in EMA it was metabolic in two and respiratory in one buffalo. In EA group, a lower pH was strongly correlated to higher $PaCO_2$ ($r = -0.82$, $p < 0.001$), whereas in EMA, a lower pH was weakly correlated to the higher $PaCO_2$ ($r = -0.63$, $p = 0.01$). In neither of the two groups, a lower pH was correlated to lactates or bicarbonates ($p > 0.05$).

Electrolytes were within ruminant ranges [258]. In one buffalo of group EA, an extremely elevated value of potassium was recorded (8.8 mmol/l), although since no major other severe alteration, except high glucose, were recorded in the same individuals, it suggests that an analytical error might have occurred. Ionized calcium was lower than cattle reference values (1.2 – 1.6 mmol/l) in 10 buffalos in EA and 6 in EMA. Creatinine, BUN and anion gap were also within bovine range values [258], and hematocrit and hemoglobin were in line with values reported in free-ranging African buffalos [259]. Glucose was elevated (> 75 mg/dl) in both groups, but significantly higher in EMA ($p = 0.002$), whereas BUN was lower in EMA ($p = 0.009$). Cardiac troponin I (CTNI) was detected in 13 individuals out of 31 examinations, and range of values were 0.01 – 0.03 ng/ml. Buffalos immobilized with EA combination had significantly higher levels of CTNI (0.008 ± 0.01 Vs. 0.02 ± 0.004). The mean values and standard deviations of electrolytes, selected biochemistry and CTNI divided by group are represented in Table 6.

Variable	EMA		EA	
	Mean	SD	Mean	SD
Na (mmol/l)	139.5	2.0	139.9	2.8
K (mmol/l)	5.0	0.5	5.3	0.9
Cl (mmol/l)	102.3	2.5	103	2.8
iCa (mmol/l)	1.2	0.05	1.2	0.06
Glu (mg/dl) *	124.8*	22.3	95.7*	29.6
BUN (mg/dl) *	10.4*	2.3	13.3*	3.7
Crea (mg/dl)	1.09	0.16	1.03	0.17
Hct (%)	36.0	9.1	40.1	14.1
Hb (g/dl)	12.2	3.2	13.6	4.8
Anion Gap (mmol/l)	19.4	3.6	19.6	2.8
Cardiac Troponin I (ng/ml) *	0.0025*	0.004	0.008*	0.010

Table 6. Electrolytes, selected biochemistry and cardiac troponin I values in EMA and EA groups, showed as mean values and standard deviation (CHEM8 and CTNI cartridges, I-Stat1, Abbott Laboratories). * = statistically significant difference between EMA and EA group ($p < 0.05$).

The changes observed in the levels of some of the blood analytes appeared to have some correlations. In EMA group, the higher lactates detected in some individuals were correlated with other alterations such as higher hematocrit (r = 0.58, $p = 0.003$), hemoglobin (r = 0.58, $p = 0.03$), and anion gap (r = 0.71, $p = 0.01$), and lower calcium (r = -0.69, $p = 0.01$), and the same values were correlated to lower bicarbonates (respectively, r = -0.60, $p = 0.02$; r = -0.60, $p = 0.02$; r = -0.71, $p = 0.009$; r = 0.67, $p = 0.01$). Lower bicarbonates were indeed correlated with higher lactates in EMA (r = -0.74, $p = 0.005$), and also in EA (r = -0.71, $p < 0.001$). In EA higher lactates were instead not correlated to hematocrit and hemoglobin, but to higher glucose (r = 0.43, $p = 0.05$), and anion gap (r = 0.51, $p = 0.02$), whereas lower bicarbonates were correlated only to higher anion gap (r = -0.58, $p = 0.006$). In EA, a lower pH was correlated to higher calcium (r = -0.48, $p = 0.02$) and lower creatinine (r = 0.52, $p = 0.01$). In addition, in EMA, lower bicarbonates (r = -0.64, $p = 0.01$), lower $PaCO_2$ (r = -0.71, $p = 0.006$), and higher PaO2 (r = 0.79, $p = 0.001$), were correlated to higher HR. However, higher HR was not influenced by different doses of medetomidine, or induction scores or times ($p > 0.05$). HR was furthermore correlated to higher RR (r = 0.85, $p = 0.01$) and even if not significant, there was a weak correlation with lactates levels (r = 0.54, $p = 0.06$). In EA, HR did not account for any correlations, but a higher PaO_2 was instead correlated to lower bicarbonates (r = -0.57, $p = 0.02$), similarly to EMA. Furthermore, in this group a high rectal temperature was correlated to lower $PaCO_2$ (r = -0.65, $p < 0.001$), and higher pH (r = 0.68, $p < 0.001$), and with elevated hematocrit and hemoglobin (both r = 0.44, $p = 0.04$).

In EMA group, the difference that occurred for medetomidine dose per-kg, due to errors in body weight estimation before darting, accounted for some correlations. A higher dose was correlated to lower lactates (r = -0.57, $p = 0.04$), potassium and chloride (both r = -0.54, $p = 0.04$), and higher bicarbonates (r = 0.75, $p = 0.002$). Although a wide range of doses per-kg of etorphine and azaperone was administered, no significant correlations were seen with blood gases and analytes in neither of the two groups.

Correlations were also observed regarding the age of the animals. IN EMA an older age was correlated to higher BUN (r = 0.58, $p = 0.02$) and creatinine (r = 0.80, $p < 0.001$), whereas in EA older buffalos had increased anion gap (r = 0.45, $p = 0.04$) and lower calcium (r = -0.61, $p = 0.003$) values. No significant correlations were observed for the gender.

Diprenorphine was given at 3 times the etorphine dose in both groups (range 6 – 10 mg), whereas atipamezole varied between 3 and 5 times the dose of medetomidine (range 5 – 7.5 mg). However, in EMA the time for recovery was not influenced by the different atipamezole doses. Antagonists were administered on average 22.8 ± 14.9 minutes in EA and 20.3 ± 9.8 minutes in EMA from the start of the recumbency. The wide range of the duration of the immobilizations was due to the fact that more buffalos were darted simultaneously, therefore some individuals were not reached immediately when recumbency occurred. After the antagonization, in both the groups recoveries were quick (1.2 ± 0.4 minutes in EMA Vs. 1.8 ± 0.9 in EA) and qualitative, scored as 1 (1 – 2) in EMA Vs. 1 (1 – 3) in EA. In EA, slightly prolonged recoveries (3 – 4 minutes) occurred in six buffalos, whereas in EMA were all in less than 2 minutes. In EA, worse induction scores were correlated to worse recovery scores (r =

0.4, $p = 0.02$) and longer recovery times (r = 0.51, $p = 0.009$), but not in EMA. Furthermore, in EA, lower bicarbonates and higher lactates were correlated to longer recovery times (r = -0.55, $p = 0.004$, r = 0.45, $p = 0.02$). Regardless of the physiological alterations observed, no complications including regurgitation and injuries occurred and all buffalos recovered well.

4. Discussion

Etorphine-medetomidine-azaperone combination was evaluated in this study for the first time in a wild bovid species, and was compared with the commonly used etorphine-azaperone combination.

Since etorphine has several adverse effects on the physiologic function, including respiratory depression, and these are dose-dependent [260], in EA group we used etorphine at doses of 0.010 mg/kg combined with 0.11 mg/kg azaperone, which are on the low end of the suggested doses for the immobilization of African buffalo [34,136]. The use of low doses can on the other hand result in longer inductions and excitement, and as a consequence of the overexertion, in acid-base and metabolic alterations that increase the risk of capture myopathy [92]. In this study, excitement was surprisingly marginal and seen only in few animals in etorphine-azaperone combination, but inductions were overall prolonged, and the animals walked in semi-sedated state for quite a distance in rough terrain, thus increasing the risk of accidental injuries and causing logistic challenges for the capture team. The restraint was also in most cases unacceptably unsafe, as many buffalos were only partially sedated and were aggressively reacting to the stimuli. A poor level of immobilization is undesired as it can further increase the risk of capture morbidity, since it triggers a stress cascade that results in catecholamines release, hyperthermia, reactive oxygen species production, and cell damage [41,47,87].

When medetomidine was added to the dart mixture, inductions were significantly smoother and less excitement was observed. The time to reach recumbency was almost halved compared to etorphine-azaperone combination, even though it was still longer than in protocols in which high doses of opioids are administered, which is usually 3 – 4 minutes [34,92,261,262]. The quality of the immobilization was significantly improved in EMA combination, as was characterized by adequate anesthetic plane and satisfactory myorelaxation. The fact that the greater sedation observed in EMA was obtained despite the lower doses of etorphine used in this group is a significant finding, and even more significant considered that also medetomidine was administered in ultralow doses. The range of medetomidine used in this study was between 0.002 and 0.005 mg/kg based on estimated body weight, and was indeed at least 10 times lower compared to what is normally used in free-ranging ungulate immobilization. For example, within butorphanol-azaperone-medetomidine combination, medetomidine is given at a minimum dose of 0.08 mg/kg in wild bison and 0.14 mg/kg in blesbok [263,264], at 0.05 mg/kg in impalas immobilized with etorphine and azaperone [94], and at 0.06 mg/kg in bison and elk immobilized with nalbuphine, medetomidine and azaperone [265,266]. In a variety of non-domestic ruminants, medetomidine was used at minimum rates of 0.04 mg/kg when combined with ketamine [267]. The same combination of etorphine, medetomidine and azaperone is described only in plains zebras, where the doses of all the three drugs were although higher, respectively 0.017

mg/kg, 0.017 mg/kg and 0.24 mg/kg, compared to our study [168]. Lower doses in our study were although similar to those used in domestic cattle [268].

Thanks to α2-agonist's anesthetic sparing effect with opioids [160–164], indeed medetomidine, even at low doses, allowed to efficiently and safely reduce the doses of etorphine, and its adverse effects, and on the other hand, provided also a better sedation. In addition, the similarity of the pre-darting startle response observed in the two groups excludes the possibility that different degrees of psychological stress biased the evaluation of the two protocols. This further supports that the improvement of inductions and immobilizations in EMA combination is a result of the potent sedative and myorelaxant effects of medetomidine, further enhanced by the synergism between the drugs in this combination [35,160,161]. This is the first study evaluating a combination of these three drugs together in wild bovids, since commonly etorphine is combined with either azaperone or an α2-agonist. Azaperone is a tranquilizer and is believed to work in synergism with opioids. Its presence in the combination might have further increased the spare effect of the three drugs, thus supporting the great sedation achieved despite the low dose used in this study.

However, within EMA group, the higher pre-darting startle response seen in two individuals, to which were also administered, as a coincidental error in weight estimation, lower doses of medetomidine compared to the other buffalos, resulted in suboptimal restraint. This finding highlights the importance of avoiding underdosing with α2-agonists due to the sharpness of its dose-dependent effect, especially when there is underlying stress. Indeed, the poor restraint in these two individuals might be explained with the competitive agonism between medetomidine and catecholamines at α2-receptors, which might delay the achievement of sedation when these are massively released as a consequence of fear and stress such as in case of higher startle response [160]. Through the same mechanism, sensitivity to sound and noxious stimulation might also result in sudden arousals in α2-agonist based protocols, especially when low doses are given [35]. Except for these two individuals, the fact that no sudden arousals were observed in any other buffalo of EMA group, despite sample collection created a significant disturbance (e.g. conjunctival swabs), might be attributed to the potent synergism and appropriate doses of medetomidine, etorphine and azaperone [269].

The reduction in etorphine dose requirement obtained thanks to synergistic drugs sparing effect is important as it not only demonstrated to improve immobilization quality, but can also reduce capture morbidity by limiting the potent dose-dependent opioid adverse effects on the physiological function [161]. Etorphine-mediated respiratory depression, characterized by severe hypoxemia and hypercapnia, is indeed of particular concern. Through its agonism at μ- and δ-receptors, opioids reduce the respiratory drive response to increased carbon dioxide, resulting in hypoventilation, whereas as a consequence of pulmonary hypertension, other gas-exchange alterations can occur, such as ventilation perfusion mismatch, shunting and oxygen diffusion impairment [107,109]. Hypoventilation and hypoxemia have also been associated with the use of α2-agonists, particularly in ruminants [161,167]. However, these effects are mostly observed with less specific α2-agonists and are dose-dependent [160,161,167].

In our study, respiratory rates were similar to those found in other immobilized wild bovids and were within physiological rates in both groups, although overall slightly higher in EMA [261,265,270–272]. Also mean arterial carbon dioxide and end tidal carbon dioxide were within physiological ranges in both the groups, but mild to moderate hypercapnia was seen in several buffalos in EA group. Carbon dioxide was indeed significantly more elevated in EA group, whereas in EMA the higher respiratory rates, presumably thanks to a reduction of the amount of etorphine administered, resulted in normocapnia. A2-agonist mediated hypoventilation was instead not observed in our study. This might be explained with the ultralow dose of medetomidine used in this study, which were significantly lower compared to other studies on wild ungulate capture, [94,168,263–267].

Hypoxemia (PaO_2 < 70 mmHg) was instead observed in both combinations. When the delta between measured and expected PaO_2 for the altitude was calculated, it showed that slightly more severe hypoxemia occurred in EMA group. However, the slightly higher oxygen values of EA group seem to be caused by the presence of hyperoxygenation in three individuals in this group, whereas overall oxygenation values are similar in the two groups. On the other hand, pulse-oximetry was slightly more elevated in EMA; however this method showed little accuracy in predicting hemoglobin oxygen saturation in our study, similar to other wild herbivores [81,106]. Although hypoxemia was severe in several individuals in both combinations, it was less severe compared to many other opioid-based immobilizations in species of wild ruminants [94,262,265,273].

The mean alveolar arterial gradient was significantly more elevated in EMA, whereas it was within physiological levels (< 20 mmHg) in many individuals in EA. In general, an elevated alveolar-arterial gradient suggests that the cause of hypoxemia is within intrapulmonary factors, such as ventilation-perfusion mismatch, right-to-left intrapulmonary shunting or oxygen diffusion impairment, rather than to hypoventilation [94,115]. The occurrence of dead-space ventilation usually results in a reduction of carbon dioxide elimination, and can be detected through a decrease of $ETCO_2$ compared to $PaCO_2$ [94,104]. Even though nasal capnometry has not been validated in African buffalos, its use has been described in another wild bovid, the muskox [145]. In our study, the small gradient between $PaCO_2$ and $ETCO_2$ observed in both groups, suggests that dead space ventilation was not involved in the mechanism of the hypoxemia [94,104]. Ventilation-perfusion mismatching, shunting and oxygen diffusion impairment are all believed to be the result of capture drug adverse effects, pathophysiological alterations due to capture, and recumbency in large-sized herbivores. The less increased alveolar-arterial gradients seen in EA support that hypoventilation occurred in this group, presumably caused by the higher doses of etorphine. Indeed, in most of the buffalos that had carbon dioxide beyond physiological levels, the alveolar-arterial gradient was not increased. On the other hand, the fact that carbon dioxide was not elevated in those hypoxic buffalos in EA that had an increased gradient, and in buffalos of EMA group, suggests that oxygen diffusion impairment might have been instead the primary mechanism for hypoxemia in those individuals where hypoventilation did not occur. Indeed, since CO_2 is twenty times more soluble in water than oxygen, it is less likely to be affected by diffusion limitation, and as such hypercapnia is uncommon in this condition [115]. When hypoxemia is mediated by oxygen diffusion impairment, it normally improves when an increased fraction of oxygen is provided. Similar to other studies where even worse hypoxemia was

reported, oxygen therapy might have been able to fully correct hypoxemia in these individuals, and its use should be evaluated in association with this protocol [262].

Medetomidine might have also contributed to the hypoxemia since the peripheral vasoconstriction mediated by intravascular α2-receptors can cause a delay in blood flow through peripheral tissues, that results in increased oxygen extraction and less oxygenated blood that returns to pulmonary capillaries [274]. To contrast this mechanism, the addition of azaperone to the protocol might have been beneficial. Indeed not only azaperone produces, through its α1-antagonism, vasodilatation which has been shown to be effective in counteracting hypertension caused by etorphine-mediated sympathetic stimulation [70,154]. But, since both α1-and α2-post-synaptic adrenoceptors are located in peripheric arteries and veins, and both subtypes mediate vasoconstriction, azaperone might also counteract α2-agonist peripheral vasoconstriction, thus resulting in the mitigation of hypoxemia triggered by this mechanism [160,275]. However, the fact that oxygenation was not substantially worse in EMA group, and the fact that carbon dioxide elimination was not impaired with this combination, creates evidence that the adjunct of medetomidine did not enhanced, but mildly reduced respiratory depression in our study.

Arterial lactates can be an indicator of adequacy of oxygen supply to the body and of blood perfusion under anesthesia [276]. However, in wildlife capture, that is performed in drastically different conditions compared to usual anesthetic- or physiologic-focused studies, elevation in lactates is more likely to be linked to insufficient oxygen supply during the pre-anesthetic increased physical activity, and less to the pathophysiological effects of the drugs themselves. However, the choice over the drug combination used can still indirectly influence lactic acid production, and other metabolic changes, in the sense that it affects the duration and intensity of the exertion. In our study lactates were similar in the two groups, and overall only marginally elevated, suggesting that although hypoxemia was recorded, it did not significantly affect the cellular aerobic metabolism. Although lactate levels were not overall correlated with acidosis in neither of the two groups, in a few individuals slight lactic acidosis occurred. In these individuals, lactates increased together with hematocrit and hemoglobin in EMA and glucose in EA, that are all indicators of capture stress [43,75,83], which support the hypothesis that lactates increased as a consequence of the increased physical activity rather than the drug-mediated hypoxemia. However, the fact that in EMA group, the pH was higher, and within physiologic limits in most individuals, further support that a functional respiratory compensatory mechanism through carbon dioxide elimination was maintained with this drug combination, whereas in EA carbon dioxide elimination was impaired in many buffalos. In EA, the strong correlation between hypercapnia and low pH suggests that the higher doses of etorphine used in this group not only led to hypoventilation, but also resulted in slight to moderate respiratory acidosis, or transformation of lactic acidosis, in those few cases where it occurred, into mixed acidosis [193].

Metabolic acidosis was however surprisingly only a marginal complication in this study. Metabolic acidosis, often severe, is a common consequence of capture of free-ranging ungulates, and being involved in the development of capture myopathy is of particular concern [47]. Commonly, severe acidosis with values of pH sometimes close or lower than 7.0 and associated with significantly

increased lactates and decreased bicarbonates is observed in immobilized individuals [262,265], whereas is this study lactic acidosis was slight and occurred in few individuals. In this study, the fact that the capture site was a highly touristic area where buffalos are used to vehicles resulted in limited startle response, which combined with overall marginal induction-induced excitement, especially in EMA, might explain the low occurrence of metabolic acidosis in both groups.

The bradycardic effect associated with α2-agonists presumably accounted for the significantly lower heart rates observed in EMA compared to EA group. Bradycardia is a result of diminished sympathetic tone and a reflex to increased vascular resistance caused by the α2-agonists, and can produce a severe decrease in cardiac output. However, perfusion and oxygenation to vital organs are normally maintained, and if bradycardia is not severe, it might help to decrease myocardial oxygen demand and workload, and as a result the whole body oxygen requirements under anesthesia, whereas tachycardia increase the oxygen demand [276]. Heart rates of buffalos of EMA group, although lower in respect to those recorded in EA, were not decreased compared to buffalos resting rates, and were within physiological ranges in all individuals [136,165]. Instead, heart rates seen in EA group were significantly elevated compared to physiological rates and most buffalos were tachycardic. This might be a consequence of sympathetic stimulation resulting from higher etorphine doses [35], and longer and more rough inductions that resulted in increased physical activity [223]. Furthermore, different to EMA group where medetomidine might have reduced the stress, and the release of catecholamines, thanks to its anxiolytic effect [160], in EA the excited restraints have also likely contributed to elevated heart rates.

The fact that we did not observe different arterial oxygenations as a consequence of the significantly different myocardial work load, and presumably oxygen consumption, in the two groups, might be explained with samples being collected at the beginning of the immobilization, and to the bias placed by the physical activity that occurred before recumbency [224]. Even if the addition of medetomidine would have resulted in this improvement, it may have been challenging to see it under our study conditions. On the other hand, lowered heart rates in EMA did not have pathological consequences in the short monitoring period of this study, since these did not result in a lower pH or increased lactates, that might have been caused if insufficient perfusion and tissue oxygen delivery had occurred due to the bradycardic-mediated reduction in cardiac output. In general, the fact that lower doses of α2-agonists may be more associated with predominant central nervous system effects, whereas cardio-respiratory alterations might be seen mainly when higher doses are administered [161,167,277] is in agreement with the results of our study, where physiological function was better preserved compared to studies were higher doses or less selective α2-agonist were used [167,262].

The correlations observed in EMA group between higher heart rates and higher respiratory rates, arterial oxygenation and lower $PaCO_2$ are interesting since higher heart rates were not associated with lower medetomidine doses and vice versa, or other drug doses that might have explained a respiratory depression. The heart rate was also not correlated to any other possible influencing factor such as induction scores or times. However, in this group, higher heart rates were correlated to lower

bicarbonates, and although not significantly, had an association with increases in lactates. In wildlife capture, lactates usually increase, and bicarbonates decrease, as a results of pre- or post-darting physical activity [95,106,278], as supported in our study by their correlation with worse inductions in both groups. Therefore, a higher physical activity before the recumbency might have accounted for the increased heart rates, and as a physiological response to exercise to the increased ventilation. Although in most wildlife capture pre-dart chase or post-dart excitement are often strenuous and lead to dangerous metabolic alterations, in this study where inductions, although sometimes long, were calm, might have provided benefits in terms of improving gas exchanges. This is further supported by the fact that also in EA, although HR was not correlated, lower bicarbonates accounted for higher oxygenation.

According to our results, it seems that a slow gait during induction prevented the shift to the anaerobic metabolism, or enhanced a rapid lactate clearance [95,96], which is supported also by the only marginally increased lactates and rare metabolic acidosis. On the other hand, higher medetomidine doses were associated with shorter inductions, and lower lactates and higher bicarbonates, and lower potassium and chloride. Although short and smooth inductions are considered a goal in wildlife immobilization, as these also decrease the risk of injuries other than exertion, these might otherwise limit the pre-recumbency hyperventilation. However, in our study, the blood gases were collected at the beginning of the recumbency, where changes are largely explained by the prior activity and less by the anesthetic effect, therefore it is not possible to state if a prolonged calm induction is preferable to quick inductions without the evaluation of serial blood gases, which were not available in this study.

Shifts in electrolytes, anion gap, glucose, hematocrit, hemoglobin, creatinine and BUN can be indicators of increased exercise, or fright, occurring during wildlife capture [43,75,76,83,85,218,219]. The fact that these metabolites were within physiological ranges highlights, in agreement with the absence of metabolic acidosis, and the marginal increase in rectal temperature that is an indicator of fright response [41], that capture stress was limited in this study. In both groups, calcium was correlated to indicators of acidosis, but since the analysis of ionized calcium is impacted by a more acid pH, this finding might have little clinical significance [279]. However, the slightly higher levels of BUN seen in EA group might be explained with longer and more rough inductions, since BUN can increase after strenuous exercise [75,76,85]. Even though hyperglycemia following the release of catecholamines after a stressor is well described [43,64], the significantly higher levels of glucose in EMA group are instead most likely explained with the hyperglycemic effects of the α2-agonists through the inhibition of insulin secretion [160,280]. The clinical significance of the higher levels of cardiac troponin I in EA group is unclear. Cardiac troponin is released into the circulation after cardiac myocytes damage (e.g., after ischemia), and have been observed after intense exertion in dogs and racehorses, without indicating cardiac pathology [86,281,282]. The CTNI cut-off for cattle is set at 0.07 ng/ml, whereas the higher value recorded in this study was 0.03 ng/ml [283]. However, since the plasmatic level peak occurs several hours after the cardiac damage or the exertion, even a small increase shortly after the stressor might represent a clinical finding, and would require further investigation [87,220].

Even though α2-agonist can influence the endocrine system by reducing the stress-related hormones, in our study we did not overall observe benefits since most of the indicators of capture stress were similar, and not altered in neither of the two groups. However, in EA group, temperature was higher when lower dose of etorphine were administered, and its correlation with lower $PaCO_2$, higher pH, hematocrit and hemoglobin suggests that it increased in individuals that had higher stress or exertion. The fact that hyperthermia is a major complication in free-ranging buffalo immobilization, and that α2-agonist can affect thermoregulation [284], is promising and might be explained by the ultralow dose used in this study.

In addition, differences in stress hormones in response to capture seems to be age-dependent in African buffalos, since older buffalos mount a greater stress response [91]. Although we did not measure post-capture fecal corticosteroids, the elevations of metabolic indicators such as creatinine, BUN and anion gap in older individuals might be in agreement with previous findings and show that higher exertion occurred in these individuals perhaps as a consequence of higher psychological stress.

Recoveries were overall quick and smooth in both groups. However, in a small number of buffalos in EA recoveries were slightly prolonged, which seemed to be influenced by the occurrence of greater exertion during the induction phase. Instead in EMA, despite the variability in the ratio of antagonist administered, all recoveries were smooth, further highlighting the safety of this protocol.

A limit of this study was the fact that it was not possible, due to the nature of the field study, conducted within an opportunistic approach, to standardize the times of blood samples and physiological monitoring. Furthermore, the unavailability of serial samples and cardio-respiratory monitoring over time, affected the ability to evaluate the trend of the physiological alterations observed. However, all samples were obtained within a short time limit of 5-10 minutes since recumbency was achieved. The values that might have been more affected by different sampling times are the gases, since these are more susceptible to quick alterations resulting from exertion at the beginning of the immobilization, and to the drug effects later. On the other hand, the wide numerosity in our study, and the narrow range of values observed within different individuals support that these factors did not represent a significant bias for the evaluation of the two protocols.

5. Conclusion

Several benefits were observed with the addition of medetomidine to the etorphine-azaperone combination. The more evident was the improved immobilization level, that allowed to work safely on the animals, which had an adequate sedation, myorelaxation and stable cardio-respiratory function. In second place, the adjunct of medetomidine allowed to decrease the dose of etorphine, thus decreasing the occurrence of hypoventilation-mediated hypercapnia and respiratory acidosis. This however did not result in more induction-induced excitement, but instead reduced the time for recumbency, and smoothened the inductions. Thanks to limited excitement, and combined with almost no startle response, metabolic acidosis was marginal in this study, as well as metabolic alterations, especially in EMA combination. This is the first study evaluating arterial blood gases and metabolic indicators in

immobilized buffalos, but when compared to other wild ruminant capture, overall alterations were limited, and were further reduced in EMA. The safety of recoveries, and the fact that these were even slightly quicker in medetomidine combination, further support the safety of this combination.

In different capture settings where higher startle response is expected, such as in less touristic areas or in helicopter darting, higher doses of medetomidine, or more selective α2-antagonists such as dexmedetomidine might be beneficial and would need further evaluation. Furthermore, since hypoxemia was not improved in EMA combination, but was still severe, the adoption of nasal oxygen therapy, and the investigation over a further decrease of etorphine doses is suggested as it might lead to an improvement of gas-exchanges.

4 DISCUSSION

Chemical immobilization of East African, free-ranging, large-sized herbivores is characterized by a high morbidity and mortality risk. Their large size is among the key factors contributing to these risks by creating logistical challenges during field capture, as well as their delicate physiology which becomes altered by changes in recumbency [34,104,105]. In addition, their susceptibility to capture stress [30,41,55,234] and opioid adverse effects [70,104,106,107,126,172] are also major factors which play a role in morbidity and mortality risk. These factors are intrinsic and cannot be completely avoided, but their consequences can be reduced by the availability of safer, species-specific capture methods and immobilization protocols [26,30,41]. Indeed the assumption that the same physiological alterations occur across all wild herbivores, as often assumed in the field, without taking into account the species-specific difference in anatomy, physiology, and behaviour, and the use of techniques and drug protocols extrapolated from other species, enhance the risk of complications [26,27].

Given that many large-sized herbivores are threatened with extinction [1,2], and that there is an increasing need to capture them for conservation-related purposes [21,53], in this thesis I aimed to improve the understanding of the physiological impact that occur during immobilization of large-sized East African herbivores. The focus was on Eastern black rhinoceros, African buffalo, and two giraffe subspecies, Maasai and reticulated giraffe, because preliminary results conducted during my PhD research on a wide variety of East African species showed that major capture morbidity occur in these three select study species. I assumed that improving the knowledge on the species-specific physiological alterations that occur as a result of their capture, and the respective predisposing factors, would result in targeted strategies of prevention and treatment, and lead to an improvement of the short and long-term immobilization safety. My hypothesis about the existence of different mechanisms that account for different predominant physiological alterations in response to capture stress and drugs in

each species was confirmed in this thesis and these findings are vital to successfully developing specific strategies to reduce capture risk in each of the selected species.

The results of my PhD research represent one step forward towards the achievement of safe immobilization in the selected species. Specific factors of risk for capture morbidity were identified for each species, and if considered during capture operations, will prevent or reduce the severity of some physiological alterations, such as the insurgence of acid-base imbalance. Advances on intra-anesthetic monitoring techniques, such as nasal capnometry, were achieved and will help in early detecting anesthetic complications. An improved understanding of the species-specific mechanism of physiological impact of capture helped identify improved pharmacological strategies to treat these complications, and to prevent them through the use of drug synergistic combinations that are able to reduce opioid doses and in turn their adverse effects.

The main achievements of this PhD study were:

1. **Identification of species-specific physiological alterations under etorphine-azaperone immobilization (Study I-II-III-IV)**

   Major physiological derangement occurred in this study, including metabolic and cardio-respiratory alterations, during the immobilization with a combination of etorphine and azaperone in all the three species. The severity of the homeostatic derangement observed is not surprising, as significant physiological alterations that increase the risk of mortality and of long term complications, are well described across the wildlife literature [26,30,43,55,78,218]. The alterations observed are also consistent with the use of the combination of etorphine and azaperone. Indeed, despite azaperone reduces etorphine's hypertension and gas-exchange impairment, and provides muscle relaxation, tranquilization and presumably shorter inductions, severe alterations in rhinoceroses are still reported [104,106,116,147,157–159]. Instead, this combination was not previously evaluated in giraffe or buffalo. The alterations observed were not only a result of drug adverse effects, but also species specific response to capture stress and changes due to recumbency. Patterns of physiological disturbance vary with species, method of capture and drugs used [26,30,43,218], and obtaining detailed and species-specific knowledge on physiological effects was essential in this study to infer strategies to prevent or treat intra-anesthetic complications.

   Within the metabolic and cardio-respiratory alterations that were observed, acidosis and hypoxemia were focused on as, in my opinion, the degree of these conditions best represents the presence of broader pathophysiological alterations that occur in captured wildlife. The occurrence of acidosis and hypoxemia is of particular concern since they are often unnoticed when only basilar monitoring is available since they have unspecific symptoms, and if acidemia and hypoxemia are combined, this can result in even greater acute or delayed mortality [26,138]. In addition to gas exchange and acid-base status, attention was also given

to electrolytes and selected biochemical analytes, combined with physiological function monitoring, as the change of their values can also be short-term indicators of the complex pathophysiologic processes that occur during the immobilization period, and predicting factor for delayed complications [30,43,76,80].

In this study, the most severe acidosis occurred in the Eastern black rhinoceroses, and in reticulated giraffes darted from helicopters. This acidosis was of lactic origin in both species, and the degree of alteration was consistent with other studies, as the alterations caused by the huge stress triggered by helicopter-darting are well described [104,106,116,126,187]. The helicopter chase resulted in initial hyperoxemia and hypocapnia in the giraffes, while in the rhinoceroses hyperoxemia occurred in only few animals but hypoxemia was observed in 84% of them, in half of which was severe. This difference observed between the two species might be due to the fact that in giraffes the first sample was withdrawn a few seconds after the end of the physical activity, since the excited giraffes were roped down to recumbency, and etorphine was already antagonized in this species, whereas in rhinoceroses, after the chase had stopped and physical activity ended, then drug-mediated respiratory depression kicked in, further worsening the oxygen debt resulting from the pre-darting run. The severity of the hypoxemia observed in some of the rhinoceroses, which was similar to other opioid-based immobilization studies [104,106,116], further highlights the need for capture protocols and intra-anesthetic treatments that reduce opioid-mediated respiratory depression.

In ground darted animals, where the amount of stress induced by the capture method was less intense, the main alterations observed were depended on the species and etorphine doses used. In Masai and reticulated giraffes, metabolic acidosis still occurred due to increased physical activity that was a result of induction-induced excitement, whereas no giraffes startled in response to vehicle-darting. However, since this triggered an exercise-mediated respiratory activation [128,222,224], the hypocapnia partially compensated the metabolic acidosis, which was slight. Etorphine-mediated excitement is particularly severe in giraffes and easily leads to overexertion, especially when underdosing occurs [34,125,126]. When etorphine doses were increased with the intent to limit this process, excitement was reduced but acidosis was even more severe, because respiratory acidosis occurred and complicated the homeostatic derangement. In buffalos, metabolic acidosis was instead surprisingly almost absent, different to other wild ruminant species where severe metabolic acidosis is often observed [94,265]. In buffalos however, limited drug-induced excitement also occurred, which explains the marginal metabolic acidosis observed in comparison to giraffes. On the other hand, slight to moderate respiratory acidosis was observed in buffalos and was interpreted as a result of etorphine-mediated hypoventilation. In buffalo, the main pathological alteration was on the respiratory side, as demonstrated with the significant hypoxemia, presumed to occur as a result of both hypoventilation, and oxygen diffusion impairment that is a common consequence of etorphine-mediated pulmonary hypertension [109]. In both reticulated and Masai giraffe, hypoxemia was of little concern since it was severe in only a few animals. In giraffes that were

administered a higher dose of etorphine, hypercapnia was more of an issue than hypoxemia. This finding might suggest that, in this species, hypercapnia might not only result from respiratory failure, but also from an increased carbon dioxide production as a result of exercise [285], which highlights that future studies should evaluate how to decrease excitement through the use of drug combinations that also have less effect on respiratory compensation. Giraffes are particularly susceptible to hypoxemia when immobilized [126,187,195,197], and the fact that initial, severe hypoxemia was found only in a few giraffes, might be mainly attributed to the early etorphine antagonization, that allowed them to maintain adequate gas exchange, although to the detriment of their welfare.

Since the results of the blood gas analysis obtained immediately after recumbency reflect a combination of the consequences of both strenuous chase and of the anesthetic effect, resulting in complex and dynamic pathophysiological alterations, it is fundamental to evaluate trends over time to understand the pathological scenario. Trends were evaluated in the rhinoceroses and in ground darted reticulated giraffes. In giraffes, after early antagonization is performed, they are awake and physically restrained, therefore the trends overtime might reflect changes that can occur as a result of manipulation stress or recumbency or of azaperone, but not of etorphine. In black rhinoceroses the trend was instead evaluated before and after nasal administration of oxygen and a post-induction treatment (butorphanol and doxapram, or butorphanol) therefore the trend evaluation reflected mainly the effects of these treatments. However, in both giraffes and black rhinoceroses I observed that, similar to other species [95,106,224], initial hyperoxygenation and hypocapnia tend to stabilize and sometimes become pathological in the second sample. This suggests that initial hyperoxygenation should be interpreted with caution and not considered optimal *a priori* [224]. Lactates decreased over time in black rhinoceroses, but not in reticulated giraffes. An active recovery phase, which in the rhinoceroses occurred because inductions were mostly calm, has been suggested to clear accumulated blood lactate faster than in passive recovery [95,96]. This process might have occurred also in buffalos, where calm inductions were correlated with low lactates. In both sub-species of giraffe, the abrupt shift from gallop to recumbency likely affects this recovery phase and highlights that other alterations might follow this slow lactate clearance, such as acidosis that did not improve. This further supports the need for advances in giraffe immobilization that will allow calmer induction and limit excitement, while reducing etorphine doses to avoid respiratory depression.

Shifts in electrolytes, anion gap, glucose, hematocrit, hemoglobin, creatinine and BUN variously occurred in the different species, similar to previous studies, where these changes were associated with physiological derangement due to either exercise or fright [43,75,76,83,85,218,219]. In particular, potassium elevation is a major component in the pathogenesis of capture myopathy [43], and the high levels of potassium observed in helicopter darted giraffes, and in a few rhinoceroses, were of concern as hyperkaliemia can induce changes in neuromuscular and heart excitability which can lead to ventricular fibrillation and

death [74,75]. Other electrolytes were not altered in rhinoceroses, or in buffalos and Masai giraffes, but in reticulated giraffes chloride was elevated, in a dependent manner with glucose levels. Chloride usually increases as a compensatory response to bicarbonate loss in acidosis characterized by non-increased anion gap, but elevations in chloride have also been observed in captured wild species [218,219]. Hyperglycemia occurred in black rhinoceroses, reticulated giraffes and buffalos in a dependent manner with higher lactate levels. Hyperglycemia can be increased due to an acute stressor, or after exercise, due to the hyperglycemic effect of catecholamines and glucocorticoids [43,64], therefore its rise in the studies of this thesis is not surprising. BUN was elevated in reticulated giraffes and buffalos likely as a result of increased activity, in agreement with other studies in different wildlife species after capture [75,76,85]. Creatinine, which also has been linked with capture stress [76,85] was only elevated in reticulated giraffes, and I observed that its rise occurred in correlation to both psychological and physical stress. These analytes were not measured in Masai giraffes since a different analyzer was used. In general, in reticulated giraffes elevations of lactates, electrolytes, glucose and BUN seems associated with increased physical activity. Hematocrit and hemoglobin instead seemed to be more associated to psychological stress in ground darted reticulated giraffes regardless of physical activity, probably as a consequence of fear-mediated splenic contraction [83], although they also increased in the case of increased exercise.

The use of cardiac troponin I is flourishing in veterinary medicine as a biomarker for myocardial injury, but it has been observed that it can increase after strenuous exercise without indicating cardiac pathology [86]. A cut-off of 0.08 ng/ml is suggested for a variety of zoo mammals [86], and 0.03 ng/ml in horses with peak elevation usually recorded 2 – 6 hours after exercise or capture [220]. Except in two giraffes, where sample collection was delayed of 30 and 60 minutes, and values of 0.04 and 0.07 ng/ml were respectively measured, in all other animals levels not higher than 0.03 ng/ml were measured. Although it can't be excluded that these small elevations are due to the clinical condition of the giraffes, because both of them were immobilized for a limb injury, this finding might suggest that cardiac troponin I might be a biomarker for exercise-related or myocardial stress as a result of capture in giraffes. Although not included in this study, high levels (0.25 ng/ml) of cardiac troponin I were observed in one Southern white rhinoceros that was chased for a long period by helicopter prior to darting and died a few days after the capture. The death was presumably a result of a chronic infection (which treatment was the purpose of its immobilization) or as a result of capture myopathy – which was not confirmed since the carcass had been scavenged before a post-mortem examination could be done. These observations suggest that more research on the use of cardiac troponin I in wildlife is needed, to understand its role as a predictive factor, and eventually establishing the cut-off values for capture-related morbidity.

Alterations in these analytes, and in particular lactate, glucose, potassium, calcium and sodium, have been considered predictive factors for higher risk of mortality [88]. In the studies where a longer-term follow up was possible (Study I-II-III) no mortality or signs of morbidity such

as capture myopathy occurred, therefore possible predictive values and cut-off were not assessed. In my research the post-capture follow-up was only observational and qualitative, therefore, techniques that include non-invasive monitoring to determine chronic stress or reproductive alterations would be advisable for future studies in order to evaluate not only the occurrence of mortality, but also to quantify delayed morbidity.

2. Predisposing factors of capture morbidity (Study I-II-III-IV)

The retrospective analysis of short-term morbidity, measured in terms of physiological alterations, in relation to physiological status, capture methods and drug doses, and environmental factors, proved to be a valuable tool to detect species specific factors of risk that need to be considered to prevent complications when capturing wildlife. In addition, the use of descriptive score scales, based on video recordings, used to evaluate in a standardized manner the reaction before and after the darting was useful to understand individual or species susceptibility to stress and drug effects, and its correlation to morbidity. I observed not only that there are distinct species differences for physiological effects of opioids, as previously highlighted [27], but that species are also differently susceptible to predisposing factors of physiological alterations and capture morbidity.

The main factors influencing capture morbidity detected in this study were the stress response to the capture methods, the drug-induced excitement and its relations with opioid doses. The highest startle response was, not surprisingly, observed during helicopter darting in Eastern black rhinoceroses and reticulated giraffes, and in these species it coincides with a high speed run, probably as a result of both psychological and physical stress. In particular, the intensity and length of the chase – mainly dictated by logistical needs and not by the animals' reaction – were the main predisposing factors for the severe lactic acidosis seen in these animals, whereas induction times or drug doses did not have an influence. When the helicopter stops the chase after successful darting, most animals calm down, and in giraffes less excitement was observed compared to the ground darted reticulated giraffes in this study. In helicopter darted giraffes, a higher startle response accounted also for increases in metabolites commonly associated with capture stress, such as sodium, chloride, glucose, hematocrit and hemoglobin [43,47,64,75,76,85] with some of these values substantially increased. Although sometimes helicopter darting is indispensable because it allows spotting shy animals, reaching remote locations or herding animals out of thick bush [104,116], these results highlight that there is a need to rethink strategies, such as through the adoption of new technologies, to reduce pre-dart chase and stress in order to avoid life-threatening metabolic alterations.

During my research it was observed that individuals that are located in highly touristic areas are less prone to sprint when approached by vehicles for darting, likely as a result of habituation [41]. In preliminary data, collected in other ungulates (plains zebra, mountain bongo and common eland) found in less touristic areas, or where poaching pressure is elevated, extremely high startle responses occurred when the animals were approached by the darting vehicle and

resulted in severe lactic acidosis [286]. A limited startle response accounted instead for minimal metabolic alterations in buffalos, in which overall physical activity did not happen because also drug-induced excitement was rare. This finding highlights the advantage of using techniques that keep the stress to a minimum when immobilizing wild herbivores.

In giraffes, I used a scoring system to evaluate the behavioural response to the stressor (the darting method) separately from the physical activity, as a hypothetical measure of psychological stress. The primary response of giraffe when alarmed due to perceived danger (e.g. presence of predators) is often to first observe (likely due to their marginal susceptibility to predation) and then walk away or run but only when seriously threatened. Major attention was hence given to their behavioural response when approached for darting. When the startle response was considered in ground darted reticulated giraffes only, a higher stress – that did not involve any increased physical activity – was not associated with acid-base alterations, different to helicopter darted giraffes, but it had an effect on hyperthermia, hematocrit and hemoglobin. Stress can indeed be the primary cause of capture-induced hyperthermia, and the magnitude of this stress-induced hyperthermia exceeds that of exercise-induced hyperthermia or that due to the environment [41,82]. Instead, increases in hematocrit and hemoglobin can results from splenic contraction due not only to exertion, but also to fear [41,83,84]. In my research, this is supported by the fact that in helicopter darted rhinoceroses that underwent extensive chasing, the hematocrit, hemoglobin and rectal temperature rose but not in a correlated manner to more intense chase, or longer induction times. Although I could not separate signs of psychological stress from physical activity in this species, these might have been correlated to individual susceptibility to stress regardless of the physical activity. Since capture-induced hyperthermia, that in my studies seemed to be related to stress regardless of the physical activity, can contribute to the development of capture myopathy [41,47,82], it is important to consider the startle response when darting free-ranging wildlife, and adopt techniques to keep it to a minimum also during ground darting, including the use of anxiolytic drugs.

Induction-induced excitement was marginal in buffalos, as it was observed in only a few animals, and was not dose-dependent in regards to etorphine. In rhinoceroses, induction-induced excitement was also marginal, and the severity of the initial homeostatic derangement appeared not to be correlated to time of inductions, and was not dependent on opioid dosages, as doses were similar in all rhinoceroses. Severe induction-induced excitement was instead observed in ground darted Masai and reticulated giraffe, consistent with previous reports [125,126]. Regardless of the initial stress, our results show that the higher the excitement, the higher degree of bicarbonate loss and, indirectly, metabolic acidosis in Masai and reticulated giraffes, and this was associated with lower etorphine doses. In reticulated giraffes it was also associated with hyperglycemia, and increase in heart rate. However, although higher doses of etorphine seem to decrease the degree of induction-induced excitement and shorten the induction length, this approach results in gas-exchange impairment and hypercapnia, which

can significantly affect the essential compensatory mechanism that is needed after exertion, and which resulted in worrisome acid-base complications. My results show that the acidosis presumably resulting from the etorphine-mediated respiratory depression was more serious compared to the metabolic acidosis triggered by higher excitement, and in addition showed less improvement over time despite gas-exchange being rapidly restored with antagonization. These findings suggest that overdosing etorphine in order to produce a lesser excitement does not improve the physiological safety of giraffe immobilization.

High doses of opioids are generally administered in wildlife immobilization to have inductions as rapid as possible as this is generally believed a method to decrease the risk of post-induction excitement and thus overexertion [34]. In buffalos, quicker inductions resulted in lower heart rates and higher carbon dioxide and worse hypoxemia. Although serial blood gas analyses were not available to evaluate the trend over time, this finding might suggest that quicker inductions might be associated with more severe opioid respiratory depression, perhaps as a result of higher individual sensitivity. Although no correlations in regard to higher doses were observed in buffalos, in Masai giraffes and black rhinoceroses, I observed more severe respiratory depression in those individuals which were given higher doses of etorphine as a result of erroneous weight estimation. Longer and calmer inductions might otherwise, as observed in other wild ungulates [95] allow to a better lactate and acidosis compensation, supported in my study by the better lactate clearance seen in rhinoceroses after calm inductions compared to giraffes where an active recovery phase does not exist. The use of a combination of synergistic drugs that allows a calm, but not ultrashort, induction and limited excitement, but on the other hand allow to reduce etorphine doses and its respiratory depression might improve giraffe and black rhinoceros immobilization safety, as demonstrated in this thesis by the addition of medetomidine to buffalo immobilization protocols.

Anecdotal evidence from the field suggests that different subspecies of giraffes are differently sensitive to capture drugs. According to my observations, Masai giraffes are more sensitive to etorphine-azaperone combinations, as despite the lower etorphine doses that were administered to them, the first signs of sedation and recumbency were achieved earlier, and the degree of excitement was less intense and resulted in less severe acidosis. Gas exchange was more affected as oxygenation was slightly worse, despite a higher respiratory rate being observed. A possible explanation, other than a different susceptibility to stress, would be the evolutionary divergence of subspecies of giraffes. Masai and reticulated giraffes diverged 1.25 to 2 million years ago [8], so it would not be surprising to find differences, for example, in drug sensitivity and, as is recognized for other similar species [11,74], perhaps due to genetically mediated individual differences in sensitivity to opioids [199–201]. Since taxonomic classifications are under review, and Masai giraffes and reticulated giraffes could emerge as different species and further be separated from those found in other African regions [8–11], it is important to begin to have a species-specific approach from a veterinary point of view, and account for the clinical differences observed.

Although I initially assumed that a history of previous immobilization might have triggered a worse stress response and worse metabolic alterations, in black rhinoceros individuals that were known to have been captured in the past, it did not result in a longer chase, longer induction time or variation in blood gases and acid-base values. This finding is important since life-historical, extreme stress events, such as a previous capture, can predispose individuals to capture myopathy [71], although this result might have been biased by the small sample size (n=10). Older age was instead correlated with higher indicators of stress, although only in buffalos and similar to a previous study [91], and to longer time to induction in rhinoceroses. These findings suggest that older age should be considered when immobilizing buffalos, and should further be evaluated in other species.

3. Field monitoring techniques to allow early prediction of physiological alterations (Study I-II-III-IV)

In the field, the evaluation of the immobilization safety is mostly based on basilar monitoring, which can often be misleading, as for example, ventilation might not reflect the actual gas exchanges [104,116]. The analyses of gas exchanges and acid-base status, and of selected hematological and biochemical analytes through the use of portable blood analyzers enabled the detection of severe alterations that would have otherwise gone unnoticed by only relying on basilar monitoring. Cardio-respiratory function was stable and hyperthermia was not severe in most animals, despite the gas exchanges and acid-base status alterations. Furthermore, the analysis of trends over time is important as it allows to evaluate the effect of postinduction treatments (Study III) and to understand how the physiological function varies considering that the initial values are highly affected by the physical activity prior to recumbency [224] (Study II). However, even though the use of blood gas analysis is highly recommended for intra-anesthetic monitoring to evaluate the physiological status, in the field, due to the reduced number of personnel and quickness of immobilizations, samples are often only analyzed when the animal has already recovered, usually as a retrospective evaluation of the protocol, or for research purposes.

Nasal capnometry can be a valid clinical monitoring tool to evaluate continuously gas exchange dynamics in the field. Nasal capnometry is non-invasive and easily usable in the field as does not require endotracheal intubation. Its use has been spreading in human medicine [144,230], whereas only a few studies report its use in wildlife, and its accuracy in predicting arterial carbon dioxide has not been evaluated [104,145]. My findings suggest that, in giraffes and rhinoceroses, where $PaCO_2$ and $ETCO_2$ were recorded simultaneously, it may be difficult to predict the absolute value of $PaCO_2$ from the $ETCO_2$ due to the wide limits of agreement, presumably also as a result of their large size [119,140]. In contrast, nasal capnometry proved

to be a useful clinical tool that allows early detection of ventilation-related complications through the evaluation of $ETCO_2$ trends.
In both rhinoceroses and giraffes, the nasal capnometer rarely underestimated the level of arterial carbon dioxide, which can often occur in the case of acidosis, or dilution with ambient air [142]. This finding is clinically important since it is essential to detect hypercapnia, given that it is a common complication of opioids and can result in respiratory acidosis.
The capnometry was more accurate in predicting $PaCO_2$ values in reticulated giraffes compared to rhinoceroses, but in both species the accuracy was lower at the beginning of the immobilization compared to the second sample. This change in accuracy may be due to the fact that at the beginning of the recumbency, greater and more dynamic ventilation alterations happen due to a combination of exertion and initial drug effects, which might affect more the reading of the capnometer [230]. The fact that giraffes were awake when the second blood gas sample was obtained might explain why in this species the accuracy increased over time. Indeed, being awake, less respiratory dynamic alterations that might increase capnometer inaccuracy (e.g. dead space ventilation or ventilation-perfusion mismatch) likely occurred, compared to the fully immobilized black rhinos. In rhinoceroses, overestimated values of $ETCO_2$ were measured in the case of hypoxemia, and most of the overestimations occurred when quick fluctuation of values were observed and were concomitant with hypopnea and apnea. In contrast, in giraffes there was a correlation between higher arterial oxygenation and overestimation of $ETCO_2$ but it was unrelated to respiratory rates. The overall overestimations of the capnometry compared to $PaCO_2$ are presumably due to a reduction in functional residual capacity resulting from the increased pressure on the diaphragm by abdominal organs during lateral recumbency, similarly to the mechanism in pregnant and obese human patients, or rebreathing of carbon dioxide due to increased anatomical dead space [141,249]. When the respiratory cycle is altered and the inspiration time is prolonged compared to the expiration time, which can happen as a reflex to increase alveolar recruitment [105,251], the chances of accumulation of carbon dioxide in the upper respiratory tract, or even in the capnometry sampling tube, are increased [285]. More research on the optimal nasal capnometry sampling technique is needed, as a smaller size tube might increase the accuracy of the records [143,144,249].

In my studies, nasal capnometry seemed more reliable in detecting ventilatory alterations compared to pulse-oximetry. The pulse-oximeter resulted in poor accuracy and both over- and under-estimations of PaO_2 values in all the species, similar to previous studies in large-sized herbivores [106]. Dark skin pigmentation and skin layer thickness might be among the more important limits [26,138], and other pulse-oximetry techniques (e.g. transflectance probes) should be evaluated as they might provide more accurate readings in species with these traits.

Since accuracy of non-invasive blood pressure is limited in large-sized herbivores [119], in this thesis I used an aneroid manometer attached to a intra-arterial catheter inserted in the auricular artery of both reticulated giraffes and rhinoceroses to measure invasive mean blood

pressure [129]. This method is inexpensive and portable, and is highly recommended for field use when an anesthetic monitor is not available due to the higher costs or dimensions. Although the measurement of invasive blood pressure might not be always feasible in the field since it requires operator training, it is important to research the safety of protocols, especially in large-sized herbivores where an inadequate pulmonary and peripheral tissue perfusion might increase the risk of ventilation perfusion mismatch and tissue hypoxia. The monitoring of blood pressure is even more important in giraffes, since, due to their unique anatomy and physiology, their pressure regulatory mechanism is delicate and can result in pathology in case of hypotension [119,123].

A meticulous basilar monitoring remains a cornerstone in the field, since blood gas analysis is not always available as the device is expensive, sensitive to ambient temperature, and demands specialized training to avoid pre-analytical errors during sample collection. In this study I have observed that, in reticulated giraffes, an initial heart rate equal or higher than 80 bpm was linked to greater homeostatic alterations, in particular with acidosis and hyperglycemia. Although this value does not represent a significant elevated heart rate compared to giraffe resting rates (40-60 bpm [117,206]) and therefore might be not taken into account, in my opinion it should instead be considered as an indicator for greater physiological derangement during field immobilizations. This finding might furthermore suggest that the establishment of clinical cut-off values for basilar monitoring variables based on their correlations with alterations observed with the blood gas analysis, especially in studies based on larger sample size, might be helpful in order to detect alterations in field immobilizations.

4. The use of intra-anesthetic pharmacological treatments and nasal oxygen insufflation to treat drug-related adverse effects (Study I-II-III)

Intra-anesthetic treatments were administered in Masai and reticulated giraffes, and in black rhinoceroses to decrease etorphine-mediated adverse effects.

In both the subspecies of giraffes, early antagonization resulted in clinical improvement. Regardless of the antagonist given or doses used – which were lower than recommended doses in relation to the amount of etorphine administered – no substantial difference was observed in the physiological function and optimal recoveries were observed, although reticulated giraffes were generally more excited during the manual restraint, whereas Masai giraffes were calm enough to be safely loaded. It is commonly assumed that, after antagonization, no major complication occurs since the giraffes are awake. However, physical restraint can trigger a stress cascade that can be more deleterious than the adverse effects of the drugs used for chemical immobilization [41,87]. In addition, the recumbency might easily impair the giraffe delicate blood pressure regulatory system or the ventilatory dynamic, as seen in other megaherbivore species [104,105,120,122,123,215,216]. For these reasons I investigated, for the first time, if the early antagonization does actually improve the acid-base and gas exchange

function in awake and physically restrained Masai and reticulated giraffes. Early antagonization resulted in an improvement of gas-exchanges, but not of the acid-base balance, whereas cardio-respiratory function was stable. Even though early antagonization provides improvement in gas exchanges in giraffes, the manual restraint is rough, as observed in the study in reticulated giraffes where the clinical procedures exposed them to algic stimuli, and poses safety concerns for both the giraffe and capture team. Following the evaluation of these results, an alternative protocol that includes early partial antagonization with a partial opioid antagonist, butorphanol, combined with oxygen insufflation was developed. Although it has been demonstrated that butorphanol does not improve arterial oxygenation acting on ventilation, but rather on decreasing oxygen consumption in species where opioid-related tremors occur [107,112], when it is given at higher doses, it partially antagonizes the μ-opioid receptor effects of the etorphine, creating a partial antagonization. This principle is used to produce a state of semi-sedation and to "walk" white rhinoceros into transport crates [54,174]. It is likely that etorphine cardio-respiratory adverse effects are displaced along the μ-mediated sedative effects. The use of post-induction butorphanol might be beneficial in giraffe immobilization as it would not only be able to partially reverse etorphine μ-mediated effects, but through its agonism at the k-opioid receptor would provide additional sedation and mild analgesia, but with fewer cardio-respiratory consequences. Administration of a μ- and k-antagonist, e.g. naltrexone, at the end of the procedure would instead provide full antagonization to both etorphine and butorphanol, with satisfactory recoveries and no resedations. According to the preliminary results, not included in this PhD study, it guarantees promising improvement both regarding physiological and handling safety, and future studies need to evaluate this post-induction treatment using a larger sample size.

In black rhinoceroses, the intra-anesthetic administration of a respiratory stimulant (doxapram), a partial antagonist (butorphanol) and oxygen resulted in an improvement in gas exchanges and acidosis, likely as a result of an increase in ventilation mediated by doxapram, that might have diminished the severity of intrapulmonary alterations such as ventilation-perfusion mismatch and oxygen diffusion impairment. In contrast with results obtained from white rhinoceroses, the administration of butorphanol and oxygen alone did not result in improvement of physiological function in study subjects, but the hypoxemia worsened. I hypothesized that this difference occurred because the main mechanism of hypoxemia in black rhinoceroses might not be through increased oxygen consumption resulting from tremors – which is the mechanism through which butorphanol improves hypoxemia in white rhinoceroses [107] – but it occurs because of a combination of hypoventilation and intrapulmonary alterations resulting from etorphine side effects and recumbency. However, higher doses of butorphanol have been observed, when combined with oxygen, to improve gas exchanges in other species. In preliminary results obtained in reticulated giraffes and in Grevy's zebras – not included in this PhD study – the use of butorphanol and oxygen resulted in improved hypoxemia. The difference might be explained with far higher doses of butorphanol used in giraffe (10 mg per mg of etorphine), and zebra (2-3 mg per mg of

etorphine), which might have explicated partial antagonism and thus improved the ventilation. Instead, in black rhinoceroses it is not possible to further increase butorphanol, as it would result in arousals, and therefore the low doses reported in this PhD study might explain the lack of improvement with butorphanol and oxygen alone. These findings further support the hypothesis that different species-specific response to drugs happen and that dedicated research on each species needs to be performed instead of extrapolating protocols and doses from other species.

Administration of oxygen therapy is highly recommended in wildlife immobilization. However, high flows of oxygen can be detrimental due to the occurrence of absorption atelectasis which can increase gas exchange impairment [112,157]. Within this PhD I have evaluated oxygen therapy only in black rhinoceroses, which improved oxygenation only when combined with a respiratory stimulant. However, across all studies in this thesis, major presumed causes of hypoxemia were hypoventilation, perfusion ventilation mismatching or oxygen diffusion impairment, which all respond to oxygen therapy. Therefore, the administration of oxygen, at adequate flows, may improve arterial oxygenation in other species. In addition, the administration of oxygen might be combined with respiratory stimulants in other species like buffalos that suffer from hypoxemia. This is in line with previous results I obtained in lions immobilized with ketamine-α2-agonist combinations where oxygen administration at low flow improved arterial oxygenation without depressing the respiratory drive [287]. However further species specific studies will need to evaluate if nasal oxygen administration, and which flow rates, provide benefits.

5. The use of synergistic drug protocols to reduce immobilization morbidity (Study IV)

The combination of drugs with synergistic properties allowed, in African buffalos, a decrease in drug doses, and in turn, a decrease of their side effects. The sparing effect of an ultralow dose of medetomidine resulted in an efficient reduction of etorphine dose compared to that used in the etorphine-azaperone combination. The slight decrease in etorphine dosage allowed us to reduce the occurrence of hypoventilation, hypercapnia and respiratory acidosis, but it did not impact the immobilization depth, which was instead significantly improved as the buffalos had an adequate level of sedation and myorelaxation. Furthermore, because with this combination the inductions were smoother and calm, metabolic acidosis was minimal.

This finding is important since higher mortality due to etorphine adverse effects is more likely to occur within the first ten minutes of immobilization [92,109]. Therefore, to make capture of large-sized species safer in a field setting, the availability and further research of improved protocols of immobilization – such as synergistic drug mixtures – that reduce etorphine doses and prevent the origin of alterations of the animal's physiological function is vital. Indeed etorphine-azaperone combination still causes severe morbidity, as observed in all the studies of this thesis, despite the benefits provided by the adjunct of azaperone. Post-induction

treatment can be beneficial, but is not always possible to administer it immediately when the animal is recumbent, as the animal might not be immediately reachable by the capture team, and preventing the complications is generally more efficient than their treatment [94]. In the buffalos, the hypoxemia was not improved with the medetomidine-based combination, but was still severe, and as such the adoption of nasal oxygen therapy, and the investigation of a further decrease of etorphine doses is suggested, as it might lead to an improvement of gas-exchange. In different capture settings where higher startle response is expected, such as in less touristic areas or during helicopter darting, higher doses of medetomidine, or more selective α2-antagonists such as dexmedetomidine, or other sedatives such as benzodiazepines [54] might be beneficial and would need further evaluation.

The results achieved in the buffalos with the addition of medetomidine to the dart mixture highlighted how through the use of drugs with synergistic properties, it was possible to increase the drugs desirable effects such as sedation, analgesia, myorelaxation and anxiolysis, and at the same time to keep dosages low thus reducing adverse effects. Further research is suggested in order evaluate the application of these findings to other species where opioid-mediated gas exchange impairment and metabolic derangement resulting from the capture are life-threatening, such as in other large-sized herbivores, where the use of non-opioid alternatives is often not possible.

The opportunistic research approach adopted in this thesis might have resulted in some limitations, due to logistics and planning factors. These limitations include a small sample size in some of the studies (e.g. in giraffes), a narrow monitoring time due to the short duration of the immobilizations (e.g. in buffalos), or poor distribution regarding the capture sites (e.g. in rhinoceroses). However, despite the limitations, this approach successfully resulted in important species-specific knowledge regarding mechanism of capture morbidity. The findings of my research suggest targeted strategies to improve the immobilization safety of the three select species, and paves the way to further studies that can now focus on the specific complications detected for each species.

Furthermore, with an opportunistic approach, it was possible to perform the studies in realistic conditions that represents the unpredictable and diverse settings in which field captures take place. Although studies performed in controlled conditions, such as in captivity, are essential to deeply investigate physiological mechanism or study drug effects [105,123], these do not take into account the occurrence of capture stress, which is an important part of the mechanisms that lead to capture morbidity. In addition, especially given the endangered status of Eastern black rhinoceroses and Masai and reticulated giraffes, the capture of wild animals would not be justifiable for pure research purposes, if not for operations with a direct conservation impact, such as translocations or the attachment of GPS units.

Capture mortality did not occur during the studies of this thesis, and post-capture mortality did not occur in the months of follow-up monitoring in those species where it was performed. Especially

in Critically Endangered Eastern black rhinoceroses, with less than 1000 individuals left, and in Masai and reticulated giraffes that are both Endangered, the loss of even one individual is unacceptable as it can affect the conservation efforts for the species. However, the results of this PhD highlight that mortality is not the only criteria by which to define the safety or success of an immobilization, but that severe alterations occur even when the immobilization seems stable, and can increase the risk of delayed complications.

Not only is a safe immobilization needed for animal welfare purposes and capture team safety, but this might further affect the outcome of conservation operations since the high stress developed during the capture, and its resulting pathophysiological alterations, and can result in longer-term consequences. These complications include chronic stress that might result in greater disease susceptibility and eventually delayed death, or reduced breeding performance, thus impacting conservation goals such as population size increases.

Therefore, the results of this PhD study, that have led to the detection of strategies to prevent, monitor and treat the physiological alterations caused by multifactorial elements that contribute to wildlife capture morbidity and mortality, have opened the path to further targeted studies that will not only contribute to improving the short-term, but also the longer-term safety of immobilization. By decreasing the stress response and the degree of physiological derangement, the improved immobilization will reduce the rate of delayed complications, thus improving the long-term outcome of conservation operations, and contributing to the conservation of Eastern black rhinoceros, African buffalo and Masai and reticulated giraffe.

5 CONCLUSION

The findings of this PhD study confirmed that there is variability in the physiological response to capture stress and drugs in Eastern black rhinoceros, African buffalo and Masai and reticulated giraffe, which is not only species-specific, but also occurs at subspecies and individual level. A species-specific approach should hence be endorsed when performing capture of East African mesoherbivores and megaherbivores as these differences assume a clinical importance in terms of the occurrence of capture complications.

In addition, the identification of predisposing factors should be further expanded in order to ameliorate capture techniques in a manner that considers ethological traits of each species. A better understanding of the mechanism of the physiological alterations have allowed me to identify targeted strategies to prevent, detect or treat capture morbidity, and to reduce mortality risk. Improved strategies to clinically monitor complications, such as the use of nasal capnometry was shown to be effective in the early detection of respiratory alterations during field capture, and further research on determining clinical cut-off values is encouraged.

More efficient protocols to reduce intra-anesthetic complications have been detected, such as the use of a combination of doxapram, butorphanol and oxygen in Eastern black rhinoceroses; or in preventing the origin of physiological alterations, such as through the use of a synergistic drugs combination in African buffalos. However, the improved protocols resulted from this thesis represent just the first step to produce safer immobilization in large-sized herbivores.

Starting from the knowledge gained in this thesis on the species-specific physiological impact and mechanism of capture morbidity, further research can now be aimed at investigating multi-drug protocols with balanced pharmacological properties that can both reduce the rise of a pathological stress response, and on the other hand to decrease drug adverse effects, such as opioid-induced respiratory depression.

More importantly, the results of this thesis have highlighted that, similarly in all species, there is a need to rethink the concept of the success of chemical immobilization, since the absence of mortality is an outdated indicator, whereas severe morbidity, that can lead to delayed complications, commonly occurs but is often unnoticed. New strategies of research aimed at improving the safety of wildlife capture should take into account the long-term physiological impact of capture.

In the light of my results, focus needs to be directed on quantifying longer-term morbidity and to integrate these parameters into the search for improved capture methods. A chemical immobilization, that will both reduce the physiological derangement caused by drugs and keep the short- and longer-term stress response to a minimum, will finally be successful in safely immobilizing East African meso- and mega-herbivores, thus contributing to the success of their conservation.

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