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Evaluation of faecal glucocorticoid monitoring as a non-invasive assessment of stress in captive white rhinoceros (*Ceratotherium simum*) after ACTH stimulation.

By

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Submitted in partial fulfilment of the requirements for the degree

Magister Scientiae

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November 2007

To my mum.

Acknowledgements

I would like to thank:

My parents for giving me the chance to study for a Masters degree at the University of Pretoria and for their love and support throughout these years of study.

Dr. Joseph Chamunorwa and Professor Gerry Swan, my supervisor and co-supervisor for taking my project onboard, helping and guiding me to produce the best that I can.

The Smithsonian Institution for their invaluable assistance in processing my samples. I am forever grateful to them for accepting to help with the lab analysis and very much pushing my project forward.

Hans Koi and the team at Thaba Manzi Wildlife Services, Johannesburg Zoo and Douw Grobler and the crew at Hammanskraal for allowing me to use their animals and for their amazing assistance and support in collecting samples.

Lizette Becker for her encouragement, support and friendship throughout the many hours of boiling samples. As well as Dr. Cindy Harper for her time and assistance in the lab.

The Faculty of Veterinary Science of University of Pretoria provided funding for the project and the SAVF, South African Veterinary Foundation is acknowledged for funding laboratory costs.

And my very good friends for their fantastic advice and support, not to mention the conversations in the cafeteria which kept me smiling.

Declaration

I, L. Riato, do hereby declare that the research presented in this dissertation, was conceived and executed by myself, and apart from the normal guidance from my supervisors, I have received no assistance.

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

This dissertation is presented in partial fulfilment of the requirements for the degree MSc in veterinary research.

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Signed……..

L Riato

Date……..

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Abstract

Capturing and holding of white rhinoceros (*Ceratotherium simum*) for the re-introduction to new reserves or breeding in zoos often involves a risk of mortality. Non-invasive techniques to monitor the stress experienced by these animals may guide the selection of management techniques that reduce risks to animal well-being. The aim of the study was to evaluate the biological relevance of a developed technique to monitor stress hormone metabolites in faecal samples of wild-caught and captive-bred white rhinoceros. Faecal corticosterone concentrations were measured via radioimmunoassay (^{125}I RIA), in seven white rhinoceros (3 males and 4 females), at three sites, before and after an adrenocorticotropic hormone (ACTH) challenge test and control saline injection. Administration of ACTH resulted in a significant increase in faecal corticosterone concentrations (up to 350% above pre-ACTH baseline) within 1-2 days of injection, returning to baseline 4 days post-injection. It was found that individual baseline corticosterone concentrations fluctuate naturally and vary between individual animals, suggesting that an adequate baseline period of faecal sampling is needed in order to accurately assess responses to ACTH stimulation. Furthermore, the technique proved sensitive enough to detect elevations in faecal corticosterone concentrations due to environmental stressors. Data of faecal corticoid concentrations were correlated with gastrointestinal transit (GIT) times before and after ACTH and saline treatment by using art glitter as a digestive marker. This showed that gut passage times correlated to the ACTH-induced time to peak. Overall the results confirm that measurements of faecal corticosterone metabolites with the validated ^{125}I RIA is a useful diagnostic tool to monitor adrenocortical activity in white rhinoceros. This study can therefore provide a methodology for examining chronically heightened adrenal activity in these animals and consequently be used to inform management strategies that aim to improve the welfare of white rhinoceros in captivity.

CHAPTER 1

1. INTRODUCTION

The white rhino (*Ceratotherium simum*) exists in two subspecies, the northern (*Ceratotherium simum cottoni*) and the southern (*Ceratotherium simum simum*) white rhinoceros. Once ranging in large numbers throughout north-central Africa south of the Sahara, free-ranging northern white rhino populations have been reduced to only 30 individuals located in Garamba National Park in D.R.C.

In contrast to the critically endangered northern subspecies, the southern white rhinoceros is currently one of the most abundant surviving rhinoceros species. In 1895 only 20 southern white rhinos were left as a result of intensive hunting and the reclamation of land for agriculture. Since then, as a result of sustained and concerted efforts by various conservation agencies, populations have risen to a most recent estimate of 11,670 in 2001 (Brooks, 2002). A map of the current and historic distribution for *C. simum* is shown in Figure 1.1.

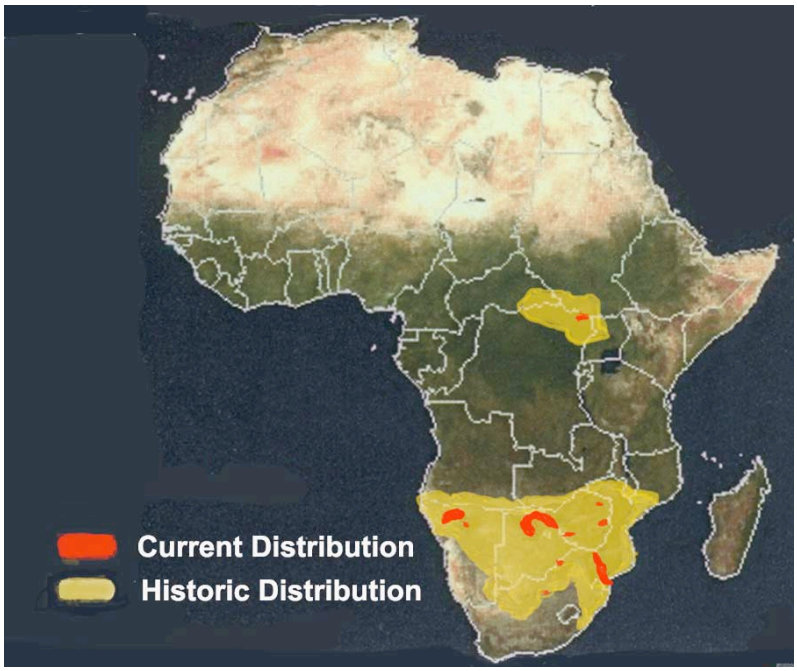


Figure 1.1 Map of Africa showing the current and historic distribution of *Ceratotherium simum*.

The World Conservation Union (IUCN) red list (Version 3.1, Friedmann and Daly, 2004) lists *C. simum simum* as Least Concern. 94% of the present wild population of *C. s. simum* lives in South Africa. Small populations also exist in Botswana, Namibia, Swaziland, Zambia and Zimbabwe (WWF, 2005).

The southern white rhino is relatively safe from future threats given the wide distribution throughout southern Africa and high number of individual populations. Although the population is now thought to be stable, illegal poaching for the horn of the animal, which in some Asian cultures is believed to possess medicinal properties, continues (Roth *et al.*, 2001). Future lack of conservation budgets and capacity could lead to increased poaching (Friedmann and Daly, 2004).

Anti-poaching operations are certainly a vital tool for the successful management of white rhino populations. However, conservation breeding in zoos and the re-introduction of these animals to new reserves to supplement the genetic stock of established populations are also essential strategies for their long-term survival. With wildlife capture being expensive, time consuming and often involving a risk of mortality, emphasis on the need to improve capture, holding and translocation techniques, both for humane and economic reasons is essential (Kapke *et al.*, 1999; Kock *et al.*, 1999). One of the major difficulties involved with capturing and holding white rhinos is to get the animals to feed (Booth and Coetzee, 1988; Player, 1967). About one third of white rhinoceros captured in the wild refuse to feed while they are temporarily housed in solid enclosures, locally referred to as bomas, until ready for translocation. Subsequently, these animals are released back into the wild at the great expense of game capture management.

An ideal indicator for unseen stress related issues is the level of physiological stress (Möstl and Palme, 2002), which may contribute to the non-habituation of white rhinos in bomas. Exposure to stressors can result in an increase in adrenocorticotropic hormone, ACTH, and subsequently an increase in circulating glucocorticoids (Touma and Palme, 2005). However, prolonged periods of high glucocorticoid concentrations

in response to chronic stressors such as game capture, may have biological costs (Carlstead and Brown, 2005).

Non-invasive techniques of measuring faecal metabolites of glucocorticoids has become a useful way to assess adrenal function in captive and wild species (Millspaugh and Washburn, 2004; Möstl and Palme, 2002; Wielebnowski *et al.*, 2002), offering the advantage that faeces can be easily collected while imposing minimal stress on the animal (Stead *et al.*, 2000). Thus, monitoring faecal corticoid concentrations can provide a useful measure of stress when evaluating the response of captured rhinos to changes in management strategies.

One of the strongest methods to physiologically validate non-invasive techniques are pharmacological stimulations of steroid hormone release (Goymann, 2005). Secretions of corticosteroids are pharmacologically induced by the administration of high doses of long-acting ACTH, which in turn will increase synthesis of corticosterone by the adrenals (Palme *et al.*, 2005). Examples of studies describing ACTH challenge experiments involving various species of mammals are compiled in Table 1.1. Although there have been many articles published dealing with faecal glucocorticoids in various species of mammals, convincing validation experiments have been performed on only a few species (Touma and Palme, 2005), white rhino not included (Table 1.1). Hence, the primary aim of the study was to evaluate the rate and extent of faecal corticosterone excretion after ACTH stimulation in captive bred and wild-caught southern white rhino.

White rhino were darted with ACTH with the assumption that corticosterone concentration would rise after ACTH stimulation. It was hypothesised that after ACTH stimulation, the faecal glucocorticoid concentrations, measured by a validated radioimmunassay (RIA) kit, would differ from baseline glucocorticoid concentrations. The appropriate null hypothesis was set: After ACTH stimulation, the faecal glucocorticoid concentrations, measured by a validated RIA kit, do not differ significantly from baseline glucocorticoid concentrations.

Table 1.1 Examples of studies describing experiments to physiologically and biologically validate techniques to measure faecal glucocorticoid metabolites as a non-invasive tool to assess adrenocortical activity in different species of mammals (Touma and Palme, 2005).

Species		Sample size				
Scientific name	Common name	Male (M)	Female (F)	Physiological validation (pharmalogical treatment)	Biological validation (challenging procedure / situation/ condition)	Ref.
Primates						
Macaca fascicularis	long-tailed macaque		10	ACTH (data for 6 shown)		Wasser *et al.* (2000)
Papio cynocephalus	yellow baboon		2	ACTH		Wasser *et al.* (2000)
Rodentia						
Mus musculus f.domesticus	laboratory mouse	36	36	ACTH	saline injection, diurnal variation (6M/6F, each)	Touma *et al.* (2004)
Rattus norvegicus f.domesticus	laboratory rat	24		ACTH	saline injection, handling (6M, each, no effects)	Bamberg *et al.* (2001)
Carnivora						
Crocuta crocuta	spotted hyena	4	2	ACTH	translocation (1M) agonistic interactions (1M/1F)	Goymann *et al.* (1999)
Ursus acrtos horribilis	grizzly bear	1	1	ACTH		Hunt and Wasser (2003)
Helarctos malayanus	Malayan sun bear		1	ACTH		Wasser *et al.* (2000)
Ursus thibetanus	Himalayan black bear	1		ACTH	aggression (1F), no consistent effects	Young *et al.* (2004)
Canis lupus *f.familiaris*	domestic dog	5	5	ACTH		Schatz and Palme (2001)
Canis lupus	wolf		2	ACTH (data not shown)		Sands and Creel (2004)
Canis rufus	red wolf	1		ACTH	various stressors (1M/1F), inconsistent effects	Young *et al.* (2004)
Lycaon pictus	African wild dog	2	3	ACTH		Monfort *et al.* (1998)
Acinonyx jubatus	cheetah	2	2	ACTH		Terio *et al.* (1999)
		2	2	ACTH (data for 1 shown)		Wasser *et al.* (2000)
			1	ACTH	anesthesia	Young *et al.* (2004)

Table 1.1 (*continued*) Examples of studies describing experiments to physiologically and biologically validate techniques to measure faecal glucocorticoid metabolites as a non-invasive tool to assess adrenocortical activity in different species of mammals (Touma and Palme, 2005).

Species		Sample size				
Scientific name	Common name	Males (M)	Females (F)	Physiological validation (pharmalogical treatment)	Biological validation (challenging procedure / situation/ condition)	Ref.
Neofelis nebulosa	clouded leopard	2	2	ACTH		Wielebnowski *et al.* (2002)
		2	1	ACTH	anesthesia (1F)	Young *et al.* (2004)
		3	2	ACTH (data for 1 shown)		Wasser *et al.* (2000)
Felis silvestris f.catus	domestic cat	5	5	ACTH		Schatz and Palme (2001)
		1	1	ACTH	anesthesia (1F), no clear effect	Young *et al.* (2004)
Suricata suricata	slender-tailed meerkat	2		ACTH		Young *et al.* (2004)
Enhydra lutris kenyoni	Alaskan sea otter	1		ACTH		Wasser *et al.* (2000)
Mustela nigripes	black-footed ferret	4	6	ACTH	restraint, saline injection	Young *et al.* (2001)
Eumetopias jubatus	Stellar sea lion	1	2	ACTH (faeces & plasma)		Mashburn & Atkinson (2004)
Proboscidea						
Loxodonta africana	African elephant	1		ACTH		Ganswindt *et al.* (2003)
			4	ACTH (faeces & plasma)	effects of enclosure size	Stead *et al.* (2000)
			2	ACTH (data for 1 shown)		Wasser *et al.* (2000)
Perissodactyla						
Equus caballus	domestic horse	3	3	ACTH (faeces & plasma)		Möstl *et al.* (1999)
Dicernos bicornis	black rhinoceros	4		ACTH		Brown *et al.* (2001)
		1	1	ACTH (data for 1 shown)		Wasser *et al.* (2000)

Table 1.1 (*continued*) Examples of studies describing experiments to physiologically and biologically validate techniques to measure faecal glucocorticoid metabolites as a non-invasive tool to assess adrenocortical activity in different species of mammals (Touma and Palme, 2005).

Species		Sample size				
Scientific name	Common name	Male (M)	Female (F)	Physiological validation (pharmalogical treatment)	Biological validation (challenging procedure / situation/ condition)	Ref.
Artiodactyla						
Bos taurus	cattle	3	3	ACTH (3 doses)		Palme *et al.* (1999)
		5		ACTH (faeces & plasma)		Morrow *et al.* (2002)
Ovis ammon f.aries	domestic sheep	3	3	ACTH (faeces & plasma)		Palme *et al.* (1999)
Cervus elaphus	elk/red deer	6		ACTH	saline injection	Huber *et al.* (2003)
Cervus elaphus roosevelti	Roosevelt elk	1	1	ACTH (data for 1 shown)		Wasser *et al.* (2000)
Litocranius walleri	gerenuk	2		ACTH (data for 1 shown)		Wasser *et al.* (2000)
Oryx dammah	Scimitar-horned oryx	2		ACTH (data for 1 shown)		Wasser *et al.* (2000)
Sus scrofa f.domestica	domestic pig	3	3	ACTH		Möstl *et al.* (1999)

Additionally, a control experiment was performed to investigate effects of the injection procedure itself. Animals were darted with saline solution, following the exact experimental design to the ACTH challenge experiments. It was hypothesised that animals treated with saline would show an increase in faecal glucocorticoid excreted from the baseline glucocorticoid concentrations. A suitable null hypothesis was appointed: After saline injection, the faecal glucocorticoid concentrations, measured by a validated RIA kit, do not differ significantly from baseline glucocorticoid concentrations.

Moreover, it was hypothesised that animals treated with saline would show faecal glucocorticoid excretion patterns similar to those observed after ACTH stimulation. However, peak values of faecal glucocorticoid concentrations excreted should be lower than when animals are injected with ACTH. A further null hypothesis was established: After saline injection, faecal glucocorticoid concentrations excreted will not differ significantly from the faecal glucocorticoid concentrations excreted post ACTH injection.

The next step of the study was to relate the profile of corticosterone excretion with gastrointestinal transit time using digestive markers in the same captive bred and transiently housed white rhinos. Digestive markers were used to estimate the transit time within the digestive tract. Known gut passage times could therefore be related to the faecal glucocorticoid metabolite excretion pre and post ACTH and saline treatments. This may provide valuable information on the digestive pattern of white rhinos with differing animal management techniques. The final null hypothesis was set: The gastrointestinal transit time has no significant effect on the pattern of faecal corticosterone excretion.

Managing and feeding rhinos in enclosures is costly as is the value of each individual animal. Evaluating a non-invasive technique to monitor stress could conceivably be used in the future to monitor the success of drugs or management techniques in captured or captive bred white rhinos. The objective of the study was therefore, to evaluate a non-invasive technique to assess stress in white rhinos by pharmacologically inducing physiological changes in circulating glucocorticoid concentrations using an ACTH challenge test, and to evaluate whether these changes were reflected in measured concentrations of faecal glucocorticoids after treatment, using a validated RIA kit.

Furthermore, to analyse any possible links between stress and digestive rates in white rhinos, data of faecal corticoid concentrations was correlated with gastrointestinal transit times before and after treatments. Information on the digestive pattern of white rhinos in captivity can be used for future developments in dietary recommendations to improve their wellbeing. Hence, this research not only provides health and welfare benefits for these animals but is of economical importance to parks, zoos and game sanctuaries too.

CHAPTER 2

2. LITERATURE REVIEW

2.1 Introduction

Faecal steroid analysis are becoming increasingly popular amongst both field and laboratory scientists. The benefits associated with sampling procedures that do not require restraint, anaesthesia, and blood collection include less risk to subject and investigator, as well as the potential to obtain endocrine profiles that are not influenced by the sampling procedure itself (Möstl *et al.*, 2005).

However, although sampling may be relatively easy to perform and free of feedback, a careful consideration of various factors is necessary to achieve proper results that lead to sound conclusions (Palme, 2005). In this review, general analytical issues regarding sample collection and storage, transit time, extraction procedures, and immunoassay selection are discussed. Thus, a careful consideration of the techniques used will ensure that the measurement of faecal glucocorticoids can be used as a useful diagnostic tool to assess adrenocortical activity in white rhino.

2.2 The Concept of Stress

Defining the term 'stress' has led to extensive debates amongst scientists (Pottinger, 1999). W.B. Cannon originally described the concept of stress in the early 20th century and defined the 'fight or flight' response to a perceived threat (Sapolsky *et al.*, 2000). Seyle (1973) used the term *stress* to refer to the response itself and the word *stressor* to refer to the stimuli that evoked the stress. Thus, he considered stress as a reaction involving many systems of the body, and termed this progressive series of reactions the General Adaptation Syndrome. However, Rushen (1986) maintains that Seyle's term *stressor* did not give any definition independent of its ability to elicit the response**.** He further states that the terms *stress* and *stressor* are merely convenient shorthand descriptions that add nothing further to a description of the physiological response itself and of the events that produced the response.

Developments in the stress concept resulted from the work of Mason (1971). He reviewed evidence, that part of the stress response was the result of emotional arousal elicited by psychological stimuli such as novelty or anticipation. Hence, the study of stress moved from the field of physiology to psychophysiology. Mason implied that a variety of events produced the same physiological response in the animal because they induced the same emotional response. Continuing these studies, Dantzer and Morméde (1983) suggested that it is the subjective experience of the animal that results in the physiological response and that these subjective experiences must be unpleasant in order to elicit the response.

Caution is required when using behaviour as an index of stress. An animal that is described as under stress might be expected to behave in a certain way (Toates, 1995). For instance, the stressed animal might make repeated attempts to escape from its situation. It might pace up and down in a ritualistic fashion (Mason, 1991). Or in the case of some freshly captured rhinos placed into an enclosure, it might go into a corner and stand apathetically, ignoring such needs as food and water. It is clear that there is no behaviour response that can be used as an indicator of stress (Toates, 1995). Any behaviour needs to be evaluated firstly, what the animal achieves by this behaviour, in terms of both the external environment and its internal state; and secondly, what would happen if the animal was prevented from behaving in this way (Toates, 1995). 'Abnormal' behaviour might be indicative of stress, but the animal might be even more stressed if it was kept in a similar environment but failed to 'discover' such behaviour, or was prevented from showing it (Mason, 1991).

From the viewpoint of a physiological index of stress, Rushen (1986) believes that changes in plasma cortisol concentrations can be used as an indicator of the degree of stress in animals when they are subjected to unpleasant treatment. However, he also states that a rise in cortisol concentrations may be the result of the animal being exposed to a novel and unpredictable event. On the other hand, Möstl and Palme (2002) consider the response characterised as stress to be a physiological mechanism, which results from a stimulus that is not necessarily unfavourable. Cortisol is released in response to situations that are not normally regarded as stressful, including courtship, copulation and

hunting. The fact that physiological adaptations are commonly observed in apparently healthy animals suggests that a more sensitive means of assessing welfare should be used (Dawkins, 1980). Hence, the use of the term *stress* and the physiological responses observed when an animal responds to a *stressor* must be interpreted carefully, especially when applied to animal welfare (Rushen, 1986).

2.3 Stress hormones

2.3.1 Anatomy of the Adrenal Cortex

The adrenal gland, cranial to the kidney, consists of the outer cortex, and inner medulla. It is contained within a capsule of connective tissue. In turn the adrenal cortex has three histological distinct zones: the outer *zona glomerulosa* (the site of mineral corticoid production); the central *zona fasciculate;* and inner *zona reticularis*; the latter two mainly responsible for glucocorticoid synthesis (Buckle, 1983). In addition to the two major groups of steroid hormones, the gland also produces small quantities of sex steroids, principally in the *zona fasciculata* and *zona reticularis* (Buckle, 1983).

2.3.2 Metabolism and Excretion of Glucocorticoids

Within seconds of the body detecting a physical or psychological stressor, the hypothalamus releases corticotrophin-releasing hormone, CRH, into the hypopheseal portal blood supply (Engelmann *et al.*, 2004; Sapolsky *et al.*, 2000). CRH travels to the pituitary gland stimulating it to release adrenocorticotropic hormone, ACTH (Engelmann *et al.*, 2004; Sapolsky *et al.*, 2000). In mammals, the adrenal cortex is stimulated by ACTH to secrete glucocorticoids (Möstl and Palme, 2002). Circulating glucocorticoids vary amongst species as cortisol, cortisone or corticosterone and are metabolised in the liver and excreted via the urine and bile conjugated to glucuronic acid (Morrow *et al.*, 2002). Intestinal bacteria in the gut deconjugate most of the metabolites, which are then partially reabsorbed into the blood (Palme *et al.*, 1996). Steroids, which are not reabsorbed, are eliminated via faeces. In most species, including the white rhinoceros, steroid metabolites are predominantly excreted in the faeces as deconjugated forms (Schwarzenberger *et al.*, 1996; Hindle and Hodges, 1990). Hindle and Hodges (1990) showed that 92.4% of steroids are present as deconjugates in the faeces of white rhinos.

Furthermore, Turner *et al.* (2002) found faeces in the same species to yield corticosterone: cortisol ratios of 2.2:1.0 respectively.

2.3.3 Effects of Glucocorticoids

Physiological and behavioural stress responses are initiated and coordinated by the central nervous system. The stress responses can be broadly sub-divided into two effector pathways – the autonomic nervous system and the neuroendocrine system (Moberg, 2000). Stimulation of the sympathetic branch of the autonomic nervous system results in the release of catecholamines, eliciting a 'fight or flight' response (Wiepkema and Koolhaas, 1993). The glucocorticoids are so-called because they stimulate hepatic gluconeogenesis, increasing the production of carbohydrates from amino acids, and in so doing, liberating energy (Sapolsky *et al.*, 2000). With an increase in blood glucose concentrations, as well as an increase in the breakdown of fats, known as lipolysis, the effects of glucocorticoids give rise to a provision of readily metabolisable fuels, thus preparing the body for action (Toates, 1995).

The 'fight or flight' response can be described as short-term stress if it is of relatively short duration and allows the animal to remove itself from a stressor (Wiepkema and Koolhaas, 1993). However, when circumstances do not allow an appropriate behavioural reaction, the sympathetic tone and subsequently the concentration of glucocorticoids, can be elevated for long periods. This is a state of chronic stress (Moberg, 2000).

Chronic stress, more specifically, heightened concentrations of glucocorticoids, ACTH, and corticotropin releasing hormone can have detrimental effects, including immunosuppression and loss of muscle mass (Creel, 2005). Glucocorticoids stimulate the breakdown of muscle into amino acids and decrease protein synthesis rates, both of which result in muscle atrophy (Young *et al.*, 2004).

Elevated concentrations of glucocorticoids resulting from chronic stress may also cause depression, gastrointestinal ulceration, circulatory disorders, inhibition of growth and a decrease in reproductive success (Young *et al.*, 2004; Sands and Creel, 2004). The effects of chronic stress on the reproductive system of mammals may be manifested in a number

of ways including, delays in the onset of puberty, behavioural alterations, failure or delay in ovulation, spontaneous abortion or infant mortality and reduced fecundity (Pottinger, 1999).

2.3.4 Post Capture Conditions

Abnormal external influences such as capture, transportation and handling of wild and domestic animals can result in capture myopathy. This non-infectious condition is characterised by damage to skeletal and cardiac muscles and associated with physiologic imbalances following extreme exertion, struggle and stress (Williams and Thorne, 1996). Basson and Hofmeyr (1973) best documented the occurrence of capture myopathy in African ungulates, and the black rhinoceros, *Diceros bicornis*. Unwillingness to feed (Booth and Coetzee, 1988), along with other common post capture conditions including hyperthermia, profuse sweating, and hyperventilation (Smith *et al.*, 1996; Ebedes, 1993) are specifically notable in the rhinoceros (Basson and Hofmeyr, 1973).

Exposure to the above stressors such as translocation can be detected as increases in glucocorticoid secretion (Carlstead *et al.*, 1993a). Hence fluctuations of adrenocortical concentrations in an animal can be used as a useful diagnostic tool to measure the response to the animal being handled (Carlstead *et al.*, 1993a).

2.3.5 Methods of Measuring Corticosteroids

While various physiological signals are widely accepted as being appropriate indicators of stress levels in captive individuals, the sampling protocols themselves often superimpose additional stress on the animals, thus confounding the results (Miller *et al.*, 1991; van Heerden and Bertschinger, 1982).

Plasma cortisol concentrations have been widely used as an indicator of the stress related to capture and translocation (Kock *et al.*, 1999; Smith *et al.*, 1996; Morton *et al.*, 1995). Morton *et al*. (1995) collected blood during capture and translocation from 18 wildlife species including the white rhinoceros. Cortisol concentrations in the white rhino increased significantly as a result of the stress of capture (Morton *et al.*, 1995).

A further study by Kock *et al.* (1999) showed haematological data during routine translocation of free ranging black rhinos in Kenya. A variety of adverse effects were demonstrated on these animals after captivity and translocation, with severity of effects related to time in transit.

Since frequent blood sampling is not feasible without the use of capture drugs in most free-ranging wild animals, this would obviously and unavoidably affect cortisol concentrations (Stead *et al.*, 2000). This was observed by Reinhardt *et al.* (1991) who measured serum cortisol concentrations in adult rhesus macaques (*Macaca mulatta*) comparing values between samples collected by venipuncture in restraint apparatus versus in their home cages. Alternatively, non-invasive techniques of sampling and determining urine glucocorticoids have been investigated in domestic (Pol *et al.*, 2002; Graham and Brown, 1996; Jones *et al.*, 1990) and non-domestic species (Owen *et al.*, 2004; Brown *et al.*, 1995; Carlstead *et al.*, 1993a; Carlstead *et al.*, 1992), offering the advantage that they can be easily collected while imposing minimal stress on the animal. However, urine samples are usually difficult to collect and may often require some manipulation of individuals (Möstl and Palme, 2002). Furthermore, supplementary time-consuming procedures are sometimes necessary to separate urine from the soil (Brown *et al.*, 2001; Brown, 2000).

Alternatively, the collection of faecal samples offers several advantages. Faecal collection is more reliable and practical, particularly when dealing with free-ranging animals (Stead *et al.*, 2000) and sampling is feedback free because there is no need to capture and handle the animal (Möstl *et al.*, 2005). Therefore, repeated sampling of the same individual is possible without affecting the animal's behaviour or its endocrine status (Touma and Palme, 2005). This method allows the monitoring of short-term hormonal changes in reaction to specific situations or social encounters (Touma and Palme, 2005), as well as assessing long-term endocrine profiles (Carlstead and Brown, 2005) or in this case, day-to-day hormonal changes to treatments.

In addition, circulating hormone levels in the faeces are integrated over a certain period of time (Palme, 2005; Goymann, 2005; Palme *et al.*, 2005). Hence, faecal hormone

metabolite concentrations reflect the production rate, that is, the cumulative secretion and elimination of hormones, over several hours (Palme *et al.*, 2005; Goymann, 2005). Therefore, unlike blood samples, faecal samples are less affected by episodic fluctuations or the pulsatility of hormone secretion (Möstl and Palme, 2002). Depending on the question asked, an integrated measure of steroid concentrations over a longer period of time may be more desirable than a plasma point in measure (Goymann, 2005). Conversely, dampening of short peaks of hormone secretion can also be a disadvantage if faecal samples are used to monitor these short-term changes (Touma and Palme, 2005; Goymann, 2005).

2.3.6 Extraction Procedures

Prior to analysis, faecal glucocorticoid metabolites must be extracted from the faecal matrix (Touma and Palme, 2005). As nearly all faecal corticosterone metabolites in white rhinos have a polarity similar to unconjugated steroids (Hindle and Hodges, 1990; Schwarzenberger *et al.*, 1996), the selection of an appropriate extraction procedure is essential (Goymann, 2005; Möstl *et al.*, 2005; Whitten *et al.*, 1998). Various studies have recommended extracting faecal steroid metabolites simply by suspending and shaking a certain amount of homogenised faeces; 0.5g of wet or dry faeces in 5mL of 80% methanol yielded highest recoveries for virtually all species tested so far (Möstl *et al.*, 2005; Palme, 2005).

Alternatively, Wasser *et al*. (2000), described an extraction system based on 0.2 g fully lyophilised, powdered faeces, boiled in 90% ethanol: 10% distilled water that has proved successful in many species, including white and black rhinoceros (Brown *et al.*, 2001). Brown *et al*. (2001) extracted corticosterone metabolites successfully by boiling in 90% ethanol: 10% distilled water followed by centrifuging and re-extraction of the pellet by vortexing and re-boiling in 90% ethanol: 10 % distilled water. The two extraction methods – wet faeces/methanol or dried faeces/ethanol, both seem to yield good recovery levels (Möstl and Palme, 2002; Wasser *et al.*, 2000).

2.3.7 Fluid Variability and Dietary Effects on Steroid Concentrations in Faeces

Conflicting results have been reported concerning the need to eliminate the effect of fluid variability on steroid concentration in the faeces (Ziegler *et al.*, 1996). Bamberg *et al.* (1991) reported that diarrhoea in gorillas did influence estrogen concentrations. He states that conditions like severe diarrhoea can alter the concentrations of steroids in faeces by substantially increasing the volume of output and proportionally decreasing estrogen concentration. Even less severe or obvious changes in the solid-liquid ratio in faeces, could cause variability.

Shideler *et al.* (1993) found that diarrhoea did not reduce the steroid concentrations in daily, morning faecal samples from captive cynomolgus monkeys maintained in individual cages. He believes that a 'better' relation between desiccated sample mass and excreted hormone concentrations is no more valid than for direct analysis of wet faecal mass; fluctuations in dry biomass and commonly suggested indexing compounds, such as bile salts, are just as independent with respect to steroid excretion as is water. Nevertheless, Shideler *et al.* (1993) does maintain that diets for free-ranging animals may offer more variation and consequently necessitate an approach to compensate for variations in faecal concentrations.

Ziegler *et al.* (1996) also suggests that fluid removal from the faeces does not alter steroid profiles, since steroid concentrations between frozen and lyophilised faecal samples are highly correlated.

On the other hand, Wasser *et al.* (1993) found that lyophilizing faecal samples from yellow baboons to correct for fluid variability, improved faecal to serum correlation of steroid concentration. As previously discussed in 2.2.2**,** in most species including the white rhinoceros, voided faeces contain a higher percentage of free than conjugated steroids (Schwarzenberger *et al.*, 1996; Hindle and Hodges, 1990) and therefore, are relatively hydrophobic (Wasser *et al.*, 1993). For this reason, Wasser *et al.* (1993) lyophilised samples to stabilize steroid concentration in relation to faecal weight and thus, exclude any possibility of variability due to differences in the proportion of fluid to solid faecal material.

Brown *et al.* (1994) tested the potential of shortening the faecal extraction process by eliminating the drying and pulverising steps. Although there were significant positive correlations between steroid metabolite levels in wet and dry faecal samples in wild felids, data were variable. This may have been influenced by the faecal steroid distribution, which was found to be uneven throughout a sample (Brown *et al.*, 1994).

In addition, undigested grass material can be removed easily from the faecal powder, after lyophilisation. Hence, expressing samples on a per-gram dry-weight basis seems to be more advantageous (Kretzschmar *et al.*, 2004), especially when diets are variable (Wasser *et al.*, 1993), or in cases of gastrointestinal distress (Brown and Wildt, 1997).

Diet variability may also be considered a cause of variance in faecal steroid concentrations (Millspaugh and Washburn, 2003; Möstl and Palme, 2002). Wasser *et al*. (1993) reported a decrease in faecal progestogen concentrations as dietary fibre increased, in captive baboons. This may have occurred because de-conjugated steroid excretion decreased as increased dietary fibre reduced gastrointestinal transit time, and consequently increased the proportion of conjugated steroid (Wasser *et al.*, 1993).

In addition, the influence of feed intake can also be regarded a cause of variance in faecal steroid concentrations (Möstl and Palme, 2002). Nevertheless, Rabiee *et al*. (2001) demonstrated that different amounts of grass intake of dairy cows did not influence faecal progesterone metabolite concentrations significantly.

2.3.8 Faecal Hormone Assays

Non-invasive methods of measuring faecal steroid metabolites to assess an animal's endocrine status were pioneered in the late 1970's in birds (Czekala and Lasley, 1977) and early 1980's in mammals (Möstl *et al.*, 1984; Möstl *et al.*, 1983). During the past two decades, non-invasive techniques have been established in an increasing number of species, ranging from laboratory animals, companion and farm animals, to wild animals in zoo and in the field (Touma and Palme, 2005). More specifically**,** it constitutes an important and useful diagnostic tool for the monitoring of reproductive activity of free-range black (Garnier *et al.*, 2002; Garnier *et al.*, 1998) and white rhinoceros

(Kretzschmar *et al.*, 2004), investigating reproductive endocrinology in the Indian (Schwarzenberger *et al.*, 2000) Sumatran (Roth *et al.*, 2001; Heistermann *et al.*, 1998), white (Radcliffe *et al.*, 1997; Hindle and Hodges, 1990) and black rhinoceros (Radcliffe *et al.*, 2001; Berkeley *et al.*, 1997; Schwarzenberger *et al.*, 1993), and the assessment of stress in black and white rhinos (Carlstead and Brown, 2005; Turner *et al.*, 2002).

Non-invasive methods are now widely used to investigate hormone-behaviour relationships as well as questions in the fields of reproduction, animal welfare, ecology, conservation biology and biomedicine (Palme *et al.*, 2005; Touma and Palme, 2005; Millspaugh and Washburn, 2004; Wasser *et al.*, 2000; Schwarzenberger *et al.*, 1996). However, until recently, assays for faecal glucocorticoid metabolites have proven difficult to develop compared to those used to measure reproductive steroids (Wasser *et al.*, 2000). Faeces contain multiple glucocorticoid metabolites because the main glucocorticoids, cortisol and corticosterone are rapidly and extensively metabolised before excretion (Bahr *et al.*, 2000). Moreover, there are considerable species-and sex-specific differences in the types of glucocorticoid metabolites formed, resulting in a characteristic pattern of glucocorticoid metabolites present in the faeces of a given species (Möstl *et al.*, 2005; Palme *et al.*, 2005). Therefore, it is crucial to select an appropriate assay system that includes an antibody capable of detecting most, or at least a considerable proportion of the respective glucocorticoid metabolites in the faeces of the species investigated (Goymann, 2005; Millspaugh and Washburn, 2004).

Turner *et al.* (2002) has compared concentrations of cortisol and corticosterone in white and black rhino under various conditions of captivity, including translocation. High-performance liquid chromatography (HPLC) was used to determine the presence of different faecal metabolites, however these results were not correlated to an immunoassay concentration.

Wasser *et al.* (2000) investigated three different commercially available radioimmunoassays (RIA) for cortisol and one assay for corticosterone in a variety of wildlife mammals, including the black rhino for the measurement of faecal cortisol metabolites. For corticosterone and cortisol tested with the corticosterone antibody (MP

corticosterone, formerly ICN Biomedicals), cross-reactivity of 100% was measured for corticosterone and less than 1% for cortisol.

Brown *et al*. (2001) developed an RIA analysis method to measure adrenal activity in four black rhinos after administering a slow release ACTH gel (800 IU custom preparation; Medicine Shop, VA) using a pole syringe. Two RIA's were used for assessing corticoid activity, a solid phase ^{125}I RIA for cortisol (Diagnostic Prod. Corp.) and a double-antibody ^{125}I RIA which measured corticosterone (MP Biomedicals, formerly ICN Biomedicals). Detectable concentrations of corticoids in faecal extracts after ACTH were only measured when the corticosterone assay was used.

The MP corticosterone antibody has been shown to exhibit good cross-reactivity to faecal metabolites in a range of species, including carnivores (Young *et al.*, 2004; Wielebnowski *et al.*, 2002; Monfort *et al.*, 1998; Graham and Brown, 1996, 1997), birds and herbivores (Goymann, 2005; Möstl *et al.*, 2005; Wasser *et al.*, 1997; Wasser *et al.*, 2000). These studies suggest that the MP corticosterone antibody reliably detects ACTH-induced adrenal activation in the faeces of black rhinoceros. This has not been confirmed in white rhinos.

2.3.9 Collection and Storage of Faecal Samples

Sampling and storing is an important issue for steroid analysis. Any problems arising during this process usually cannot be compensated for by analytical skills afterwards (Möstl *et al.*, 2005). For example, if glucocorticoid metabolites are not evenly distributed throughout the entire faecal mass, intra-sample variability may be high, and subsequently may affect one's ability to detect treatment effects (Millspaugh and Washburn, 2004). Wasser *et al*. (1996) examined the distribution of radio-labelled estrogen and progesterone metabolites in African elephant faeces and found concentrations were higher on the outside of the sample compared to the inside. He concluded that this could be reduced by extracting well-mixed faecal powder from freeze-dried samples, taken from the central part or premixed portion of the entire wet sample (Wasser *et al.*, 1996). In the rhinoceros, collecting samples from the central part of a bolus or premixing wet faeces has provided satisfactory homogenous samples for hormone metabolite analysis

(Galama *et al.*, 2004; Kretzschmar *et al.*, 2004; Wasser *et al.*, 2000; Heistermann *et al.*, 1998).

Faecal glucocorticoids are reported to be further metabolized by bacterial enzymes after defeacation, hence, time of collection and time until analysis can influence the steroid concentrations. (Möstl *et al.*, 2005; Palme *et al.*, 2005). For this reason, the best option is to collect a sample shortly after defeacation and to freeze it immediately (Palme, 2005).

A variety of steroid preservation methods have been used in the field, the most reliable method using a freezer or liquid nitrogen canister if available on site. Another effective method is to lyophilise (freeze-dry) samples directly (Lynch *et al.*, 2003). However, lyophilisers are not widely available, and therefore investigators have turned to a variety of other faecal storage methods, including preservation in ethanol, and/or drying the faeces with silica, ovens, solar radiation and fires, to avoid further metabolism of the steroids (Millspaugh and Washburn, 2004; Lynch *et al.*, 2003; Khan *et al.*, 2002; Terio *et al.*, 2002). Yet, some of these preservation methods may result in significant changes in immunoreactive hormone concentrations, depending on the preservation technique used (Hunt and Wasser, 2003). For example, it has been reported that the addition of alcohol to the sample will initiate steroid extraction and subsequently bias the results (Palme, 2005). Whitten *et al*. (1998) observed that the majority of steroid is extracted into the solvent when faeces are suspended for prolonged periods in 90% ethanol at ambient temperatures. Hence, care must be taken into account for the effects of the solvent on steroid partitioning (Touma and Palme, 2005).

There seems to be conflicting reports regarding faecal collection and storage methods of samples from several species. Depending on the storage method, the storage duration, the metabolite studied and the species under investigation, the hormone concentrations can both increase or decrease (Möstl *et al.*, 2005; Millspaugh and Washburn, 2004; Hunt and Wasser, 2003). Lynch *et al*. (2003) demonstrated the effects of sample storage on hormone concentrations between various hormones. Short-term freezing of samples from wild baboons maintained stability in faecal progestagen, but led to a significant decline in faecal glucocorticoids; in contrast, a combination of ambient temperature storage

followed by freezing led to a significant increase in faecal progestagen but no change in faecal glucocorticoids (Lynch *et al.*, 2003). Khan *et al*. (2002) studied metabolites from baboons in 95% ethanol stored in the freezer and ambient temperatures and found that there was a rise in glucocorticoids after 120 days.

Conversely, Möstl *et al*. (2002) observed a decrease in faecal cortisol metabolites of cows after storage at room temperature for longer than four hours. In deer, a decrease in faecal cortisol metabolite concentration was detected only when samples were collected later than 6 hours post-defecation (Huber *et al.*, 2003).

A study by Galama *et al*. (2004) discusses storage methods of black rhino faecal progestagen samples. He concluded that samples can be stored outdoors for up to 180 days when dried in a solar box cooker or mixed with 80% methanol, without affecting progestagen concentrations. Faecal samples that were not preserved showed marked differences in hormone concentrations and the ability to accurately reflect reproductive events. Such variations may have been caused by bacteria or other micro-organisms in the faeces, transforming the available hormone metabolites so that they could not be detected by the progestagen assay used in the study (Galama *et al.*, 2004).

Turner *et al*. (2002) briefly mentions a slight degradation of corticosterone in black and white rhinoceros faeces after leaving samples for 0 – 16 h at 27°C. Further studies by Morrow *et al*. (2002) concludes that immunoreactive corticosterone detected by the MP corticosterone antibody are stable for up to 12h when stored at room temperature and for up to 24h when stored on ice.

In view of the above marked species differences in the effect of certain preservation methods, results indicate the need for important guidelines when collecting and storing samples for faecal hormone studies for a particular species (Hunt and Wasser, 2003). It is crucial to determine the effects of storage conditions as well as the range of storage duration upon each steroid analysed for the species of interest. This will ensure the optimal compromise for storage protocol when analysing faecal steroid metabolites for that particular species (Lynch *et al.*, 2003).

2.3.10 Timing of Sample Collection

The activity of the pituitary-adrenocortical system shows cyclic changes over a 24 h period, which is manifested in variations of the cortisol concentration in plasma (Koopmans *et al.*, 2005). Fluctuations of serum corticosteroid concentrations have been demonstrated for numerous diurnal species (Koopmans *et al.*, 2005; Capitanio *et al.*, 2004; Turner *et al.*, 2002; Turner, 1984), where concentrations peak before the onset of activity and decline to a late afternoon to early evening trough (Touma and Palme, 2005; Whitten *et al.*, 1998). In nocturnal animals, circadian periodicity in plasma shows high cortisol concentrations in the evening (Koopmans *et al.*, 1993).

Likewise, diurnal variation of glucocorticoids should also be taken into account for the monitoring of hormone metabolites from faecal samples (Touma and Palme, 2005). A distinct circadian rhythm of glucocorticoid metabolites has been observed in faecal samples of some mammalian and bird species (Touma *et al.*, 2004; Carere *et al.*, 2003; Kotrschal *et al.*, 2000). However, in species with a relatively long gut passage time e.g. hind-gut fermenters such as the white rhino, it might be impossible to detect diurnal changes of circulating glucocorticoid concentration in the faeces (Touma and Palme, 2005). Nevertheless, to avoid any possible fluctuations caused by diurnal variations in glucocorticoid concentrations, faecal samples should be collected at approximately the same time each day if viable (Touma and Palme, 2005).

2.3.11 Kinetics of Faecal Corticosteroids after ACTH Stimulation

In vertebrates, ACTH administration mimics a natural adrenal stress response by causing a rapid rise in circulating glucocorticoids in the blood, followed by a return to baseline within a few hours (Wasser *et al.*, 2000). The same pattern should also occur in faeces with the onset of the peak excretion delayed by the species-specific excretion lag time (Wasser *et al.*, 2000). Characterisation of lag times is important when attempting to determine the timing of physiological events (Wasser *et al.*, 1994) or to test the relation of hormone concentrations to behavioural events and states (Whitten *et al.*, 1998) in this case a faecal corticosterone response to ACTH stimulation.

Significant correlations between serum and faecal levels of glucocorticoids have been demonstrated in several species (Stead *et al.*, 2000; Möstl *et al.*, 1999; Cavigelli, 1999; Carlstead *et al.*, 1992; Miller *et al.*, 1991), including the white rhinoceros (Turner *et al.*, 2002). There is an appreciable lag time between the circulation of corticosteroids in plasma and their appearance in faecal samples, which approximately correlates with the time necessary for the intestinal passage of bile to the rectum (Palme *et al.*, 1996). The delay time from release to peak excretion of steroids is approximately 12 to 24 h in ruminants (Möstl *et al.*, 2002; Wasser *et al.*, 2000; Kapke *et al.*, 1999), 12 h to more than 48 h in cats (Brown *et al.*, 1996) and 24 h to more than 48 h in horses (Möstl *et al.*, 1999; Palme *et al.*, 1996), pigs (Palme *et al.*, 1996), primates (Barrett *et al.*, 2002; Foley *et al.*, 2001), elephants (Wasser *et al.*, 2000; Wasser *et al.*, 1996), and black and white rhinoceroses (Brown *et al.*, 2001; Hindle and Hodges, 1990).

Wasser *et al*. (2000) injected a black rhino with 1.5 IU/kg ACTH (800 IU total). The MP corticosterone antibody revealed a clear peak of 110 ng/g dry mixed faeces in glucocorticoid metabolite excretion 25 h post stimulation. Concentrations returned to a baseline of 45 ng/g dry mixed faeces, 75 h after ACTH injection. Furthermore, Brown *et al*. (2001) demonstrated a peak faecal corticoid excretion 2 days post ACTH injection in four black rhinos, with concentrations reaching baseline 4 days post stimulation. Timing of peak excretion in both studies is consistent with known gut passage time in the white rhinoceros (Hindle and Hodges, 1990).

Corticosteroids are secreted in a pulsatile fashion in many species and so accurate assessment of circulating activity can only be made by collecting multiple blood samples over time (Brown *et al.*, 1995). For this reason, faecal monitoring may provide a better picture of overall stress than serum corticosteroids because the excreted sample represents cumulative secretion over a number of hours rather than minutes (Whitten *et al.*, 1998).

The route of excretion varies amongst species and also between individuals within the same species (Schwarzenberger *et al.*, 1996). Variations in diet might be matched by changes in digesta passage and digestibility, with possible selective retention of harder to

digest items (Remis and Dierenfeld, 2004). Gastrointestinal transit, GIT, time can be influenced by the rate of passage of digesta as a result of gastrointestinal distress (diarrhoea, constipation). Varying transit time will affect the concentration of corticoid metabolites absorbed and subsequently, the concentration of metabolite excreted in the faeces. It is therefore important to measure the GIT time of white rhinos when comparing the concentration of cortisol metabolites with other studies or with various study sites with differing management and feeding techniques within the same study.

2.3.12 Types and Limitations of Digesta Passage Markers

Digestive markers are used routinely to calculate faecal output and to estimate kinetics within the digestive tract. Gastrointestinal physiology and kinetics show a wide variation among species. For this reason, markers must be appropriately selected for each species and each circumstance (Bernard *et al.*, 1994).

Despite imprecision in marker procedures, inherent variation may be small relative to other sources of variation such as diet, environment and feed intake (Owens and Hanson, 1992). Even though absolute values may be imprecise and inaccurate, marker based estimates usually provide reliable information about the direction and extent of kinetic changes induced by treatments.

These markers can be placed into one of two broad categories - internal markers that are inherent in feeds such as silica, lignin and acid insoluble ash. Each of these feed fractions does have limitations in usefulness (Owens and Hanson, 1992). External markers may be administered, for example adding acetate beads to the feed (Edwards and Ullrey, 1999). Various types of plastics with different physical properties have been useful in measuring passage events in the gastrointestinal tract of ruminants (Welch, 1990). Nevertheless these markers also have their limitations. Because they are inert, they cannot undergo the hydration, density and size changes that occur. Brown *et al*. (2001) used cake-decorating dye, which, after adding several colours of cake-decorating dye to the centre of apples, found the colour change subtle in rhino faeces, thus making samples difficult to identify. Art glitter appeared to be the most successful faecal marker, which were easily fed and

detectable in faeces. However, concern over feeding this synthetic product for months discouraged its use for her study (Brown *et al.*, 2001).

CHAPTER 3

3. MATERIALS AND METHODS

3.1 Experimental Design

3.1.1 Animals and Housing

Faeces were collected from four female and three male white rhinoceroses in South Africa. Pairs of male and female rhinos were group-housed at Thaba Manzi Wildlife Services - Group 1 (Warmbaths, Northern Province) and Johannesburg Zoo - Group 2 (Johannesburg, Gauteng). Animals at Wildlife Assignments Ltd. - Group 3 (Hammanskraal, Gauteng) were housed individually. The level of visual, auditory and olfactory exposure to conspecifics and heterospecifics varied for each study group. Figure 3.1 shows the locations for each study group in South Africa. All animals in Group 3 and one female in Group 1 were of sub-adult age; all other rhinos were adults. Details of study animals are presented in Table 3.1.

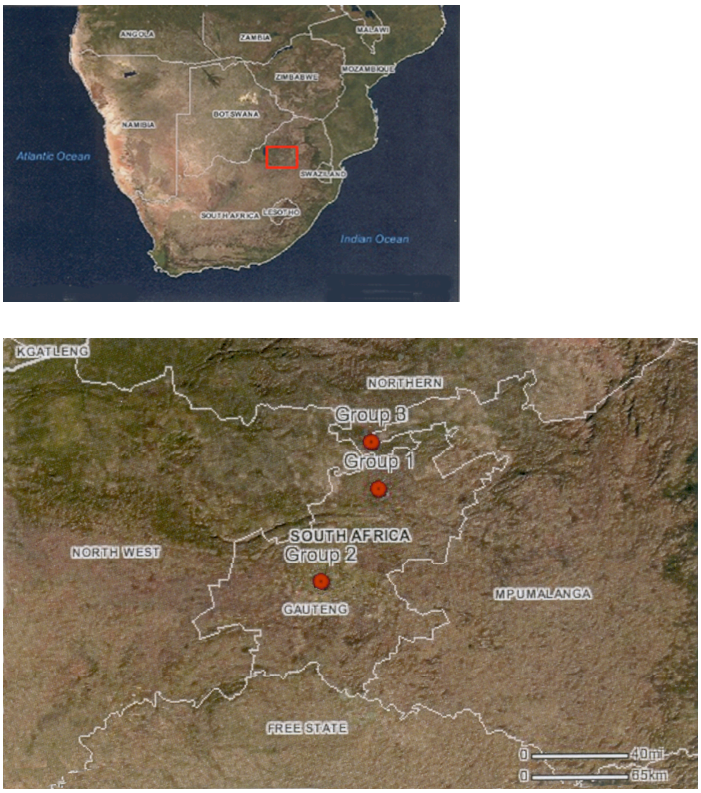


Figure 3.1 Map of the location of each study group where samples were collected in South Africa. Group 1 - Warmbaths, Northern Province; Group 2 – Johannesburg, Gauteng; Group 3 – Hammanskraal, Gauteng.

Table 3.1 Details of white rhinoceros involved in the study.

Group	Institution	Enclosure Size (m^2)	Age (yr) [a]		Weight (kg) [b]		ACTH IU/kg		Total Dose (IU)	
			M	F	M	F	M	F	M	F
1	Thaba Manzi Wildlife Services	1,000	20	5	2100	1000	0.75	1.5	1575	1500
2	Johannesburg Zoo	15,000	11	11	2100	1600	1.5	1.5	3150	2400
3	Wildlife Assignments Ltd.	96	3	4.5/4.5	1100	1500/1500	1.5	1.5	1650	2250/2250

[a] Age (or estimated age)

[b] Estimated Weight

Health care of all the rhinos was under the supervision of a veterinarian throughout each trial period. Two days preceding the study, Group 1 male and female rhinos were semi-free ranging on the 60 hectare Thaba Manzi Wildlife Services farm for a period of 12 months and 4 months, respectively. Prior to semi free-ranging at Thaba Manzi, the female was captured and placed in a boma in Kruger National Park (KNP), Mpumalanga Province, for one month; the male was formerly free-ranging on a reserve in the Eastern Cape Province.

The main diet for Group 1 rhinos consisted of grazing on farm grass and two bales of lucerne given once daily inside an open boma, allowing animals to feed adlib with access in and out of the enclosure. Two days prior to the trial, animals were enclosed in the boma. Throughout the study, animals were fed in the morning and afternoon, sharing a total of three bales of lucerne a day.

Group 2 male and female rhinos at Johannesburg Zoo were bred in captivity at Thaba Zimbi, North-West Province prior to being held in the enclosure at Johannesburg Zoo for 9 years. Total daily feed distributed between the two rhinos consisted of 2 bales of teff grass supplemented with 20 kg of chopped vegetables and Boskos pellets in the morning.

Group 3 animals at Wildlife Assignments were originally from KNP. The male was caught immediately and transported with the females who had been held in a boma for one month in KNP. Animals were held in individual housing for 3 months prior to study.

One bale of lucerne was given to each animal every morning and half a bale in the late afternoon at Group 3 study site.

For all study animals, access to fresh water was available adlib. All enclosures were cleaned each morning by the animal keepers. The presented work complies with current regulations covering animal experimentation in South Africa. The experiments were approved by the local authority as well as by the Faculty of Animal Use and Care Committee, AUCC, of the University of Pretoria.

3.1.2 ACTH Challenge

Each animal received both ACTH and normal saline (0.9% NaCl) in a crossover manner. All animals received ACTH administered in a series of intramuscular, (i.m.), injections of a slow-release gel to induce a sustained corticosterone secretion (Porcine ACTH, Sigma-Aldrich, Steinham, Germany synthesised by Animal Production Laboratory, Faculty of Veterinary Science, Onderstepoort, Pretoria). Sustained adrenal activation is especially important in species that excrete a large faecal mass to prevent short-lived changes in adrenal status from becoming too diluted in the gut for faecal detection (Wasser *et al.*, 2000). ACTH dosages used for each animal are given in Table 3.1. All animals received an ACTH dosage of 1.5 IU/kg body weight, excluding the Group 1 male due to a dart of ACTH gel exploding before entering the animal. Subsequently, there was not enough ACTH on site to give the animal full dosage, thus, the male received a half dosage of ACTH (0.75 IU/kg body weight). Treatments were placed into 7.5 mL volume, (810 IU) gel-collared drop out darts (Pneu-Dart Inc., Williamsport, P.A., see Figure. 3.2) and administered by a trained veterinarian using a Daninject® system (Wildlife Pharmaceuticals, Fort Collins, Colorado) into the deep muscle of the hindquarters. In the case of Group 3 rhinos, treatments were given via stick pole into the neck as requested by the site veterinarian.

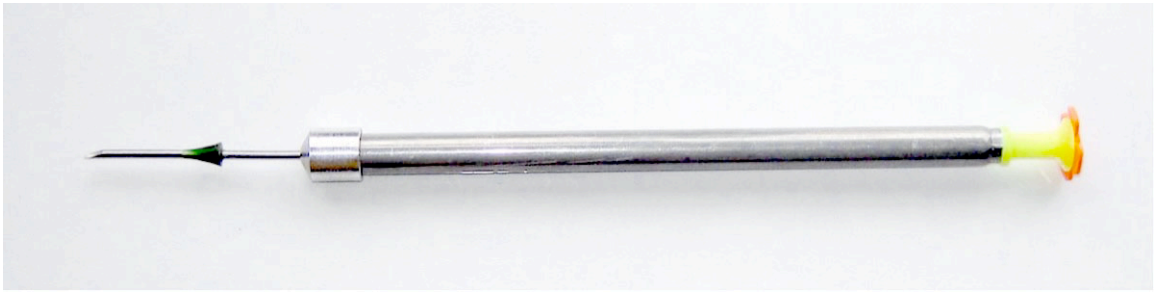


Figure 3.2 Photo of 7.5 mL gel-collared, drop out dart (Pneu-Dart Inc.) used in a Daninject® system to administer ACTH treatments.

Two injection sites were used in each of the Group 1 rhinos, Group 2 male and female rhinos were given four and three injections respectively; and Group 3 male and female rhinos received two and three injections, respectively. ACTH treatments were administered on Day 0 and saline, NaCl, treatments on Day 11, with no sample collection on Day 5 and 6. All animals received treatments in the morning, with the exception of Group 1 animals that were darted in the late afternoon. Table 3.2 shows details of the trial schedule.

Table 3.2 Trial schedule for sampling procedure

Procedure	**FS**	**FS**	**FS**	**FS**	**ACTH**	**FS**	**FS**	**FS**	**FS**	**-**	**-**	**FS**	**FS**	**FS**	**FS**	**NaCl**	**FS**	**FS**	**FS**	**FS**
Day	-4	-3	-2	-1	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15

FS – day of faecal sample collection

3.1.2.1 Effects of the Injection Procedure

Since the injection procedure of ACTH is a relatively stressful event for the rhino and therefore likely to influence the animals' glucocorticoid concentrations, a control experiment was conducted to investigate effects of the injection procedure itself on the pattern of excreted faecal steroid metabolites. The experimental design for the treatment of sterile isotonic saline solution was identical to that of the ACTH challenge.

3.1.3 ACTH Gel Preparation

The ACTH gel was prepared in the lab at the University of Pretoria prior to sampling at each study site. The following protocol described below, yields 46 mL of ACTH gel at a concentration of 108 IU/mL. To produce the required amount of ACTH (mL) for animals at each study site, the following values of reagents were calculated accordingly and formulated in the lab, adhering to sterile laboratory procedures.

0.625 g of sodium carboxymethylcellulose (Sigma-Aldrich, Steinham, Germany, lot# 20K0237, medium viscosity) was weighed and added slowly to 40 mL of swirling distilled water in an Erlenmeyer flask. Once mixed for 20-30 minutes, the dissolved carboxymethylcellulose solution was autoclaved for one hour at 180°C. 0.3125 g of liquid phenol (Sigma-Aldrich, Steinham, Germany, lot#80K0812, VSP grade) was added to a small sterile beaker, along with 2.565 mg of dextrose (Sigma-Aldrich, Steinham, Germany, lot# 30K0208, VSP grade) and 10 mL of distilled water. Dextrose was mixed until completely dissolved. The dextrose/phenol solution was then poured into the container containing 5,000 IU ACTH powder. This was mixed for 5 minutes until completely dissolved. Using the sterile technique, the ACTH solution was withdrawn into a 12 cc syringe. This was then replaced with a sterile 0.22 µ filter, allowing the liquid to drip into the flask containing the sterile, swirling carboxymethylcellulose solution. Once added, the ACTH gel was mixed for 5 minutes to ensure homogeneity. A drop of the solution was placed onto pH paper to ensure the pH ranged between 4.5-6.5. Using sterile glass pipettes, the ACTH gel was withdrawn into a sterile vial and stored at 4°C until ACTH administration.

3.1.4 Gastrointestinal Passage Marker

Art glitter was applied as a digestive marker to measure the gastrointestinal transit time in relation to data of faecal corticoid concentrations pre and post-treatment of ACTH and saline. Glitter was given to each animal on day of treatment of ACTH or saline and on a pre-treatment day as the control; a random day of the trial was chosen for the control. All rhinos were provided with green glitter (six 40 mL bottles), distributed evenly throughout a bale of lucerne or teff, and were allowed time to eat a sufficient quantity of feed mixed with glitter before darting.

3.1.5 Sample Collection and Storage

Faecal samples were collected 4 days before and 4 days after each treatment. Approximately 20 g of faecal samples were collected twice a day, in the morning and afternoon from all rhinos, but occasionally only one sample was collected due to a lack of feasibility. Samples from group-housed rhinos were collected from animals after direct observation of defeacation, whilst samples from Group 3 individually housed animals

were collected within 1 h after defeacation to minimise sample degradation. The faecal samples were collected from the central bolus and placed in a plastic bag. Samples were then stored immediately after collection in a freezer on site at -20°C until preparation for hormone extraction and RIA analysis.

3.2 Extraction and Determination of Faecal Steroid Metabolites

3.2.1 Faecal Steroid Extraction

Faecal samples were extracted according to the method described by Brown *et al*. (2001). Frozen faeces were lyophilized, pulverized, and the faecal powder was stored at -20°C until steroid extraction. A 0.2g aliquot of well-mixed powder was boiled twice in 5 mL of 90% ethanol: 10% distilled water for 20 minutes in a water bath at approximately 94°C. After centrifuging at 500 g for 10 minutes, supernatants were recovered and dried in a nitrogen bath at 36°C.

3.2.2 Faecal Radioimmunoassay

To determine the concentration of faecal glucocorticoids, an established double antibody ^{125}I RIA kit (MP Biomedicals, Irvine, CA) for corticosterone was used. The antibody in this assay was raised in rabbits against corticosterone-3-carboxymethyloxime and has a high affinity for corticosterone and the various faecal metabolites of corticosterone. The manufacturers' reported cross-reactivities are shown in Appendix I. The antisera cross-reacts 100% with corticosterone.

The RIAs were performed according to manufacturer's instructions by the Conservation and Research Centre, (C.R.C)., Smithsonian Institution, Virginia. This assay has proved well suited to assess corticoid metabolites in extracted faecal samples of white rhinoceros (Carlstead and Brown, 2005) and has been used extensively in the C.R.C. laboratory for white rhino faeces.

Reagents were brought to room temperature prior to use. Extracted faecal samples were re-dissolved in 1 mL methanol, vortexed for 1 minute and diluted 1:10 in diluent buffer provided by the manufacturer, before RIA analysis. 300 µL of steroid diluent was added to the No Sample Blank, (NSB), tubes. 100 µL steroid diluent was then added to the

tubes labeled "0". To create an additional standard, 110 µl of both steroid diluent and the "25" standard was added to a separate glass tube, and vortexed. This became the 12.5 ng/mL standard. 100 µL corticosterone calibrators (12.5-1000 ng/mL) were added to the appropriately labelled tubes. 100 µL of controls (High and Low) were added to the respective tubes. 50 µL of extracted faecal sample was then added to their prepared tubes. 50 µL of steroid diluent was added to all sample tubes, excluding the Standards or Controls. Next, 100 µL corticosterone ^{125}I (blue reagent) was added to all tubes. 100 µL anti-corticosterone (yellow reagent) was then added to all tubes except to the Totals and NSBs. All assay tubes were vortexed and incubated at room temperature (22-25°C) for 2 h. After incubation, 250 µL precipitant solution (red reagent) was added to all tubes, vortexed thoroughly and centrifuged for 15 minutes at 2500 rpm, excluding the Totals. All supernatants were decanted carefully and the precipitates counted in a gamma counter, except for Totals.

Finally, each sample was assayed in duplicate yielding a within-sample coefficient of variation, CV. If the within-sample CV was higher than 10%, samples were re-assayed in an attempt to reduce the CV, following standard control procedures.

3.2.3 Standard Curve

The standard curve was produced using Packard computer software connected to the gamma counter, provided by the C.R.C lab. Averages of all duplicate tubes were calculated. The average NSB counts were then subtracted from the averages obtained. This yielded the corrected values. The corrected values were then divided by the corrected zero calibrator value to obtain the percent bound- refer to Appendix II for the formula provided by the manufacturer. Percent bound versus the concentration of corticosterone for all the calibrators (12.5-1000 ng/mL) was plotted and the sample values were calculated from the curve in ng/mL.

3.2.4 Assay Validation

The assay was validated for white rhinoceros faecal extracts by demonstrating 1) parallelism between serial dilutions of sample and the standard curve; and 2) significant mass recovery of exogenous steroid added to samples before analysis. Although an

extraction efficiency for assay validation was not performed, this assay used in the C.R.C. laboratory has shown good extraction efficiency with faecal extracts of white rhino in the past (data not shown).

3.2.4.1 Parallelism

A collection of 20 sample extracts were pooled for a "neat" extract pool. The neat faecal extract was diluted serially two-fold in assay buffer. 200 µL of buffer was added into tubes labelled neat, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, and 1:128. Next, 200 µL of the neat sample was added and mixed with the 200 µL in the 1:2 tube; this was then vortexed. 200 µL was taken out of the 1:2 and placed in the 1:4 and so on up until the 1:128. Standards and reagents were prepared according to assay protocols. The sample was run on the MP corticosterone RIA. % binding of samples was plotted by choosing an arbitrary concentration for the neat sample and halving the concentration for each dilution. If the sample curve paralleled the standard curve with the neat sample reading below 50%, the sample hormone being measured, in this case corticosterone was immunologically similar to the standard and could be measured in that assay. The correct dilution to run samples was at approximately 50% binding.

3.2.4.2 Accuracy Check

Two random freeze dried faecal samples were chosen for accuracy checks. From each sample, 0.2 g was weighed and placed into 4 test tubes. 100, 300 and 600 µL of 1000 ng/mL of corticosterone in ethanol was added consecutively to the 3 test tubes. The fourth test tube was not spiked with a hormone; this was used to measure the background baseline of the sample. The remainder of the extraction was performed as discussed in 3.2.1. The samples were then assayed as described in 3.2.2. If the samples that were spiked did not yield binding close to 50% on their initial run, they were diluted further and run on another assay. The formula below was used to calculate the % recoveries of the spiked hormone from each sample.

Amount expected = concentration of spiked hormone plus (+) value of background baseline sample, measured by an RIA kit

Amount observed = concentration of spiked sample, measured by an RIA kit minus (-) concentration of background sample

% Recovery = (amount observed / amount expected) multiplied (*)100

3.2.5 Data Analysis

Hormone data calculated in ng/mL as described in 3.2.3 were corrected for dilution and weight of faecal material extracted, and expressed as nanogram per gram, (ng/g), of dry faecal mass. Since the obtained data was not normally distributed, the data were analysed by non-parametrical statistics. All tests were calculated using BMDP Statistical Software by the Department of Statistics, University of Pretoria. Average data are presented as mean ± standard error of the mean, SEM. For all analyses, the level of significance defined for statistical tests was $P<0.05$.

3.2.5.1 ACTH/ Saline Treatment Analysis

For all study animals, individual pre-ACTH and pre-saline treatment baselines were calculated as the average faecal samples collected on all days before the injection (i.e. Day -4 to -1 and Day 7 to 11, respectively). Post-treatment rise in corticosterone are expressed as a percentage of the pre-treatment baseline, with the baseline value being equivalent to 100%.

Several statistical tests were used to analyse faecal hormone values throughout the trial period for all animals investigated: (1) variability in pre-ACTH baseline corticosterone concentration means (Day -4 to -1) using Friedman (2) differences between pre- and post-ACTH corticosterone concentration means using Wilcoxon test (3) gender differences using the overall mean of all samples for the collection period for each animal tested by Mann-Whitney *U*-test (MWU-test) (4) site differences using the overall mean for each animal tested by Kruskal-Wallis *H*-test (KWH-test). The area under the corticosterone concentration (ng/g) versus time (days from ACTH/saline treatment) curve were determined for pre-ACTH v. post-ACTH treatment, pre-ACTH v. post-saline treatment and pre-ACTH v. pre and post saline treatment, using Wilcoxon test for statistical comparison. Due to limited data, the area under the curve, AUC, time to peak and peak mean of the faecal corticosterone concentrations could not be related to the measured gastrointestinal transit, GIT, time for statistical comparison between all study animals.

CHAPTER 4

4. RESULTS

4.1 Administration of the Treatments

Treatment using a Daninject® system with 7.5 mL volume, gel-collared Pneu-darts was found to be efficient in administering the ACTH slow-release gel and saline in groups 1 and 2. However, it must be noted that an abscess developed in the female of Group 2 from a dart needle that became embedded in the hindquarters after saline administration. The dart needle was removed by the animal keeper and the abscess treated each morning and afternoon for the following three days until healed.

In the case of Group 3 rhinos, where treatments were given via stick pole into the neck, this was found to be most practical and all treatments were administered successfully.

4.2 Assay Validation

4.2.1 Parallelism

Serial dilutions (neat to 1:128) of a pool of faecal extracts from study animals yielded displacement curves (y = -0.1912x + 75.009; r^2 = 0.9124) parallel to the corticosterone standard curve (y = -0.2082x + 81.005; r^2= 0.9149, Figure 4.1). Sensitivity of the assay at maximum binding was 12.5 ng/mL.

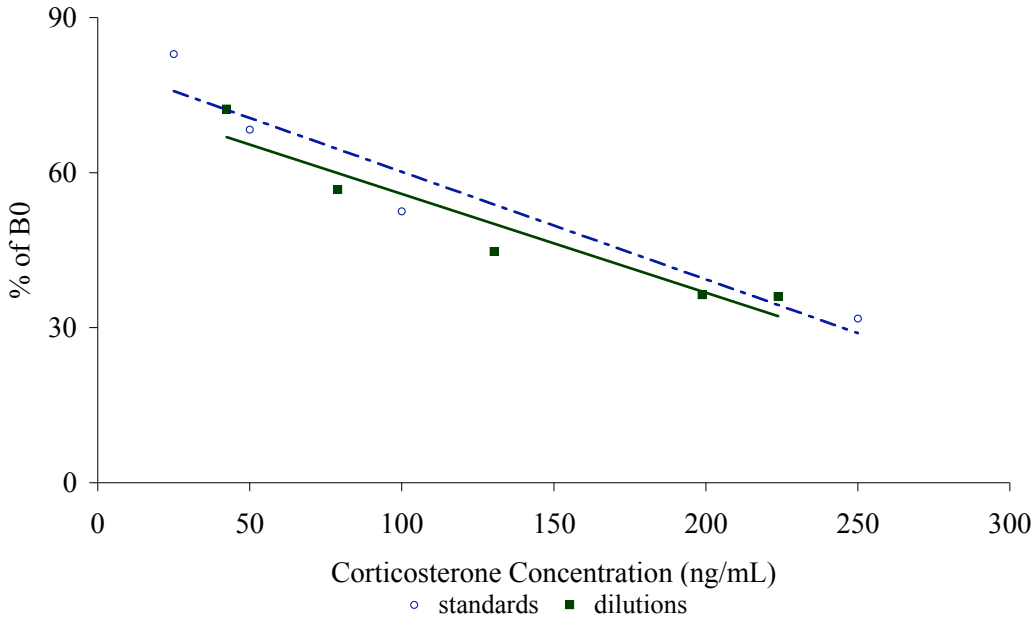


Figure 4.1 Displacement curves for the corticosterone standard and serial dilutions of a neat pool of faecal extracts from study animals, analysed by a double antibody ^{125}I radioimmunoassay (MP Biomedicals). B0 = maximum binding.

4.2.2 Accuracy Check

The amounts expected, measured and recoveries were calculated using the background baseline concentrations (44.6 and 31.2 ng/g) measured from two faecal samples before spiking with corticosterone (refer to 3.2.4.2 for method of calculating % recoveries). Recoveries were designated as the amount of spiked corticosterone measured, expressed as a percentage of the amount of corticosterone added before extraction and MP-corticosterone assay analysis. Overall mean recovery of corticosterone (range, 100–600 µL of 1000 ng/mL) added to 0.2 g aliquots from two random freeze-dried faecal samples collected from the animals investigated, was 67.2 ± 5.6 % where $y = 1.63x - 95.6$, $r^2 = 0.95$ (Figure 4.2).

Table 4.1 Recovery data from 0.2 g aliquots from two random freeze-dried faecal samples.

Spike* (µL of 1000 ng/mL of corticosterone in ethanol)	**Mean Recovery (%)**
100	69.45
300	70.95
600	61.1

*Spike refers to the concentration of corticosterone (100, 300 and 600 µL of 1000 ng/mL) added to the two freeze-dried faecal samples. Mean recovery is the amount observed expressed as a percentage of the amount expected for the two faecal samples (see 3.2.4.2 for calculations).

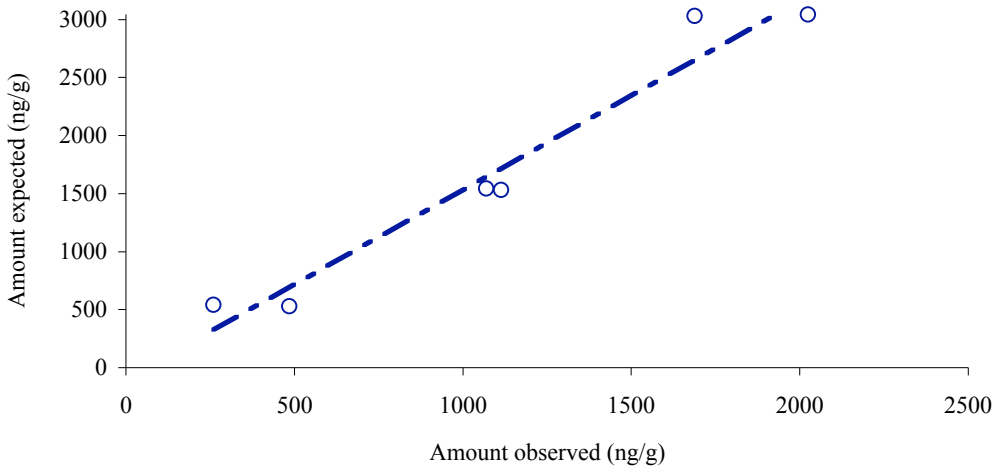
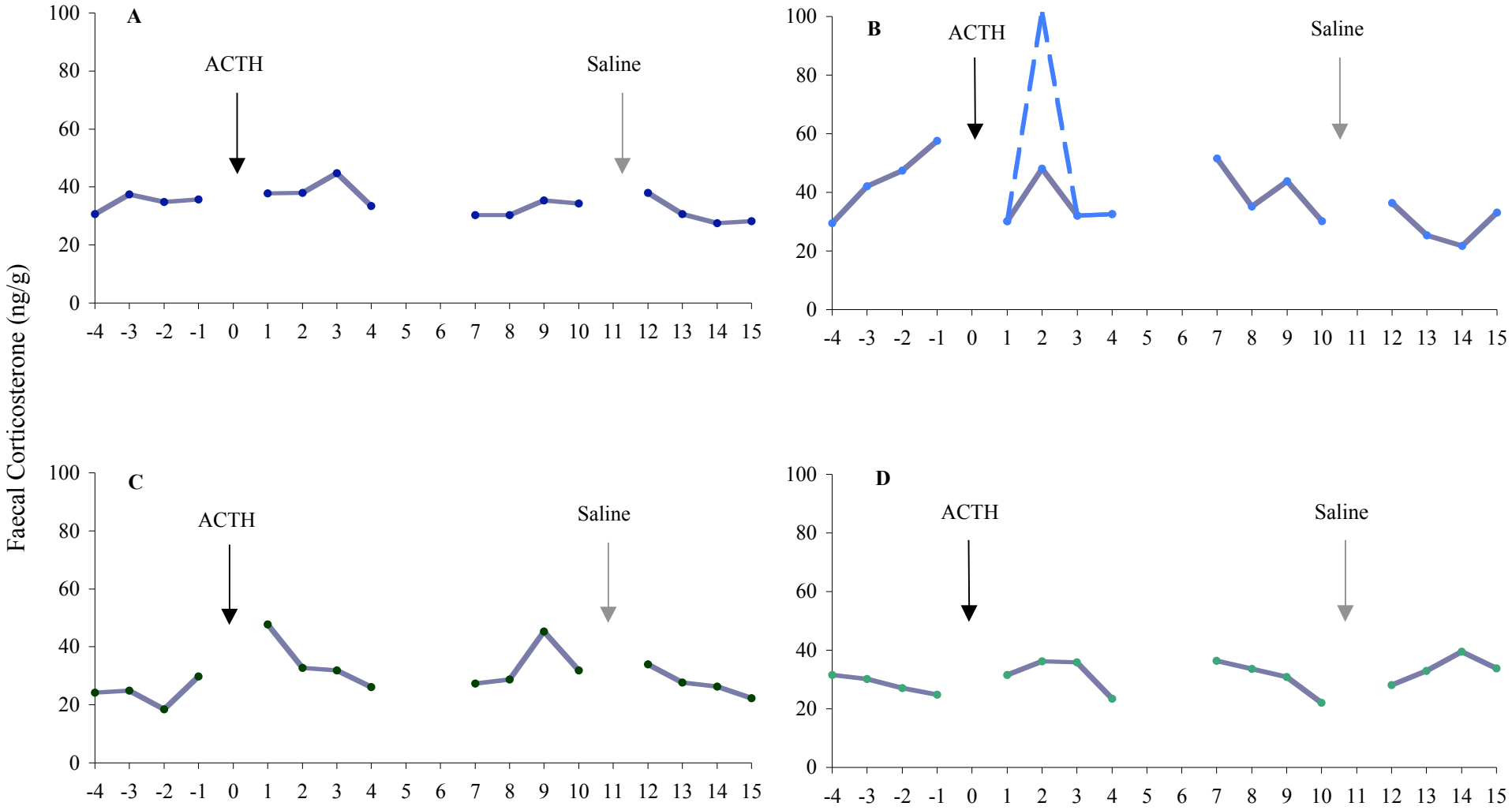


Figure 4.2 Linear regression obtained after spiking 0.2 g aliquots from two random freeze-dried faecal samples spiked with 100, 300 and 600 µL of 1000 ng/mL solution of corticosterone in ethanol, analysed by a radioimmunoassay (MP Biomedicals). Amount expected and amount observed were calculated as described in 3.2.4.2.

4.3 Results of Faecal Radioimmunoassay

To account for variation in the number of defeacations from individual animals throughout the trial, morning and afternoon faecal samples were grouped and averaged according to each day. Daily mean faecal corticosteroid excretory profiles for animals from Group 1 to 3 are depicted in Figure 4.3 A–G. Samples from these group housed animals were usually collected after direct observation of defeacation. However, in Group 1, a faecal sample was collected after defeacation, with the identity unknown, two days post ACTH treatment. A test for estrogen or testosterone to establish the identity of the sample was considered unreliable due to seasonal fluctuations in gonadal hormones in both male and female rhinos (Schwarzenberger, 2005 Pers. Comm.). Furthermore, a DNA analysis of the faecal sample produced inconclusive results, thus the identity remains unknown. However, an assumption that the unidentified faecal sample belonged to the female was made, for the reason that throughout the four days prior to ACTH treatment, a steadily increasing concentration of corticosterone was observed in the female (29.5-57.7 ng/g) as opposed to a slight elevation in the male (30.7-37.5 ng/g, Figure 4.4). Subsequently, higher pre-ACTH treatment means and standard deviations (SD) were measured in the female than in the male (44.2 ± 5.7 ng/g, SD = 11.7 and 34.7 ± 2.8 ng/g, SD = 2.7, respectively, Table 4.2). Furthermore, the female received a full

dose of ACTH (1.5 IU/kg body weight), whereas the male only received half the dose. And so for all the above reasons, the high corticosterone concentration of 154.7 ng/g measured in the unidentified sample is more than likely to belong to the female. Individual responses to ACTH and saline treatments are discussed below.


A
B
C
D
ACTH
Saline
Faecal Corticosterone (ng/g)
Days Post ACTH/Saline Treatment

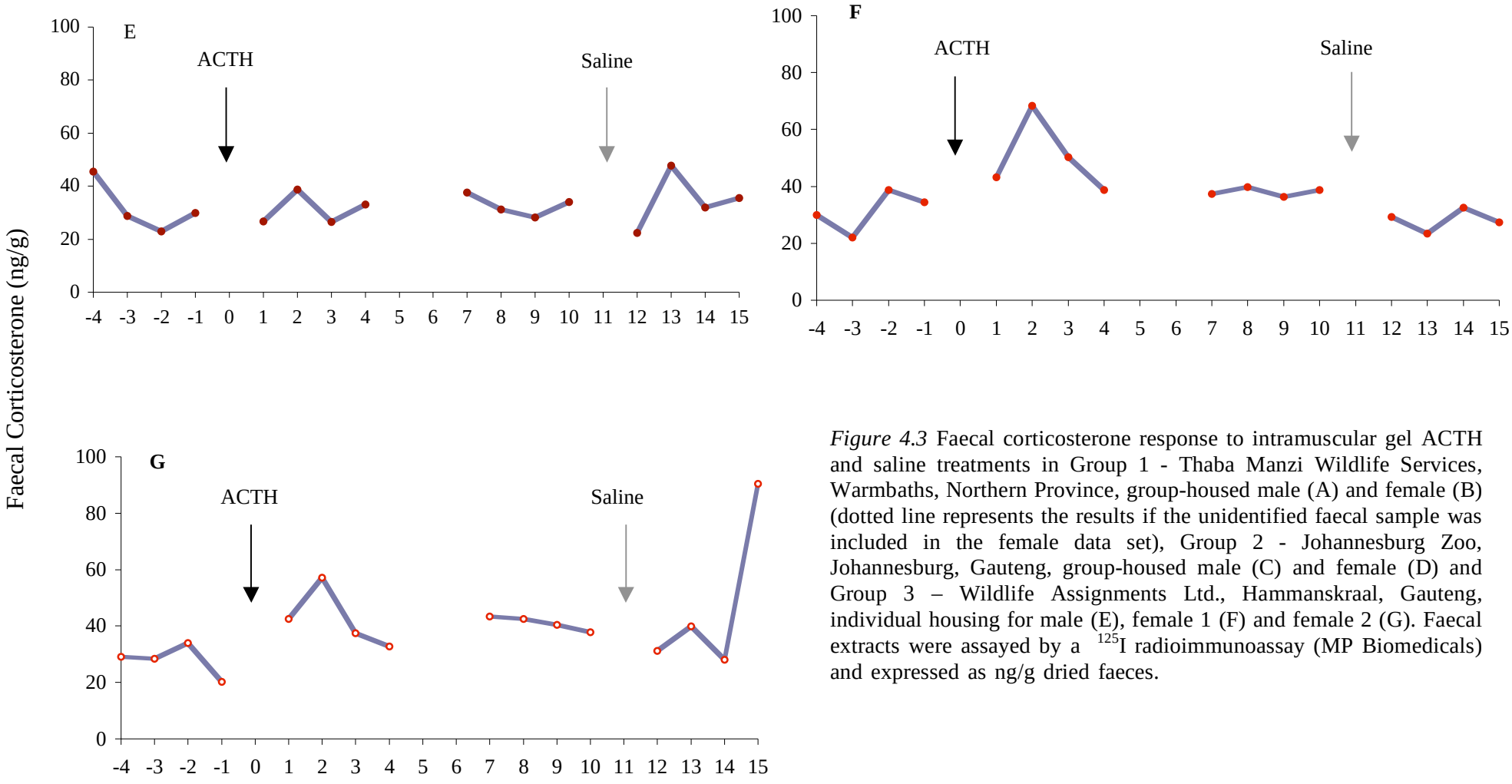


Figure 4.3 Faecal corticosterone response to intramuscular gel ACTH and saline treatments in Group 1 - Thaba Manzi Wildlife Services, Warmbaths, Northern Province, group-housed male (A) and female (B) (dotted line represents the results if the unidentified faecal sample was included in the female data set), Group 2 - Johannesburg Zoo, Johannesburg, Gauteng, group-housed male (C) and female (D) and Group 3 – Wildlife Assignments Ltd., Hammanskraal, Gauteng, individual housing for male (E), female 1 (F) and female 2 (G). Faecal extracts were assayed by a ^{125}I radioimmunoassay (MP Biomedicals) and expressed as ng/g dried faeces.

4.3.1 Pre-ACTH Treatment Baselines

Although baseline concentrations of corticosterone did not differ significantly between pre-ACTH treatment days in any of the study animals (-4 to -1 day before treatment Day 0, Friedman test statistic = 0.26, n = 7, P = 0.9679), the MP-corticosterone assay revealed a large variability between individuals during this period (range, 18.5-57.7 ng/g) (Figure 4.4). Lowest pre-ACTH treatment means and SD were observed in Group 2 male and female (24.4 ± 1.5 ng/g, SD = 4.6 and 28.4 ± 1.7 ng/g, SD = 3.0 respectively), in contrast to Group 1 female showing the highest pre-ACTH baseline mean concentration and SD of 44.2 ± 5.7 ng/g, SD = 11.7 (Table 4.2).

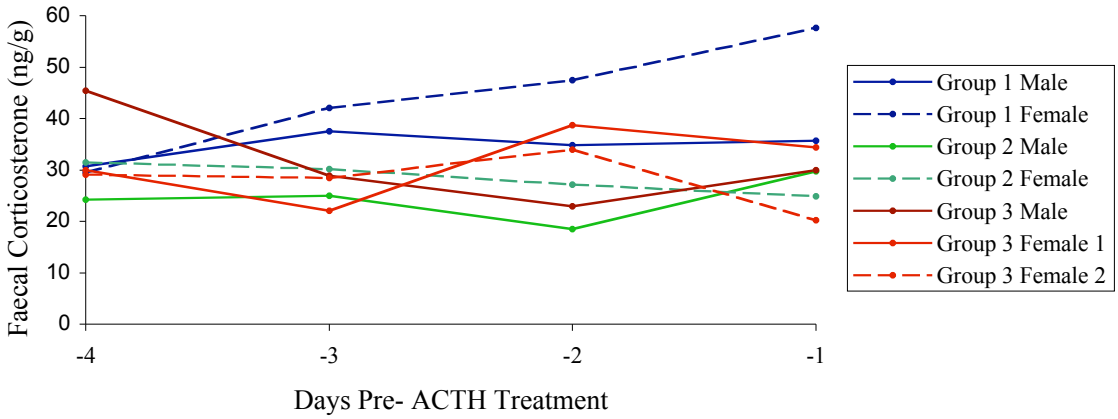


Figure 4.4 Baseline faecal corticosterone concentrations (ng/g) in all study animals pre-ACTH treatment (-4 to -1 days before treatment Day 0). Data represent either single samples or daily means.

4.3.2 ACTH Challenge

The MP-corticosterone assay successfully detected the expected increase in corticosterone faecal concentrations following the treatment of ACTH, although the degree of response differed between individual animals, as discussed below (Figure 4.3). Concentrations of faecal corticosterone during the pre-treatment and at the ACTH-induced peak are shown in Table 4.2. Mean corticosterone concentrations in seven white rhinos showed a significant difference between the control periods prior to treatment (-4 to -1 day before treatment Day 0) and peak excretion days (Wilcoxon statistic = 0.0, n = 7, df = 6, P = 0.0156). Significant net output in faecal corticosterone concentrations above the pre-ACTH treatment baseline were also measured in all study animals, assuming the unidentified sample belonged to Group 1 female (Wilcoxon statistic = 0.0,

$n = 7$, $df = 6$, $P = 0.0156$). However, it must be noted that even with the exclusion of this unknown sample, absolute concentrations of corticosterone excreted post-ACTH injection remained significant, albeit, with a slightly lower probability ($P<0.11$).

The greatest ACTH-induced rise from pre-treatment baseline concentrations was observed in Group 1 female (assuming the unidentified sample belonged to this animal, Figure 4.3B). Pre-ACTH treatment mean was measured at 44.2 ± 5.7 ng/g (Table 4.2). After the ACTH injection, mean corticosterone concentrations increased 350% above pre-ACTH baseline to 154.7 ng/g, with peak values occurring 2 days post-injection (Figure 4.5A, Table 4.2). Concentrations returned to baseline 3 days after treatment. In contrast to Group 1 female's pronounced rise in response to the ACTH injection), the elevation in concentration in Group 2 female and Group 3 male showed no clear peaks (Figure 4.5A), whilst Group 2 male and Group 3 female 1 and 2 showed a less pronounced increase above the pre-ACTH baseline mean (range, 196-230%), with smaller, less well defined peaks occurring between 1 to 2 days after treatment (Figure 4.5A, Table 4.2). Furthermore, when analysing each group as opposed to individual responses to the ACTH treatment, Group 2 animals peaked one day before the other groups (Figure 4.5B) although net output of corticosterone excreted did not differ significantly between groups (KWH-test, $n = 3$, $H = 3.93$, $P = 0.1403$).

Overall, a mean corticosterone increase of 184% above pre-ACTH treatment baseline was measured for all seven white rhinos, coinciding with the ~24 to 48 h excretion lag times in faeces measured by Hindle and Hodges (1990) where concentrations declined to baseline 4 days post ACTH treatment (Figure 4.5C).

Table 4.2 Post-ACTH/saline treatment rise in faecal corticosterone concentrations for each study animal.

Group	Sex	Treatment	Pre-Treatment Mean (ng/g) (±SEM)	Standard deviation (SD)	Peak Post Treatment Value (ng/g)	Post-Treatment Rise (% pre-treatment mean)
1	M	ACTH	34.7 ± 2.8	2.7	44.8	129
		Saline	32.5 ± 1.9	2.6	38	117
	F	ACTH	44.2 ± 5.7	11.7	48.1/ *154.7	109/*350
		Saline	40.1 ± 4.7	9.5	41.7	104
2	M	ACTH	24.4 ± 1.5	4.6	47.8	196
		Saline	33.3 ± 3.7	8.2	37.1	111
	F	ACTH	28.4 ± 1.7	3	37.4	132
		Saline	30.7 ± 2.0	6.2	40.2	131
3	M	ACTH	31.8 ± 3.9	9.6	38.8	122
		Saline	32.8 ± 1.8	4.1	53	162
	F1	ACTH	31.3 ± 3.5	7.1	68.4	219
		Saline	38.0 ± 1.7	1.5	43.2	114
	F2	ACTH	27.9 ± 2.9	5.7	64.2	230
		Saline	41.0 ± 1.3	2.5	90.5	221

Pre-ACTH and saline treatment mean represents the average corticosterone concentrations for pre-treatment samples (ng/g of dried faeces), 4 days before treatment Day 0 and Day 11, and are expressed as means ± standard error. Data are presented as a percentage of the pre-ACTH/saline treatment mean, which was designated as 100%. * represent the results if the unidentified sample was included in the Group 1 female data set.

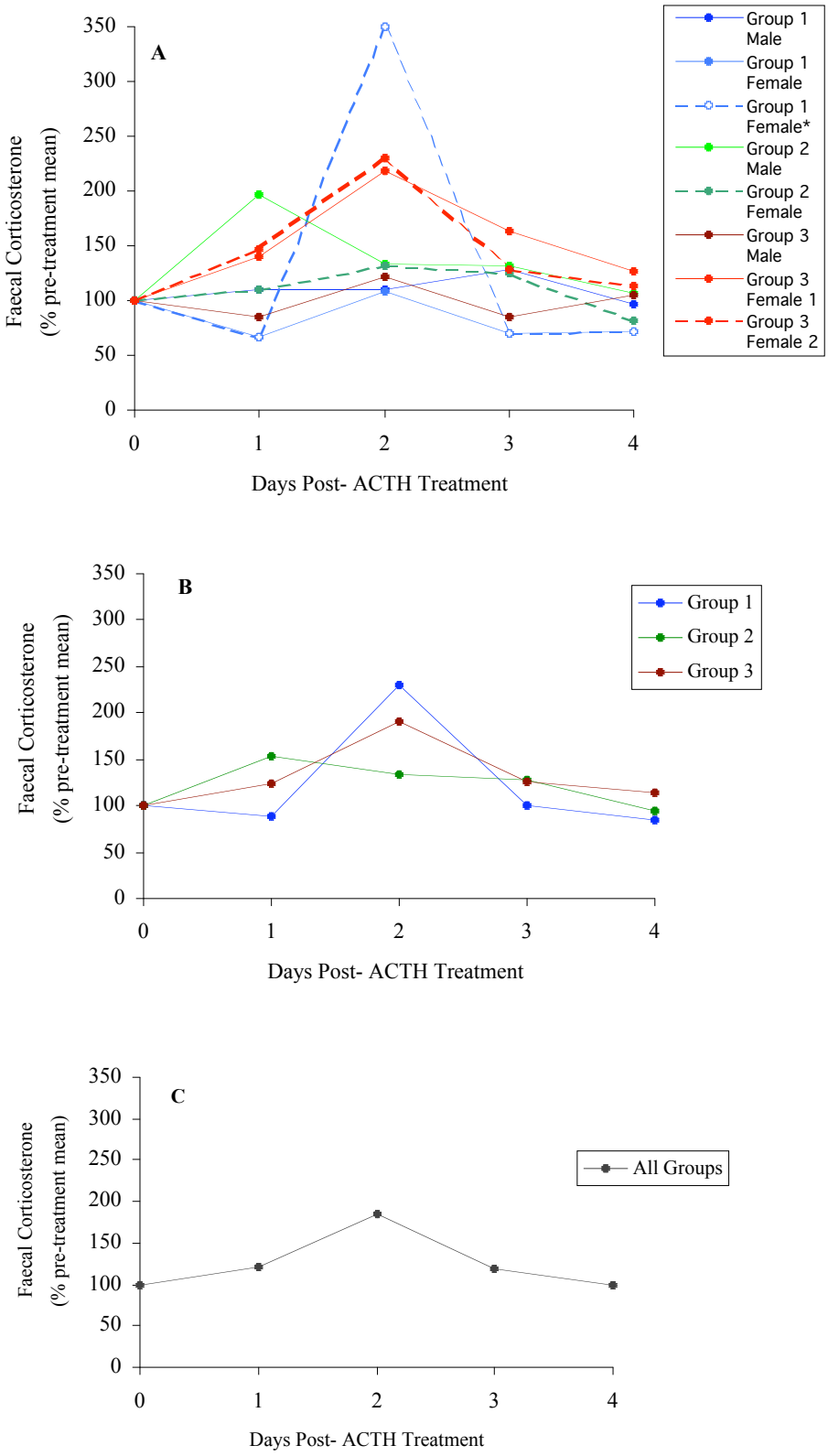


Figure 4.5 Mean faecal corticosterone response to ACTH treatment in (A) individual animals (Group 1 female * includes unidentified sample), (B) in Groups 1, 2 and 3, and (C) in all study animals. B and C assume unidentified sample belongs to Group 1 female. Data are presented as a percentage of the pre-ACTH treatment mean, which was designated as 100%.

4.3.3 Effects of the Injection Procedure

Unlike the response reported in the ACTH challenge test, no significant changes were observed in faecal samples in all seven white rhinos used in the experiment to evaluate effects of the injection procedure itself (Wilcoxon statistic = 5.0, n = 7, df = 6, P = 0.1563). In Group 1 male, Group 2 female and Group 3 female 2, concentrations of corticosterone excreted post saline treatment were almost equal to the mean corticosterone concentrations percentage increases above pre-ACTH and saline treatment baselines (Table 4.2), thus confirming that in neither of these animals was the post-injection measured four days after treatment significant. In most of the other animals, percentage increase post saline treatment was less than the percentage increases post ACTH treatment (Table 4.2), however percentages did not differ significantly (P>0.05). Although Group 3 male measured a slightly higher percentage increase from pre-saline treatment to peak value (162%) than during the ACTH challenge (122%) (Table 4.2), there was no significant difference in pattern of excretion as a reaction to the injection procedure, as the increase observed was within the range of baseline fluctuations.

Whilst Group 3 female 2 excreted the highest post-saline treatment concentration of corticosterone, at a peak of 90.5 ng/g, this peak was observed 4 days after saline treatment and was unlikely to be induced by the saline injection itself. Possible reasons for this peak are discussed in 5.1.3.

Comparison of the pre-ACTH treatment corticosterone concentrations and pre- and post-saline treatment concentrations revealed no significant difference between these experimental periods (Wilcoxon statistic = 9.0, n=7, df= 6, P= 0.4688), demonstrating that the control experiment was within the range of baseline variability. Finally, when comparing gender, there was no significant difference between overall means of faecal corticosterone excreted throughout the trial period (MWU-test, U= 2.00, df= 1, P= 0.1573).

4.4 GIT Time

The GIT times that were measured after ACTH and saline treatments, and after a baseline day as the control, are presented in Table 4.3. Due to limited data, the net output of corticosterone excreted, time to peak and peak mean of the faecal corticosterone concentrations post ACTH and saline treatments, could not be related to the measured GIT time for statistical comparison between individual treatments. Nevertheless, from the data that was obtained, it was noted that gut passage times correlated to the ACTH-induced time to peak (Figure 4.5B, Table 4.3), and were in accordance to the ~24 to 48 h excretion lag times in faeces measured by Hindle and Hodges (1990). Group 2 animals displayed the fastest GIT times throughout all the glitter trials, however there were marginal differences in GIT times between post-ACTH, saline and control days in all the groups (Table 4.3).

Table 4.3 Individual GIT times after ACTH and saline treatments, and on baseline days during the trial.

Group	Animal	Gastrointestinal transit time after individual treatments (hours)		
		ACTH	**Saline**	**Baseline**
1	M	no data	no data	no data
	F	36	37	no data
2	M	27	28	30
	F	28	30	32
3	M	no data	no data	39
	F1	43	~24	40
	F2	44	no data	42

The green art glitter added to the daily feed was easily consumed although not always detectable in the faeces; on several occasions was the marker not observed - Group 1 male after ACTH and saline treatment, Group 3 male after ACTH and saline treatment, and female 2 after saline administration. GIT times after a non-treatment day were not recorded from Group 1 animals because the control part of this experiment was only decided upon after the trial had ended, at which time the animals had been re-released onto a game reserve.

In the case of measuring GIT times post-saline treatment in Group 3 male and female 2, early morning access into the enclosure was restricted as animals had to be held inside

the main part of their enclosure where the midden was situated, whilst buffalos in a neighbouring enclosure were being moved into a vehicle for translocation. By the time access was permitted, the faecal piles from the previous night and early morning had been trampled and urinated on by each animal, thus making it extremely difficult to detect any glitter within the faecal bolus. No glitter was found following subsequent checks of faecal stools.

Although access into the enclosure was also limited for Group 3 female 1 for the same reasons described above, a trace of glitter had been observed in fresh early morning faeces in a scattered pile of faeces, first excreted in the very early hours of the morning until the time of access into the enclosure. Whilst an exact GIT time could not be determined, it was estimated that the time to excrete the marker was at approximately 24 h post-saline treatment.

CHAPTER 5

5. DISCUSSION

5.1 Introduction

The major objective of the study was to prove the suitability of a developed technique to monitor stress hormones non-invasively in faecal samples of white rhinos. Therefore, it was tested whether adrenocortical function can be monitored accurately by analysing faecal corticosterone using a ^{125}I radioimmunoassay (MP Biomedicals) in captive bred and boma-housed white rhinos.

Like conventional assays for hormones in plasma, each assay for steroid metabolites in faeces requires analytical validation steps to demonstrate parallelism and accuracy (Goymann, 2005). These analytical validations ensure that the assay system works properly and that there are no substances present in the extracts disturbing the binding properties of the antibodies used (Goymann, 2005). Furthermore, the importance of proving the biological validity of these non-invasive hormone assays is essential for the reason that hormone metabolites rather than the actual hormone are measured; hence it must be proved that the antibody is indeed capable of detecting the metabolites of the hormone in question (Touma and Palme, 2005). Therefore to evaluate the biological relevance of this non-invasive approach for measuring concentrations of corticosterone, an experiment was performed using pharmacological stimulation of the adrenal cortex in each study animal. A control experiment was then performed to investigate the effects of the injection procedure itself.

5.1.1 Faecal Radioimmunoassay

The ^{125}I radioimmunoassay (MP Biomedicals) was validated by demonstrating firstly, parallelism between displacement curves for the serial dilutions of a neat pool of faecal extracts and the corticosterone standard curve (Figure 4.1); and secondly, mass recovery of exogenous corticosterone added to samples before analysis (Figure 4.2 and Table 4.1). Based on the data obtained, the validated RIA, currently favoured for monitoring faecal

corticoids in white rhinos (Carlstead and Brown, 2005; Brown *et al.*, 2001), reliably detected individual variations of concentrations of corticosterone, pre- and post-injection with ACTH and saline, in both captive bred and boma-housed white rhinos.

5.1.2 ACTH Challenge

Validation of RIA (MP Biomedicals) detected an increase in white rhino faecal corticosterone, released in response to exogenous administration of ACTH. In the present study, ACTH induced a maximum rise of 350% above mean pre-treatment baseline concentrations of corticosterone (Figure 4.5A, assuming the unidentified sample belonged to Group 1 female, 1.5 IU/kg of body weight, 1500 IU ACTH)). This compares with the results of a similar study that was done in four male black rhinos, using the RIA (MP Biomedicals) and 800 IU ACTH (Brown *et al*., 2001). In another study that used the commercial corticosterone RIA in a male and female black rhino (Wasser *et al*., 2000) the results (mean ~255% above baseline, as calculated from graph, 1.5 IU/kg, 800 IU ACTH), were comparable with those measured in Group 3 female 1 and female 2 (219% and 230 %, respectively, Table 4.2). Predictably, Group 1 male who received a half dosage of ACTH (0.75 IU/kg, 1575 IU ACTH, for reasons discussed in 3.1.2.) showed a less prominent rise following an ACTH challenge (129% above mean baseline).

As discussed earlier in Chapter 2, the adrenal cortex rapidly synthesizes and secretes glucocorticoids in response to ACTH (Palme, 2005; von Holst 1998). Although serum corticoid concentrations in mammals rise within 10-30 min (Brown *et al.*, 1995), the appearance of elevated concentrations of glucocorticoid metabolites in faeces following ACTH injection is much slower (Young *et al.*, 2004). This species-specific lag time between hormonal events in the blood and the appearance of the respective signal in the faeces was found to be related to the animals' intestinal transit time (Palme *et al.*, 1996). The present study also supports this finding, as the delays between ACTH administration and the appearance of peak corticosterone concentrations in faeces (1-2 days post-ACTH treatment, Figure 4.3 and 4.5) were consistent with known gut passage times in white rhinos (~24-48 h, Hindle and Hodges, 1990). Knowledge of this gut passage time can therefore allow extrapolation from collection dates to determine relative contribution of

potential stressors (e.g. response to conspecifics, caretakers) to corticosterone concentrations in faecal samples (Mashburn and Atkinson, 2004).

5.1.3 Effects of the Injection Procedure

The saline experiment served to evaluate possible effects of the injection procedure itself, since this is likely to be a stressful event for a rhino and therefore could influence the animal's glucocorticoid concentrations (Kretzschmar *et al.*, 2004). The absence of significant elevations (when using Wilcoxon statistical test comparing all seven study animals) in glucocorticoid metabolites following saline treatment suggested that the injection procedure may not have been as stressful for the individuals examined in this study. However, adrenocortical responses are known to reflect interanimal variation in the perception of a stimulus, and the biological responses evoked to cope with a stressor depend on factors such as previous experience, genetics, age and physiological state (Moberg, 2000).

When observing individual responses to the saline treatment, it must be noted that Group 3 male elicited a greater rise (162% above pre-saline treatment mean) in faecal corticosterone concentration 2 days post saline injection, than after an ACTH challenge (122% rise above pre-ACTH treatment mean, Table 4.2). A likely reason for this pronounced rise in corticosterone concentration could be a response to the arrival of burnt elephants rescued from another reserve on the same day of treatment and placed in the enclosure adjacent to the male. Along with the introduction of heterospecifics, came a notable amount of human disturbance, all of which perhaps elevated concentrations of corticosterone. The timing of the peak (2 days post arrival of elephants) corresponded with the ~24-48 h excretion lag times in white rhino faeces (Hindle and Hodges, 1990), allowing us to extrapolate from the collection date, that this stressor was more than likely the reason for such an elevation in corticosterone concentration.

Furthermore, Group 3 female 2 excreted a peak of 90.5 ng/g, 4 days post saline treatment, that was also unlikely to be induced by the saline injection itself (Figure 4.3G). A plausible explanation for this peak would most likely be the same social stressor (i.e. the elephants) which elevated corticoid concentrations in Group 3 male. Another load of

elephants were placed into adjacent enclosures 2 days before the peak in Group 3 female 2, which also correlates to the ~24-48 h excretion lag times previously reported (Hindle and Hodges, 1990). If this was indeed, the reason for these post-saline injection peaks, it implies that the corticosterone detectable with the RIA (MP Biomedicals) is a measure of the animal's response to environmental events, that are not associated with an ACTH challenge or the injection procedure.

5.2 Individual Variability

Many authors who investigated a substantial number of animals reported considerable individual variation, both in baseline and ACTH induced concentrations of faecal glucocorticoid metabolites (Touma and Palme, 2005). Such was observed in the laboratory mouse (Touma *et al.*, 2004), cattle and domestic sheep (Palme *et al.*, 1999), long-tailed macaque (Wasser *et al.*, 2000), Adélie penguins (Nakagawa *et al.*, 2003) and more specifically, in the black and white rhino (Carlstead and Brown, 2005; Brown *et al.*, 2001). On the basis of weekly faecal sample analysis over a 12-month period in black and white rhinos, Brown *et al*. (2001) observed fluctuations within and among individuals (Figure 5.1). Within species, there were no gender differences in overall mean corticoid concentrations in black or white rhino. However, overall mean concentrations were significantly higher in the black (41.8 ± 3.0 ng/g) compared to the white (31.2 ± 1.7 ng/g) rhino, with this difference being more prominent in the males (Figure 5.1A). Whether this is related to species or age differences in adrenal function or stress responsiveness remains to be determined (Brown *et al.*, 2001).

Similarly, results in the present study showed no difference between sexes in faecal corticosterone excreted throughout the trial period in the white rhinos. Furthermore, mean corticosterone concentrations in pre-ACTH treatment baselines in most of the study animals (Figure 4.4) were consistent with the overall mean corticoid concentrations in the white rhinos measured by Brown *et al*. (2001) (Figure 5.1).

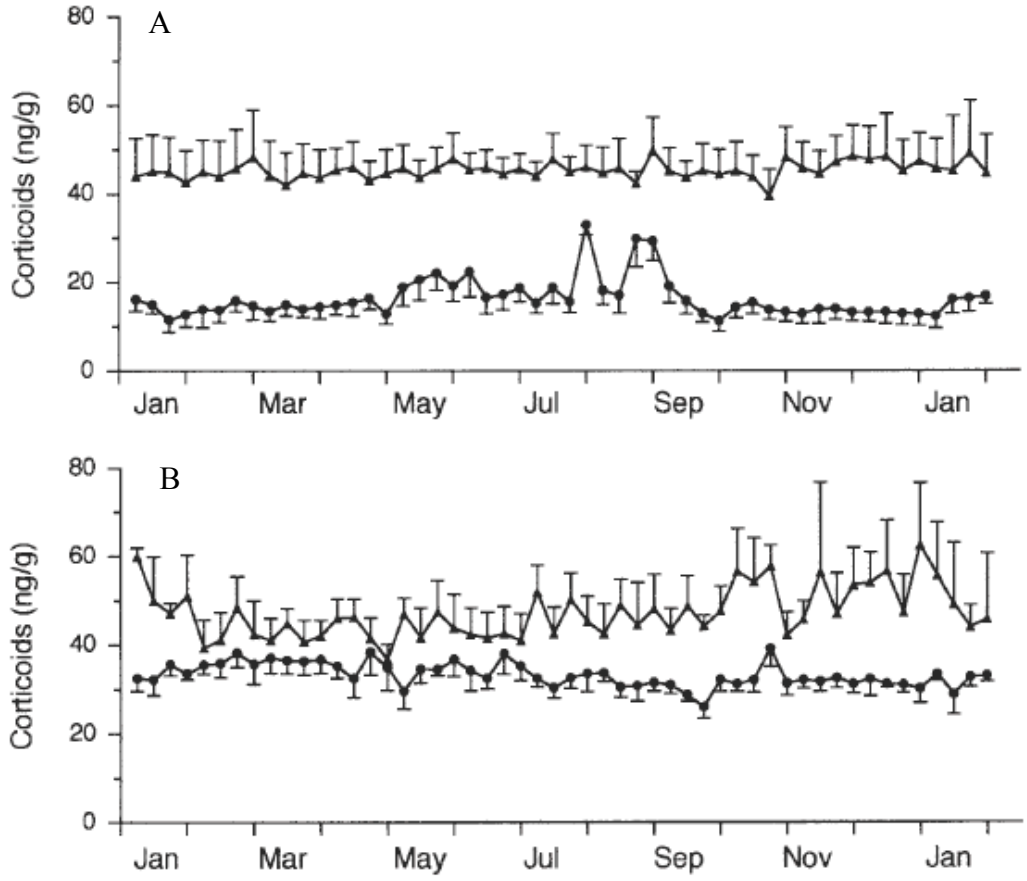


Figure 5.1 Weekly mean (±SEM) profiles of faecal corticoid concentrations in male (A) and female (B) black (solid triangles; *n* = 10) and white (solid circles; *n* = 6) rhinoceros (Brown *et al.*, 2001).

In this study, a good example of individual variability was demonstrated in Group 1 female, whose pre-ACTH treatment mean (44.2 ± 5.7 ng/g, Figure 4.4), albeit statistically insignificant, was slightly higher than the other animals in the present study, as well as the overall mean corticoid concentrations of white rhinos investigated by Brown *et al.* (2001) (Figure 5.1) Group 1 female also demonstrated the greatest response to the ACTH injection (350% rise above pre-ACTH treatment mean, assuming the unidentified sample belonged to the female) compared to the other study animals (range, 122–230 % rise) possibly indicating that this individual may have been demonstrating adrenal hyperactivity. Research on laboratory and farm animals using ACTH challenges have shown that animals that are experimentally subjected to chronic stressors may not differ from control animals in baseline corticoid concentrations (Carlstead and Brown, 2005). Instead, they appear to habituate to the chronically present stressor, but exhibit hyperactivity of the adrenal cortex in response to a new, acutely presented stressor

(Carlstead and Brown, 2005; Carlstead *et al.*, 1993b). Whether the female was showing adrenal hyperactivity to an acute stressor (i.e. the ACTH injection) after being subjected to chronic stressors (enclosed two days prior to the trial with an increased level of human interference during sample collection) remains unknown but is highly plausible.

In the case of wild-caught animals placed into captivity, such as those in Group 1 and 3, previous studies suggest that time spent in captivity influences their baseline corticoid concentrations and responsiveness to stimuli (Millspaugh and Washburn, 2004). If captive animals have not had sufficient time to habituate to their new environment, their response to treatment may not be as reliable as those who have had time to acclimate (Millspaugh and Washburn, 2004). Unlike Group 3 animals that were placed into individual housing three months prior to the study, Group 1 animals were held in their enclosure two days before the trial. Thus, it is possible that in Group 1, the female did not have sufficient time to habituate to it's new environment, and therefore could be a likely explanation for the elevated pre-ACTH treatment baseline and pronounced response to ACTH administration.

Moreover, it would appear in some instances, that accurate identification of a true adrenocortical response to exogenous ACTH might be questioned due to the high day-to-day variation in concentrations of faecal corticosterone. For example, mean corticosterone concentrations following ACTH administration of Group 2 male (47.74 ng/g) exceeded values for significance (when using Wilcoxon statistical test comparing all seven study animals). However it was difficult to determine whether these elevations were true responses to the ACTH stimulation because an unexplained peak in corticosterone pre-saline treatment also reached a mean concentration (45.29 ng/g) comparable to the threshold value (Figure 4.3C).

Conversely, the absence of pronounced elevations in glucocorticoid metabolites following an ACTH treatment as seen in Group 2 female and Group 3 male (Figure 4.3 D, E) suggests that maximal amplitude of adrenal activity in the faeces was not reached, even though the same dosage administered to other animals in this study provided their own benchmark in which responses to other stimuli could be compared. Such a

benchmark allows for the comparison of faecal corticosterone concentrations to be used to evaluate physiological function of a species in response to a variety of potential stressors (Young *et al.*, 2004).

Since adrenal responsiveness to ACTH undoubtedly shows strong individual differences, it is likely that the ACTH dose used for Group 2 female and Group 3 male was inadequate to induce a maximal response. Alternately, it is possible that these individuals did respond to the ACTH, but the acute adrenocortical rise was undetectable. Brief or small increases in circulating concentrations of glucocorticoids are likely to be masked by the pooling of metabolites in bile and faeces (Young *et al.*, 2004). Also, faecal samples containing ACTH-induced peaks in corticosterone may have been missed due to failure to collect all stools defecated each day.

Other studies have also had varied success in detecting adrenocortical responses following treatment with exogenous ACTH (Young *et al.*, 2004). In fact, there are a few examples in the literature stating that in some species, certain individuals showed the expected pattern of faecal glucocorticoid metabolite concentrations after ACTH stimulation (Touma and Palme, 2005), whereas inconsistent results were obtained in others (Nakagawa *et al.*, 2003; Young *et al.*, 2001; Wasser *et al.*, 2000). Thus, for a proper biological validation of the technique, it is strongly recommended to use enough individuals of both sexes and not to rely upon results obtained from only a few animals of a given species (Touma and Palme, 2005). Unfortunately, in this study using more than seven animals was not financially feasible, hence each animal was used as its own control by calculating percentage increase from pre-treatment baselines to post-treatment peak corticosterone concentrations, thereby minimizing the problems of individual variability.

Moreover, because faecal corticoid concentrations fluctuate over time, an adequate sampling regimen must be used for accurate assessment of baseline adrenal activity. Conducted properly, these assessments will be most useful when data are compared with previously established baseline values to evaluate individual responses to changes in management strategies or environmental factors. With regards to the present study, increasing the period for pre-ACTH baseline sampling would perhaps provide a more

meaningful response to an ACTH challenge and saline administration as the control, and thus provide a more reliable biological evaluation of a non-invasive approach for measuring concentrations of corticosterone. Particularly in the case of wild-caught animals, whose period of habituation might be quite variable and subsequently may influence basal corticoid concentrations and responsiveness to treatments (as may be the case in Group 1 female).

Obviously, a wide variety of potential stressors could have affected the corticoid profiles observed in the animals in each of the three groups, including noises, husbandry events, sample collection, and in the case of Group 3, arrival of heterospecifics. Nevertheless, there were no significant differences between groups in overall mean faecal corticosterone concentrations excreted throughout the trial. However, in a study by Carlstead and Brown (2005), variability in corticoids, rather than the mean concentrations, may be most informative for identifying chronic stress and associated biological costs of excessive adrenal activation in white rhinos, and possibly other species (Carlstead and Brown, 2005). Studies of stress emphasise the importance of maintaining physiological homeostasis and the adaptive ability of the animal (Broom and Johnson, 1993).

Increased variability in corticoids due to responsiveness to human caretakers and conspecifics in captive white rhinos, have been associated with potentially deleterious consequences (i.e. absence of ovarian cycles) (Carlstead and Brown, 2005). In the current study, there was no significant difference between the days before ACTH treatment in all study animals although variability (SD) was highest in Group 1 female. However, if corticoids were monitored over a longer period of time, it may become apparent that individuals with more widely fluctuating concentrations have more difficulty maintaining homeostasis. Thus, variability of longitudinal faecal corticoid analysis is emerging as a valuable measure of chronic stress, that is, adrenocortical activation that has biological costs to the animal (Carlstead and Brown, 2005).

5.3 GIT Time

Faecal glucocorticoid metabolites in the white rhinos used in the present study increased within 24-48 h after injection of ACTH (Figure 4.5A). Palme *et al*. (1996) suggested that the delay of faecal glucocorticoid excretion roughly corresponds with the food transit time from duodenum to rectum in sheep, horses and pigs. In white rhinos, the presence of glitter in the faeces was found approximately 27-44 h after consumption and treatment of ACTH (Table 4.3). Thus, taking GIT time as a measure of food passage time between duodenum and rectum, the time lag of glucocorticoid excretion in faeces appears to correspond with food transit time in white rhinos. Moreover, the results in the present study were consistent with known gut passage times in white rhinos (Hindle and Hodges, 1990).

Although there was only slight difference in GIT times post-ACTH and saline treatments as well as control days in each individual, there was a notable difference between groups whereby Group 2 animals displayed the fastest GIT times throughout all the glitter trials. A plausible explanation for this could be due to the higher feed intake and differences in diet composition where Group 2 animals were fed chopped vegetables, Boskos (supplementary fibre source) and teff grass whilst the other groups were fed on bales of Lucerne. Previous studies have shown that an increase in feed intake and dietary fibre may increase gut passage time (Goymann, 2005; Morrow *et al*., 2002). This could therefore, be a likely reason for the difference in GIT times in Group 2 compared with the other groups.

As previously mentioned in 4.4, measuring GIT times using art glitter added to food was often unreliable, where no trace of glitter could be found inside the faecal bolus. Even when glitter was detected after defecation, results were not always dependable. Such as in the case of Group 3 female 1, where a spec of glitter was observed on a scattered pile of faeces at approximately 24 h post-saline treatment. However, this data was not conclusive for the reason that the glitter may have been attached to the animal and carried from the feeding area to the midden. When access into the enclosure was not feasible, by the time entry was possible, often the stools had been trampled and urinated on thus making it difficult to detect any glitter.

Overall, using glitter as a digestive passage marker to measure gastrointestinal transit time was not always reliable. However the data that was collected of the time between consumption and first appearance of the marker in the faeces, correlated to ACTH-induced peaks. Therefore the GIT time of glucocorticoid excretion post-ACTH treatment corresponded to the time of the initial appearance of the digestive marker after consumption. Known gut passage times can therefore be correlated to peaks of glucocorticoid excreted in an event of an acute or chronic stressor. However it must be highlighted that there is a need to consider conditions known to affect gut transit time (e.g. feed intake, feed composition) when deciding to measure GIT times in relation to the stressor of interest.

CHAPTER 6

6. CONCLUSION

The present study showed that 1) after ACTH stimulation, the faecal glucocorticoid concentration, measured with the validated ^{125}I RIA (MR Biomedicals), differed significantly from baseline glucocorticoid concentrations in white rhinos, 2) after saline treatment, the faecal glucocorticoid concentrations did not differ significantly from baseline glucocorticoid concentrations, and 3) after saline treatment, faecal glucocorticoid concentrations excreted did not differ significantly from faecal glucocorticoid concentrations excreted post ACTH treatment.

However I can conclude that stimulation of the adrenal cortex is reflected accurately by means of corticosterone measurements in faecal samples of captive bred and transiently housed white rhinos. Furthermore, the RIA technique not only proved sensitive enough to detect effects of the ACTH treatment in most of the animals used in the study, but also naturally occurring elevations of glucocorticoids in response to environmental stressors. Thus, the results confirm that measurement of faecal corticosterone with the validated ^{125}I RIA (MR Biomedicals) offers a practical alternative for investigators wishing to non-invasively monitor adrenocortical activity for improving the health and well being of white rhinos.

While this technique has immediate applications for the assessment of welfare in permanently captive and transiently-housed white rhinos, a large unresolved issue impeding the use of this non-invasive technique to monitor stress hormones, is an understanding of what range of faecal glucocorticoid concentration, over what period of time, is deleterious to the animal (Mashburn and Atkinson, 2004). An increase in glucocorticoid secretion does not automatically equate to a state of distress (Moberg, 2000). Elevated concentrations might forewarn of possible harm (Creel, 2005), however care should be taken to avoid confusing physiological evaluations with detrimental responses.

For rhinos in captivity, where space is restricted, problems have been identified that suggest compromised animal well-being (e.g. poor reproductive performance, disease susceptibility and high mortality rate, Brown *et al.*, 2001). Determining whether these environmental conditions or management practices facilitate or compromise the animal's ability to cope is key to ensuring animal welfare. Thus, future studies should focus on integrating faecal corticoid analyses with other evaluations based on behaviour, reproductive physiology, immunology and pathology to provide a more meaningful measure of stress and associative factors affecting health and well-being of white rhinos in captivity. A successfully established and validated non-invasive technique to monitor stress hormones in faecal samples of white rhinos can therefore open new perspectives in biomedical and behavioural science in wildlife.

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Appendix I

Source and Cross-reactivity Data for MP Biomedicals Corticosterone ^{125}I RIA (MP Biomedicals, Instruction manual).

Manufacturer	**Catalogue No**	**antigen**
MP Biomedicals	07814201.6	corticosterone

Steroids	**% Cross Reaction**
Corticosterone	100
Desoxycorticosterone	0.34
Testosterone	0.1
Cortisol	0.05
Aldosterone	0.03
Progesterone	0.02
Androstenedione	0.01
5a-Dihydrotestosterone	0.01
Cholesterol	<0.01
Dehydroepiandrosterone	<0.01
Dehydroepiandrosterone-sulfate	<0.01
11-Desoxycortisol	<0.01
Dexamethasone	<0.01
20a-Dihydroprogesterone	<0.01
Estrone	<0.01
Estradiol-17a	<0.01
Estradiol-17b	<0.01
Estriol	<0.01
Pregnenolone	<0.01
17a-Hydroxypregnenolone	<0.01
17a-Hydroxyprogesterone	<0.01

Appendix II

Average counts of duplicate – CPM calculation (MP Biomedicals, Instruction manual).

$$\%B/Bo = \frac{CPM\ (sample) - CPM\ (NSB)}{CPM\ (0\ calibrator) - CPM\ (NSB)} X100$$

CPM = Average counts of duplicates

Sample = Particular faecal sample or calibrator being calculated

NSB = Non-specific binding tubes (also known as the blank tube)

0 Calibrator = 0 tube (also known as 100% binding tube)