Monitoring adrenocortical function as a measure of stress in blue wildebeest (*Connochaetes taurinus*)

By

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Declaration

I, the undersigned Adél R. de Haast, whose name appears on the title page of this dissertation, do hereby declare to be the author of this work.

The author hereby declares that the research presented in this dissertation was conceived and executed by herself and, apart from the normal guidance from my supervisor, she had received no assistance.

Neither the substance, nor any part of this dissertation has been submitted in the past, or is to be submitted for any other degree at this University or any other University.

The applicable research ethics approval has been obtained for the research described in this work.

The author declares that she has observed the ethical standards required in terms of the University of Pretoria's Code of ethics for researchers and the Policy guidelines for responsible research.

This dissertation is presented in partial fulfilment of the requirements for the Master of Science (Veterinary Science) degree in the Department of Paraclinical Science, University of Pretoria.

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List of Abbreviations:

ACTH – Adrenocorticotropic hormone

ANOVA – Analysis of variance

ANS – Autonomic nervous system

CNS – Central nervous system

DW – Dry weight

EIA – Enzyme immunoassay

fGCM – Faecal glucocorticoid metabolites

GCM – Glucocorticoid metabolites

HPA – Hypothalamic – pituitary – adrenal system (axis)

PNS – Parasympathetic nervous system

RIA – Radioimmunoassay

SAM – Sympatho – adrenomedullary system (axis)

SNS – Sympathetic nervous system

Chapter 1. Abstract

Like many other wildlife species, blue wildebeest (*Connochaetes taurinus*) are routinely captured for relocation purposes. Several studies have shown that physiologically this is a very stressful period for wild animals and can affect several aspects of their well-being. Little is known about the level of physiological stress experienced by blue wildebeest during capture and confinement before transport. A non-invasive approach to assess adrenocortical function as a measure of physiological stress would be preferable, as animals are not disturbed during sample collection, and therefore sampling is feedback free. Currently, however, such a non-invasive measure has not been evaluated for any wildebeest species.

An adrenocorticotropic hormone (ACTH) challenge test was performed on one sub-adult male and one adult female. We examined the suitability of five enzyme immunoassays (EIAs), detecting faecal glucocorticoid metabolites (fGCM) with a 5β-3α-ol-11-one (3α,11oxo-CM), 5α-pregnane-3β,11β,21-triol-20-one (37e), 11β,21-diol-20-one (corticosterone), 11,17,21-trihydroxypregn-4-ene-3,20-dione (cortisol), and 11,17-dioxoandrostane (11,17-DOA) structure respectively, for monitoring stress-related physiological responses in blue wildebeest. After evaluating all five EIAs, it was concluded that the EIA measuring 11,17-DOA performed the best for detection of fGCMs in blue wildebeest with a 21 fold increase above the baseline in the male and a 4,7 fold increase above the baseline in the female at 12 hours post ACTH injection.

Under field conditions the use of the 11,17-DOA EIA allowed the monitoring of fGCM alterations when wildebeest were captured using a mass capture technique; fGCM concentrations were elevated by 1,5 times in samples collected from animals restrained for up to 10 hours in a truck compared to those collected directly after capture ($P < 0,001$). No significant increase in fGCM concentrations was detected in faecal samples collected directly after capture from animals of the same herd captured either on day 1 or day 2 of mass capture, indicating that the presence of a helicopter during the two days of capture was not associated with a profound stress response in wildebeest that were not captured at the time but were in the vicinity of the capture.

Storage of faeces in labelled plastic containers at ambient temperature for up to 48 hours post-defecation resulted in a significant decrease in 11,17-DOA levels from 8 to 48 hours after defecation. Therefore sample collection for 11,17-DOA determination in wildebeest can occur for up to 8 hours post-defecation without the risk of substantial decreases in 11,17-DOA concentrations. By identifying a suitable assay for determining 11,17-DOA concentrations in blue wildebeest, it can now be used by researchers, wildlife managers and veterinarians to reliably monitoring the physiological stress responses to capture or other management procedures with minimum interference to the animals. We have also shown that the presence of a helicopter during capture does not appear to have a lasting influence on wildebeest 11,17-DOA concentrations, but prolonged restraint for 10 hours does.

Chapter 2. Literature review

2.1 Introduction

For management purposes the capture and confinement of wild animals is routinely carried out in South Africa, and other parts of the world, and is associated with a high incidence of morbidity and mortality that can often be attributed to the subsequent effects of physiological and psychological stress (Knox et al. 1990). The inability to adapt to confinement often results in a marked loss of condition and exhaustion from constant pacing and refusal to eat and drink. These effects are most likely caused by the effects of prolonged stress from exposure to an unfamiliar environment, food and water delivery systems (Fowler & Miller 1999, Knox et al. 1990).

Both free-ranging and zoo-housed animals show a physiological stress response during capture and periods of adaptation to new or changed surroundings and conditions. Although this stress response does not have one specific cause and cannot precisely be measured by objective means, evaluation of changes in behavioural and physiological coping mechanisms allows some quantification of the magnitude of the stress response (Fowler & Miller 1999, Knox et al. 1990). Behavioural coping mechanisms include movement from a threat to a more favourable location, vocalization, increased locomotion, aggression, and stereotypic behaviour (Broekman 2013, Hattingh 1988, Kock et al. 1990, Moberg & Mench 2000).

Because capture and translocation procedures are non-avoidable in the wildlife management process, it is essential, from a welfare perspective, to understand the related effects, and therefore a quantifiable measure of the stress response is very important. Observation of changes in behaviour and measures of heart rate, blood catecholamine and glucocorticoid concentrations may be used to quantify the stress response and reflect the "stress status" in animals (Knox et al. 1990, Clippinger et al. 1998). However, behavioural measures are influenced by observer bias and heart rate and blood samples can only be measured if animals are restrained or immobilized. Therefore it is important to be able to quantify a stress response using non-invasive means and faecal glucocorticoid metabolite measurement allow for this (Sheriff et al. 2011, Ganswindt et al. 2012).

However, due to the fact that steroid metabolism is highly species specific, methods for determining glucocorticoid metabolites (GCM) using faeces as hormone matrix need to be properly validated for each species monitored (Palme et al. 2005, Touma & Palme 2005). The wildebeest is a common game ranching species that is often captured and translocated. However, no non-invasive technique to reliably monitor faecal glucocorticoid metabolite alterations, as an indicator of perceived stress, have so far been validated for free-ranging blue wildebeest.

2.2 Wildebeest biology

The wildebeest, genus *Connochaetes,* are part of the Bovidae family. They belong to the subfamily Alcelaphinae where their closest relative is the hartebeest (*Alcelaphus* spp.) (Estes 2014). Two types of wildebeest are recognised within the genus *Connochaetes*: the black wildebeest (*C. gnou*) and the blue wildebeest (*C. taurinus*) (Wilson & Reeder 2005). Furthermore, five subspecies are currently recognised for the blue wildebeest, whereas no subspecies are recognised for the black wildebeest (IUCN 2016, ITIS 2016).

Black wildebeest prefer the open grassland areas and do not migrate over long distances while blue wildebeest live in a wide variety of habitats and readily migrate when there are food and water shortages (IUCN 2016, Napier 1971, von Richter 1974).

The black wildebeest's historical range included South Africa, Swaziland and Lesotho but they were hunted to near extinction in the latter two countries in the 19th century (IUCN 2016). They have now been reintroduced to these countries and also into Namibia where they have become well established. They inhabit open plains, grasslands and Karoo scrublands in both mountainous regions and lower hills at varying altitudes from 1,350 and 2,150 m (IUCN 2016). The blue wildebeest can be found in a variety of habitats from open woodlands to dense bush areas and thrive in areas that have a moderate rainfall and are not too dry. They are a notable feature of the Serengeti National Park in Tanzania and the Masai Mara Game Reserve in Kenya. They also commonly occur in Botswana, Zambia, Zimbabwe, Mozambique, South Africa, Swaziland and Angola. They no longer inhabit Malawi, but have been reintroduced into Namibia (IUCN 2016).

The blue wildebeest was given its scientific name *Connochaetes taurinus* by William John Burchell in 1823 (Pickering 1997). *Connochaetes taurinus* has five subspecies: *C.t. albojubatus* range extends from northern Tanzania to central Kenya, *C. t. cooksoni* is restricted to Zambia, *C.t. johnstoni* is extinct in Malawi but still occurs in Mozambique to Tanzania, *C. t. mearnsi* is found in northern Tanzania and Kenya, and *C. t. taurinus* is found from Namibia to South Africa and Mozambique (IUCN 2016, ITIS 2016).

The blue wildebeest inhabit grassland areas that contain surface water as they like to drink twice a day. They are herbivores and feed primarily on short grasses and therefore are commonly found in savannah grasslands and plains. If no grass is available, they will also feed on foliage from shrubs and trees. Blue wildebeest are commonly associated with zebras (*Equus quagga*) as the latter eat the lesser nutritious grass canopy leaving the lower greener material for the wildebeest. The wildebeest's broad mouth is very well adapted for this lower more nutritious material. The wildebeest is present in abundance in Africa and is also the main prey item for larger carnivores including the lion (*Panthera leo*) and spotted hyenas (*Crocuta crocuta*). They play a pivotal role in maintaining the ecosystem especially in big ecosystems like the Serengeti where their migratory patterns have long been a big tourist attraction. They are commonly found on most game ranches in Africa and are hunted for biltong and trophies (Taylor et al. 2015).

A major concern for this species are anthropogenic factors which affect their numbers through degradation of their preferred habitat, drying up of water sources, poaching and expansion of human settlements. The implementation of fences that interrupt traditional migratory movements between wet and dry season ranges has resulted in mass deaths during droughts. A study by Ottichilo et al. (2001) revealed that wildebeest populations in the Masai Mara ecosystem have undergone a decline of 80 % from about 119,000 in 1977 to around 22,000 twenty years later, due to the expansion of agriculture (Ottichilo, de Leeuw & Prins 2001). However, the International Union for Conservation of Nature (IUCN) rates the blue wildebeest conservation status as being of "Least Concern" as the total number of blue wildebeest throughout Africa is estimated to be around 1,550,000. Population numbers in the Serengeti National Park (Tanzania) have increased to about 1,300,000 and they have also been introduced into a number of private game farms, reserves and conservancy areas throughout Southern Africa (IUCN 2016).

2.3 The wildlife industry in South Africa

In the early 20th century, wildlife became a burden to landowners as they competed with livestock for grazing and harboured diseases. Therefore, wildlife was either neglected or deliberately eradicated (Bond et al. 2004). In the 1950's, ecologists determined that the productivity of wildlife from the land could be higher than that of domestic livestock and could provide an alternative healthy source of meat. Land could also be utilized more efficiently, especially in marginal agricultural areas (Mossman & Mossman 1976). With a new democratic government in South Africa in 1994, the profit margins for traditional farming practices declined due to decreasing subsidies and the emergence of intensive feedlot production systems. There was also an increase of intensive breeding of high valued species on wildlife ranches (van Zyl et al. 2001). These are just some of the main factors that played a role in promoting the switch from stock farming to wildlife ranching.

The wildlife industry has grown tremendously over the past 30 years. The use of wild animals has promoted game farming and wildlife ranching as forms of sustainable utilization. Game farming in Southern Africa has been stimulated by the availability of suitable natural habitat and the diversity of endemic wild animal species, together with the initiatives taken by conservationists, business personnel and farmers (Fowler & Miller 1999).

During the last $10 - 20$ years the wildlife industry has changed in Southern Africa. Previously the main incentive for farming with wild animals was trophy hunting and buying animals at auctions purely for this purpose. In recent years the focus has shifted towards conservation and ecotourism (McKenzie 1993) and currently there is a great interest in breeding and trading in colour variants. In many parts of Africa there has been an alarming decline in wild animal populations during the last century. Certain species have been in imminent danger of becoming extinct, because of inadequate protection. This situation has resulted in an increase in the need to move animals to national parks and game reserves. Normally animals are captured, transported then housed in bomas before they can be moved into their new environment (Fowler & Miller 1999, Hofmeyr & de Bruine 1973, Dickens et al. 2010).

Data provided in 2014 by provincial nature conservation departments, determined that wildlife ranches covered $170\,419$ km^2 in South Africa. In a study of several wildlife ranches (n = 251)

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covering an area of 13 775 km^2, 38 % of the properties bred high value wildlife species and 23 % of properties bred colour variants in camps. Sixty-three percent of properties conducted live sales during 2014 which was estimated at 225 200 animals which were sold (Taylor et al. 2015).

Live sales, trophy hunting and culling of game for meat play a large economic role in the wildlife industry and make a tremendous contribution to the South African economy. In 2014 alone, it was calculated that private sales equated to R 2.453 billion, while the total value of meat extracted from wildlife was estimated at R 611.5 million. Trophy and biltong hunting generated revenues of R 1.96 and R 0.65 billions respectively (Taylor et al. 2015).

As a result of the growing wildlife industry, the total estimated number of jobs created by the wildlife ranching sector in 2014 was 65 172. This number excludes people employed by industries reliant on the wildlife ranching sector e.g. wildlife translocation, fencing businesses and taxidermists (Taylor et al. 2015).

The term "wildlife ranching" and "game farming" both refer to the management of wildlife on private land for commercial purposes. These terms are often used interchangeably, although game farming generally refers to smaller properties (<5,000 ha) where some form of constant management is necessary, while wildlife ranching generally refers to larger properties (>5,000 ha) where management interventions are less frequent. The game farming and wildlife ranching sectors are supported by four main "pillars"; a) ecotourism, b) live sales and breeding of high value species and colour variants like golden wildebeest, c) trophy hunting and hunting for meat, and d) processed game products (Taylor et al. 2015).

Game breeding generally takes place in intensive or semi-intensive environments (Kock & Burroughs 2012). The three main species supporting this sector over the last 30 years have been sable (*Hippotragus niger*), roan (*Hippotragus equinis*) and disease-free African buffalo (*Syncerus caffer*), while more recently the industry has expanded to include colour variants of plains game species including impala (*Aepyceros melampus*), springbok (*Antidorcas marsupialis*) and blue wildebeest. Taylor et al. (2015), determined that 31 ranches out of 251 studied in South Africa were breeding for the "golden" colour variant, of a blue wildebeest called the golden wildebeest (Taylor et al. 2015). The popularity of this colour variant was second only to that of black impala (38 ranches). Golden wildebeest are high value individuals and breeding is often conducted under conditions that reduce the risks of predation, generally in fenced camps

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or enclosures of varying size. Live antelope and bovine species, including the blue, black and golden wildebeest may be bought from auctions, catalogues or directly from wildlife ranches. Trophy hunting also forms a big part of the game ranching industry and some species, like the blue and black wildebeest are valuable trophy species due to their horns and other characteristics. Taylor et al. (2016) found that of 5 704 animals that were hunted for trophies on wildlife ranches covering a total area of 510 724 ha, 2 % were black wildebeest and 5 % were blue wildebeest (Taylor et al. 2015).

Game ranches generally have a combination of several herbivore species which is believed to keep the ecosystem stable. From the results obtained from a recent study involving 306 135 wildlife ranches, more than 20 % of herbivores were black wildebeest and more than 60 % were blue wildebeest (Taylor et al. 2015). Based on these studies it is clear that wildebeest play an important role in the wildlife ranching industry.

2.4 Stress

The concept of "stress" has been defined for a long time and dates back to the ancient Greeks (Touma & Palme 2005). Stress deals with physiological and hormonal changes that occur when daily social and non-social stimuli challenge or threaten the survival, health and reproductive success of an animal (Touma & Palme 2005). A "stressor" can be defined as the environmental stimulus that leads to an imbalance in homeostasis and the corresponding defence reaction of an animal as the "stress response" (Möstl et al. 2002). Metabolic, immunological and neuroendocrine mechanisms form part of the physiological responses to stress. Adrenocorticotropic hormone (ACTH), glucocorticoids, catecholamines and prolactin are the primary hormones involved in the neuroendocrine response to a stressful stimulus. The adrenal glands play a key role in the hormonal response to stress as they are involved both in the hypothalamic-pituitary-adrenocortical (HPA) axis and the sympatho-adrenomedullary system (Touma & Palme 2005, Möstl et al. 2002, Ganswindt et al. 2010). Therefore stress induced by environmental stimuli (stressors) can result in physiological responses. The effects of a stressor can be monitored by measuring behavioural responses, which tend to be subjective in nature, or by measuring physiological responses which are more objective (Ganswindt et al. 2010).

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When an animal experiences physiological stress, two major systems in the body are involved to deal with this situation. The hypothalamic-pituitary-adrenal (HPA) system (axis) and the sympatho-adrenomedullary (SAM) system (axis; Figure 1). The HPA system is 'slow' acting and is seen as the body's main stress system by controlling levels of cortisol and other hormones that regulate a stress response. The hypothalamus sends messages to the pituitary gland, which leads to the production of ACTH. The adrenal cortex is stimulated, which then produces steroid hormones like cortisol or corticosterone. These steroid hormones provide the steady flow of 'fuel' necessary to deal with the demands of stressful situations that might be on-going, for example prolonged confinement. The SAM system is the 'fast' reaction of the body to sudden perception of a stressor. The hypothalamus activates the sympathetic branch of the autonomic nervous system (ANS) which stimulates the adrenal medulla and leads to the release of adrenaline and noradrenaline. This acute response is known as the 'fight or flight' response. This response leads to the physiological changes associated with an acute stress response e.g. increased heart rate, raised blood pressure and shaking (Moberg & Mench 2000, Meyer et al. 2008).
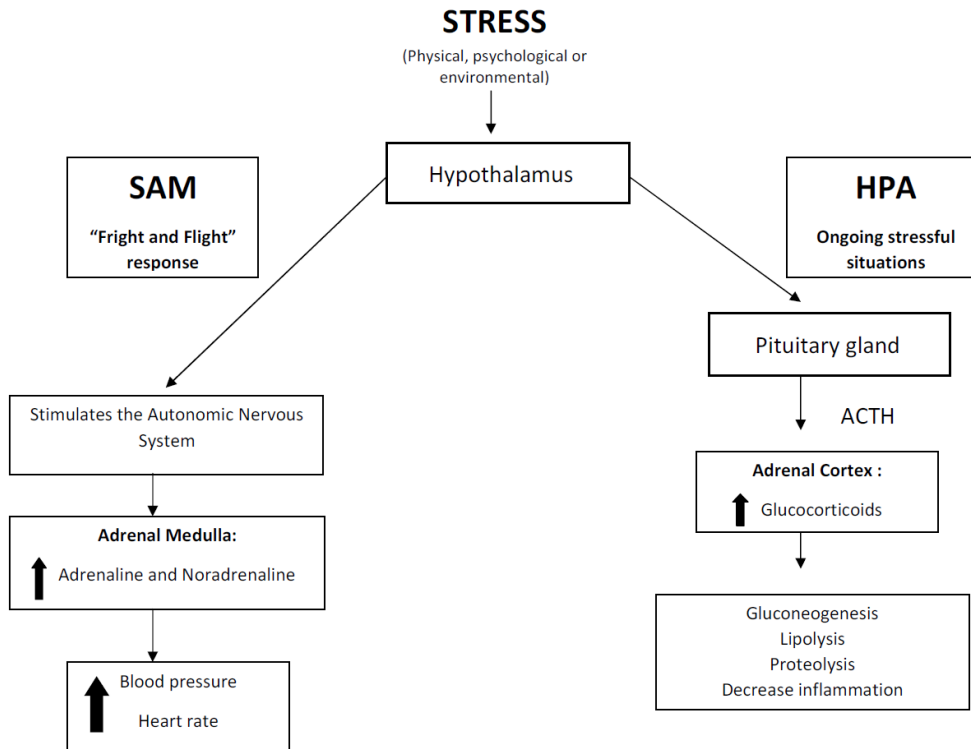
HPA = Hypothalamic-pituitary-adrenal axis; **SAM** = Sympatho-adrenomedullary axis; **ACTH** = Adrenocorticotropic hormone

Figure 1: The physiological responses to stress through the hypothalamic-pituitary-adrenal axis and sympatho-adrenomedullary axis with examples of these axes effects. Figure redrawn from Burdick et al. (2011). Interactions between Temperament, Stress, and Immune Function in Cattle. *International Journal of Zoology*, Volume 2011, pg 1-9.

2.4.1 Stress response

The stress response can be divided into three general stages as depicted in figure 2;

1. the recognition of a stressor (homeostasis disturbance),

2. the stress response (behavioural and physiological responses described above) and

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3. the consequences of the stress response.

The last stage of the stress response is the most important and will determine if an animal suffers from distress or adapts to it without abnormal changes in homeostasis (Moberg & Mench 2000). When an animal encounters a stressor that disrupts homeostasis, the HPA axis will be stimulated further to respond to this stressor. It generally takes 3-5 minutes for this pathway to result in measurable increases in glucocorticoid concentrations. After 15-30 minutes the glucocorticoid levels will reach peak levels in the blood and return to basal levels after 60 – 90 minutes after exposure to the stressor (De Kloet et al. 2005).

However, the severity of the stressor will determine the level at which glucocorticoids are elevated. When the stressor is acute and not sustained (few seconds to a few hours), the feedback mechanism (HPA) operates efficiently and the system returns rapidly to normal. When an animal is exposed to a chronic stressor, feedback signals are weak and the system remains activated for longer periods. Chronic stress can therefore lead to negative consequences on health, reproduction and growth (Sheriff et al. 2011, Touma & Palme 2005, Möstl et al. 2002, Sapolsky 1992).

Both the magnitude and the duration of glucocorticoid secretion are biologically important in free-range animals. The biological effects of the stress response result from the released hormones' interactions with organs and other tissues over the entire time course of the response and not just at the peak of glucocorticoid release. When studying the effects of stressors in free-range animals it is very important to study an integrated stress response, as animals may experience a number of different stressors at any given time (Sheriff et al. 2011). When a stress response truly threatens the animal's well-being the animal experiences "distress". Distress will have a deleterious effect on an individual's welfare (Moberg & Mench 2000). A model for defining and recognizing distress in animals is depicted in figure 2 under consequences of stress.
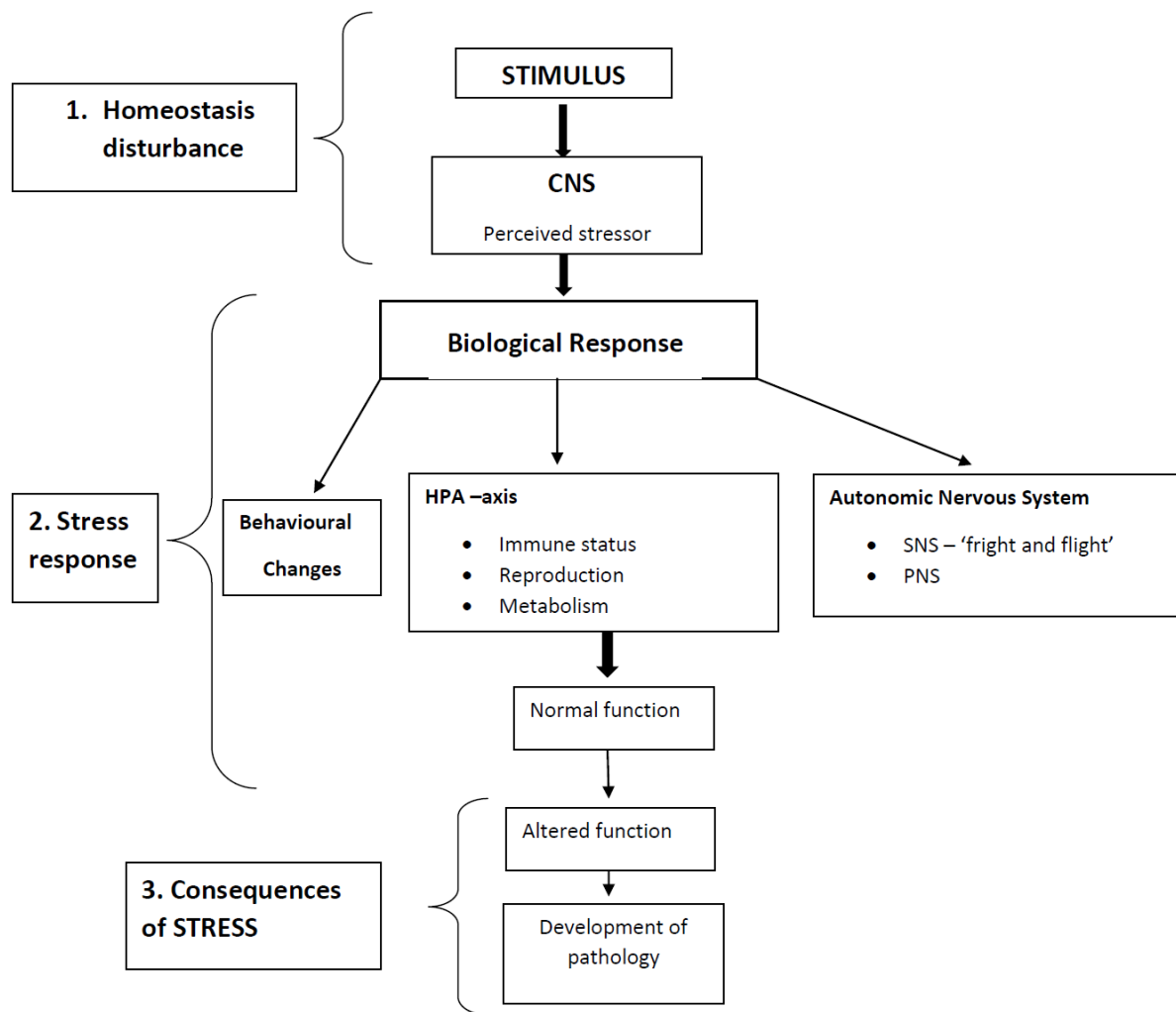
HPA = Hypothalamic-pituitary-adrenal axis; **SNS** = Sympathetic Nervous System; **PNS** = Parasympathetic Nervous System; **CNS** = Central Nervous System

Figure 2: A model of the biological response of animals to stress that can lead to distress. Figure redrawn from Moberg & Mench (2000). *The Biology of Animal Stress*. Chapter 1 pg 4-7.

2.4.2 Stress-related challenges during capture and housing in bomas

Bomas, which are high-walled enclosures, play a crucial role in wildlife capture and translocation and can be used to either capture animals or temporarily confine them. Boma housing is essential to help animals to adjust or habituate to their new environment before they are released or translocated. The longer animals are kept in bomas, the more time they will have to adjust and thus their stress response to stressors will be reduced (Meyer et al. 2008). However, the initial time spent in a boma, before adjustment has occurred, often causes a severe chronic stress response which can result in morbidity and mortality (Kock & Burroughs 2012, Kock et al. 1990).

Bomas are usually constructed in an environment close to either the capture or release site and are constructed using either temporary material for example shade or sail cloth or permanent materials including sisal or gum poles. Airflow through the boma is very important to reduce heat stress. The minimum size of enclosures for temporary holding is calculated at 2 m^2/50 kg live mass of animal. If these minimum measurements are not adhered to, a stress response to overcrowding can occur and animals might escape or injure each other (Kock & Burroughs 2012). Management of animals in bomas is very important for their health; enough shade, food, water and cleaning of the bomas is essential (Kock & Burroughs 2012).

Before animals are placed in bomas they need to be captured from the wild. One of the greatest problems encountered during capture and handling of wild animals is that of stress and overstraining (Fowler & Miller 1999, Swan 1993). The failure of translocations projects are usually caused by the effects of chronic stress during the adaptation phase when animals are moved to and housed in bomas (Dickens et al. 2010). A high percentage of deaths that occur during the capture and translocation process are attributable to untreatable injuries in newly captured animals that are "stressed" and "panic stricken" (Fowler & Miller 1999). These deaths occurred especially in the past where capture procedures were undertaken in a manner that was extremely stressful to animals, where they were sometimes chased until exhaustion, which often caused post-capture complications including capture myopathy. Abnormal increases in body temperature (hyperthermia) were also identified as a common consequence of capture in impala (Meyer et al. 2008). This hyperthermia was found to mainly occur due to physiological responses

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to stress (Meyer et al. 2008). Other physiological changes that occur post-capture, or during confinement, include increases in biochemical and hematologic parameters like creatine phosphokinase (CPK), lactate dehydrogenase (LDH), aspartate aminotransferase (AST), glucose, cholesterol, calcium, blood urea nitrogen (BUN), haemoglobin (Hb), packed cell volume (PCV) and white blood cells (WBC's) (Broekman 2013, Hattingh 1988, Kock et al. 1990, Hattingh et al. 1990).

With the development of mass capture techniques and immobilizing and long-acting tranquilizing drugs, the stress induced by capture and confinement, and the resulting complications thereof, have been reduced since the first wildlife capture and translocation operations (Hofmeyr & de Bruine 1973). Nonetheless, mortality associated with transport and boma housing of wildlife still remain high.

2.5 Glucocorticoids

Changes in glucocorticoid (i.e., cortisol and corticosterone) concentrations are commonly used as a hormonal measure of the magnitude of a stress response in wild and captive animals (Millspaugh & Washburn 2004). The type of glucocorticoid secreted by the adrenal gland differs between species, but cortisol is primarily secreted in primates, carnivores and ungulates whereas corticosterone is the main hormone in rodents, birds and reptiles (Palme et al. 2005, Touma & Palme 2005).

In most, but not all species glucocorticoids are usually secreted at relatively low concentrations in a pattern that follows a circadian rhythm, but this rhythm may be abolished by acute and prolonged stress responses (Möstl et al. 2002). Thus, detection of rhythmicity and episodic secretions demand frequent sampling. In the liver, glucocorticoids undergo metabolism and conjugation and some are recycled via the enterohepatic pathway (Figure.3). The majority of conjugated glucocorticoids are excreted in bile and urine (Möstl et al. 2002).

The main route of excretion might differ between species and sex of a given species (Palme et al. 2005), but essentially glucocorticoids are mostly excreted via faeces or urine or both, although

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other routes (saliva, milk and feathers) can occur (Möstl & Palme 2002). Therefore, plasma glucocorticoid concentrations can be assessed indirectly and non-invasively by measuring glucocorticoid metabolites in either urine or faeces.
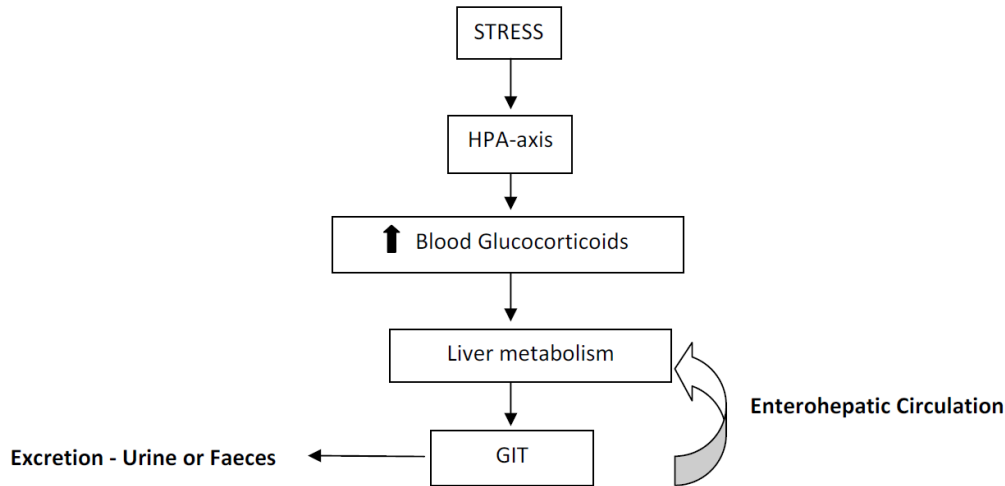
HPA = Hypothalamic-pituitary-adrenal axis; **GIT** = Gastrointestinal tract

Figure 3: Entero-hepatic circulation and excretion pathways of glucocorticoids. Figure redrawn from Möstl and Palme (2002). Hormones as indicators of stress. *Domestic Animal Endocrinology,* Volume 23 pg 67-74.

2.5.1 Determining glucocorticoid concentrations in blood

Glucocorticoids have been quantified in blood for many years (Millspaugh & Washburn 2004). Assays are available to measure the concentrations of glucocorticoids from serum or plasma. These glucocorticoids normally stimulate biological responses in an animal (Wasser et al. 2000, Wingfield & Monk 1994). However, when measuring glucocorticoids, a distinction between "free" and "bound" hormone needs to be made. A large proportion of total circulating glucocorticoids are bound to a plasma protein, corticosteroid binding globulin (CBG). It is the concentration of free, unbound hormone that determines how much glucocorticoid diffuses out of the capillaries and reaches the tissue to cause a biological response (Sheriff at al. 2011). The use of serum or plasma to measure a stress response has its limits especially in wild animals where an animal first needs to be captured to collect blood using methods that normally induce an additional stress response, thus potentially compromising an accurate assessment of the initial stress response (Moberg & Mench 2000). Remote blood sampling can be used but is not readily available for free-ranging animals. Furthermore, blood measures only provide a snap shot of a stress response, which may not be representative of the overall long-term response due to the

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pulsatile and circadian secretion pattern of glucocorticoids in blood (Windle et al. 1998, Harper & Austad 2000). However, blood measures are useful when evaluating only the acute adrenal responsiveness to capture and restraint protocols (Wingfield & Monk 1994).

2.5.2 Determining glucocorticoid metabolite concentrations in faeces

Glucocorticoid metabolite concentrations can be determined using alternative matrices like urine or faeces and those non-invasive approaches have gained popularity over the past 30 years as a more practical approach for assessing adrenocortical activity in intractable wildlife and domestic species (Ganswindt et al. 2012, Palme et al. 2005). However, respective assays for non-invasive hormone measurements need to be carefully validated in terms of applicability for the species-specific hormone matrix of interest to ensure a reliable quantification of respective glucocorticoid metabolites (Ganswindt et al. 2012).

As mentioned above, plasma glucocorticoids are naturally metabolised in the liver and eliminated by either urine or faeces (Figure 3). The amount of glucocorticoid metabolites excreted via the faeces varies between species and can range from as little as 7 % in pigs to about 86 % in cats (Palme et al. 2005). Sex differences related to the route of excretion also occurs. Touma et al. (2003) demonstrated in mice that the amount of the overall corticosterone metabolites recovered in faeces of males was higher than in females, however, both sexes excreted most of their corticosterone metabolites via the faeces (Palme et al. 2005). There is also a species specific delay in time from when an animal experiences a stressor to when glucocorticoid metabolites are measured in the faeces. This delay corresponds mainly to an animals' faecal intestinal transit time from the duodenum to the rectum. Pigs, sheep and ponies had an average delay of between 13 – 48 hours (Palme et al. 2005). The delay time for many wildlife species has been determined by a number of studies and it generally ranges from 12 – 26 hours depending on the species (Palme et al. 2005).

Faecal sampling for the determination of faecal glucocorticoid metabolite concentrations offers several advantages over blood sampling. Faeces can be collected easily and over long periods of time without disturbing an animal, therefore avoiding the confounding effects of the stress induced by capture. Faecal hormone metabolites also reflect the production rate of

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glucocorticoids and their metabolites over several hours. Palme at al. (2005) found that faecal concentrations of cortisol metabolites reflected the total amount excreted over several hours and therefore reflected overall cortisol secretory patterns better than blood concentrations, which generally change quickly (Palme et al. 2005). In addition, the secretion of glucocorticoids occurs in a pulsatile fashion and therefore the blood concentrations can change by a factor of 10 or more within minutes (Palme, Wetscher & Winckler 2003, Palme et al. 1996). Compared to blood concentrations of glucocorticoids, faecal glucocorticoid metabolite concentrations have been shown to more accurately reflect the magnitude of a stress response because only the biological active portion (the free glucocorticoid fraction not bound to blood proteins) of the total released amount in the blood is available for metabolism and excretion (Palme et al. 2005).

2.6 Determining glucocorticoid concentrations

The measurement of hormones and their metabolites are usually carried out by immunological procedures using hormone- or hormone-group specific antibodies (Kleiman et al. 2010). Two main types of immunoassays are available; radioimmunoassays (RIA) and enzyme immunoassays (EIA). Radioimmunoassays use radio-labelled hormones as the competitive tracer, whereas EIAs use either enzyme- or biotin- labelled preparations in the process of quantification. Enzyme immunoassays have the advantage that they are non-isotopic and do not make use of radioactive particles, therefore disposal is easier, safer and less costly. Furthermore, the end point of the reaction is a colour change that is simple to quantify and makes use of less expensive instrumentation (Kleiman et al. 2010). A range of test-systems for the analysis of faecal glucocorticoid metabolites have been established over the last few years in various mammal, bird, reptile and fish species (Millspaugh & Washburn 2004). Any respective assay for species specific hormone measurement needs to be validated for each species to ensure correct quantification of the faecal glucocorticoid metabolites (Ganswindt et al. 2012).

The main criteria of assay validation are: a) sensitivity – minimum amount of substance that can be detected, b) precision – within and between assay repeatability, c) accuracy – ability to detect the correct amount of steroid in the sample, and d) specificity – degree of specificity of the antibody used and the possible influence of interfering substances (Kleiman et al. 2010). Prior to

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analysis, a number of additional factors, such as stability of faecal metabolites post-defecation and efficiency of steroid extraction, have to be considered and if necessary assessed to ensure a representative result is obtained (Millspaugh & Washburn 2004).

Although respective assays have been reliably used to detect endogenous changes in adrenocortical activity in many wildlife species like the African elephant (*Loxodonta africana*), black rhinoceros (*Diceros bicornis*), elk (*Cervus canadensis*), oryx (*Oryx gazella*), Alaskan sea otter (*Enhydra lutris*), cheetah (*Acinonyx jubatus*) and clouded leopard (*Neofelis nebulosa*), to name a few (Wasser et al. 2000, Schwarzenberger 2007), no assay has been so far validated for determining glucocorticoid metabolite alterations in blue wildebeest faeces.

2.6.1 ACTH Challenge test

The adrenocoticotropin hormone (ACTH) challenge test measures how well the adrenal glands respond to ACTH. By administering a high dose of a synthetic ACTH, we mimic a stress response in the body and therefore stimulate the release of glucocorticoid metabolites which then can be measured in faecal samples. The ACTH challenge test is also used for validation of the metabolites tested in faeces as these may differ between sex and species (Touma & Palme 2005). After administering ACTH, the changes, or expected changes, in blood glucocorticoid concentrations can be correlated with, or evaluated against faecal glucocorticoid metabolites concentrations to determine which the ideal metabolite to measure is. To perform these tests faeces are collected for 2 – 3 days prior to injection of ACTH and then for 5 – 7 days afterward, which allows for the measurement of ACTH-induced changes in glucocorticoids and their metabolites in the faeces. This sampling regime also allows for the determination of the time-course in these changes. In order to correlate fluctuations in glucocorticoids with a stressor, it is vital to know this time-course, which is the length of time it will take from the time of a stressor to the spike in glucocorticoid metabolites concentrations to appear in the faeces (Keay et al. 2006).

2.7 Aims and specific objectives of the study

The primary aim of my study was to validate a non-invasive technique to reliably monitor adrenocortical activity in blue wildebeest using faecal glucocorticoid metabolite measurements. An additional aim was to determine the changes in faecal glucocorticoid metabolite concentrations in wildebeest in response to capture and confinement.

Objectives

a) To determine the best suited EIA for measuring GCM to indicate stress-related physiological responses in blue wildebeest faeces by performing an adrenocorticotropic hormone stimulation test (ACTH challenge test).

b) To examine the stability of faecal glucocorticoid metabolites in blue wildebeest faeces post defecation.

c) To characterise changes in faecal glucocorticoid metabolite concentrations linked to blue wildebeest capture and confinement.

Chapter 3. Materials and Methods

3.1 Animals and study sites

Two blue wildebeest, one male (20 months of age, 160kg) and one female (40 months of age, 180kg), were housed in individual bomas (82.5 m^2 each) that allowed visual and olfactory contact with each other but not with the rest of their herd mates. These animals, used for the adrenocorticotrophic hormone (ACTH) challenge, were housed at Emerald Casino Game Park, Gauteng, South Africa. Throughout the experiment the animals were fed fresh teff hay and 0.5 kg of concentrate pellets (Wildlife Pellets, Epol®, Rustenburg) daily with water available *ad libitum*.

In addition, a population of blue wildebeest (n = 79) at Mokala Game Reserve (196 km^2), Northern Cape, South Africa were sampled for two days in June 2015 during a planned mass-capture event performed by SANParks' capture team. Mokala Game Reserve is classified as Kalahari Thornveld and is composed of grassland, scrubland/thicket, and *Acacia* open woodland habitat (Estes 2014).

The study was performed with the approval of the University of Pretoria Animal Use and Care Committee (Reference V055-14).

3.2 Adrenocorticotropic hormone (ACTH) challenge test

3.2.1 Study design

Individual faecal samples were collected from every dropping event for nine days prior to ACTH administration in order to determine baseline faecal glucocorticoid metabolite levels in the two wildebeest. On day 10 of the study, each animal received 400 IU (1 - 2 IU/kg) of synthetic ACTH (Synacthen Depot$^{©}$, Novartis South Africa (Pty) Ltd, Johannesburg) intramuscularly in the gluteus muscle using a pressurized dart syringe (Dan-Inject, Skukuza). During the following five days, a sample of each voided faecal dropping was collected for faecal glucocorticoid metabolite (fGCM) analysis. On day 16 of the study, the two animals were released and allowed to join their herd mates.

3.2.2 Capture of study subjects

The two animals for the ACTH challenge were captured from the same herd ($n = 12$ animals) by means of chemical immobilization via darting with a combination of etorphine hydrochloride (2 – 3mg, M99, Novartis South Africa (Pty) Ltd, Johannesburg), thiafentanil oxalate (2 – 3 mg, Thianil, Wildlife Pharmaceuticals, White River), xylazine hydrochloride (10 – 15 mg, Xylavet 2%, MSD Animal Health SA, Isando) and azaperone (40 – 50 mg, Stresnil, Janssen Pharmaceutica, Sandton). Once immobilised these animals were loaded onto a utility vehicle and transported within 20 minutes after capture to a boma (holding enclosures used to house wild antelope) 3 km from capture site. Once in the boma the effects of the immobilization were reversed with diprenorphine (4 – 6 mg, M50/50, Novartis South Africa (Pty) Ltd, Johannesburg) and animals were monitored until full recovery.

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3.2.3 Faecal sample collection

At immobilization and capture, one rectally-extracted faecal sample was taken from each wildebeest. Further collection occurred for the next 16 days between 07:00 and 18:00 from their boma enclosures where every dropping was sampled. Approximately 50 g of homogenised faecal material was taken from the centre of the dropping to avoid possible contamination from urine or soil. Animals were observed throughout the day (07:00 to 18:00) so that samples voided were collected approximately within 20 minutes of defecation. Enclosures could not be accessed at night; therefore samples voided overnight (18:00 – 07:00) were collected the next morning. Only samples that were warm to the touch were included as early morning samples (7:00) for that day. A total of 142 samples (n = 79 male and n = 63 female) were collected during this period. Collected faecal material was immediately placed on ice and then, within 20 minutes, placed in a -20°C freezer until further processing.

3.3 Faecal glucocorticoid metabolite study during mass-capture

3.3.1 Study design

In order to determine whether the EIA identified in the ACTH stimulation test would detect a response to a known stressor like mass capture, samples were collected during a mass capture event at Mokala Game Reserve. As animals were caught, they would defecate and fresh samples were collected for analysis. Samples were collected whenever wildebeest were brought into the capture boma during the capture operation over 2 days. By measuring the concentration of faecal glucocorticoid metabolites we could assess the magnitude of the stress response experienced by the wildebeest for the duration of capture and transport.

3.3.2 Capture of study subjects

Wildebeest capture at Mokala Game Reserve started at 9:00 am on the 23 June 2015. Animals were chased by means of a helicopter from within a 12 km radius into a collapsible man-made mass-capture boma. This boma was constructed from double layered sheets made out of plastic and shade cloth and was constructed into a large funnel shape using poles and wires (see figure 4). The animals were herded into the mouth of the funnel by helicopter and once inside a plastic sheet curtain (curtain 1, Figure 4) at the opening was closed behind them. As the animals were chased towards the neck of the funnel two other plastic sheet curtain were closed behind them (curtain 2 and 3, Figure 4). The neck of the funnel, the chute, was connected to a solid steel handling passage which had a ramp. Once all handling procedures were complete, the animals were pushed up the loading ramp and into a suitable game transport vehicle (Figure 5 and 6). Faeces voided during their time in the handling area (chute) were then collected. All faecal samples were collected in individual plastic containers and were marked with the time and date of collection. Multiple species were caught on this day and included Red Hartebeest (*Alcelaphus buselaphus*), sable, zebra and impala. Capture continued on the 24 June 2015. Multiple species were also captured on that day using the same mass-capture boma and loading procedures. Thirty nine wildebeest were captured on day 1 and these animals were transported immediately to Loxton in the Karoo. On day 2, 32 wildebeest were captured. All these animals were left in the transport trucks overnight and transported the following day to the Eastern Cape.
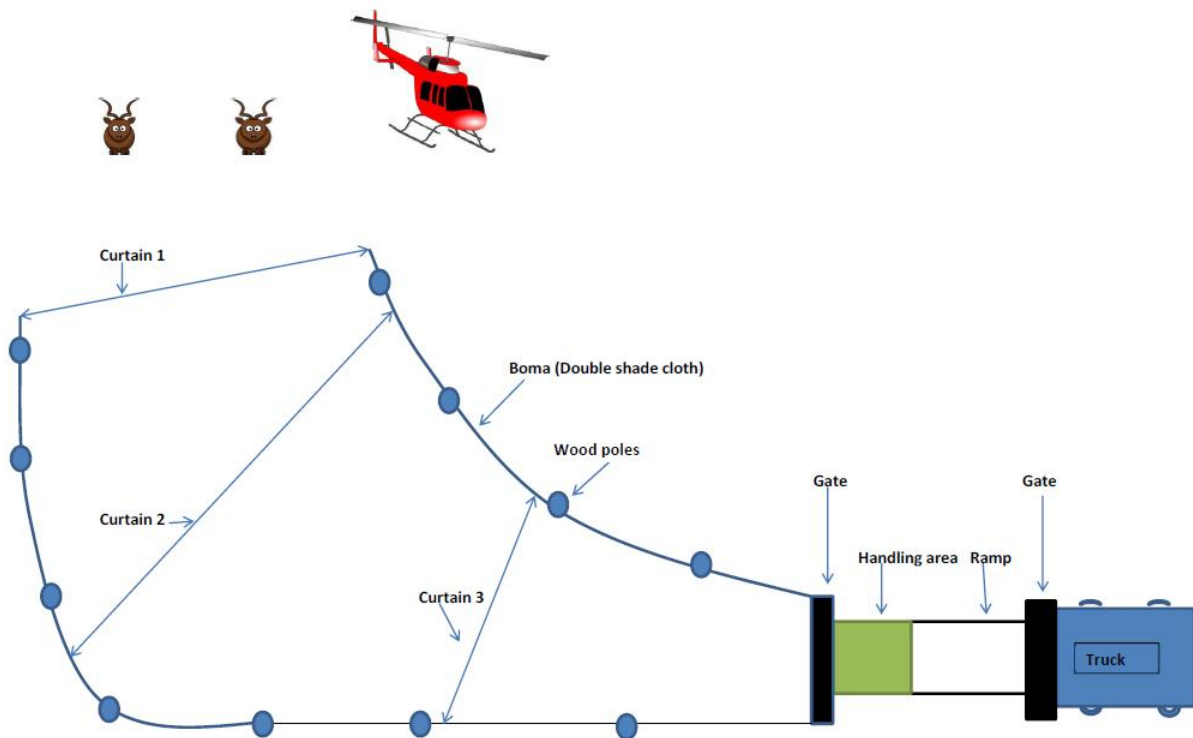
Figure 4: Sketch of the funnel-shaped constructed boma

Figure 5: Holding area (chute) and the ramp to the transport vehicle

Figure 6: Holding area (chute), ramp and transport vehicle

3.3.3 Faecal sample collection

Faecal samples that were defecated spontaneously as the animals moved through the neck of the funnel and handling area were collected. Animals that were not transported immediately were left in the truck for several hours at a time. Once these animals were ready for transport, they were moved to another truck and spontaneous defecated material was sampled in this truck. This sampling was done at random and animals were not identified or directly linked to the sampled material. A total of 63 samples (n = 26 on day 1 and n = 37 on day 2) were collected. All samples collected on day 1 were taken immediately after capture. On day 2, 16 samples were collected immediately after capture. Three fresh samples were collected after animals spent 3 hours in the truck and 17 fresh samples were collected after the animals had spent 10 hours on the truck, thereafter the animals were transported. Collected faecal material was placed on ice immediately and within 20 minutes they were placed in a -20°C freezer and stored until further processing.

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3.4 Faecal sample processing and extraction

Within four days of collection, frozen samples were lyophilized, pulverized, and sieved through a mesh to remove fibrous material (Fieß, Heistermann & Hodges 1999). To extract the faecal glucocorticoid metabolites approximately 0.1g of the faecal powder was vortexed for 15 minutes with 3 ml of 80 % ethanol. Following centrifugation for 10 minutes at 1,500 g_n, supernatants were transferred to glass tubes and stored at -20°C until analysis (Fieß, Heistermann & Hodges 1999).

3.5 Measuring faecal glucocorticoid metabolites

The extracts from the faeces were analysed to measure immunoreactive faecal glucocorticoid metabolite (fGCM) concentrations using enzyme immunoassays (EIAs) to measure 11,17-dioxoandrostanes (11,17-DOA) as well as fGCMs with either a 5β-3α-ol-11-one (3α,11oxo-CM), a 5α-pregnane-3β,11β,21-triol-20-one (37e), a 11,17,21-trihydroxy-20-one (cortisol) or 11β,21-diol-3,20-dione (corticosterone) structure. Details of the EIAs, including the cross-reactivities, are described by Möstl et al. (2002) for 3α,11oxo-CM, by Touma et al. (2003) for 37e and by Palme (1997) for 11,17-DOA, cortisol and corticosterone.

For steroid hormone determination, 50µl aliquots of standards (range: 0.976 – 250 pg for the 11,17-DOA EIA), quality controls and diluted faecal extracts were pipetted in duplicate into microtiter plate wells. Then 50 µl of biotinylated 11,17-DOA label and antiserum were added and the plates were incubated overnight at 4 °C. Following incubation the plates were washed four times with buffered saline and 150 µl (20 ng) of streptavidin-peroxidase was added to each well. Following incubation in the dark for 30 min at 4 °C, the plates were washed again before 150 µl peroxidase substrate solution was added and the plates were incubated further for 30-60 min. The reaction was terminated by adding 50 µl of 4N H_2SO_4 and the absorbance measured at 450 nm. Serial dilutions of extracted faecal samples gave a displacement curve that was parallel to the respective standard curve.

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Sensitivity of the assays were 0.6 ng/g dry weight (DW) for 11,17-DOA, 3α,11oxo-CM and cortisol, 1.5 ng/g DW for 37e, and 1 ng/g DW for corticosterone. Intra- and inter-assay coefficients of variation determined by repeated measurements of high- and low-value quality controls ranged between 4.8 and 5.8 % for 11,17-DOA, 6 and 8 % for 3α,11oxo-CM, 6.9 and 11.6 % for cortisol, 4.9 – 6.2 % for 37e and 6.3 – 9.1 % for corticosterone. Faecal samples from Mokala Game Reserve were analysed with the 11,17-DOA EIA only. Respective intra- and inter-assay coefficients of variation for Mokala Game Reserve samples ranged between 4.8 and 15.3 %. Assays were performed on microtitre plates as described by Ganswindt et al. (2012).

3.6 Data analysis

Descriptive statistics were used to determine alterations in fGCM concentrations following ACTH administration. To achieve this, an iterative approach was used to determine individual baseline fGCM values as described by Brown et al. (2001). For that, all fGCM concentrations of an individual data set exceeding the mean plus two standard deviations (S.D.) were excluded, the average recalculated, and the elimination process repeated until no hormone concentrations exceeded the mean plus 2 S.D. To determine changes in fGCM concentration post-defecation, the relative change (%) of fGCM concentration post-defecation were calculated for each subsample separately, using the mean fGCM value determined at t = 0 as 100 %. Differences in relative alteration rate between samples stored at t = 0 h and 1 – 48 h post-defecation were examined by one-way repeated measures ANOVA, followed by post hoc analysis using Bonferroni t – test, with application of Bonferroni correction. Respective data subsets were tested for normality using Shapiro-Wilk test. Comparison between two groups was made by using Mann-Whitney U test. Data were statistically analysed using SigmaPlot 12.5 and significance was considered achieved when P < 0.05.

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Chapter 4. Results

4.1 ACTH challenge

All five EIAs detected increases in fGCM concentrations approximately 20-25 hours post-ACTH injection in both the male and female wildebeest (Figure. 7 - 16). In the male, all five EIAs used showed a peak increase in fGCM concentrations (11,17-DOA: 2126 %; 3α, 11oxo-CM: 968 %, 37e: 457 %, Cortisol: 253 %, Corticosterone: 202 %, Figure 7 - 11) following ACTH administration of at least 100 % above fGCM concentrations measured before the ACTH injection (baseline values). In the female only three of the five EIAs revealed an overall increase in fGCM concentrations of 100 % above baseline concentration in the female (11, 17-DOA: 474 %, 3α, 11oxo-CM: 60 %, 37e: 263 %, Cortisol: 310 %, Corticosterone: 72 % Figure 12 - 16). The 11,17-DOA EIA showed the greatest response post ACTH injection in both sexes, and therefore this assay was subsequently used for monitoring capture-induced alterations in fGCM in free-ranging wildebeest.

Figure 7: The response displayed by the 11,17-DOA (mean ± S.E.M.) enzyme immunoassay in the male wildebeest following ACTH administration (arrow).

Figure 8: The response displayed by the 3α, 11oxo-CM (mean ± S.E.M) enzyme immunoassay in the male wildebeest following ACTH administration (arrow).

Figure 9: The response displayed by the 37e (mean ± S.E.M) enzyme immunoassay in the male wildebeest following ACTH administration (arrow).

Figure 10: The response displayed by the cortisol (mean ± S.E.M) enzyme immunoassay in the male wildebeest following ACTH administration (arrow).

Figure 11: The response displayed by the corticosterone (mean ± S.E.M) enzyme immunoassay in the male wildebeest following ACTH administration (arrow).
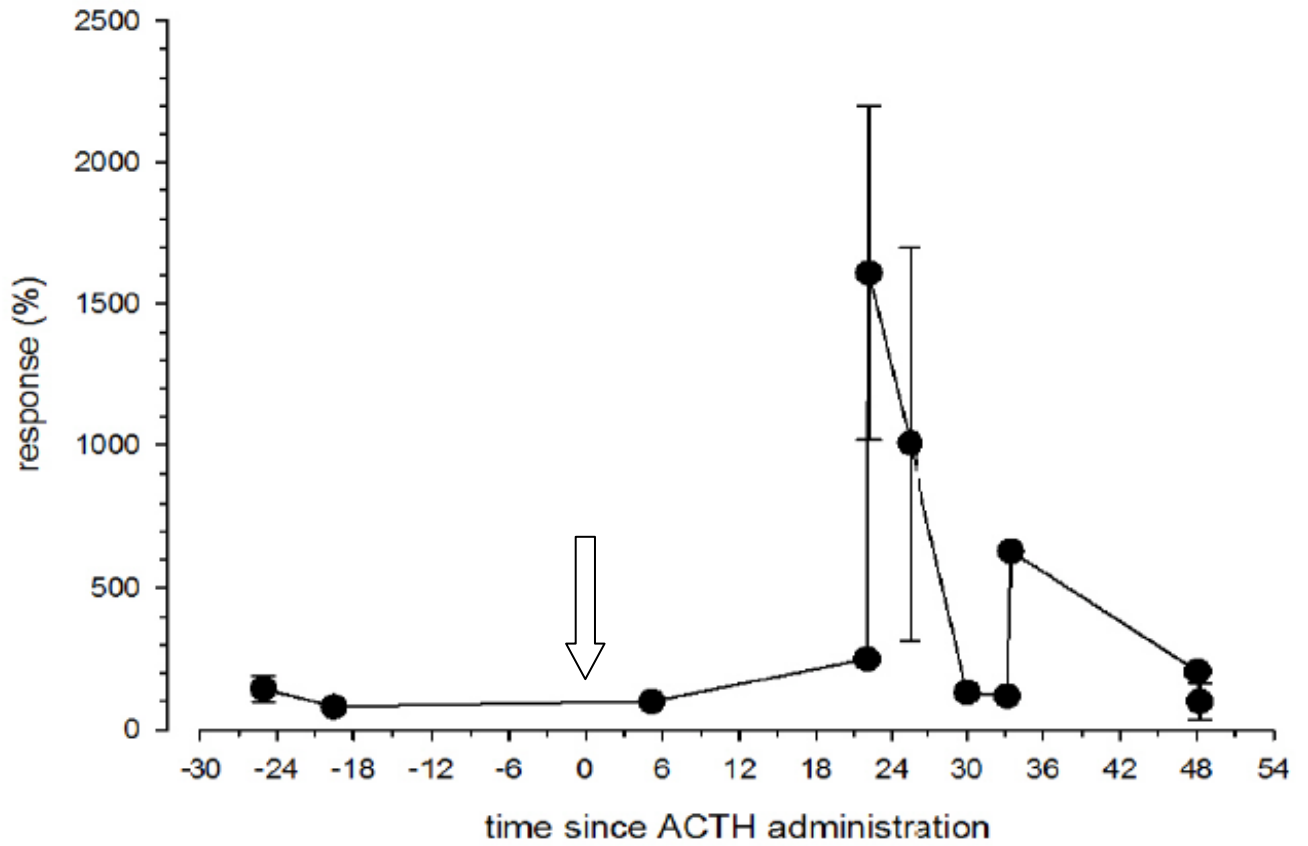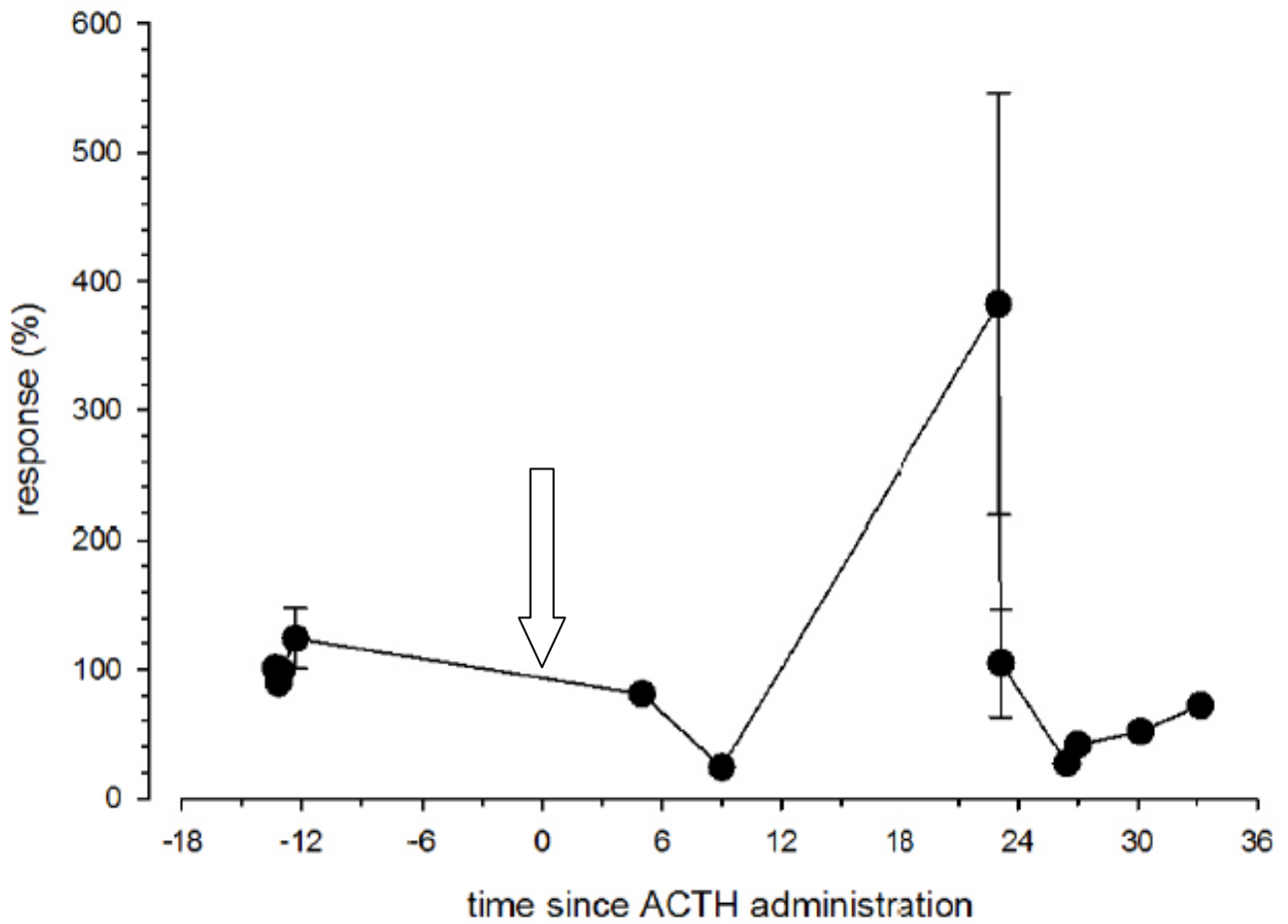
Figure 12: The response displayed by the 11,17-DOA (mean ± S.E.M) enzyme immunoassay in the female wildebeest following ACTH administration (arrow).
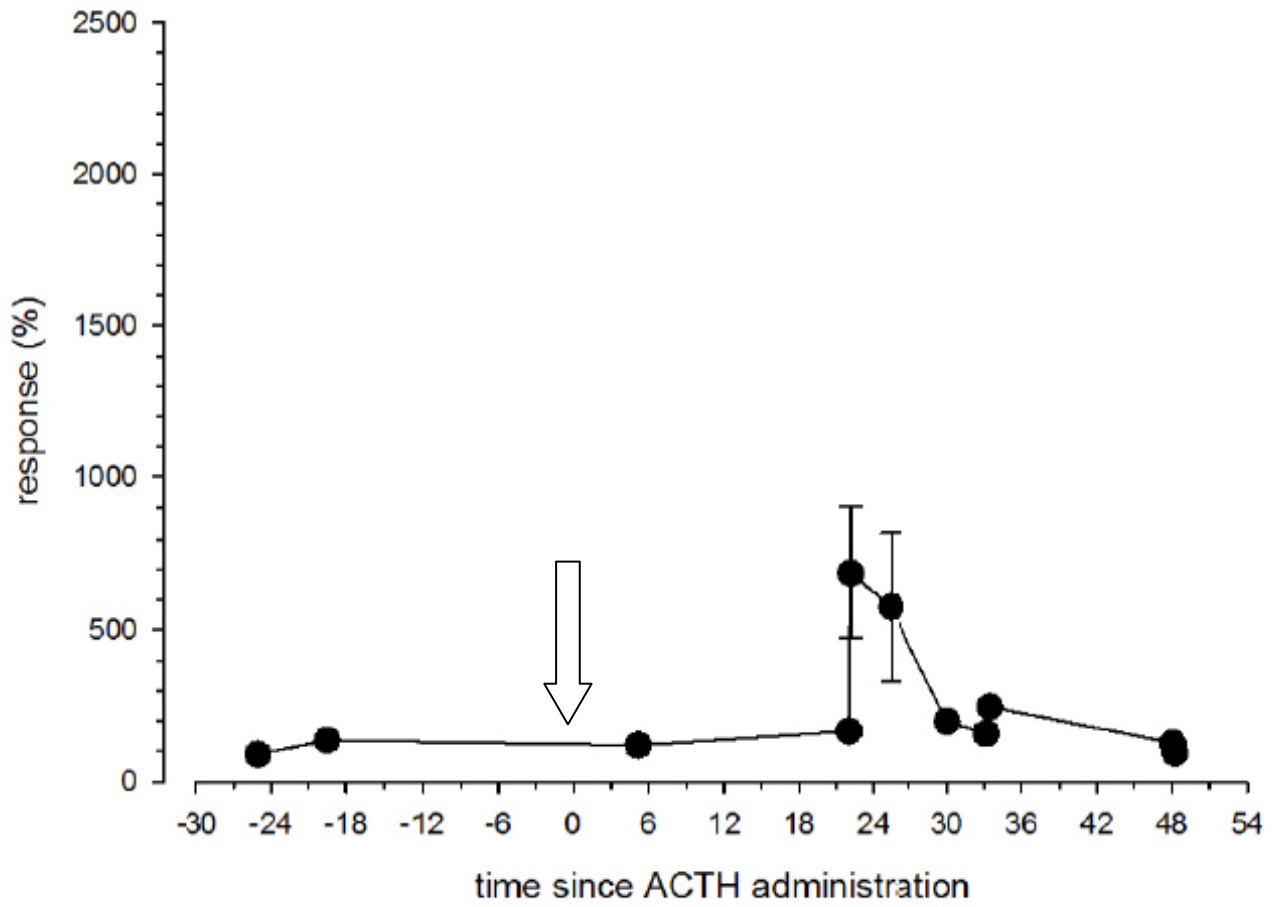
43
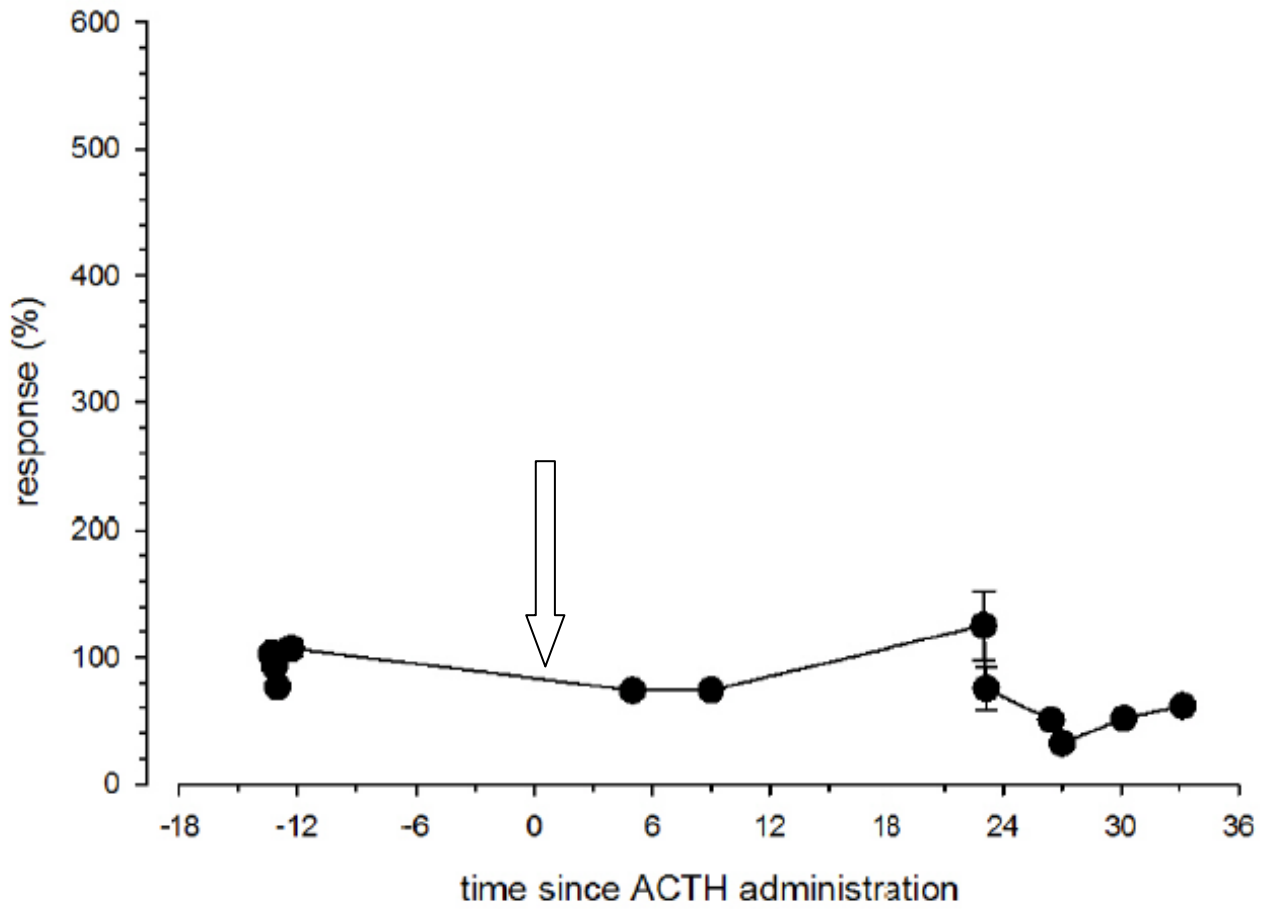
Figure 13: The response displayed by the 3α,11oxo-CM (mean ± S.E.M) enzyme immunoassay in the female wildebeest following ACTH administration (arrow).
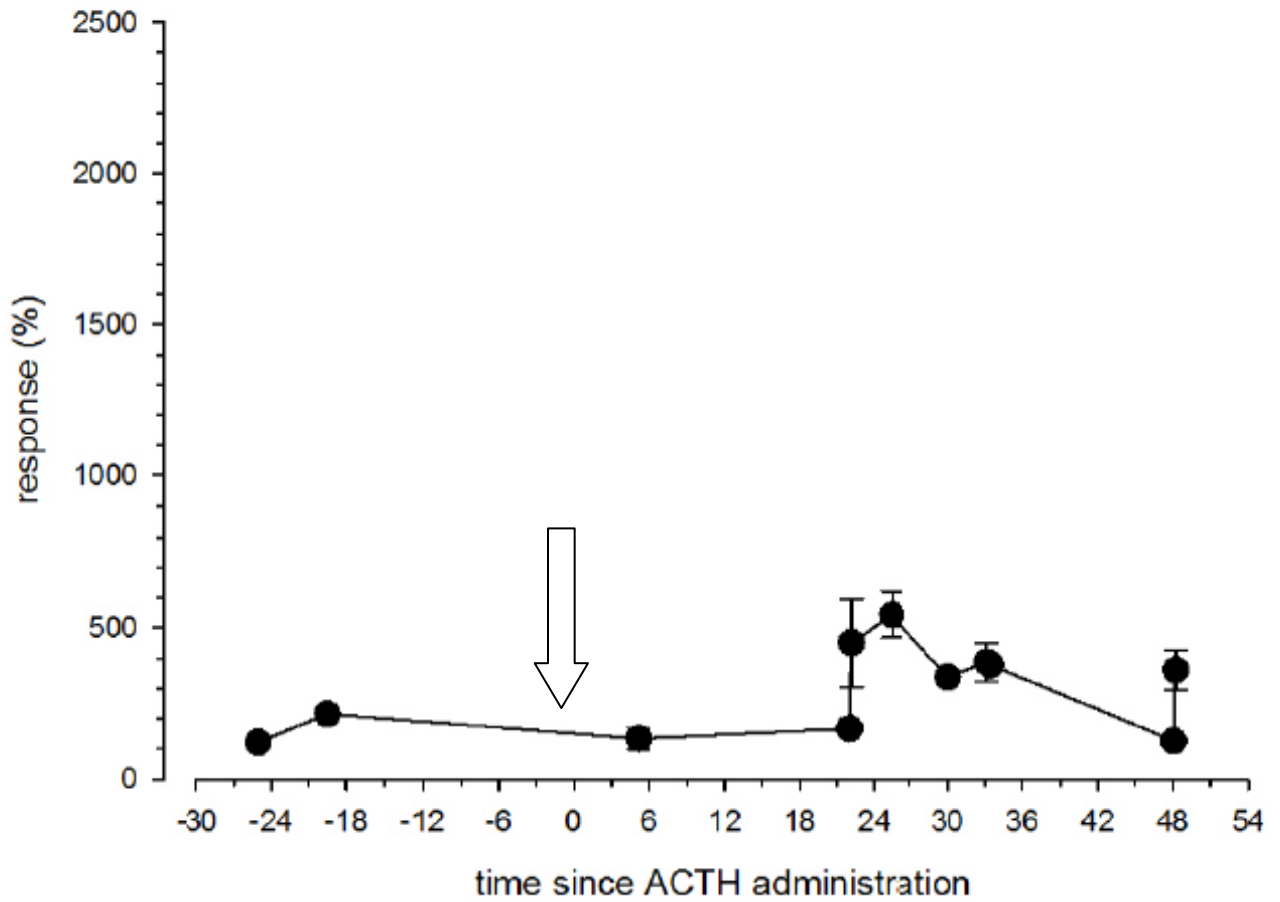
44
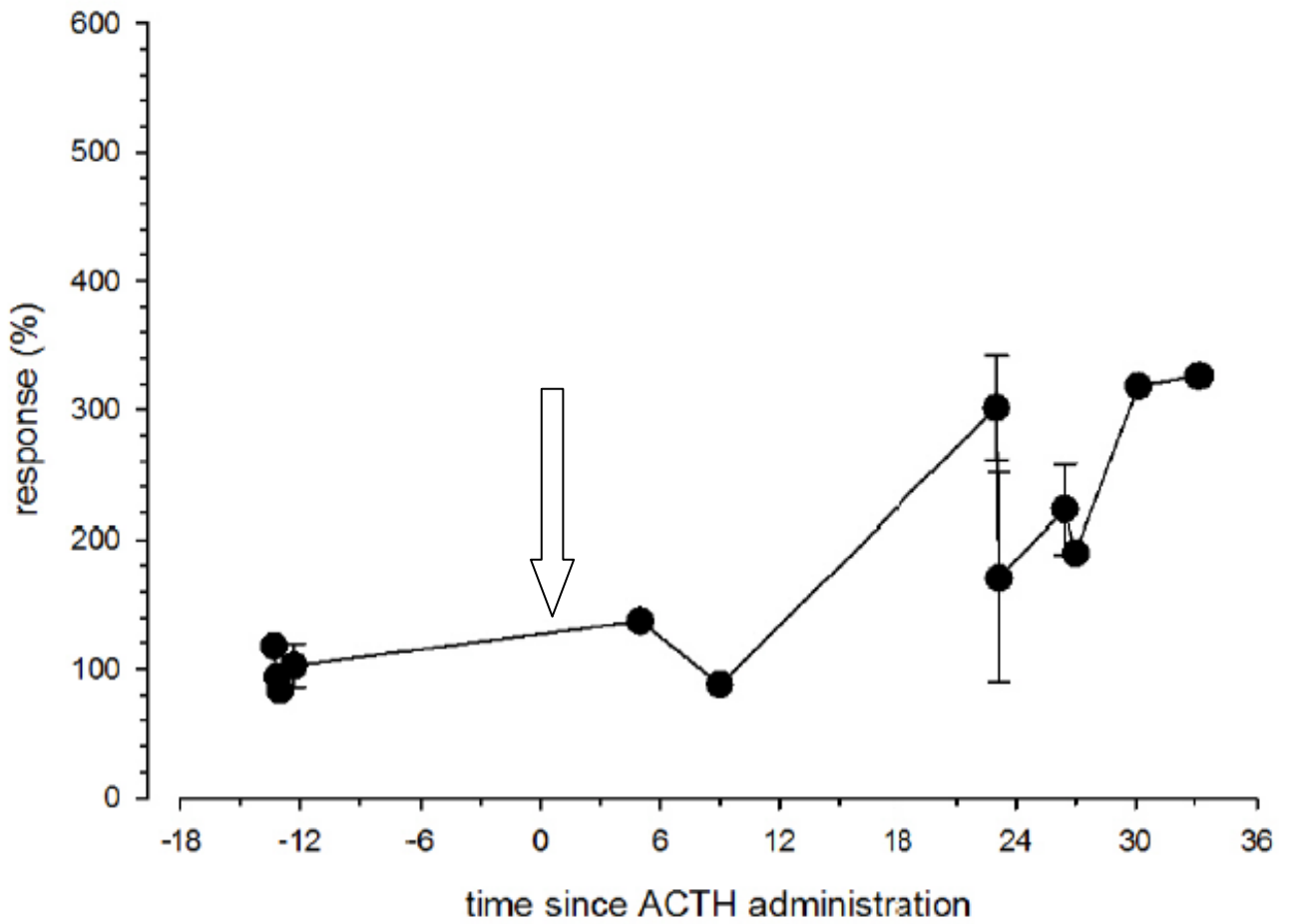
Figure 14: The response displayed by the 37e (mean ± S.E.M) enzyme immunoassay in the female wildebeest following ACTH administration (arrow).
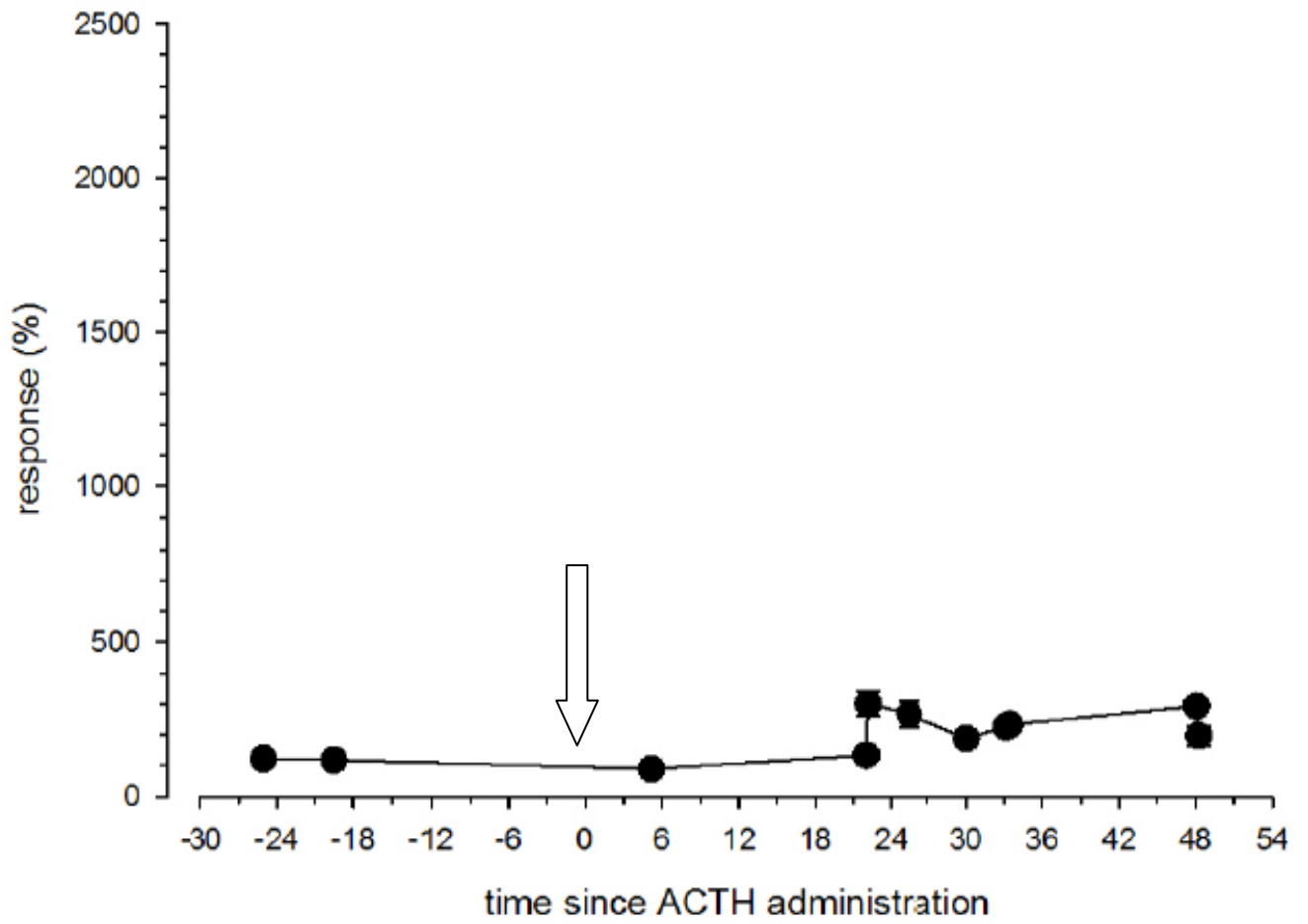
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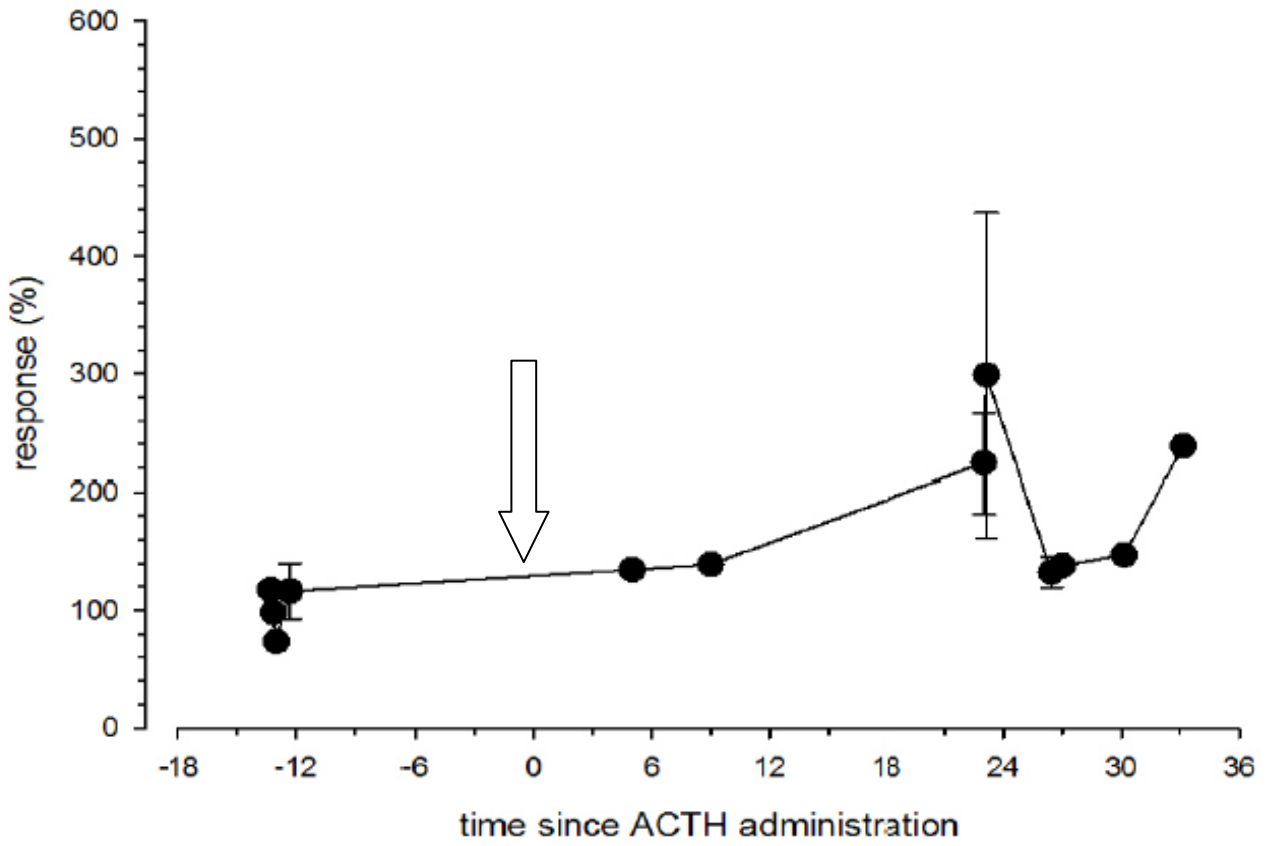
Figure 15: The response displayed by the cortisol (mean ± S.E.M) enzyme immunoassay in the female wildebeest following ACTH administration (arrow).
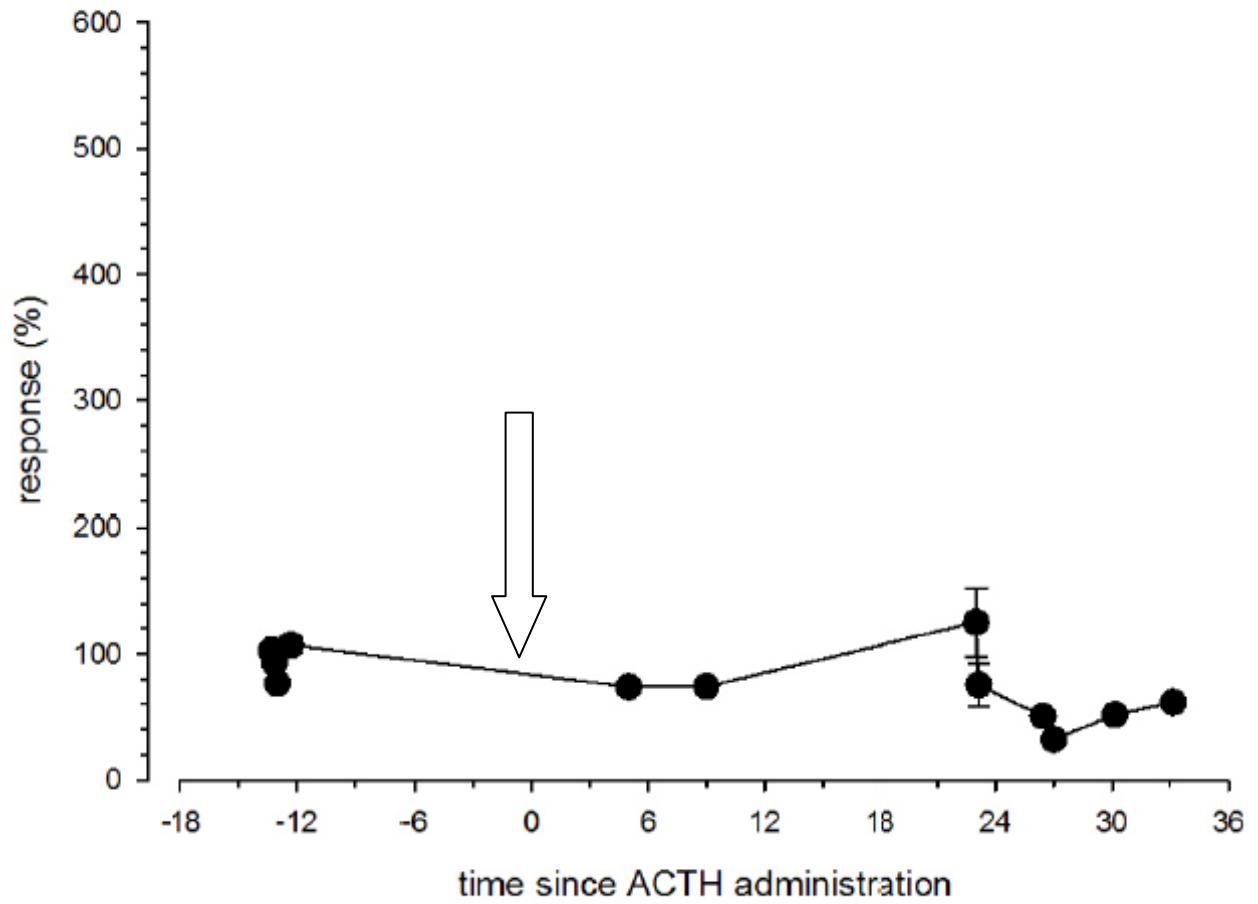
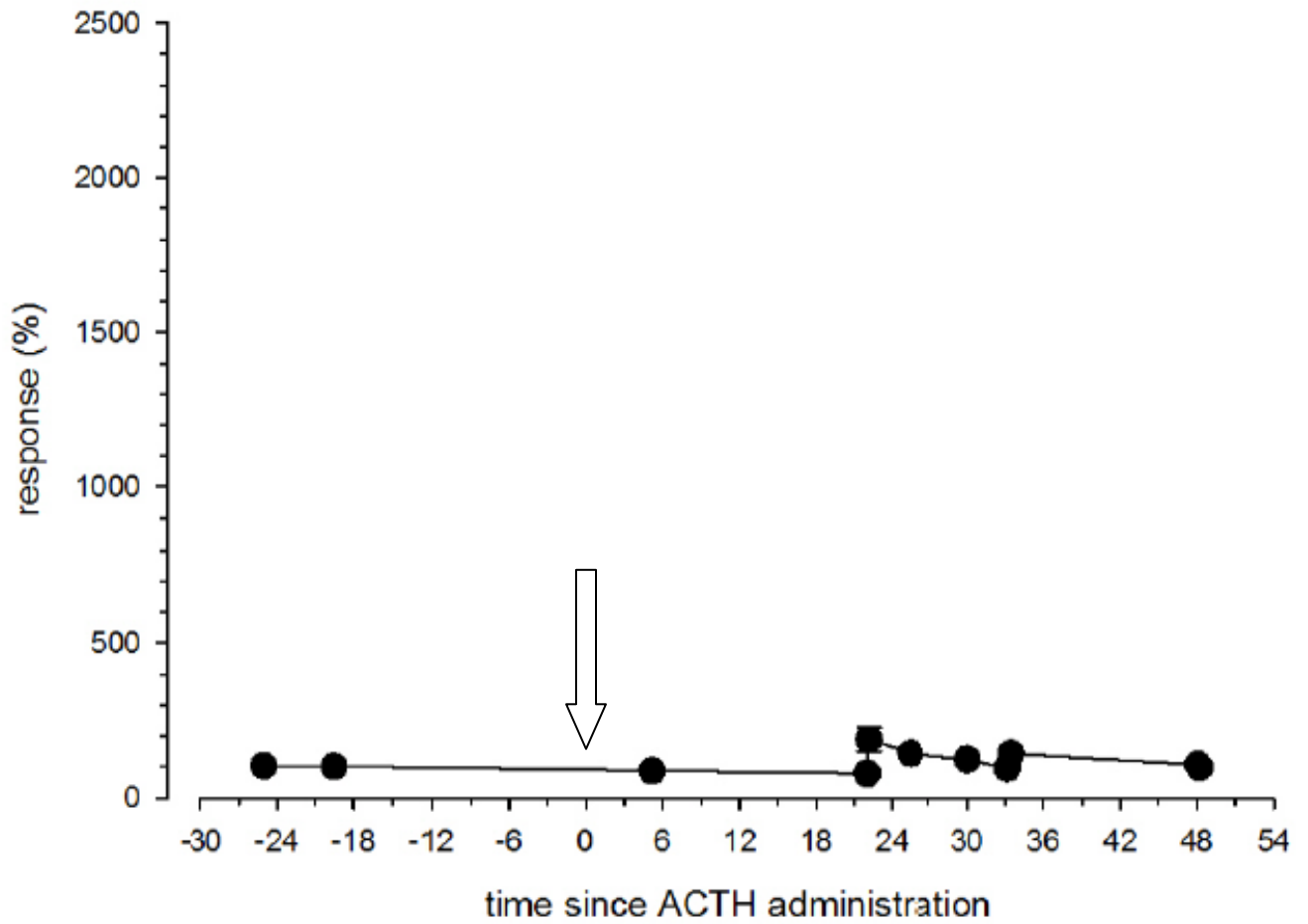Figure 16: The response displayed by the corticosterone (mean ± S.E.M) enzyme immunoassay in the female wildebeest following ACTH administration (arrow).

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4.2 Stability of fGCM concentrations post-defecation

When compared to concentrations measured from faeces collected and frozen directly after defecation (time = 0), the 11,17-DOA concentrations increased up to 12 % in samples collected and frozen 2 - 8 hours post-defecation (Figure 17). Mean 11,17-DOA concentrations dropped to 71 % after 16 hours, and to 44 and 30 %, of initial concentration, after 24 hours and 48 hours post-defecation, respectively. Faecal glucocorticoid metabolite concentrations from samples collected and frozen at 1, 2, 4 and 8 hours were no different to those directly after defecation, but samples collected and frozen 16 - 48 hours post-defecation were significantly lower (F = 35.72, P < 0.001, *post hoc* analysis: P < 0,001 - 0,011 for 16, 24 and 48 hours, respectively). The variation in fGCM concentration between triplicate subsamples ranged from 2 − 8 % for respective measuring points in time.
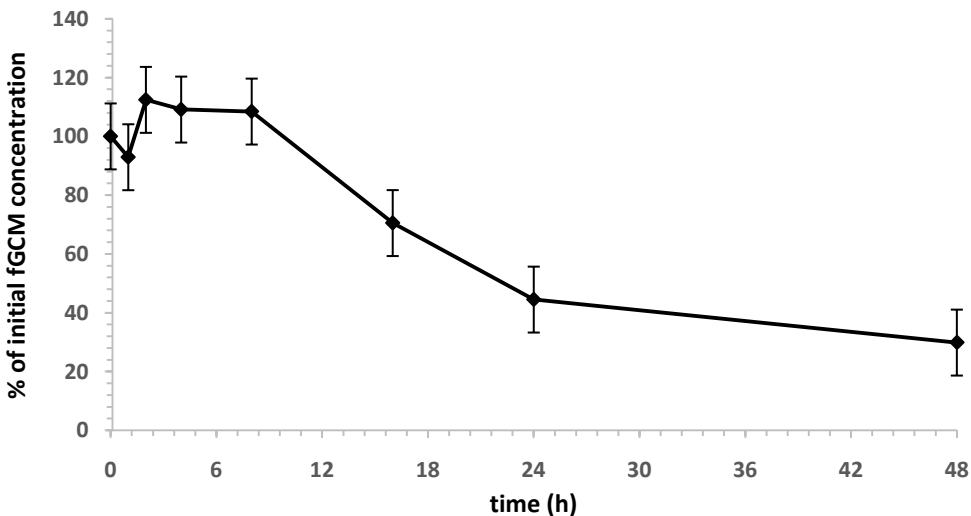
Figure 17: Relative change (%) of 11,17-DOA concentrations (mean ± S.E.M.) in blue wildebeest faeces over time (0, 1, 2, 4, 8, 16, 24, 48 hours post defecation)

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4.3 Comparison of fGCM concentrations between study groups

4.3.1 Influence of capture day

There was no statistically significant differences in fGCM concentrations measured from the faeces of free-ranging wildebeest that were captured on day 1 compared to day 2 (Mann-Whitney Test: $T_{16,26} = 357$; $P = 0.75$, Figure 18) from the same population at the same capture site.
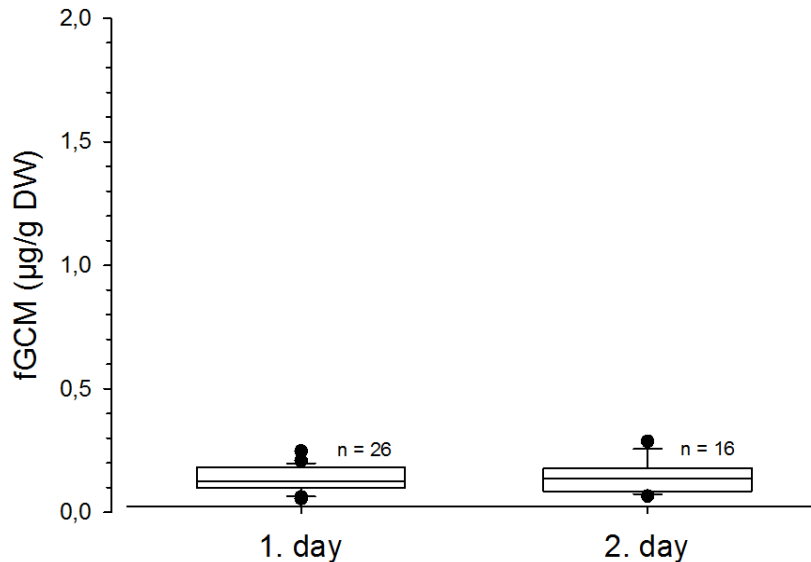
Figure 18: Boxplots of fGCM concentrations (determined by the 11,17-DOA EIA) of animals from one free-ranging population captured once on two consecutive days. Boxes show median, 25 and 75 percentiles, whiskers show 10/90 percentiles, and dots show outliers.

4.3.2 Influence of prolonged confinement

Faecal samples collected from wildebeest that were confined for 10 h on a truck post capture had a 1.5 fold elevation of fGCM concentrations compared to faecal samples collected from them directly after they were captured (Mann-Whitney test : $T_{16,21} = 176$; P <0.001 (Figure. 19).
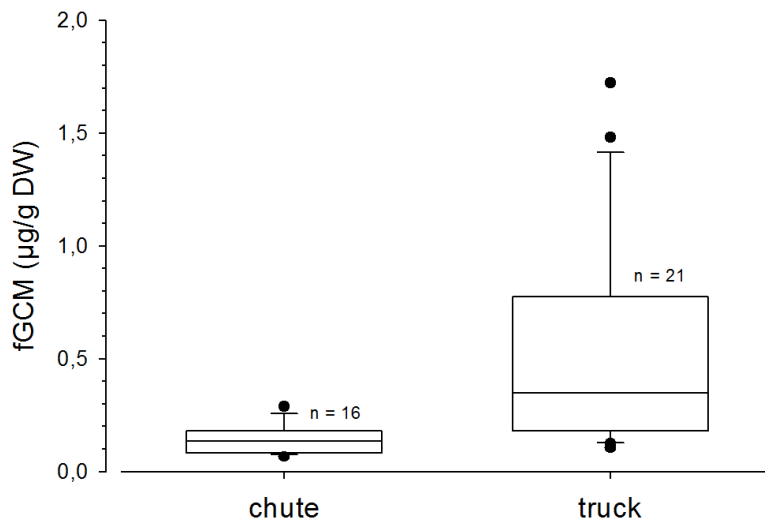
Figure 19: Boxplots of fGCM concentrations (determined by the 11,17-DOA EIA) from animals directly after capture, when standing in the chute, and subsequently after confinement for 10 hours on a vehicle. Boxes show median, 25 and 75 percentiles, whiskers show 10/90 percentiles, and dots show outliers.

Chapter 5. Discussion

The ACTH test in one male and one female blue wildebeest both resulted in an appropriate response, with elevations in fGCM concentrations above 100 % of pre-administration concentrations after ACTH injection. However, of the five EIAs used to detect changes in fGCM concentrations, all five showed an adequate response in the male, but only three did so in the female wildebeest. The EIA detecting 11,17 dioxoandrostanes showed the most distinct response to the ACTH challenge in both animals and thus seems to be most suited to monitor a stress response in wildebeest.

There was a distinct quantitative difference between fGCM 11,17-DOA in the studied male and female. In the male blue wildebeest, fGCM concentrations post ACTH challenge was 2126 % above baseline values and in the female the fGCM concentrations rose to only 474 % above baseline. It was clear from our study that the immature male blue wildebeest had much higher fGCM 11,17-DOA concentrations, and other metabolite differences, throughout the ACTH stimulation test, which like other studies indicates a sex difference in fGCM in this species too.

Males and females may differ in terms of their steroid metabolism (Touma & Palme 2005) and thus sex-related qualitative differences in response to an ACTH challenge can be expected. Several studies investigating fGCM in both sexes report higher concentrations in females (e.g. domestic dog (*Canis lupis*), mouse (*Mus musculus*), African wild dog (*Lycaon pictus*), cheetah), males (e.g. rat (*Rattus rattus*), Steller sea lion (*Eumetopias jubatus*), domestic chicken (*Gallus gallus*) or no difference between the sexes (e.g. wolf (*Canis lupis*), black rhinoceros, white rhinoceros (*Ceratotherium simum*) (Touma & Palme 2005). Different factors may be responsible for these sex-specific differences. Some females have higher plasma glucocorticoid concentrations because of a higher capacity of steroid-binding globulins expressing affinities to glucocorticoids (Breuner & Orchinik 2002). Palme et al. (2005) also showed that a larger fraction of metabolites are excreted via the urine of female, compared to male mice, horses and cats. The structure and quantity of fGCM formed may also differ significantly between males and females (Touma & Palme 2005). Because the non-specific binding of the antibodies used in

a given assay strongly depends on the biochemical structure of the steroid, these differences are likely to bring about different concentrations of fGCMs in males and females.

When testing stability of metabolite concentrations post-defecation, immunoreactive fGCM concentrations determined with an EIA measuring 11,17-DOA revealed only minor changes of up to 12 % after 8 hours, indicating a fair stability of this metabolite concentration for that time interval. However, at 16 hours post-defecation, fGCM concentrations dropped by 71 %, making an interpretation of other impacting variables rather challenging if faeces are collected at this time. Therefore, taking faecal samples greater than 8 hours after defecation may not provide an accurate measure of fGCM.

The early changes in fGCM concentrations post-defecation in the wildebeest were similar to studies in species like the bovine where there was an increase in measured cortisol metabolites after storage for 1 hour (Möstl et al. 2002). In porcine and equine there was only an increase in fGCM after 24 hours and 4 hours respectively (Möstl et al. 2002, Messmann et al. 1999). A recent study by Ganswindt et al (2012), determined that there was a significant increase in 11,17-DOA levels 2 hours after defecation in African buffalo (Ganswindt et al. 2012). Bacterial enzymes present in faeces may metabolize fGCM (Möstl et al. 2002, Millspaugh & Washburn 2004) and the resulting metabolites may or may not cross-react with the antibodies used in the different enzyme immunoassays thus causing either a decrease or increase in measured fGCM concentrations (Lexen et al. 2008).

In our study, overall the variance in fGCM concentrations between subsamples ranged from 2 – 12 %. This degree of variance has been seen in other similar studies and is regarded as acceptable (Ganswindt et al. 2012). This variance occurred despite our careful handling of samples and therefore highlights that an adequate amount of sample should be collected and it must be sufficiently handled and homogenized.

When we collected samples from free-living wildebeest directly after capture on two consecutive days, we found no significant difference in fGCM concentrations in the two groups. Therefore, the animals that were captured on the second day (21 hours after the first capture) did not perceive the capture of their counterparts, which were herded by a helicopter the day before, as a prolonged stressor. In contrast, fGCM concentrations of animals determined directly after

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capture and after confinement in a truck for 10 hours post capture, differed significantly, with higher levels after prolonged confinement. One possible explanation for this could be due to increased GIT transit time of fGCM during confinement (Palme et al. 2005).

Although studies measuring the stress response in animals due to herding by a helicopter have not yet been done, studies have been done to assess animal's stress responses due to capture, veterinary treatment and transportation. Dehnhard et al. (2001) determined that capture, veterinary treatment and transportation of roe deer (*Capreolus capreolus*), resulted in a 7.5-fold increase in faecal metabolites (Dehnhard et al. 2001). In gray mouse lemurs (*Microcebus murinus*), there was an average 3.3 fold increase from baseline over 3 days post capture and handling (Hämäläinen et al. 2013).

Since there is a lag time from determining stress-related changes in glucocorticoid concentrations in plasma and GCM concentrations in faeces due to gut passage time (Möstl et al. 2002, Palme et al. 1996, Palme et al. 2005) a stress response is usually only detected in faeces a number of hours post a stressor. The lag seen in the wildebeest was similar to those seen in other wildlife species (Palme et al. 2005).

Although we have shown that fGCM 11,17-DOA can be used in free-living blue wildebeest to indicate a stress response to a known stressor, further improvements to this aspect of the study could have been made which may have given us more information on the extent and the time course of this stress response. These improvements should include sampling from individuals rather than a pooled sample during capture and confinement, and more regular and longer sampling post-capture. Unfortunately, these improvements could not have been achieved from this part of the study as samples were collected from a capture event that was part of SANPark's normal capture operations. Therefore, more regular sampling and finer manipulations could not be made, and samples could only be collected opportunistically and follow-up samples could not be collected as these animals were sold and transported by private buyers. An improved study could have been achieved by getting more frequent samples from individuals over a longer period of time by placing animals in a boma directly after capture and transport.

A further definite limitation of our study was that during the ACTH stimulation test we only tested one male and one female. However, currently using a small subset of animals for ACTH

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testing is common practice in wildlife (Moberg & Mench 2000) due to practical and ethical reasons.

Increased welfare and conservation concerns for wildlife species have resulted in more studies determining the stress responses to different management practices (Ganswindt et al. 2012, Ganswindt et al. 2010). Capture of animals to obtain blood samples for cortisol measurement, however, causes stress and virtually none of the results obtained in this manner represent true resting baseline cortisol levels. However, non-invasive techniques using faecal glucocorticoids metabolites have become standard practice for measuring stress responses in domestic livestock (Palme & Möstl 1997) and wildlife (Sheriff et al. 2011, Schwarzenberger 2007, Bahr et al. 2000, Goymann et al. 1999, Graham & Brown 1996). These non-invasive techniques have the benefit of not inducing a stress response and therefore do not interfere with faecal cortisol metabolites concentrations measured, and are thus a better reflection of stress experienced by an animal.

Capture of wildlife species are dependent on a few factors like season, time of day, type of wildlife species and the area the wildlife occupy. Therefore capture operations often need to be extended over a number of days which means that the animals in the vicinity of a capture site may be disturbed particularly if a helicopter or other vehicles are used. No research has been done to determine if these vehicles, that are used during capture cause stress in animals that are in the vicinity of the capture but are not captured themselves. We made use of a mass capture technique with the help of a helicopter over two separate days and were able to assess the impact of the helicopter on the animals in the vicinity of capture site. From our results it was clear that the wildebeest either developed an acute stress response which was minor and short-lived or no stress response at all since the fGCM for day 1 and day 2 were similar.

When different capture methods are used to capture animals, we can expect different quantitative and qualitative physiological responses. Chemical immobilization of free-ranging grizzly bears by remote injection from a helicopter resulted in longer induction times and higher drug dosages than did chemical immobilization in bears captured and physically restrained by leg-hold snares prior to chemical immobilization (Cattet et al. 2003). In general, high circulating levels of catecholamines (adrenaline and nor-adrenaline) modify the effects of immobilization and increase drug requirements. Biochemical data from bighorn sheep obtained after four different capture methods (Drop-net, Drive-net, darting, net-gun) revealed that cortisol levels did not

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differ significantly in the animals caught by the different methods except between the drop-net capture and chemical immobilization methods (Kock et al. 1987). None of the four methods were without a stress response, but chemical immobilization caused the greatest stress response. Further studies need to be performed on African wildlife species to determine differences in stress responses to different capture methods used in Africa.

Several consequences exist when wildlife are captured. One of the most common side effects is an increase in body temperature during and after capture. This may lead to lethal hyperthermia. An increase in body temperature also increases cellular oxygen consumption (Meyer et al. 2008). In chemical immobilization, animals can have a negative oxygen balance from drug-induced hypoxia and a high metabolic demand from intense activity during escape attempts (Meyer et al. 2008). If cellular energy production cannot keep up with normal cellular needs, cellular function and integrity are disrupted, which can lead to organ failure and capture myopathy. In studies on impala it was shown that psychological stress is known to induce hyperthermia which causes an acute rise in body temperature (Meyer et al. 2008). Temperatures could not be monitored in the wildebeest in this study as we did not have direct access to the animals. However, it would be of interest in future studies to validate whether hyperthermia can also be used in wildebeest to determine an acute stress response to capture, as has been done in impala, and whether changes in fGCM can be correlated with these effects.

All capture procedures, whether done by chemical immobilization, mass capture or trapping are "stressful" for animals as they cause a disturbance resulting in a 'fright and flight' response which causes a disruption to their homeostasis. By assessing the stress response in a non-invasive way, we can get a clearer idea of which capture methods induce less stress in animals. This assessment has been done in multiple studies in some zoo and wildlife species (Knox et al. 1990, Ganswindt et al. 2012, Ganswindt et al. 2010, Meyer et al. 2008, Hattingh et al. 1990). The least stressful capture method can then be used to capture animals thereby limiting the side effects of capture, improving animal welfare and decreasing mortality. In our capture study the presence of a helicopter on day 1 did not appear to affect the stress experienced by the animals that were subsequently captured on day 2. However, capture caused a stress response in these animals that was detected by an increased fGCM concentrations measured at 10 hours after capture. Following these animals in the truck when they were confined and collecting samples

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after transport would have given us a better indication of their stress response to the combination of capture and prolonged confinement. Our results therefore indicate that blue wildebeest appear to tolerate the noise and presence of a helicopter in their environment without developing a stress response that can be detected using fGCM. However, in this species the capture procedure did elicit a stress response and therefore efforts to reduce stress should be focused on minimizing the duration and magnitude of this "stressful" stimuli caused by these procedures.

Despite blue wildebeest been commonly captured, the effects of capture in this species has not been studied much. Future studies should continue to focus on how to improve welfare during capture by investigating methods that reduce the stress response experienced by these animals. Whether long-acting tranquilizers and sedatives can be used to reduce the capture-induced stress response or the chronic stress response to long-term confinement in transport vehicles and bomas, also needs to be determined. Thus far there are several combinations of these sedatives and tranquilizers that have been anecdotally investigated for this purpose in several wildlife species (Taylor et al. 2015, Kock & Burroughs 2012). However, their efficacy has been poorly assessed and mainly human drugs have been used extra-label (off-label). Newer veterinary specific drugs are been developed and their efficacy in reducing these stress responses needs to be properly assessed.

Chapter 6. Conclusion

The ability to reliably assess adrenocortical function in blue wildebeest now provides a solid basis to further examine endocrine responses to putative stressful circumstances in this species. Potential applications could be the ability to measure potential variability of fGCM levels in natural populations of blue wildebeest facing ecological challenges such as shortages of grazing or water sources.

Our study on blue wildebeest has been the first of its kind. By determining which fGCM assay is the best to assess a stress response in this species, it can now be used by researchers, wildlife managers and veterinarians to accurately measure stress responses to capture and other management procedures in blue wildebeest. We have also shown that the presence of a helicopter during capture does not appear to have a lasting influence on wildebeest in the vicinity of a capture site, unless they are captured. The changes in fGCM concentrations induced by a mass capture involving a helicopter and a temporary boma capture system have also been characterized in blue wildebeest for the first time. This type of capture induces a stress response that could be reduced if further research in this field is done.

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Addendum

UNIVERSITEIT VAN PRETORIA
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Animal Ethics Committee

PROJECT TITLE	**Assessing the efficacy of the long-acting neuroleptic zuclopenthixol in impala (Aepyceros melampus) and wildebeest (Connochaetes gnou)**
PROJECT NUMBER	**V055-14**
RESEARCHER/PRINCIPAL INVESTIGATOR	**Dr. A de Haast**

STUDENT NUMBER (where applicable)	**240 25 705**
DISSERTATION/THESIS SUBMITTED FOR	**MSc**

ANIMAL SPECIES	**Impala (Aepyceros melampus)**	**Wildebeest (Connochaetes gnou)**
NUMBER OF ANIMALS	**30**	**32**
Approval period to use animals for research/testing purposes		**July 2014-July 2015**
SUPERVISOR	**Dr. L Meyer**	

<u>**KINDLY NOTE:**</u>

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

APPROVED	Date	28 July 2014
CHAIRMAN: UP Animal Ethics Committee	Signature	

UNIVERSITY OF PRETORIA

FACULTY OF VETERINARY SCIENCE

APPLICATION FOR APPROVAL OF TITLE OF DISSERTATION OR THESIS

PLEASE NOTE:

This form must be completed in **TYPING.**

Please send the application form to: **HEAD: ACADEMIC ADMINISTRATION.**

Name of candidate:	Dr Adel de Haast
Student number:	24025705
Degree:	MSc (Vet. Science)
Course code:	VWE 804
Department:	Paraclinical Sciences
Name of supervisor/Leader:	Prof Leith Meyer
Name of co-supervisor(s)/co-leader(s)	Prof Andre Ganswindt

Protocol approved : Yes ☐X No ☐ **Date submitted/approved:** _14/_8_/2014

Please attach a summary/abstract. **Protocol Number V055/14**

Title of dissertation/thesis:

Monitoring Adrenal Cortical function as a measure of stress in Blue Wildebeest (Connochaetes taurinus)

SIGNATURES OF:

CANDIDATE:		Date of submission: Sep 16
SIGNATURE OF SUPERVISOR/PROMOTER:		DATE: 14/4/16
APPROVED BY HEAD OF DEPARTMENT:		DATE: 14/4/16
APPROVED BY DEPUTY DEAN:		DATE: 30/05/16

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