UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Determining adrenocortical activity as a measure of stress in domestic pig (*Sus scrofa domesticus*) based on salivary and faecal analysis

By

Nobert Mangwiro

Submitted to the Faculty of Veterinary Science, University of Pretoria in partial fulfilment of the requirements for the degree of
Master of Veterinary Science

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Research conducted in the Department of Production Animal Sciences
Faculty of Veterinary Science
University of Pretoria
Onderstepoort

Supervisor: Prof. F. O. Fasina

Co-Supervisor: Prof. A. Ganswindt

March 2016

© University of Pretoria

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

ACKNOWLEDGEMENTS

I would like to acknowledge the following persons and institutions for their most appreciated input and support:

Firstly, I would like to thank UPBRC for their cooperation and availability as study site.

Sincere thanks to my supervisors Prof. Fasina, F. O. and Prof A. Ganswindt for all their advice and guidance regarding the project.

Additionally I would like to thank UPBRC staff for their generous logistical support of the ACTH study and Stefanie Ganswindt and her team for the analysis of samples and their invaluable guidance.

Special thanks go to my wife Rumbidzai, family and friends for their personal support.

I acknowledge the assistance of my late Supervisor DR P J Loock of Department of Agriculture, Limpopo, my employer who always permitted my travel and facilitated my work.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

DECLARATION

I, Nobert Mangwiro, 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 (Veterinary Science) in Production Animal Studies.

I hereby grant the University of Pretoria free license to reproduce this dissertation in part or as whole, for the purpose of research or continuing education.

Signed……
Nobert Mangwiro

Date……

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

LIST OF TABLES

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

LIST OF FIGURES

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

LIST OF APPENDICES

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

LIST OF ABBREVIATIONS

- 11,17-DOA 11,17 dioxoandrostanes
- 3α,11-oxo-CM Cortisol metabolites with a 5β-3α-ol-11-one structure
- ACTH Adrenocorticotropic hormone
- AVMA American Veterinary Medical Association
- CRH Corticotropin-releasing hormone
- CV Coefficients of variation
- DW Dry Weight
- FAO Food and Agricultural Organisation
- GAS General adaptation syndrome
- GCs Glucocorticoids
- GCM Glucocorticoids metabolites
- fGCM Faecal Glucocorticoid Metabolites
- HPA Hypothalamic–pituitary–adrenocortical
- EIA Enzyme Immunoassay
- ONF Oral-nasal-facial
- ng nano gram
- *UPBRC* University of Pretoria, Biomedical Research Centre
- US-NPPC U.S. National Pork Producers Council

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

ABSTRACT

A pig is any listed animal species in the genus *Sus*, within the family Suidae of even-toed ungulates. The domestic pig (*Sus scrofa domesticus*) (also referred to as swine or hog) is a large, domesticated, even-toed ungulate that has its ancestry in the wild boar. Domesticated pigs are commonly raised as livestock by farmers for pork, hams, gammon or bacon), as well as for leather. For the Pig industry, chronic stress posed by gestation crates and its often negative consequences are a welfare concern, since stressors can negatively affect animal production as well as the health of pigs.

When confronted with a stressor, an individual displays a stress response consisting of a suite of physiological and behavioral alterations to cope with the challenge. In pigs, the assessment of physiological responses to stress, like the related alterations in glucocorticoid (GC) concentrations, has already been done, but only by using an invasive approach, with the disadvantage of a possible handling-induced stress response. So far, no validated non-invasive method for determining stress-related responses in pigs seems available. Therefore the study aimed to determine validated stress-related physiological responses in domestic pigs' saliva and faeces through the performance of an adrenocorticotropic hormone stimulation test (ACTH challenge test), the effect of circadian rhythm on faecal GC levels and to investigate the effect of storage on the rate of metabolism of faecal glucocorticoid metabolites post-defaecation.

An ACTH challenge was performed on four pigs and with two pigs as saline controls, at University of Pretoria Biomedical Research Centre (UPBRC), South Africa. Faecal GC metabolites (fGCM) were validated against four enzyme immuno-assays including the 3α,11-oxo-CM, 3β,11β-diol-CM, 11,17-DOA and cortisol. The 3β,11β-diol-CM method was subsequently selected and used to investigate for salivary and faecal GCM. The results indicated that acute salivary cortisol levels can increase up to ~600 % above the pre-injection baseline levels within 40 to 60 minutes post-injection. Faecal glucocorticoid metabolites

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

(FGCM) levels in the 4 ACTH inoculated pigs reached 2-fold peaks of pre-injection levels within 7 to 36 hours following treatment.

Comparatively, the early morning saliva and faecal samples have higher glucocorticoid concentrations compared with the late afternoon samples. In addition, fGCM in faecal samples were relatively stable for over 48 hours demonstrating that faecal sample storage may not affect outcomes of non-invasive hormone monitoring to assess adrenocortical function in pigs. This method provides an empirically sound basis for a non-invasive monitoring of adrenocortical function in pigs and serves as template for further studies which may have value for the monitoring of adrenocortical function in populations of pigs in different housing units and other stressful conditions.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

CONTENTS

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

1. INTRODUCTION

There are over 1 billion pigs worldwide (FAO, 2009; CIWF, 2015), and a large proportion of these pigs are intensively managed in confined pens and sow crates with animal welfare implications in especially the intensive piggeries (Kittawornrat et al. 2010, McGlone 2013b, Salak-Johnson&McGlone 2007) In this regard, animal welfare issues are often evaluated by monitoring proxies for intrinsic and extrinsic stress perceived by pigs (Turner, Hemsworth & Tilbrook 2005) Potential stressors could be periods of weaning, transportation, mixing, and confinement, as well as events like castration and tail docking among others (Ruis et al. 1997). Consumers of finished products (pork, bacon, ham and their bye-products) paying increasingly attention to farming practices used in modern food production and making informed choices (Tonsor, Olynk & Wolf 2009). Due to animal welfare concerns and consumer interests as well as to ease the management of sows in intensive care settings, the housing conditions of pregnant pigs in crates and its potential stress-inducing effects are of utmost interest to the industry (McGlone 2013a, Salak-Johnson&McGlone 2007).

In response to a perceived stressor, corticotropin-releasing hormone (CRH), released from the hypothalamus will stimulate the pituitary to release adrenocorticotropic hormone (ACTH), which in turn stimulates the adrenal cortex to release glucocorticoids. Glucocorticoids can therefore be seen as key elements in the neuroendocrine stress axis, as their measurement can give insight into an animal's well-being (Sheriff et al. 2011). In pigs, the predominant glucocorticoid secreted is cortisol (Bottoms et al. 1972, Smith&Vale 2006). So far, blood, saliva, and to a quite lesser extent urine have been used as hormone matrix for monitoring glucocorticoid alterations in pigs (Broom&Fraser 2007, Moestl 2014, Mostl et al. 1999a, Ruis et al. 1997). Although blood is still wildly used for determining glucocorticoid concentrations in a wide range of species including pigs, respective results are often of limited value as determined steroid levels are usually affected by episodic fluctuations and the pulsatility of hormone secretion (Touma&Palme 2005). In addition, blood collection often requires some sort of animal restraint, which is usually perceived as a stressful event in itself (Broom&Johnson 1993). Consequently, minimal or even non-invasive monitoring of steroid hormone metabolites, using alternative matrices like saliva, urine,

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

and especially faeces, have become an increasingly popular technique (Hodges K, Brown J, Heistermann M, 2010, Hodges, Brown & Heistermann 2010). In other developments the determination of medium- and long-term stress response with the use of hair matrix as a non-invasive technique has also been proposed (Bacci et al. 2014) as this hair matrix is a reliable medium for measuring basal cortisol concentrations (Antonides et al. 2015). The collection of saliva, however, still requires some manipulation of the animal and might therefore not entirely feedback free. Furthermore, it can only be applied to a limited extent in free-moving animals (Möstl&Palme 2002, Touma&Palme 2005). Also the absorbent material used for saliva collection needs to be carefully selected, as some of them are very efficient at binding cortisol and essentially strip cortisol from the sample (Cook 2012). Finally, salivary steroid concentrations also susceptible to circadian rhythm and episodic fluctuations of hormone secretion. This will lead to the need for accurate stressor event timing which is important for the acute cortisol response (Ruis et al. 1997), therefore it is not ideal for chronic stress measurement Urine can usually be collected feedback-free (Sheriff et al. 2011) but longitudinal collection on individual level is often challenging, especially in intensive piggeries or in free moving housing settings.

Using faeces as basic sample material for determining glucocorticoid output in pigs would therefore be ideal, as it would allow a non-handling approach and therefore avoid alteration of the results due to handling stress. Further faecal samples are less affected by daily variation in hormone secretion (Touma, Palme & Sachser 2004) and can be comparatively easily collected even under free-roaming conditions. So far, however, attempts to reliably determine adrenocortical activity as a measure of stress in domestic pigs using faeces as hormone matrix have failed, leading to doubts whether glucocorticoid alterations can actually be measured in pig faeces (Mostl et al. 1999a).

Therefore the overall aim of this study was to examine the suitability of four enzyme immunoassays (EIAs) detecting cortisol, 3β,11β-diol-Cortisol metabolites, 11,17 dioxoandrostanes, and faecal glucocorticoid metabolites (fGCMs) with a 5β-3α-ol-11-one structure, respectively, for monitoring adrenocortical function in the domestic pig (*Sus scrofa domesticus)*. EIAs has been used before for other subjects but no record exist in pigs (Ganswindt et al. 2003, Ganswindt et al. 2014) More specifically, the objectives of this study were: a) to determine stress-related physiological responses in domestic pig's saliva and faeces by performing an

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

adrenocorticotropic hormone stimulation test (ACTH challenge test), b) to determine the effect of circadian rhythm in saliva GCs faecal GCM levels, and c) to investigate the effect of storage on the rate of metabolism of faecal glucocorticoid metabolites post-defaecation.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

2.0 LITERATURE REVIEW

2.1 Pigs

A pig is any listed animal species in the genus Sus, within the family Suidae of even-toed ungulates. The domestic pig (*Sus scrofa domesticus*) (also referred to as swine or hog) is a large, domesticated, even-toed ungulate that has its ancestry in the wild boar; it is considered a subspecies of the wild boar or a distinct species in its own right (Tisdell 2013). Pigs are omnivore animals and they are highly social and intelligent. Domestic pigs descended from the European wild pig, *Sus scrofa* and the Eastern Asiatic-banded pig, *Sus vittatus*, which is in turn a descendant of the Indian boar (*Sus crystatus*) (Groves 1981). Currently, there are over 78 recognised breeds of domestic pigs and the most popular among them include the Large White, Landrace, Duroc, Pietran, Large Black, Hampshire and the Yorkshire.

A typical pig has a large head with a long snout which is strengthened by a special pre-nasal bone and by a disk of cartilage at the tip (Figure 1). The snout is used to dig into the soil to find food and is a very acute sense organ. There are four hoofed toes on each trotter (foot), with the two larger central toes bearing most of the weight, while the outer two are used for addition stability on soft ground (Food and Agricultural Organisation , 2009) .

Figure 1. Domestic pig (*Sus scrofa domesticus*)

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

2.2 The Pig Industry

Domesticated pigs are reared livestock by farmers for the purpose of pork, hams, gammon or bacon production. In certain instances, specific types of leather are also obtained from pigs (McGlone et al. 2004). However, humans may also rear pigs especially the potbelly pigs for the pet market. Young gilts are usually serviced at the age of seven to eight months (McGlone 2001, Salak-Johnson&McGlone 2007) when they have reached a mass of about 115 kg (range: 100 – 140kg). Sows become susceptible again approximately four to six days post-weaning, and are mated within 12 to 24 hours afterward.

China has nearly half of the total global pig population with nearly 50 million individuals and there are over 6 million pigs in the US, and around 12 million pigs in the Netherlands (FAO, 2009; (Kušec et al. 2015). In South Africa, there are approximately 1.6 million pigs at any time, (FAO, 2009). The South African pig industry consist of approximately 125 000 production sows as at 2010/2011, with approximately 100 000 sows in the large and medium-scale commercial farms (Mokoele et al. 2014) while the remaining 25 000 are kept by small-scale farmers (Mokoele et al. 2014).

Approximately half of all the meat consumed in the world comes from pig (McGlone et al. 2004) and in the current pig production and husbandry systems, these animals are exposed to numerous intrinsic and extrinsic factors that often effects their health and wellbeing (Ruis et al. 1997, Tonsor, Olynk & Wolf 2009). In large and medium-scale commercial operations, pregnant pigs are often housed in crates to monitor their conditions and enhance the development of the implanting embryo (Soede et al. 2007). Crate housing seems to reduce most of the usually occurring social stress and allow individual feeding and treatment of sows (McGlone et al. 2004). On the other hand, keeping pigs in crates during pregnancy might actually increase stress due to inability to observe motility and lameness (McGlone 2001). In addition, confined pigs suffer from stereotyped behaviours, limited social interactions and therefore, there is a general negative perception on the use of crates by the European Union and USA markets (Mcglone et al. 1994).

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

2.3 Housing Systems in Outdoor and Commercial Piggery

Sow housing refers to where and how sows are accommodated. The U.S. National Pork Producers Council (US-NPPC) and the American Veterinary Medical Association (AVMA) both recognize gestation stalls and group housing systems as appropriate for providing for the well-being of sows during pregnancy.

Pigs are omnivores and traditionally were allowed to roam freely in the community and fend for themselves (Fig.2a) Pigs under improved management systems are kept either in a field (outdoors) or indoors where there are shelters and they may be fed on concentrate feed supplemented with grasses and other plants.

An outdoor system is surrounded by either a strong fence or a wall without sharp edges (Curtis et al. 2009, Hicks et al. 1998, Johnson, Morrow-Tesch & McGlone 2001) (See Figure 2b), and pigs are usually kept individually or in small groups on a muddy or concrete floor pen with a low shelter. Outdoor group pen designs differ in terms of layout, group size, and the method of feed presentations. Group pens typically hold up to 50 - 60 sows, and may provide about 1.9-5.0m^2 of space per sow,(Curtis et al. 2009, Gentry, Johnson & McGlone 2008). Some disadvantages of pen-based systems are the enhanced potential for cross-injection of internal parasites, lack of individual control of pigs, danger posed by predators, less environmental control to temperature extremes, bullying, and foot lesions (Gentry, Johnson & McGlone 2008, McGlone 2013b).

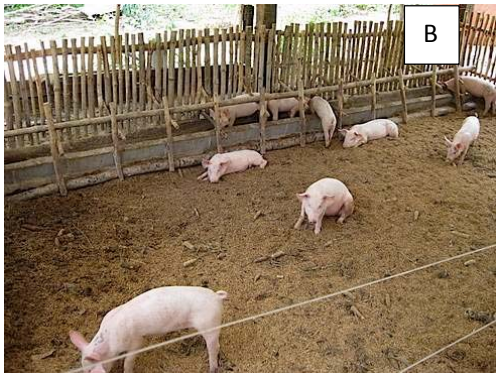
Pigs can of course also be kept indoors in groups or by using gestation crates for sows (Fig.2c,d). A gestation crate/sow stall is approximately a 2.1 m by 0.7 m metal enclosure, often used in intensive pig farming, in which a female breeding pig (sow) may be kept individually during pregnancy (Mcglone et al. 1994, Tonsor, Olynk & Wolf 2009). The crating systems in use currently as observed in the field can be divided into the following: a) Full crating I: The sows are continually kept in the gestation crate up to about 1-2 weeks before parturition when they are moved into the farrowing pens; b) Full crating II: The sows are continually kept in the gestation crate for the first 45 days of pregnancy and get transferred to a group housing setting for the remaining duration of pregnancy; c) Half crating: The crates can be accessed all the time, e.g. during feeding and watering, but individual sows have the liberty to move away from the crate by entering a group pen.

The choice of housing depends on the preference of the producer, available funds, and welfare consideration for the sows, geographic location, legal requirements, environmental and climatic factors. The overriding

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

reason in most cases however remains the economic realities of production (McGlone 2013a). It is known that a good and efficient housing makes management easier and significantly improve the survivability of live born piglets (Kraeling&Webel 2015, Whitney&Masker 2010).

http://www.sperotto-spa.com/en/pig-sheds/



http://www.naturalpigfarming.com/npfvfreerange.htm

Courtesy: AbdulKadir Gamawa

http://www.sperotto-spa.com/en/pig-sheds/

Figure 2: Different types of indoor and outdoor management systems. (a) extensive system/free range system; (b) outdoor semi-intensive management; (c) Indoor grouping of pigs (d) intensive system with individually housed pigs

While pregnant sows have been kept in sow crates (gestation stalls) or group housing systems for the well-being of sows during pregnancy, differing opinions support and oppose the different systems and the level of stress associated with them (Harris et al. 2013, Rhodes et al. 2005). However, to date, insufficient data exists on alternative housing systems and their specific effect on reproductive performance, stress and welfare (Broom, Mendl & Zanella 1995, Rhodes et al. 2005).

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

2.4 Management associated causes of stress in Commercial Piggery

Several management procedures have been confirmed to be sources of stress for pigs including weaning (Roldan-Santiago et al. 2015) transport (Sutherland, Backus & McGlone 2014, Von Borell&Schäffer 2005)

,confinement (van der Staay, F Josef et al. 2010) castration (Thun, Gajewski & Janett 2006, von Borell 2000) tail docking (Noonan et al. 1994, Prunier, Heinonen & Quesnel 2010), and crate housing (Barnett&Hemsworth 1990, Broom, Mendl & Zanella 1995). In this regard, some farmers highlighted negative consumer perception regarding some of the above listed management procedures; particularly in the European Union and USA markets (Boyle et al. 2002).

2.5 Evaluating Stress in Commercial Piggery

In the pig farming industry, amongst others, it is common practise to evaluate stress caused by management practices subjectively using indicators like vocalisation or stereotype behaviour (McGlone 2013b). Stress in pigs can be observed indirectly when animals exhibit gastric ulcers, cardiovascular effects like heart rate (Marchant, Rudd & Broom 1997) and immunosuppression which can be caused by elevated levels of glucocorticoids. Pigs when stressed will engage in Oral-nasal-facial (ONF) “stereotypic” bar-biting, an abnormal behaviour characterised by repeated mouthing movements on the metal rails of the crate. Deviations from standard production parameters like piglet birth weight, total pigs born per litter, stillborn pigs per litter, piglets born alive per litter and farrowing rates have also been used to assess stress. The challenge starts with our inability to define objectively what we mean by animal welfare (McGlone 2001) or alternatively quantifying stress. As a result the multidisciplinary approach is used in the context of other important society issues including: food safety, environmental protection, worker health and safety, economics, international trade, domestic protection, public perception and consumer economics (McGlone 2001) and these differ from society to society.

An alternative approach would be the monitoring of physiological stress-related markers; e.g. glucocorticoids. This approach is presumably more objective and less prone to homeostatic adjustments (McGlone 2013a).

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

2.6 The Stress response

Stress can be defined as a state of perturbed homeostasis following endangerment (real or just perceived) that evokes manifold adaptive reactions (physiological and behavioural), which are summarised as stress response (van der Staay, F Josef et al. 2010) .Stress can be perceived short term (acute) or long term (chronic). Acute stress may persist from seconds, minutes up to a few hours facilitating classical conditioning, while chronic stress lasts much longer (days, weeks up to months) (Turner, Hemsworth & Tilbrook 2005), and may result in reduced reproductive success as well as immunosuppression and atrophy of tissues leading to decrease in individual fitness (Möstl&Palme 2002) In piggeries, chronic stressors include the socio-environment (e.g. number of pigs housed together in a pen), stocking density (i.e. area of floor space/animal), housing systems and human–animal interactions (i.e. fear of the stockpersons) (Turner, Hemsworth & Tilbrook 2005).

Physiologically, stress leads to the activation of the hypothalamic–pituitary–adrenocortical axis (HPA) (Figure 3) and the symphatho-adreno-medullary system, which results amongst others in an increase in glucocorticoid and catecholamine secretions, which is an essential component in the physiological response to stress (Ganswindt et al. 2012, Touma&Palme 2005). The HPA axis is comprised of the hypothalamic paraventricular nucleus (PVN), anterior pituitary gland and the adrenal gland. When an animal encounters a stressor the PVN is stimulated and cause the activation of parvocellular neurones .This leads to sequential release of corticotrophin releasing hormone (CRH) and vasopressin from the hypothalamus, triggering the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary gland (Sheriff et al. 2011). ACTH stimulates the adrenal cortex to increase synthesis and secretion of glucocorticoids, including cortisol and corticosterone (Breinekova et al. 2007, Turner, Hemsworth & Tilbrook 2005).

The released glucocorticoids (GCs) have a number of effects on the body which are essential for coping with a stressor (von der Ohe&Servheen 2002). The magnitude and duration of GCs release is biologically important as the effects of a stress response result from the hormone–receptor interactions over the entire duration of the response (Sheriff et al. 2011).In addition to the cases of immunosuppression and compromised reproduction, hypothesised general adaptation syndrome (GAS) - a series of nervous-system and endocrine-gland activities

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

during the chronic phase of stress activity may result (von der Ohe&Servheen 2002).For this reason, measurement of these hormones has the potential to yield important information regarding animal well-being (von Borell 2000, von der Ohe&Servheen 2002).The HPA pathway is regulated by GCs themselves through a feedback mechanism. Stress induced concentrations of GCs interact with glucocorticoid receptors in the hippocampus, hypothalamus and pituitary to suppress the initial steps of HPA axis (Sheriff et al. 2011). In acute stress, the feedback mechanism operates efficiently and the system rapidly returns to normal and whereas in chronic stress, feedback signals are weak and the system remains activated for longer periods (Sapolsky 1992)

.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

GCs in the plasma through entero-hepatic circulation undergo extensive metabolism and conjugation in the liver and subsequent excretion into the urine via the kidneys or into the digestive tract via the bile ducts (Touma&Palme 2005). The metabolism of GCs is a complex process. Some of the GCM produced in the liver are reabsorbed into the blood stream from the digestive tract and undergo further hepatic degradation (Sheriff et al. 2011, Sheriff et al. 2011). It follows that, glucocorticoid concentrations can be determined non-invasively using either urine or faeces as the hormone matrix (Ganswindt et al. 2014)

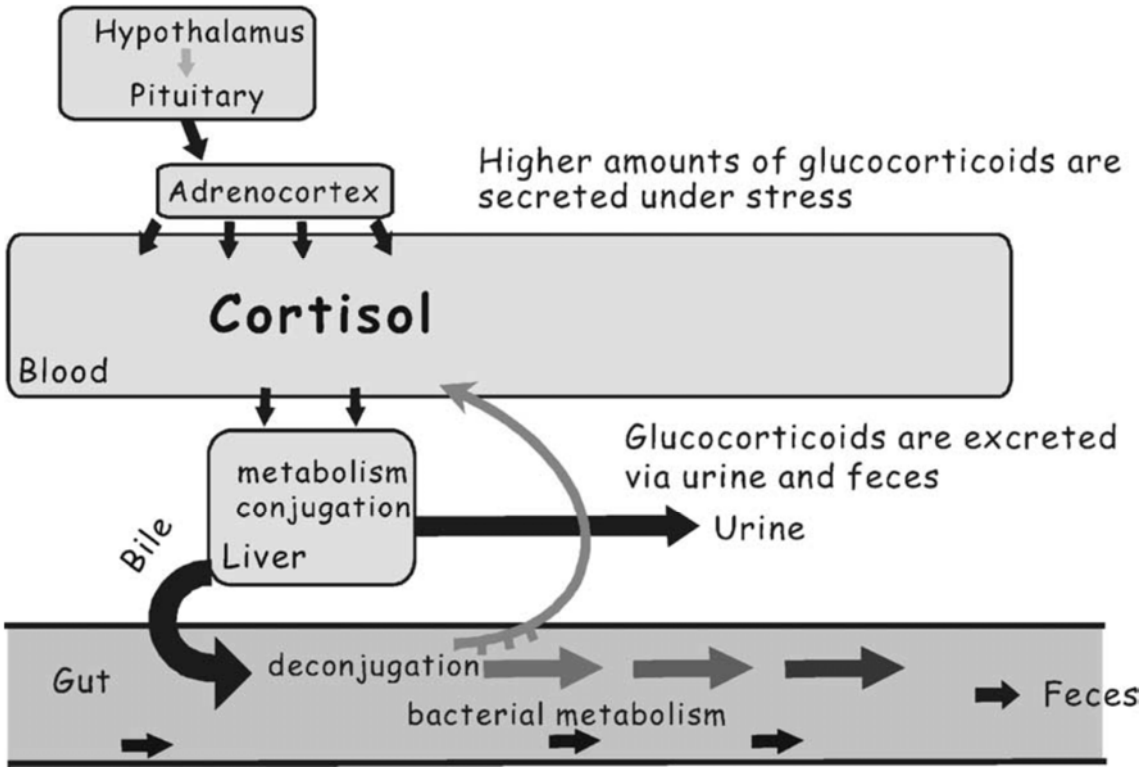


Figure 3. Scheme of the secretion, metabolism and excretion of glucocorticoids. Figure adapted from Möstl & Palme, (2002)

2.6.1 Measuring glucocorticoid concentrations in blood

Glucocorticoids can be quantified directly from blood samples obtained via either manual or automated sampling. Manual sampling is associated with direct interaction with the animal. The stress associated with animal-handling during sample collection can elevate glucocorticoid levels (Pol et al. 2002), thereby confounding the results

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

(Ganswindt et al. 2010). By contrast, automated blood sampling enables blood sampling without any interference (except for the initial catheterisation) with the animal during sampling, however, this methodology still requires preceding surgery associated with the insertion of catheters (Siswanto et al. 2008). However blood shows effects of fluctuations in corticosteroid concentrations due to brief episodes of acute or episodic synthesis of corticosteroids (Touma&Palme 2005). Previously, Relationship between pituitary-adrenal function and stress susceptibility in pigs have also been measured using muscle quality, response to halothane challenge, exposition to high temperature or physical exercise (Cassens, Marple & Eikelenboom 1975, Dantzer&Mormede 1978)

2.6.2 Non-invasive assessment of adrenocortical activity

Measures of stress may be obtained by quantifying glucocorticoid metabolites excreted in faeces and saliva (Siswanto et al. 2008) which are easy to collect without disturbing the animals or any form of restraint or capture (Ganswindt et al. 2014, Touma&Palme 2005). Faecal glucocorticoid metabolite (fGCM) assays reflect an average level of circulating glucocorticoids over a relatively longer time period (von der Ohe&Servheen 2002), rather than a time-point sample. The use of faeces may therefore provide a more accurate assessment of long-term acute glucocorticoid levels (Harper&Austad 2000). Monitoring of GC metabolites in faeces minimises the variability associated with serum samples that are prone to circadian, season and photoperiod or food intake rhythm (Monfort, Brown & Wildt 1993).

Nowadays, the assessment of adrenocortical function using faeces as hormone matrix is a widely accepted approach for monitoring responses to stressors, because faeces can be collected very easily and sampling is feedback-free due to the absence of capture and handling (Ganswindt et al. 2012, Touma&Palme 2005). Due to species-specific differences in steroid metabolism even in the closely related species, careful validation of assay methods is necessary in order to generate meaningful, accurate, consistent and quantifiable results (Schwarzenberger 2007).To ensure that such assays are suitable for non-invasive hormone measurements in animal, there is a need to carefully validate them in terms of application for species-specific hormone matrix to generate reliable quantification of respective glucocorticoid metabolites (Touma&Palme 2005)

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

2.7 ACTH challenge test

The ACTH challenge test is widely used for assessing neuroendocrine responses to stress and gets also increasingly used in animal welfare studies to demonstrate adrenal activity (Möstl&Palme 2002)The adrenocorticotropic hormone (ACTH) challenge test, as a standard physiological validation experiment, mimics neural-endocrine responses to stress (Pol et al. 2002) by stimulating glucocorticoid production. This validation experiment proves that changes in activity of the hypo-thalamic-pituitary- adrenal (HPA) axis are reflected by the levels of FGM concentrations measured by the respective assay, e.g. an increase in FGM/SGC levels following an adrenocorticotropic hormone (ACTH) challenge. ACTH is an integral component of the HPA axis and is often produced in response to perceived or real stress (Ganswindt et al. 2014)

2.8 Circadian rhythm

When studying the corticosteroid responses of animals to stressors, circadian (about 24-h) fluctuations in basal corticosteroid concentrations have to be considered. In pigs, basal concentrations of cortisol in blood are generally higher in the morning than in the afternoon and evening (Barnett, Cronin & Winfield 1981, Janssens, Helmond & Weigant 1995). As it was observed with other hormones, plasma glucocorticoids also exhibit circadian rhythm pattern in most vertebrate species (Möstl&Palme 2002, Touma&Palme 2005)Therefore differences in adrenocortical responses to stimuli may depend upon when, during the circadian cycle, a stimulus or stress event is applied or experienced (Janssens, Helmond & Weigant 1995).

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

2.9 Post-defecation metabolism

FGM levels post-defecation can be affected by unfavourable environmental conditions, age of the samples (Millspaugh&Washburn 2004) and bacterial or microbial degradation after defecation (Lexen et al. 2008, Mostl et al. 1999b). When collecting faecal samples under field conditions they often cannot be preserved immediately, and the period of time between excretion and collection may often varies (Sheriff et al. 2011) .Therefore, the preservation of faecal samples to maintain hormonal stability is very important. This should be done as soon as possible after defecation to minimise bacterial or microbial degradation of FGM. Freezing of the samples immediately after collection (followed by lyophilisation if the samples cannot be kept frozen) is arguably the best choice (Hunt&Wasser 2003, Hunt&Wasser 2003)

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

3.0 Materials and methods

3.1 Study animals and housing

This study was conducted using six Large White-Landrace F1 gilt crosses of approximately 14 weeks of age obtained from a commercial breeder in Pretoria, South Africa. The animals were transported to the University of Pretoria, Biomedical Research Centre (UPBRC), South Africa, at the beginning of the study. On arrival, the gilts were weighed (mean weight: 46kg; range: 43.7 - 47.7kg) and were clinically observed and the external environment was monitored throughout the duration of the study (Appendix 1). The pigs were thereafter allocated to a straw bedded pen (approximately 3X3m) for acclimatisation to the new environment for 3 days (Fig. 4).

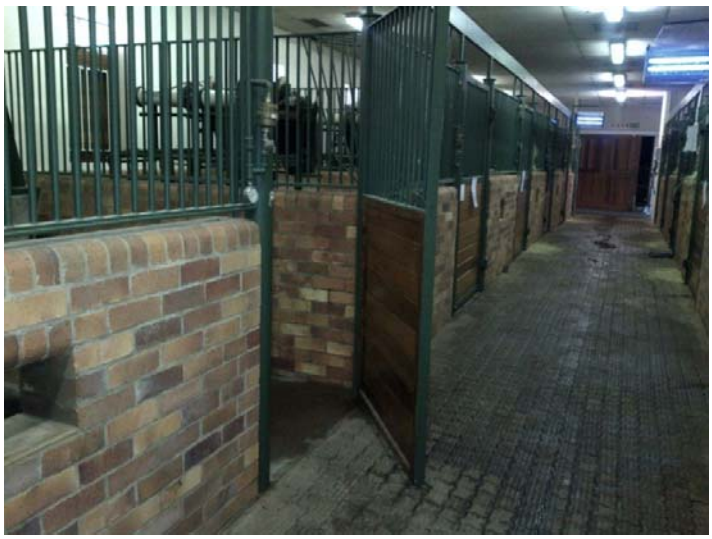


Fig. 4 Acclimatisation facilities, Biomedical Research Centre (UPBRC), Faculty of Veterinary Science, University of Pretoria, South Africa.

Subsequently, each pig was randomly assigned to an individually-housed metabolic crate and kept there until the end of the experiment (Fig. 5). The study animals were fed commercial grower ration (AFGRI, Centurion, South Africa) ad-libitum which was supplemented with fruits and vegetables (e.g cabbages, bananas, carrots, apples). Access to water was provided *ad libitum* and environmental biostimulation was provided in each metabolic crate. The metabolic crates were cleaned daily only after faecal and saliva samples have been

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

collected. Housing conditions were monitored, maximum and minimum temperatures were taken and humidity data was recorded on a daily basis.

The study was conducted under the approval and in accordance with the guidelines of the University of Pretoria Research Committee and Animal Use and Care Committee (Reference V094-13), Appendix 2 & 3 respectively.

Fig. 5 *Metabolic crate for individual pig housing at the UPBRC, Faculty of Veterinary Science, University of Pretoria, South Africa.*

3.2 Experimental design and Adrenocorticotropic Hormone (ACTH) Challenge

After the acclimatisation phase the animals were kept in the metabolic crates for 8 days prior to ACTH administration. Faecal samples were collected as soon as they were voided between 0800hrs in the morning and 1530hrs in the afternoon. Similarly saliva was collected twice a day between 0800 to 0830hrs in the morning and 1500 to 1530hrs in the afternoon. On the day of ACTH administration, the pigs were minimally restrained by blocking each animal off to a side of the metabolic crate without the use of force and four of the six animals were injected intramuscularly with approximately 50IU (10µg/kg of Synacthen®, Novartis, South Africa Pty Ltd) each. The remaining two animals served as controls and received approximately 0.5 ml physiologic saline each. On the day of ACTH/Saline administration all voided faeces were collected immediately after defecation before

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

the challenge, as soon as possible after the challenge and for the following 5 days, extended until the end of the experiment.

Similarly, salivary samples were initially collected approximately an hour prior administration of ACTH/Saline and subsequently 20, 40, 60, 90, and 120 min. post-injection. Thereafter, saliva was collected at hourly intervals until 8 hours post-administration, and further twice daily (between 0800 to 0830 hours and 1500 to 1530 hours) for the next 5 days until the termination of the experiment. Details of the faecal and salivary samples collections are described below in section 3.4.

3.3 Stability of fGCM post-defaecation

In a separate approach, pooled freshly voided faeces were collected and used to determine the stability of fGCM post-defecation. The material was collected from 3 study animals prior to the challenge, homogenised, and divided into 24 equal sub-samples. These samples were stored at room temperature and subsequently, three sub-samples were removed and frozen at -20°C at different time points (0, 1, 2, 4, 8, 16, 24 and 48 h) post-defecation (Ganswindt et al. 2012, Ganswindt et al. 2014).

3.4 Saliva and Faecal sample collection

Saliva was collected using Salivet® tubes (Sarstedt, Aktiengesellschaft and Co., Nümbrecht, Germany), which contain a sponge. The sponges were pulled out of the tube using a forceps and hanged to allow pigs to chew on the sponge. The device was held in position by a pair of forceps or suspended using transparent strings, until the sponge was thoroughly moistened within 1-2 minutes (Fig. 6). Saliva was obtained from the sponge by centrifugation for 10 min at 3000 g, identified individually and stored at -20°C until hormone analysis. A total of170 saliva samples were collected throughout the experiment.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Fig. 6 *Collection of pig saliva using the soft spongy Salivet®*

Each pig was allowed to defeacate independently and faecal samples were collected immediately after defecation using the outlets on the base and the sides of the metabolic crates. About 10-15 g of well mixed freshly voided material was taken from the core of each faecal sample, placed in a sterile container, identified individually and stored immediately at -20ºC until further processing (Fig. 7). A total of 262 faecal samples were collected during the experiment.

Fig. 7 *Faecal sample collection from study animal*

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

3.5 Sample Processing.

Saliva was extracted from the collection sponge following the protocol by Ruis and colleagues (1997). In brief, a chewed and wet Salivet® sponge was placed in a Salivet® tube and centrifuged for 10 min. at 1500 g. The retrieved saliva was then stored at -20 °C until further analysis.

Collected faecal material was lyophilised, pulverised, and sieved through a mesh to remove the fibrous material (Fiess, Heistermann & Hodges 1999). Subsequently, 0.10-0.11 g of the faecal powder was extracted by vortexing for 15 minutes with 3 ml of 80 % ethanol (Ganswindt et al. 2012). Following centrifugation for 10 min. at 1500 g, the supernatants were transferred into new microcentrifuge tubes and stored at -20 °C until analysis.

3.6 Salivary steroid analyses

Processed saliva was measured for immunoreactive salivary cortisol concentrations via enzyme-immunoassay (EIA) using an antibody against cortisol-3-CMO (Palme et al. 1997). In brief, 50-µl aliquots of standardised, quality controlled and diluted saliva was pipetted in duplicate into microtiterplate wells followed by 50 µl of bioinylated cortisol-3-CMO label and antiserum. The plates were incubated over night at 4°C. Following incubation the plates were washed and 150 µl (20ng) of streptavidin-peroxidase (Life Technologies LTC Tech South Africa (Pty) Ltd, Johannesburg, South Africa) was added to each well and again incubated in the dark for 30 min at 4°C. Following incubation, plates were washed again before 150 µl TMB substrate solution was added, and plates were further incubated for 45minat 4°C, until the maximum OD was 1.0. The reaction was terminated by the addition of 50 µL of 4N H_2SO_4 and the absorbance measured at 450 nm. Further details on the assay characteristics are described in other work of Palme et al. (1997). The sensitivity of the EIA was 0.6ng/ml, and the intra- and inter-assay coefficients of variation (CVs), determined by repeated measurements of high and low value quality controls, were 9.5%-11.0% (for Intra-CV) and 15.0%-15.2% (for Inter-CV) respectively.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

3.7 Faecal glucocorticoid metabolite analysis

A selected set of faecal extracts (n =31) from three pigs were measured for immunoreactive fGCM concentrations using 4 different enzyme-immunoassays; namely (i) a 11-oxoetiocholanolone I (detecting 11,17 dioxoandrostanes; 11,17-DOA), (ii) 11-oxoetiocholanalone II (detecting fGCMs with a 5β-3α-ol-11-one structure; 3α,11-oxo-CM), (iii) a 5α-pregnane-3β,11β,21-triol-20-one (measuring 3β,11β-diol-CM) and iv) a cortisol (detecting fGCMs with a 11,17,21-trihydroxy-20-one structure) EIA. Detailed assay characteristics, including full descriptions of the assay components and cross-reactivities were previously described by Möstl & Palme (1997) for 11, 17-DOA and cortisol, Möstl & Palme (2002) for 3α,11-oxo-CM, and by Touma et al., (2003) for the EIA measuring 3β,11β-diol-CMs. After identifying the most suitable EIA with regards to fGCM elevation post-ACTH administration (Section4.1), the entire sample set including the material from the fGCM-stability experiment was assessed using only the 5α-pregnane-3β,11β,21-triol-20-one EIA. The sensitivity of the assays were 0.6 ng/g faecal dry weight (DW) for 11, 17-DOA, 3α, 11-oxo-CM and cortisol, and 1.5 n/g DW for the 5α-pregnane-3β, 11β,21-triol-20-one EIA (Table 1). Serial dilutions of extracted faecal samples gave displacement curves that were parallel to the respective standard curve in all assays. Intra-assay CV ranged between 4.9 % and 5.9 % for 11, 17-DOA, 6.1 % and 8.0 % for 3α, 11-oxo-CM, and 9.5%-11.0% for the cortisol EIA. Intra- and inter-assay CV ranged between 4.9 % and 9.5 % for 5α-pregnane-3β, 11β, 21-triol-20-one EIA. All assays were performed on microtiter plates as described above (Section 3.6) and conducted at the Endocrine Research Laboratory at the Faculty of Veterinary Science, University of Pretoria.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Table 1: The response displayed by each of the four tested enzyme immunoassays following ACTH administration (time 0), with peak increase levels occurring 23–25 h post injection.

		EIAs detecting			
		Cortisol	3α,11oxo-CM	3β,11β-diol-CM	11,17-DOA
animal 2 (ACTH)					
baseline	median (ng/g DW)	37,93	151,51	217,85	24,22
(n=4)	mean (ng/g DW)	36,67	163,74	220,92	25,05
	SD	5,18	49,09	30,43	12,10
peak	conc. (ng/g DW)	61,8	289,3	419,5	44,9
post injection	time (h)	6,8	23,8	21,5	6,8
	fold-baseline	1,63	1,91	1,93	1,85
animal 3 (ACTH)					
baseline	median (ng/g DW)	42,38	310,12	225,08	70,41
(n=4)	mean (ng/g DW)	41,94	327,57	220,33	63,26
	SD	13,54	141,84	42,42	31,13
peak	conc. (ng/g DW)	105,8	765,1	549,01	162,0
post injection	time (h)	23,5	24,7	24,7	23,5
	fold-baseline	2,50	2,47	2,44	2,30
animal 4 (ACTH)					
baseline	median (ng/g DW)	33,00	302,69	208,76	104,45
(n=4)	mean (ng/g DW)	33,46	291,22	220,04	106,24
	SD	10,57	134,68	35,36	17,38
peak	conc. (ng/g DW)	68,6	1373,7	559,80	372,5
post injection	time (h)	23,6	25,0	23,6	25,0
	fold-baseline	2,08	4,54	2,68	3,57

3.8 Data analysis

A total of 167 salivary and 116 faecal samples collected from six individuals were analysed. Faecal glucocorticoid metabolite (FGM) concentrations were expressed as µg/g dry faeces and sGC concentration was expressed ng/ml. Pre-ACTH administration means for fGCM and sGC concentrations were calculated, for each individual with samples prior to ACTH injection. Similarly, post-ACTH administration mean fGCM and sGC concentrations for each individual, were calculated with samples post-ACTH injection. Subsequently, individual pre- and post-ACTH mean values were pooled and comparisons were calculated using repeated measures, multiway One Way ANOVA

To determine individual baseline fGCM values an iterative process where all values greater than the mean plus 2 standard deviations (SD) were removed (Brown et al. 1999) The averages were subsequently recalculated

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

and the elimination process was repeated until there were no values greater than the means plus 2 SD. The baseline values were then compared among the 6 pigs using one-way ANOVA. Initial rise, peak and fall in fGCM and sGC concentration following ACTH challenge were determined and analysed using descriptive statistics.

The relative FGM metabolism rate post-defaecation (%) was calculated on pooled samples from three pigs divided into 8 subsets, using the mean hormone value determined at t = 0 as 100 %. Differences in the distribution of FGM concentrations between sampling subsets were subsequently conducted using One-Way Anova.. (Ganswindt et al. 2014). For all statistical analyses significance was assumed when $P < 0.05$. Statistical analyses was performed using the software program (OpenEpi v.3.03a) (Dean, Sullivan & Soe 2014). and data is presented as means ± SD.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

4.0 RESULTS

4.1 ACTH challenge

Baseline salivary glucocorticoid (sGC) concentrations (2.38±1.83ng/ml, mean±SD, table 2) increased after administration of synthetic ACTH by on average six-fold (14.03±6.83 ng/ml averaged concentration reflecting 9 hours after ACTH administration), peaking 40-90 minutes after injection in all four treated animals (Figure 8c-f). Elevated cGC levels were maintained in test subjects for up to nine hours following ACTH challenge (Figure 8c-f). Administration of saline was not reflected in changes in baseline sGC concentrations (table 2, Figure 8a-b).

Table 2. Salivary glucocorticoid metabolite concentrations of gilts (pigs) prior and post administration of either synthetic ACTH or saline.

Variables (n)	Mean±SD (ng/ml)	Min (ng/ml)	Max (ng/ml)
Baseline (n=6), pre-administration t=0	2.38±1.83	0.10	6.64
Administration (n=4) ACTH, t ≤ 9 hours	14.03±6.83	2.01	30.18
Administration (n=2) Saline, t ≤ 9 hours	2.36±2.30	0.24	11.65
Post-administration (n=4) ACTH, t=24 hours	1.74±1.82	0.16	8.57
Post-administration (n=2) Saline, t=24 hours	2.21±1.17	0.61	5.06

Baseline faecal glucocorticoid metabolite (fGCM) concentration (239.91±46.03µg/g of dry faeces (mean±SD), Table 3) increased after administration of synthetic ACTH by on average 19% (286.18±45.03 µg/g faecal dry weight (DW)) within 24 hours of injection, with peak fGCM levels reaching up to 750 µg/g DW (~300% increase) about 24 hours post-injection. Faecal GCM concentrations returning to baseline (236.60±61.19 µg/g DW) within 72 hours (Figure 9c-f). Administration of saline did not result in significant changes in baseline fGCM concentrations (table 3, Figure 9a-b).

The second test animal receiving ACTH displayed an idiosyncrasy, a pattern that was inconsistent with the other members of the test group (Figure 9d). While other ACTH-challenged pigs showed peak elevations in fGCM concentrations within 24-36 hours post administration, this pig only showed highest levels after ≈76 h, also not quantitatively comparable with the fGCM elevations obtained from the other ACTH group members. However, its sGC response with regards to the ACTH change is consistent with the other animals in the test group.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Initial transport of the pigs (n=6) to the experimental facility was reflected in increased alterations of faecal glucocorticoid metabolite (fGCM) levels of up to 14.58% against subsequent baseline (239.91±46.03µg/g dry faeces (mean±SD), Table 3, Figure 9).

Table 3. Faecal glucocorticoid metabolite concentration of gilts (pigs) after transport and prior and post administration of either synthetic ACTH or saline

Faecal Variables (n)	Mean±SD (µg/g)	Min (µg/g)	Max (µg/g)
Post-transport (n=5)	274.90±52.01	208.90	370.90
Baseline ACTH (n=4), pre-administration t=0	239.91±46.03	151.90	340.60
Baseline Saline (n=2), pre-administration t=0	225.48±41.65	222.85	295.60
Administration (n=4) ACTH, t ≤ 24 hours	286.18±45.03	179.10	358.00
Administration (n=2) Saline, t ≤ 24 hours	194.32±52.32	143.70	277.00
Post-administration (n=4) ACTH, t=72 hours	236.60±61.19	142.00	365.80*
Post-administration (n=2) Saline, t=72 hours	213.65±43.66	143.20	301.10

**Note that animal number 2 had late peak response and lag into the post-administration time.*

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

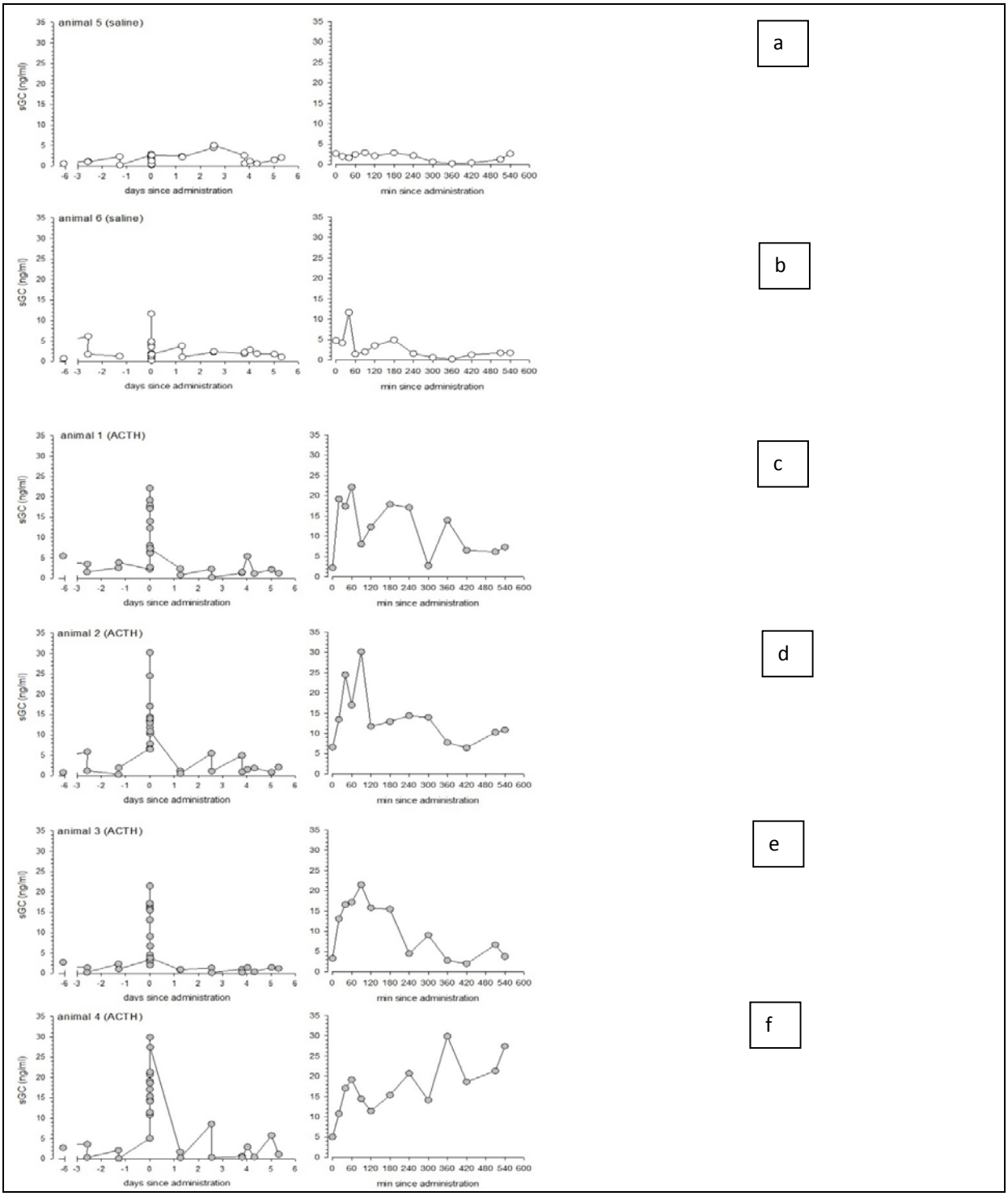


Figure 8 (a-f): Individual long-term profiles of salivary glucocorticoid metabolite concentration in gilts (pigs) prior and post administration of either synthetic ACTH (c-f) or saline (a & b), *note that bigger representation appear in the appendices (pages 49-50)*. Time point 0 indicated time of administration. The graphs on the left hand side representing the total time interval monitored (-6 to 6 days of administration), whereas the graphs on the right represent the time window of 0 to 10 hour post-injection.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

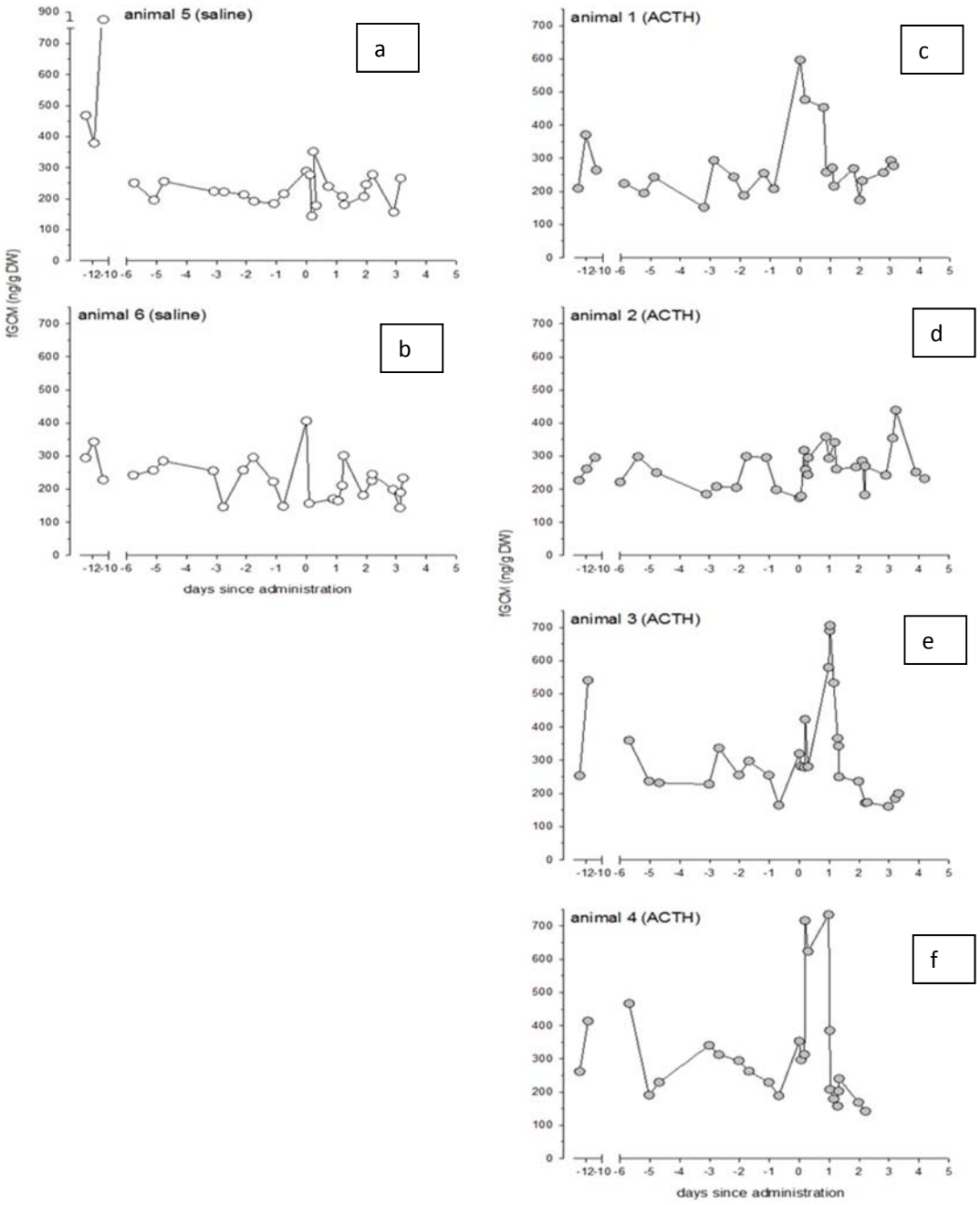


Figure 9: Individual long-term profiles of faecal glucocorticoid metabolite concentration in gilts (pigs) prior and post administration of either synthetic ACTH (c-f) or saline (a & b). Time point 0 indicated time of administration.

4.2 Stability of FGCM post-defaecation

Overall, mean fGCM levels slightly increased by 4% in samples stored frozen between 2 to 16 h following an initial decrease of approximately 10% during the first two hours of post sample collection (Figure 10). Thereafter, the mean fGCM levels gradually rose until about 8 hours post sample collection, followed by a decrease until 16 hours. After 16 h post-defecation until the end of the experiment (48 h post-defecation), fGCM levels only varied

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

by ≈7.0%. Variation in fGCM concentration between sample subsets ranged from 2.3 to 15.2% for respective measuring points in time.

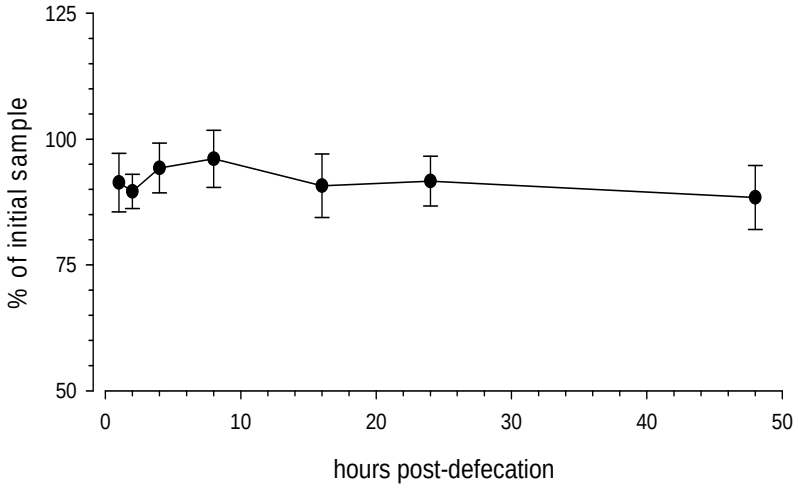


Figure 10. Relative degradation rate (%) of faecal glucocorticoid metabolites in pigs compared over time since defecation (0 to 16h). Values at respective measuring points in time are expressed as mean +/- SD

4.3 Effects of collection time

There was a significant differences in sGC concentrations in samples collected in the morning compared to when collected in the afternoon (Table 4, *P* = 0.005), with sGC concentrations in samples collected between 08:00-12:00 h (morning samples) being significantly higher that respective salivary steroid concentrations in material collected between 12.01-18:00 h hhh(afternoon samples). No significant difference was found when comparing fGCM concentrations of samples collected either in the morning (08:00-12:00 h) or the afternoon (12.01-18:00 h) (Table 4; *P* = 0.28).

Table 4: Mean sGCs and fGCMs values for morning and late afternoon samples

	Mean±SD (ng/ml)	Min (ng/ml)	Max (ng/ml)
sGC morning values (n=6) (ng/ml)	2.59±1.86	0.65	8.57
sGC afternoon values (n=6) (ng/ml)	1.20±1.00	0.16	5.06
fGCM morning values (n=6) (µg/g DW)	236.15±22.40	0.61	11.65
fGCM afternoon values (n=6) (µg/g DW)	229.03±13.31	0.24	5.06

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

5.0 DISCUSSION

Reliable monitoring of adrenocortical activity in a particular species using FGCMs requires a solid validation of the test system in question as different animal GIT systems and microbiome activities may differentially produce different fGCM; this needs to be taken into account when establishing a test system. In this regard, an ACTH challenge test can be used as physiological validation for the test system in question (Palme et al. 1996) This study establishes a non-invasive technique to monitor adrenocortical function based on measuring the concentrations of glucocorticoids or their metabolites in saliva and faeces of domesticated pigs. After the initial screening of 4 available enzyme-immunoassays (EIAs) for the measurement of glucocorticoids or their metabolites in pigs, an assay using an antibody against cortisol-3-CMO have been identified for reliably detecting sGC concentrations, whereas an assay using an antibody against 3β,11β-diol-CM have been identified to reliably determine fGCM concentrations in domestic pigs (Hodges K, Brown J, Heistermann M, 2010).

In this work, both saliva and faecal samples displayed measurable responses to exogenous ACTH challenge. A 6-fold increase in salivary glucocorticoids levels occurred within 9 hours, an observation that was consistent with the findings of (Bushong, Friend & Knabe 2000) who also observed 4-6 fold peak response of sGC following administration of ACTH in barrows. Peak fGCM concentrations post-injection showed an increase of approximately 300% compared with the baseline values about 24 hours post-injection and this peak was sustained for up to 72 hours.

The exact time of peak of fGCM could not be precisely determined, there was however a time delay between measured elevation of salivary cortisol concentrations and related increase in fGCMs .The processes by which cortisol in saliva is formed represents a time-frame of minutes, whereas the production of fGCM may be hours to days depending on the species (Cook 2012) . We measured a substantial increase in salivary cortisol 40-90 minutes after ACTH administration and detected corresponding levels in fGCM levels within 24hrs using cortisol-3-CMO and 3β,11β-diol-CM respectively. Comparable findings have been reported where peaks in faecal steroids metabolites were obtained about 12 hours (sheep), 24 hours (ponies) or 48 hours (pigs) after infusion of marked steroids (Cook 2012). Whereas sGCM was recovered rapidly following administration of ACTH, there was a delay in fGCM recovery time and this has previously been attributed to intestinal passage which causes

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

a time delay between the circulation of steroids in the plasma and their appearance in faeces (Davies et al. 2013). Whether this time delay severely affected the outcome of Animal 2 (Figure 9d) differently is not clear in this study. However, it is confirmed that idiosyncrasies do exist in animals and this particular subject comparatively paced more, grunt more, fed less and drank less prior to and during the acclimatization period.

In this work, the fGCM values rose significantly ranging from 208.90µg/g - 370.90µg/g (Mean±SD 274.90±52.01µg/g DW) in comparison to the baseline transportation values. Lexen et al (2008) have observed an up to 300-500% increase in fGCM about ten hours post transport in sheep, and in cattle the highest concentrations of fGCM have been measured about 12 hours after transport (Palme et al. 2000; (Möstl&Palme 2002, Palme et al. 2000). In a different experiment, transportation also influenced adrenocortical responses (Palme & Mostl (1997); the difference between our results and those of Lexen et al (2008) who used 11-oxoaetiocholanolone as standard of measurement may be due to the durations and conditions of transportations (Möstl&Palme 2002, Palme et al. 1997). The pigs in this experiment were transported for about 20 minutes on a smooth road whereas in the experiment of Lexen et al, the animals were subjected to approximately 60 minutes of gruelling transportation.

The stability of fGCMs post-defecation has been determined to assess any possible changes in measured hormone concentrations in unpreserved faecal material using 3β,11β-diol-CM . FGCM concentrations varied by 7.0% among all the individual animals recruited in the experiment over a period of 48h. It is concluded that there will be elevated increase in fGCM after an ACTH administration in pig faeces within 48hours post administration of an extrinsic steroid. These changes agrees with other studies that showed an increase in FGM levels within a few hours (4 - 24 h) post defecation as have been observed in cattle, horses and pigs,(Mostl et al. 1999a) and sheep (Lexen et al. 2008). However, a rather negligible change in FGM levels over several days in other species like baboons (Beehner&Whitten 2004) using a similar experimental setup and the same assay.

While results can differ distinctively even when using the same assay but different species, to date, there is no empirically conclusive explanation for this observation but bacterial enzymes and microbiome activities in different animal species man be present in the faeces and further metabolize fGCMs differently

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

(Millspaugh&Washburn 2004) (Millspaugh and Washburn, 2004; (Touma&Palme 2005) Previous studies have shown that the pH of the faeces is known to influence bacterial enzyme activities (McDermid, McKee & Marsh 1988), including the metabolism of colonic bacteria (Edwards, Duerden & Read 1985). Furthermore, the following studies have shown that with the use of 3α,11oxo-CM EIA, a pronounced decrease in hormone levels can be observed 4–8 hours post-defecation in faeces of sheep (*Ovis aries*) and brown hyaena (*Hyaena brunnea*) (Hulsman et al. 2011, Lexen et al. 2008).

Lexen et al (2008) have indicated that the varied patterns of changes in measured fGCM concentrations in unpreserved samples depend on the species and the assay used respectively. From the findings in this study, fGCM in pig faeces was relatively stable for approximately two days. The implication of this finding is that faecal materials from pigs can be collected from distant locations and transported to the laboratory for up to a total of two days without appreciable level of depreciation in fGCM.

As with many other hormones, plasma glucocorticoids have a circadian rhythm in most vertebrate species (Möstl&Palme 2002, Touma&Palme 2005), and diurnal variations of fGMs was already demonstrated for at least some mammals (Touma&Palme 2005). In pigs, the basal concentrations of cortisol in blood are generally higher in the morning than in the afternoon and evening (Ruis et al. 1997). The levels of fGCM excreted in faeces may reflect diurnal fluctuations (Keay et al. 2006). Our findings showed that there was a significant difference in sGC concentrations in samples collected in the morning compared to those collected in the afternoon. The findings compared well with other studies on pigs in terms of observed patterns in morning and afternoon saliva and blood samples (Barnett&Hemsworth 1990, Janssens, Helmond & Weigant 1995, Ruis et al. 1997). The results confirms that this effect (circadian rhythmicity of cortisol) should be taken into consideration when evaluating for stressors in pigs(Ruis et al. 1997)

The ability to reliably assess adrenocortical function in domestic pig using faeces (non-invasive method) now provides a solid basis to further examine endocrine responses to stressful circumstances in the commercially reared pigs. Potential applications could for instance be the possibility of measuring the potential effect of

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

different rearing practices and housing systems on stress in pig, or more specifically the ability to measure potential variability of fGCM levels in different pig housing set ups.

Further, the study clearly underlines the importance of non-invasive hormone measurements as a powerful tool to monitor and provide information about an animal's endocrine status. Therefore the generated information regarding pigs should help to facilitate further studies which examine endocrine responses to putative stressful circumstances in different pig environments and set ups. Furthermore, the outcome of this research can be used to plan farm animal habituation, do programming for stress in pig management; however more research may need to be engaged to compare empirical values using EIA with other evaluative methods.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

REFERENCES

Antonides, A., Schoonderwoerd, A.C., Nordquist, R.E. & van der Staay, Franz Josef, 2015, 'Very low birth weight piglets show improved cognitive performance in the spatial cognitive holeboard task', *Frontiers in behavioral neuroscience* 9 43.

Bacci, M.L., Nannoni, E., Govoni, N., Scorrano, F., Zannoni, A., Forni, M., Martelli, G. & Sardi, L., 2014, 'Hair cortisol determination in sows in two consecutive reproductive cycles', *Reproductive Biology* 14(3), 218-223.

Barnett, J., Cronin, G. & Winfield, C., 1981, 'The effects of individual and group penning of pigs on total and free plasma corticosteroids and the maximum corticosteroid binding capacity', *General and comparative endocrinology* 44(2), 219-225.

Barnett, J. & Hemsworth, P., 1990, 'The validity of physiological and behavioural measures of animal welfare', *Applied Animal Behaviour Science* 25(1), 177-187.

Beehner, J.C. & Whitten, P.L., 2004, 'Modifications of a field method for fecal steroid analysis in baboons', *Physiology & Behavior* 82(2), 269-277.

Bottoms, G., Roesel, O., Rausch, F. & Akins, E., 1972, 'Circadian variation in plasma cortisol and corticosterone in pigs and mares', *Amer J Vet Res* .

Boyle, L., Leonard, F., Lynch, P. & Brophy, P., 2002, 'Effect of gestation housing on behaviour and skin lesions of sows in farrowing crates', *Applied Animal Behaviour Science* 76(2), 119-134.

Breinekova, K., Svoboda, M., Smutna, M. & Vorlova, L., 2007, 'Markers of acute stress in pigs', *Physiological Research* 56(3), 323-329.

Broom, D.M. & Fraser, A.F., 2007, *Domestic animal behaviour and welfare,* CABI.

Broom, D.M. & Johnson, K.G., 1993, *Stress and animal welfare,* Springer Science & Business Media.

Broom, D.M., Mendl, M.T. & Zanella, A.J., 1995, 'A Comparison of the Welfare of Sows in Different Housing Conditions', *Animal Science* 61 369-385.

Brown, J.L., Schmitt, D.L., Bellem, A., Graham, L.H. & Lehnhardt, J., 1999, 'Hormone secretion in the asian elephant (Elephas maximus): characterization of ovulatory and anovulatory luteinizing hormone surges', *Biology of reproduction* 61(5), 1294-1299.

Bushong, D.M., Friend, T.H. & Knabe, D.A., 2000, 'Salivary and plasma cortisol response to adrenocorticotropin administration in pigs', *Laboratory animals* 34(2), 171-181.

Cassens, R., Marple, D. & Eikelenboom, G., 1975, 'Animal physiology and meat quality', *Advances in Food Research* 21 71-155.

Cook, N.J., 2012, 'Review: minimally invasive sampling media and the measurement of corticosteroids as biomarkers of stress in animals', *Canadian Journal of Animal Science* 92(3), 227-259.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Curtis, S.E., Baker, R.B., Estienne, M.J., Lynch, P.B., McGlone, J.J. & Pedersen, B.K., 2009, 'Scientific assessment of the welfare of dry sows kept in individual accommodations.', *Issue Paper - Council for Agricultural Science and Technology* (42), 20 pp.-20 pp.

Dantzer, R. & Mormede, P., 1978, 'Behavioural and pituitaryadrenal characteristics of pigs differing by their susceptibility to the malignant hyperthermia syndrome induced by halothane anesthesia. 1-Behavioural measures', *Ann.Rech.Vet* 9 559-567.

Davies, N., Gillett, A., McAlpine, C., Seabrook, L., Baxter, G., Lunney, D. & Bradley, A., 2013, 'The effect of ACTH upon faecal glucocorticoid excretion in the koala', *The Journal of endocrinology* 219(1), 1-12.

Dean, A., Sullivan, K. & Soe, M., 2014, *OpenEpi: Open source epidemiologic statistics for public health, version* .

Edwards, C.A., Duerden, B. & Read, N., 1985, 'The effects of pH on colonic bacteria grown in continuous culture', *Journal of medical microbiology* 19(2), 169-180.

Fiess, M., Heistermann, M. & Hodges, J.K., 1999, 'Patterns of urinary and fecal steroid excretion during the ovarian cycle and pregnancy in the African elephant (Loxodonta africana)', *General and comparative endocrinology* 115(1), 76-89.

Ganswindt, A., Palme, R., Heistermann, M., Borragan, S. & Hodges, J.K., 2003, 'Non-invasive assessment of adrenocortical function in the male African elephant (Loxodonta africana) and its relation to musth', *General and comparative endocrinology* 134(2), 156-166.

Ganswindt, A., Tordiffe, A.S.W., Stam, E., Howitt, M.J. & Jori, F., 2012, 'Determining adrenocortical activity as a measure of stress in African buffalo (Syncerus caffer) based on faecal analysis', *African Zoology* 47(2), 261-269.

Ganswindt, A., Mu¨nscher, S., Henley, M., Palme, R., Thompson, P. & Bertschinger, H., 2010, 'Concentrations of faecal glucocorticoid metabolites in physically injured free-ranging African elephants Loxodonta africana', *Wildlife Biology* 16(3), 323-332.

Ganswindt, S.B., Myburgh, J.G., Cameron, E.Z. & Ganswindt, A., 2014, 'Non-invasive assessment of adrenocortical function in captive Nile crocodiles (Crocodylus niloticus)', *Comparative Biochemistry and Physiology A-Molecular & Integrative Physiology* 177 11-17.

Gentry, J., Johnson, A. & McGlone, J., 2008, 'The welfare of growing-finishing pigs', *Welfare of pigs from birth to slaughter.Edited by Faucitano, L., Achaefer, AL, Wageningen Academic Publishers, Netherlands* 133-159.

Groves, C.P., 1981, *Ancestors for the pigs: taxonomy and phylogeny of the genus Sus,* Dept. of Prehistory, Research School of Pacific Studies, Australian National University.

Harper, J.M. & Austad, S.N., 2000, 'Fecal glucocorticoids: A noninvasive method of measuring adrenal activity in wild and captive rodents', *Physiological and Biochemical Zoology* 73(1), 12-22.

Harris, E., Berg, E., Berg, E. & Vonnahme, K., 2013, 'Effect of maternal activity during gestation on maternal behavior, fetal growth, umbilical blood flow, and farrowing characteristics in pigs', *Journal of animal science* 91(2), 734-744.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Hicks, T.A., McGlone, J.J., Whisnant, C.S., Kattesh, H.G. & Norman, R.L., 1998, 'Behavioral, endocrine, immune, and performance measures for pigs exposed to acute stress.', *Journal of animal science* 76(2), 474-483.

Hodges K, Brown J, Heistermann M, (ed.), 2010, '
Wild mammals in captivity: Principles
and techniques for zoo management

.', First edn., The University of Chicago Press, Chicago.

Hodges, K., Brown, J. & Heistermann, M., 2010, 'Endocrine monitoring of reproduction and stress', *Wild mammals in captivity: principles and techniques for zoo management* 447-468.

Hulsman, A., Dalerum, F., Ganswindt, A., Muenscher, S., Bertschinger, H.J. & Paris, M., 2011, 'Non-invasive monitoring of glucocorticoid metabolites in brown hyaena (Hyaena brunnea) feces', *Zoo biology* 30(4), 451-458.

Hunt, K.E. & Wasser, S.K., 2003, 'Effect of long-term preservation methods on fecal glucocorticoid concentrations of grizzly bear and African elephant', *Physiological and Biochemical Zoology* 76(6), 918-928.

Janssens, C., Helmond, F. & Weigant, V., 1995, 'The effect of chronic stress on plasma cortisol concentrations in cyclic female pigs depends on the time of day', *Domestic animal endocrinology* 12(2), 167-177.

Johnson, A.K., Morrow-Tesch, J.L. & McGlone, J.J., 2001, 'Behavior and performance of lactating sows and piglets reared indoors or outdoors', *Journal of animal science* 79(10), 2571-2579.

Keay, J.M., Singh, J., Gaunt, M.C. & Kaur, T., 2006, 'Fecal glucocorticoids and their metabolites as indicators of stress in various mammalian species: a literature review', *Journal of Zoo and Wildlife Medicine* 37(3), 234-244.

Kittawornrat, A., Prickett, J., Chittick, W., Wang, C., Engle, M., Johnson, J., Patnayak, D., Schwartz, T., Whitney, D., Olsen, C., Schwartz, K. & Zimmerman, J., 2010, 'Porcine reproductive and respiratory syndrome virus (PRRSV) in serum and oral fluid samples from individual boars: Will oral fluid replace serum for PRRSV surveillance?', *Virus research* 154(1-2), 170-176.

Kraeling, R.R. & Webel, S.K., 2015, 'Current strategies for reproductive management of gilts and sows in North America', *Journal of animal science and biotechnology* 6(1), 3-1891-6-3. eCollection 2015.

Kušec, G., Dovč, P., Karolyi, D. & Čandek Potokar, M., 2015, 'Local pig breeds and pork products in Croatia and Slovenia–unexploited treasure', *Poljoprivreda (Osijek)* 21(S1), 16-21.

Lexen, E., El-Bahr, S., Sommerfeld-Stur, I., Palme, R. & Mostl, E., 2008, 'Monitoring the adrenocortical response to disturbances in sheep by measuring glucocorticoid metabolites in the faeces', *Wiener tierarztliche Monatsschrift* 95(3/4), 64.

Marchant, J.N., Rudd, A.R. & Broom, D.M., 1997, 'The effects of housing on heart rate of gestating sows during specific behaviours', *Applied Animal Behaviour Science* 55(1–2), 67-78.

McDermid, A.S., McKee, A.S. & Marsh, P.D., 1988, 'Effect of environmental pH on enzyme activity and growth of Bacteroides gingivalis W50', *Infection and immunity* 56(5), 1096-1100.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

McGlone, J.J., 2013a, 'The future of pork production in the world: towards sustainable, welfare-positive systems.', *Animals* 3(2), 401-415.

McGlone, J.J., 2013b, 'Updated scientific evidence on the welfare of gestating sows kept in different housing systems.', *Professional Animal Scientist* 29(3), 189-198.

McGlone, J.J., 2001, 'Farm animal welfare in the context of other society issues: toward sustainable systems', *Livestock Production Science* 72(1-2), 75-81.

McGlone, J.J., Borell, E.H.v., Deen, J., Johnson, A.K., Levis, D.G., Meunier-Salaun, M., Morrow, J., Reeves, D., Salak-Johnson, J.L. & Sundberg, P.L., 2004, 'Compilation of the scientific literature comparing housing systems for gestating sows and gilts using measures of physiology, behaviour, performance, and health.', *Professional Animal Scientist* 20(2), 105-117.

Mcglone, J.J., Salakjohnson, J.L., Nicholson, R.I. & Hicks, T., 1994, 'Evaluation of Crates and Girth Tethers for Sows - Reproductive-Performance, Immunity, Behavior and Ergonomic Measures', *Applied Animal Behaviour Science* 39(3-4), 297-311.

McGlone, J.J., 2001, 'Farm animal welfare in the context of other society issues: toward sustainable systems', *Livestock Production Science* 72(1–2), 75-81.

Millspaugh, J.J. & Washburn, B.E., 2004, 'Use of fecal glucocorticoid metabolite measures in conservation biology research: considerations for application and interpretation', *General and comparative endocrinology* 138(3), 189-199.

Moestl, E., 2014, 'Glucocorticoids, their metabolites and their measurement in various animal species', *Medycyna Weterynaryjna-Veterinary Medicine-Science and Practice* 70(9), 524-529.

Mokoele, J.M., Spencer, B.T., van Leengoed, L.A.M.G. & Fasina, F.O., 2014, 'Efficiency indices and indicators of poor performance among emerging small-scale pig farmers in the Limpopo Province, South Africa', *Onderstepoort Journal of Veterinary Research* 81(1), 774.

Monfort, S.L., Brown, J.L. & Wildt, D.E., 1993, 'Episodic and Seasonal Rhythms of Cortisol Secretion in Male Elds Deer (Cervus-Eldi-Thamin)', *Journal of Endocrinology* 138(1), 41-49.

Möstl, E. & Palme, R., 2002, 'Hormones as indicators of stress', *Domestic animal endocrinology* 23(1), 67-74.

Mostl, E., Messmann, S., Bagu, E., Robia, C. & Palme, R., 1999a, 'Measurement of glucocorticoid metabolite concentrations in faeces of domestic livestock', *Journal of Veterinary Medicine Series A-Physiology Pathology Clinical Medicine* 46(10), 621-631.

Mostl, E., Messmann, S., Bagu, E., Robia, C. & Palme, R., 1999b, 'Measurement of glucocorticoid metabolite concentrations in faeces of domestic livestock.', *Journal of Veterinary Medicine.Series A* 46(10), 621-631.

Möstl, E. & Palme, R., 2002, 'Hormones as indicators of stress', *Domestic animal endocrinology* 23(1–2), 67-74.

Noonan, G., Rand, J., Priest, J., Ainscow, J. & Blackshaw, J., 1994, 'Behavioural observations of piglets undergoing tail docking, teeth clipping and ear notching', *Applied Animal Behaviour Science* 39(3), 203-213.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Palme, R., Möstl, E., Brem, G., Schellander, K. & Bamberg, E., 1997, 'Faecal Metabolites of Infused 14C-Progesterone in Domestic Livestock', *Reproduction in Domestic Animals* 32(4), 199-206.

Palme, R., Robia, C., Baumgartner, W. & Möstl, E., 2000, 'Transport stress in cattle as reflected by an increase in faecal cortisol metabolite concentrations.', *Veterinary Record* 146(4), 108-109.

Palme, R., Fischer, P., Schildorfer, H. & Ismail, M.N., 1996, 'Excretion of infused C-14-steroid hormones via faeces and urine in domestic livestock', *Animal Reproduction Science* 43(1), 43-63.

Pol, F., Courboulay, V., Cotte, J.P., Martrenchar, A., Hay, M. & Mormede, P., 2002, 'Urinary cortisol as an additional tool to assess the welfare of pregnant sows kept in two types of housing', *Veterinary research* 33(1), 13-22.

Prunier, A., Heinonen, M. & Quesnel, H., 2010, 'High physiological demands in intensively raised pigs: impact on health and welfare', *Animal* 4(06), 886-898.

Rhodes, R.T., Appleby, M.C., Chinn, K., Douglas, L., Firkins, L.D., Houpt, K.A., Irwin, C., McGlone, J.J., Sundberg, P., Tokach, L. & Wills, R.W., 2005, 'Task Force Report - A comprehensive review of housing for pregnant sows', *Javma-Journal of the American Veterinary Medical Association* 227(10), 1580-1590.

Roldan-Santiago, P., Mota-Rojas, D., Orozco-Gregorio, H., Borderas-Tordesillas, F., Martínez-Rodríguez, R., Mora-Medina, P., Flores-Peinado, S., Sánchez-Hernández, M. & Trujillo-Ortega, M., 2015, 'Welfare of recently weaned piglets transported on unpaved roads: the effect of age and the use of straw bedding', *Animal Production Science* 55(5), 648-653.

Ruis, M.A.W., Brake, J.H.A.T., Engel, B., Ekkel, E.D., Buist, W.G., Blokhuis, H.J. & Koolhaas, J.M., 1997, 'The circadian rhythm of salivary cortisol in growing pigs: Effects of age, gender, and stress', *Physiology & Behavior* 62(3), 623-630.

Salak-Johnson, J.L. & McGlone, J.J., 2007, 'Making sense of apparently conflicting data: Stress and immunity in swine and cattle', *Journal of animal science* 85 E81-E88.

Sapolsky, R., 1992, 'Neuroendocrinology of the stress response. In 'Behavioral Endocrinology'.(Eds JB Becker, SM Breedlove and D. Crews.) pp. 287–324', .

Schwarzenberger, F., 2007, 'The many uses of non-invasive faecal steroid monitoring in zoo and wildlife species', *International Zoo Yearbook* 41(1), 52-74.

Sheriff, M.J., Dantzer, B., Delehanty, B., Palme, R. & Boonstra, R., 2011, 'Measuring stress in wildlife: techniques for quantifying glucocorticoids', *Oecologia* 166(4), 869-887.

Siswanto, H., Hau, J., Carlsson, H., Goldkuhl, R. & Abelson, K.S.P., 2008, 'Corticosterone concentrations in blood and excretion in faeces after ACTH administration in male Sprague-Dawley rats', *In Vivo* 22(4), 435-440.

Smith, S.M. & Vale, W.W., 2006, 'The role of the hypothalamic-pituitary-adrenal axis in neuroendocrine responses to stress', *Dialogues in clinical neuroscience* 8(4), 383-395.

Soede, N., Roelofs, J., Verheijen, R., Schouten, W., Hazeleger, W. & Kemp, B., 2007, 'Effect of repeated stress treatments during the follicular phase and early pregnancy on reproductive performance of gilts', *Reproduction in domestic animals* 42(2), 135-142.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Sutherland, M.A., Backus, B.L. & McGlone, J.J., 2014, 'Effects of transport at weaning on the behavior, physiology and performance of pigs.', *Animals* 4(4), 657-669.

Thun, R., Gajewski, Z. & Janett, F., 2006, 'Castration in male pigs: techniques and animal welfare issues', *Journal of physiology and pharmacology : an official journal of the Polish Physiological Society* 57 Suppl 8 189-194.

Tisdell, C.A., 2013, *Wild pigs: environmental pest or economic resource?* Elsevier.

Tonsor, G.T., Olynk, N. & Wolf, C., 2009, 'Consumer preferences for animal welfare attributes: the case of gestation crates.', *Journal of Agricultural and Applied Economics* 41(3), 713-730.

Touma, C., Palme, R. & Sachser, N., 2004, 'Analyzing corticosterone metabolites in fecal samples of mice: a noninvasive technique to monitor stress hormones', *Hormones and behavior* 45(1), 10-22.

Touma, C. & Palme, R., 2005, 'Measuring fecal glucocorticoid metabolites in mammals and birds: The importance of validation', *Bird Hormones and Bird Migrations: Analyzing Hormones in Droppings and Egg Yolks and Assessing Adaptations in Long-Distance Migration* 1046 54-74.

Turner, A.I., Hemsworth, P.H. & Tilbrook, A.J., 2005, 'Susceptibility of reproduction in female pigs to impairment by stress or elevation of cortisol', *Domestic animal endocrinology* 29(2), 398-410.

van der Staay, F Josef, Schuurman, T., Hulst, M., Smits, M., Prickaerts, J., Kenis, G. & Korte, S.M., 2010, 'Effects of chronic stress: A comparison between tethered and loose sows', *Physiology & Behavior* 100(2), 154-164.

Von Borell, E. & Schäffer, D., 2005, 'Legal requirements and assessment of stress and welfare during transportation and pre-slaughter handling of pigs', *Livestock Production Science* 97(2), 81-87.

von Borell, E., 2000, 'Stress and coping in farm animals', *Archiv Fur Tierzucht-Archives of Animal Breeding* 43 144-152.

von der Ohe, C.,G. & Servheen, C., 2002, 'Measuring stress in mammals using fecal glucocorticoids: opportunities and challenges', *Wildlife Society Bulletin* 30(4), 1215-1225.

Whitney, M.H. & Masker, C., 2010, 'Replacement gilt and boar nutrient recommendations and feeding management', *US Pork Center of Excellence: Des Moines, Iowa* .

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

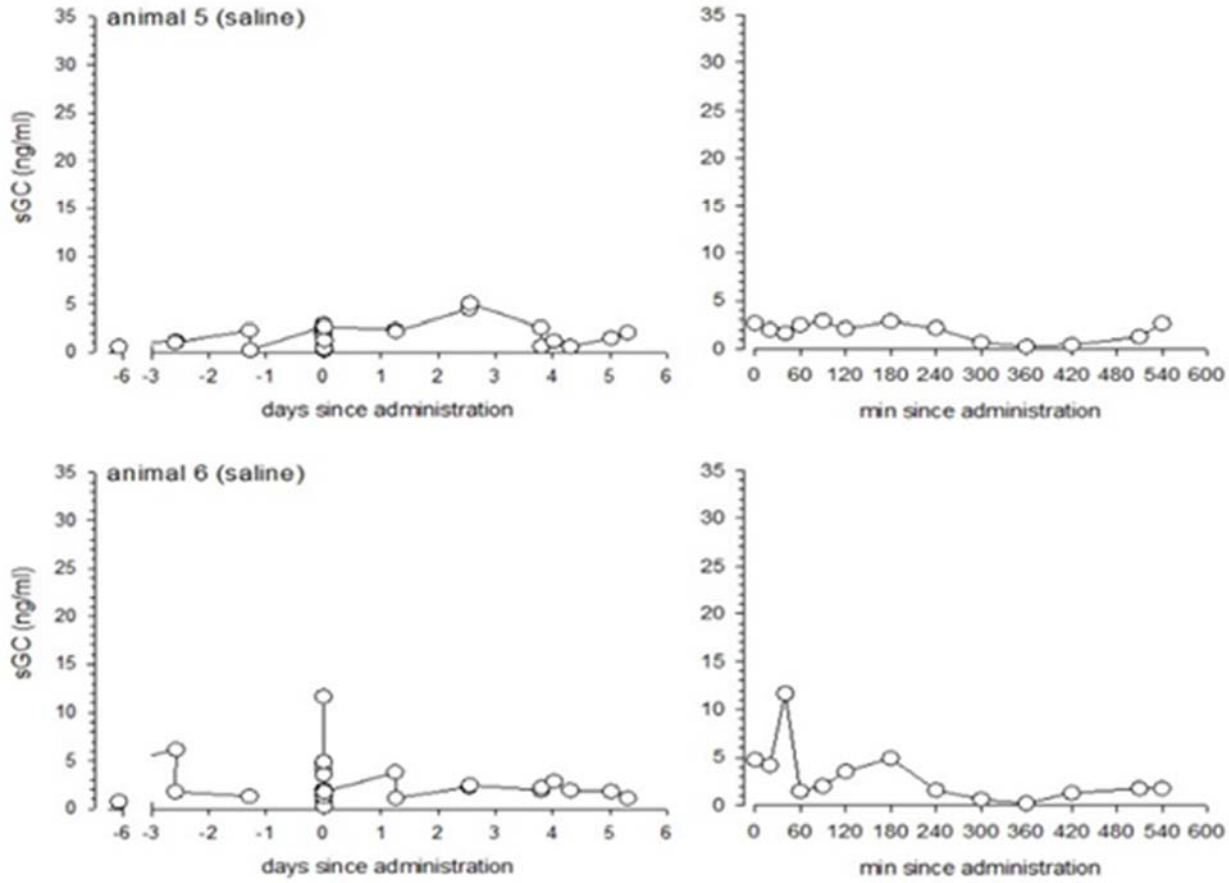


Figure 8a-b: Individual long-term profiles of salivary glucocorticoid metabolite concentration in gilts (pigs) prior and post administration of saline. Time point 0 indicated time of administration. The graphs on the left hand side representing the total time interval monitored (-6 to 6 days of administration), whereas the graphs on the right represent the time window of 0 to 10 hour post-injection.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

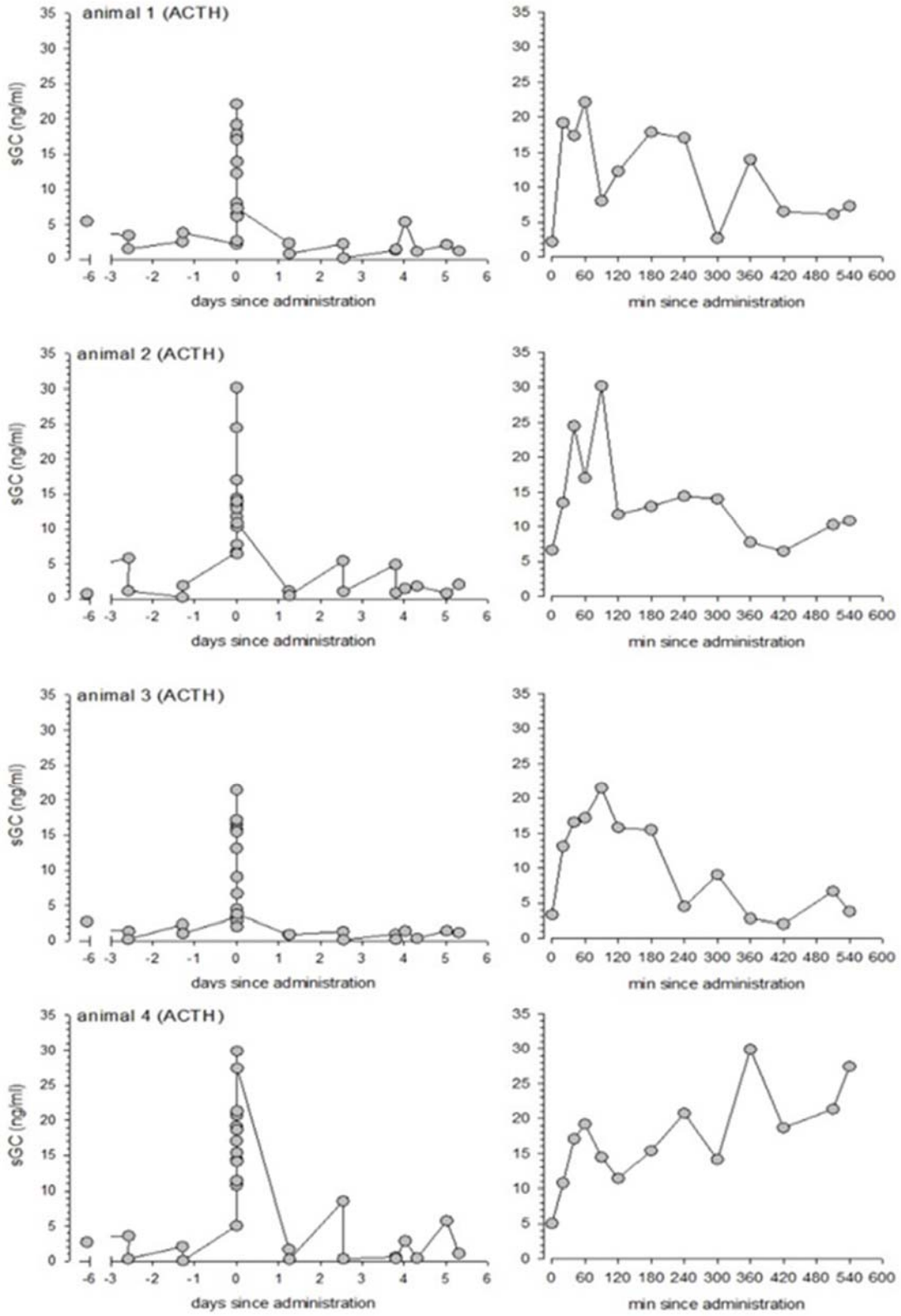
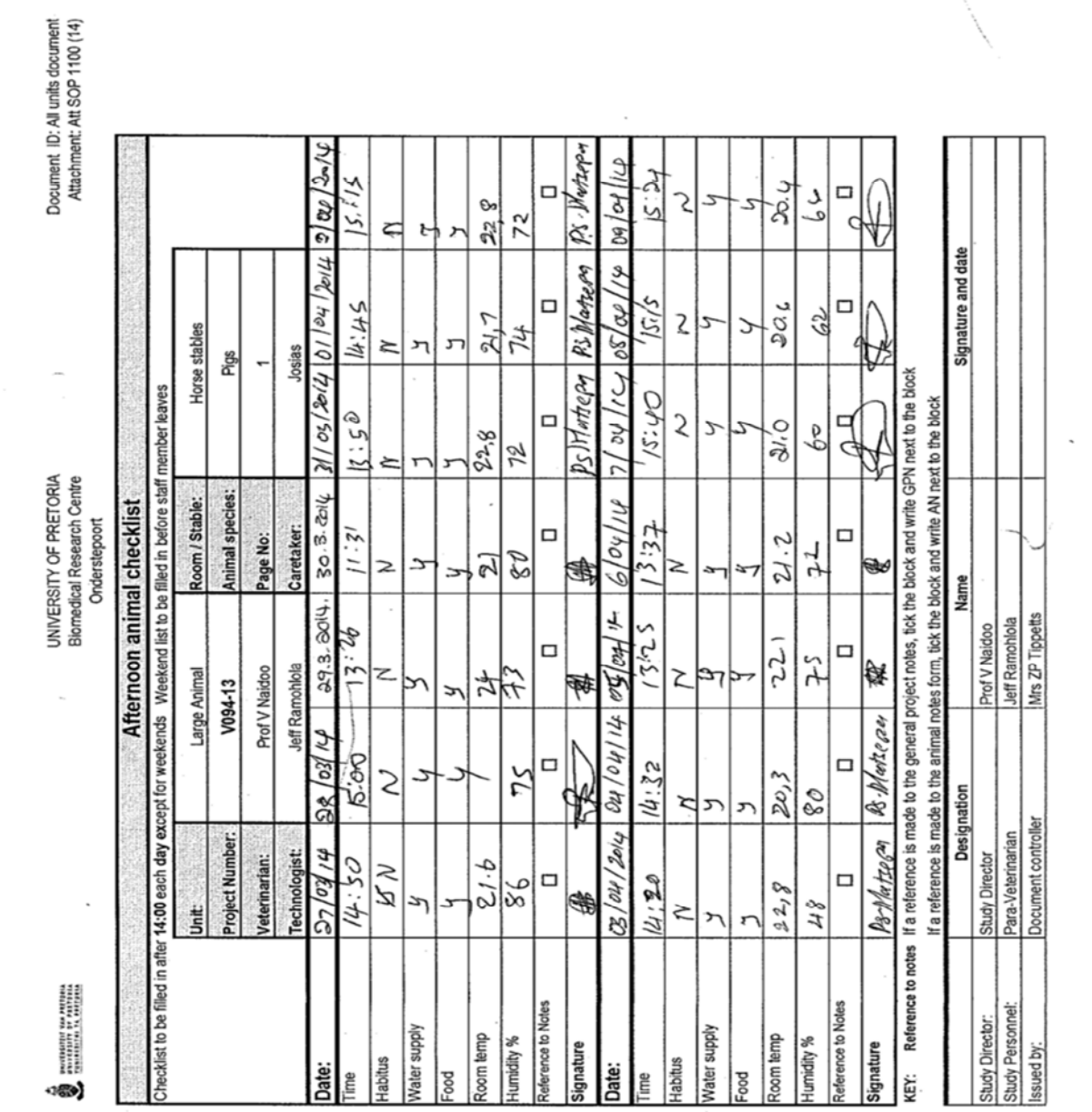


Fig 8c-f: Individual long-term profiles of salivary glucocorticoid metabolite concentration in gilts (pigs) prior and post administration of either synthetic ACTH. Time point 0 indicated time of administration. The graphs on the left hand side representing the total time interval monitored (-6 to 6 days of administration), whereas the graphs on the right represent the time window of 0 to 10 hour post-injection.

APPENDIX

Appendix 1: Clinical observation and Environmental Monitoring Indices of Experimental



UNIVERSITY OF PRETORIA
Biomedical Research Centre
Onderstepoort

Document ID: All units document
Attachment: Att SOP 1100 (14)

Afternoon animal checklist

Checklist to be filled in after **14:00** each day except for weekends Weekend list to be filled in before staff member leaves

	Unit:	Large Animal	Room / Stable:	Horse stables	
	Project Number:	V094-13	Animal species:	Pigs	
	Veterinarian:	Prof V Naidoo	Page No:	1	
	Technologist:	Jeff Ramohlola	Caretaker:	Josias	
Date:	27/03/14	28/03/14	29.3.2014	30.3.2014	31/03/2014	01/04/2014	2/04/2014
Time	14:30	15:00	13:26	11:31	15:50	14:45	15:15
Habitus	N	N	N	N	N	N	N
Water supply	Y	Y	Y	Y	Y	Y	Y
Food	Y	Y	Y	Y	Y	Y	Y
Room temp	21.6	1	24	21	22.8	21,7	22, 8
Humidity %	86	75	73	80	72	74	72
Reference to Notes	☐	☐	☐	☐	☐	☐	☐
Signature					PS Mattepan	PS Mattepan	PS Mattepan
Date:	03/04/2014	04/04/14	05/04/14	6/04/14	7/04/14	08/04/14	09/04/14
Time	14:20	14:32	13:25	13:37	15:40	15:15	15:24
Habitus	N	N	N	N	N	N	N
Water supply	Y	Y	Y	Y	Y	Y	Y
Food	Y	Y	Y	Y	Y	Y	Y
Room temp	22,8	20,3	22.1	21.2	21.0	20.6	20.4
Humidity %	48	80	75	72	60	62	64
Reference to Notes	☐	☐	☐	☐	☐	☐	☐
Signature	PS Mattepan	PS Mattepan					

KEY: **Reference to notes** If a reference is made to the general project notes, tick the block and write GPN next to the block
If a reference is made to the animal notes form, tick the block and write AN next to the block

	Designation	Name	Signature and date
Study Director:	Study Director	Prof V Naidoo	
Study Personnel:	Para-Veterinarian	Jeff Ramohlola	
Issued by:	Document controller	Mrs ZP Tippetts	

Afternoon animal checklist

Checklist to be filled in after **14:00** each **day** except for weekends Weekend list to be filled in before staff member leaves

Unit:	Large Animal		**Room / Stable:**	Horse stables		
Project Number:	V094-13		**Animal species:**	Pigs		
Veterinarian:	Prof V Naidoo		**Page No:**	2		
Technologist:	Jeff Ramohlola		**Caretaker:**	Josias		
Date:	10/04/14	11/04/14	12/04/14	13/04/14	14/04/2014	15/04/2014	16/04/14
Time	15:10	14:50	14:03	13:35	14:43	14:41	14:53
Habitus	N	N	N	N	N	N	N
Water supply	Y	Y	Y	Y	Y	Y	Y
Food	Y	Y	Y	Y	Y	Y	Y
Room temp	20,6	20,8	21.0	20.6	19,0	21,3	20,6
Humidity %	68	58	72	69	83	64	77
Reference to Notes	☐	☐	☐	☐	☐	☐	☐
Signature	P.S. Matsepa	P.S. Matsepa	E-T	E-T	P.S. Matsepa	P.S. Matsepa	P.S Matsepa
Date:	17/04/14	18/04/14	19/04/14	20/04/14	21/04/14	22/04/14	
Time	13:58	13:11	12:46	12:23	13:52	14:25	
Habitus	N	N	N	N	N	N	
Water supply	Y	Y	Y	Y	Y	Y	
Food	Y	Y	Y	Y	Y	Y	
Room temp	17,9	21,3	20,8	21,1	20,9	20.6	
Humidity %	77	66	68	70	65	60	
Reference to Notes	☐	☐	☐	☐	☐	☐	☐
Signature	P.S. Matsepa	P.S. Matsepa		P.S Matsepa	P.S Matsepa		

KEY: **Reference to notes** If a reference is made to the general project notes, tick the block and write GPN next to the block
If a reference is made to the animal notes form, tick the block and write AN next to the block

	Designation	Name	Signature and date
Study Director:	Study Director	Prof V Naidoo	
Study Personnel:	Para-Veterinarian	Jeff Ramohlola	
Issued by:	Document controller	Mrs ZP Tippetts	

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

UNIVERSITY OF PRETORIA
Biomedical Research Centre
Onderstepoort

Document ID: All units document
Attachment: Att SOP 1100 (9)

Project Number			V094-13	ANIMAL NOTES FORM	Page No.	1
Date	Time	Animal Number	1,2,3,4,5 and 6	Group / Number animals	6	
			Description of observations and any treatments administered			Signature
27.3.14	10:30	All	Pigs arrived, checked by Dr Tarsima and Nobert.			
	10:30	All	weighed, NAD			
	14:57	All	NAD			
29.3.2014	10:05	All	Active tated. Some crates, plastic sides removed by pigs			
30.3.2014	7:40	All	NAD. Active + alert.			
31.3.14	13:01	All	NAD.			
01.4.14	11:46	All	NAD			
02.4.14	10:00	All	NAD			
03.4.14	15:06	All	NAD			
	15:30	All	Plastic bottles and squeeky bygspot in.			
5/4/14	11:00	All	Some have black discarge both eyes and some Red eyes			
6/4/14	12:00	Sto	Very Restless! Trying to get out of cages, Check please.			
7/04/14	10:00	All	NAD			
8/04/14	11:50	All	NAD			
08/04/14	11:50	All	NAD			
09/04/14	08:30	All	Injected 4 pigs with 0.55ml (550ul) of ACTH & 2 pigs with 0.55ml of saline (2&4) & (1,3,5,6-ACTH) process started at 17.49 and ended at 8.28am			

	Designation	Name	Signature and date
Study Director:	Study Director	Prof V Naidoo	
Study Personnel:	Para-Veterinarian	Mr Jeff Ramohlola	
Issued by:	Document Controller	Mrs ZP Tippetts	

Project Number: V094/13 | **Animal Room:** 1-2 | **Page No:** 2 | **Unit:**

ROOM STATUS SHEET

Date	Time	Min Temp	Max Temp	Relative Humidity	Timer setting correct	Description of Enrichment	Signature	Scheduled	Stable / Cage Changed	Scheduled	Food & Water Top-up	Scheduled	Floor Cleaning	Scheduled	Cleaning of Walls; Doors	Scheduled	Bleach into open drains	Scheduled	Footbaths	Signature	Reference to General Project Notes
13/4/14	12:55	18	26	64	NA	Toys		X	✓	X	✓	X	✓	X	NO			X	✓		
14/04/14	9:15	18	26	82	NA	Toys		X	✓	X	✓	X	✓					X	✓	P.S Mokgoga	
15.4.14	11:31	17	25	74	NA	Toys		X	✓	X	✓	X	✓					X	✓	P.S Mokgoga	
16.4.14	11:30	18	26	68	NA	Toys		X	✓	X	✓	X	✓					X	✓	P.S Mokgoga	
17.4.14	7:45	18	26	81	NA	Toys		X	✓	X	✓	X	✓					X	✓	P.S Mokgoga	
18.4.2014	8:45	15	25	91	NIA	toys.		X	✓	X	✓	X	✓					X	✓	P.S Mokgoga	
19.4.2014	9:00	17	25	78	NIA	toys-Balls/Bottles		X	✓	X	✓	X	✓					X	✓	P.S Mokgoga	
20.4.2014	8:45	16	25	71	NIA	toys		X	✓	X	✓	X	✓					X	✓	P.S Mokgoga	
21.4.2014	8:45	17	25	70	NIA	toys		X	✓	X	✓	X	✓					X	✓	P.S Mokgoga	
22.4.14	8:25	18	26	77	NA	toys		X	✓	X	✓	X	✓					X	✓		

	Designation	Name	Signature and date
Study Director:	Study Director	Prof V Naidoo	
Study Personnel:	Para-Veterinarian		
Issued by:	Document controller	Mrs ZP Tippetts	

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

UNIVERSITY OF PRETORIA
Biomedical Research Centre
Onderstepoort

Document ID: All units document
Attachment: Att SOP 1100 (13)

Project Number:	V094/13	ROOM STATUS SHEET	Page No:	1
Animal Room:	1–2		Unit:	

Date	Time	Min Temp	Max Temp	Relative Humidity	Timer setting correct	Description of Enrichment	Signature	Scheduled	Stable / Cage Changed	Scheduled	Food & Water Top-up	Scheduled	Floor Cleaning	Scheduled	Cleaning of Walls, Doors	Scheduled	Bleach into open drains	Scheduled	Footbaths	Signature	Reference to General Project Notes
28.3.14	11:30	21	26	80	NIA	toys		X	✓	X	✓	X	✓					X	X		
29.3.2014	10:00	18	24	81	NIA	toys		X	✓	X	✓	X	✓								
30.3.2014	7:45	20	25	85	NIA	toys		X	✓	X	✓	X	✓								
31.3.14	13:01	19	25	74	NA	toys		X	✓	X	✓	X	✓					X	✓	PS Maitsepa	
01.04.14	11:47	19	25	79	NA	toys		X	✓	X	✓	X	✓						✓	P.S. Maitsepa	
02.4.14	10:00	19	25	71	NA	toys		X	✓	X	✓	X	✓					X	✓	PS Maitsepa	
03/4/14	9:00	19	27	73	NA	toys		X	✓	X	✓	X	✓						✓	PS. Maitsepa	
04/04/14	Ingy 10:00 11:09	19	26	73	NA	toys		X	✓	X	✓	X	✓					X	✓	P.S.Maitsepa	
5/4/14	11:01	17	26	73	NA	Empty Bottles		X	✓	X	✓	X	✓								
6/4/14	12:00	18	26	73	NA	Empty Bottle		X	✓	X	✓	X	✓					X	✓		
7/04/14	12:17	18	25	73	NA	empty bottles		X	✓	X	✓	X	✓					✓	✓		
08/04/14	11:50	18	25	86	NA	empty bottles		X	✓	X	✓	X	✓					X	✓	P.S. Maitsepa	
09/04/14	8:20	18	24	66	NA	Toys		X	✓	X	✓	X	✓						✓	PS Maitsepa	
10/04/14	8:00	19	25	73	NA	Toys		X	✓	X	✓	X	✓					X	✓	P.S. Maitsepa	
11/04/14	8:00	17	26	73	NA	Toys		X	✓	X	✓	X	✓						✓	PS. Maitsepa	
12/04/14	12:50	19	26	72	NA	Toys		X	✓	X	✓	X	✓					X	✓	E-T	

	Designation	Name	Signature and date
Study Director:	Study Director	Prof V Naidoo	
Study Personnel:	Para-Veterinarian		
Issued by:	Document controller	Mrs ZP Tippetts	

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

UNIVERSITY OF PRETORIA
Biomedical Research Centre
Onderstepoort

Document ID: All units document
Attachment: Att SOP 1100 (10)

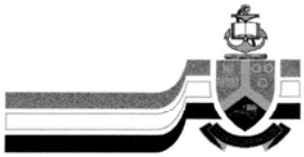
									PARAMETER:					Weight(kg)					
Project Number:		T001-14			INDIVIDUAL ANIMAL RECORD - SINGLE PARAMETER												Page No:	1	
Group Number →																			
Date	Control	1	2	3	4	5	6												
27/03/2014	5Kg	45.6	46.2	47.7	46.4	46.4	43.7												Jeff
22/04/2014	5Kg	63.2	64.7	66.8	66.7	65.4	58.0												Jeff

	Designation	Name	Signature and date
Study Director:	Study Director	Prof V Naidoo	
Study Personnel:	Para-Veterinarian	Mr Jeff Ramohlola	
Issued by:	Document controller	Mrs ZP Tippetts	

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Appendix 2: Research Committee Protocol Approval

Ref: V094/13



University of Pretoria

Faculty of Veterinary Science
Private Bag X04
Onderstepoort
0110

Tel: +27 12 529 8000
Fax: +27 12 529 8300

22 January 2014

Dr FO Fasina
Department Production Animal Studies
(dayo.fasina@up.ac.za)

Dear Prof Fasina

PROTOCOL V094/13: VALIDATION OF SALIVARY AND FAECAL GLUCOCORTICOID METABOLITES IN PIGS – *N Mangwiro*

I am pleased to inform you that the abovementioned protocol was approved by the Research Committee.

Kindly take note of the attached document.

Kind regards

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

NIESJE TROMP

SECRETARY: RESEARCH COMMITTEE

Copy: Prof JAW Coetzer, Deputy Dean: Research (koos.coetzer@up.ac.za)

Dr N Mangwiro, Researcher (mangwiron@yahoo.com)

Prof P Irons, HOD (pete.irons@up.ac.za)

Prof PN Thompson, Departmental Research Coordinator (peter.thompson@up.ac.za)

Ms M Human, Student Administration (magda.human@up.ac.za)

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

UNIVERSITY OF PRETORIA

POLICY FOR THE PRESERVATION AND RETENTION OF RESEARCH DATA

1. INTRODUCTION

Data and records are an essential part of all research projects. Disputes about intellectual property, data authenticity and data ownership have highlighted the need for all researchers at a University but in particular those involved in experimental work and other scholarly evidence based research, to take steps to secure the maintenance and storage of the raw data on which publications, theses, reports, patents and other forms of published material are based. These data need to be stored in a secure environment, in as far as possible in tamper-free form and in sufficient detail to enable the principal investigator or an independent body of experts to address all enquiries having to do with accuracy and authenticity of the research and the data on which it is based.

The policy applies to all researchers and individuals at the University involved in conducting or reporting of research, irrespective of the funding source. It is aimed at the protection of students, researchers, principal investigators and ultimately, the University against a variety of disputes concerning published or patented research. Some of the more important of these issues can be summarized as follows:

1.1 The protection of intellectual property (IP) and disputes with regard to proposed or completed patents. It is of particular importance that the time of discovery, the nature of the discovery and the individuals involved are clearly recorded.

1.2 A detailed research data record will help to resolve conflicts between students, UP researchers and external funding bodies about IP contributions if such issues are not already clearly defined in contracts.

1.3 Disputes concerning the reproducibility of research claims, accusations of falsification of the data and all other issues in which the authenticity of the published research is questioned.

1.4 Disputes between supervisors and students about research progress and the contribution made by individual students and co-workers to published and patented research. This also applies particularly to students who terminate their studies before the completion of a degree.

1.5 Keeping a detailed research data base reduces the risk that important research data may get lost because its significance was not realized at the time when it was produced. Principal

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

investigators or new researchers will have the opportunity to review unpublished data in a new light, following new discoveries.

2. DATA PRESERVATION

2.1 REQUIREMENTS FOR DATA PRESERVATION

2.1.1 All students and researchers should be aware of the fact that research data belongs to the institution. The University needs these data in order to comply with project agreements, securing intellectual property rights where applicable, protecting the rights of staff and students regarding access to data, and facilitating any investigation of charges of misconduct or conflict of interest.

2.1.2 The principal investigator of each project is charged with the responsibility of securing these data for future use. This responsibility is transferred to the School or Faculty or a duty appointed research successor if the principal investigator should leave the University.

2.1.3 Procedures should be in place to protect data from alteration and loss, including from fires or other emergencies.

2.1.4 Data is required to be stored for a minimum of ten years after the completion of the original project but if intellectual property is involved, or if there are particular statutory or contractual requirements, a longer period may well be required. Special consideration about the length of storage should also be given to cases where a potential conflict of interest or misconduct is involved. In some cases, and in particular where experiments with humans are concerned, funding bodies may require that all raw data be kept indefinitely.

2.1.5 Principal investigators have the responsibility to obtain and secure the research data from postgraduate students before degrees are awarded and especially in cases where students have abandoned the work.

2.2 MAINTENANCE OF RESEARCH DATA

There are a number of ways in which raw research data may be stored on a day to day basis but the most important of these are probably hard copy research note books and the large selection of ways and formats in which digital data can be stored on computers

2.2.1 **The note book:** The key to recording research data in a well-organized project, is the research note book. This should provide a permanent dated record of all completed research

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

and new discoveries. The record should provide the necessary facts pertaining to the methods used, observations made and results obtained. The principal investigator will have to play an important role in guiding students and other researchers as what has to be provided in terms of experimental detail. This detail should be sufficient to be able to address disputes with respect to patents, processes and designs, and deal with questions about the accuracy and authenticity of the research work. The note book remains the property of the Institution and if the principal investigator should leave the University, a copy of the note book may be given to the investigator. The original note book remains with the University. The storage of note books as PDF files on computers should also be considered. Guidelines for recording research data in a relatively tamper-proof way so that it is available for long-term storage and future inspection is provided by the University library.

2.2.2 **Electronic data storage:** The storage of research data as files on computers is already an essential way of storing large amounts of important research data and it will become increasingly more important in future. It currently complements the research note book, but in the future it may well replace it. Guidelines of how to secure and consolidate computer data that are often distributed among a range of different computers need to be developed by research groups and approved by the Faculty Research Committee.

3. DATA REPOSITORIES

3.1 Data that is in digital form or which can be converted into a digital format will be stored and curated on the digital repositories created by the University library.

3.2 All non-digital research material must be retained in appropriate repositories which are maintained and curated within the academic departments where the material was generated by research activities.

3.3 A report on the management of data repositories should be made annually by faculties to the Vice-Principal (Research and Postgraduate Studies).

RM Crewe

30 July 2007

Appendix 3: Ethics Approval

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

PROJECT TITLE	**Validation of salivary and faecal glucocorticoid metabolite in pigs**
PROJECT NUMBER	**V094-13**
RESEARCHER/PRINCIPAL INVESTIGATOR	**Dr. M Nobert**

STUDENT NUMBER (where applicable)	**122 246 78**
DISSERTATION/THESIS SUBMITTED FOR	**MSc**

ANIMAL SPECIES	**Pigs**	
NUMBER OF ANIMALS	**6**	
Approval period to use animals for research/testing purposes		**December 2013-January 2014**
SUPERVISOR	**Dr. FF Oludayo**	

KINDLY NOTE:

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

APPROVED	Date 25 November 2013
CHAIRMAN: UP Animal Ethics Committee	Signature

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA