Comparison of two CYP17 isoforms: Implications for cortisol production in the South African Merino

by
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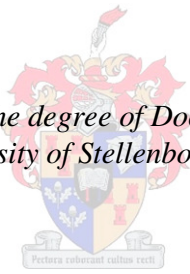

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Declaration

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Abstract

This study describes:

- the comparison of the enzymatic activities of the two ovine cytochrome P450 17α-hydroxylase/17,20-lyase (CYP17) isoforms expressed in non-steroidogenic COS-1 cells. The K_m and V_{max} values for the metabolism of pregnenolone and progesterone were determined, while time-dependent metabolism of pregnenolone, 17-hydroxypregenolone, progesterone and 17-hydroxyprogesterone was also reported. The cloning and sequencing of ovine cytochrome b_5 is reported and was co-expressed with CYP17. The results showed that the wild type 1 (WT1) isoform of ovine CYP17 produce more cortisol precursors than the wild type 2 (WT2) isoform;

- the analysis of the frequency distribution of the *CYP17* genotypes within a South African Merino population, which were divergently selected for (H-line) or against (L-line) the ability of a ewe to rear multiple offspring per birthing opportunity. It was observed that the *CYP17* frequency distribution was the same within the H- and L-line, with 78.3 % heterozygous *WT1/WT2* and 21.7 % homozygous *WT1/WT1*. No homozygous *WT2/WT2* individuals were identified;

- the development of a UPLC-MS/MS method for the separation and quantification of all thirteen adrenal steroids that are produced in the adrenal gland;

- the relative contribution of the *CYP17* genotypes in the total steroidogenic output in adult adrenocortical cells from the adrenal glands of H- and L-line sheep, with particular emphasis on cortisol production. The adrenocortical cells from the H-line sheep showed a marked higher cortisol production than the L-line, while adrenocortical cells from homozygous *WT1/WT1* sheep also produced more cortisol than heterozygous *WT1/WT2* sheep;

- the blood cortisol responses upon the stimulation of the HPA axis by insulin induced hypoglycaemia of the H- and L-line sheep with known *CYP17* genotypes. It was observed that the *CYP17* genotype and selection line are important factors affecting the cortisol responses of sheep, where L-line heterozygous *WT1/WT2* sheep showed the lowest cortisol response and glucose recovery;

- the association of the *CYP17* genotype with behavioural responses of H- and L-line sheep to flock isolation stress, as well as the association of the *CYP17* genotype with ewe reproduction and lamb output. While reproduction seemed to be unaffected by the *CYP17* genotype, the behavioural stress responses of sheep to flock isolation correlated with the *CYP17* genotype, where the heterozygous *WT1/WT2* genotype was associated with a wilder nature.

Opsomming

Hierdie studie ondersoek:

- die vergelyking van die ensiemaktiwiteite vir twee isoforme van skaap sitochroom P450 17α-hidroksilase/17,20-liase (CYP17), wat uitgedruk was in nie-steroïed genererende COS-1 selle. Die K_m and V_{max} waardes was bepaal vir die metabolisme van pregnenoloon en progesteroon, terwyl die tyd-afhanklike metabolisme van pregnenoloon, 17-hidroksiepregnenoloon, progesteroon en 17-hidroksieprogesteroon ook gerapporteer word. Die klonering en volgorde bepaling van skaap sitochroom b_5 was gedoen en gevolglik was sitochroom b_5 saam met CYP17 uitgedruk in COS-1 selle. Die resultate het gewys dat wilde tipe 1 (WT1) meer voorlopers van kortisol produseer as wilde tipe 2 (WT2);

- die frekwensie distrubusie van die *CYP17* genotipes in 'n Suid-Afrikaanse Merino populasie, waar skape in teenoorgestelde rigtings geselekteer was vir (H-lyn) of teen (L-lyn) die vermoë van 'n ooi om geboorte te gee aan veelvoudige lammers per lamgeleentheid. Die frekwensie distrubusie van *CYP17* was dieselfde in beide die H- en L-lyn, waar 78.3 % van die populasie heterosigoties *WT1/WT2* en 21.7 % homosigoties *WT1/WT1* was. Geen homosigote *WT2/WT2* individue was geïdentifiseer nie;

- die ontwikkeling van 'n UPLC-MS/MS metode vir die skeiding en kwantifisering van al dertien steroïede wat natuurlik geproduseer word in die bynier van die skaap;

- die relatiewe bydrae van die CYP17 isoforme tot die totale steroïedale uitsette vanuit die bynier kortex selle, vanaf die byniere van H- en L-lyn skape, waar klem geplaas word op die produksie van kortisol. Die bynierselle van die H-lyn skape het aansienlik meer kortisol produseer as die L-lyn, terwyl die bynierselle van die homosigotiese *WT1/WT1* skape ook meer kortisol produseer het as heterosigotiese *WT1/WT2* skape;

- die bloed kortisol in reaksie tot die stimulering van die hipotalamus-hipofise-adrenale aksis, deur insulien geïnduseerde hipoglisemiese stress, in skape van die H- en L-lyne met bekende *CYP17* genotipes. Dit was gevind dat die kortisol reaksie geaffekteer word deur beide die *CYP17* genotipe en seleksie lyn, waar L-lyn heterosigotiese *WT1/WT2* skape die minste kortisol geproduseer het en die stadigste herstel van glukose vlakke getoon het;

- die assosiasie tussen die *CYP17* genotipe en die gedrags reaksies op trop-isolasie, sowel as ooi-reproduksie en lamuitset, van die H- en L-lyn skape. Die reproduksie parameters was onafhanklik van die *CYP17* genotipe, terwyl ‘n sterk assosiasie gevind was tussen die *CYP17* genotipe en gedrags reaksies op trop-isolasie. Die heterosigotiese *WT1/WT2* skape het ‘n wilder natuur getoon gedurende trop-isolasie in vergelyking met homosigotiese *WT1/WT1* skape.

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Table of Contents

Alphabetical List of Abbreviations

11βHSD1	11β-hydroxysteroid dehydrogenase type 1
11βHSD2	11β-hydroxysteroid dehydrogenase type 2
16-OHP4	16-hydroxyprogesterone
17-OHP4	17-hydroxyprogesterone
17-OHP5	17-hydroxypregnenolone
3βHSD	3β-hydroxysteroid dehydrogenase
A4	Androstenedione
AA cells	Adult adrenocortical cells
ACAT	Acyl-coenzyme A: cholesterol acyltransferase
ACTH	Cyclic adenosine monophosphate
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
AUC	Area under the curve
CBG	Corticosteroid binding globulin
cDNA	Complementary deoxyribonucleic acid
CE	Collision energy
CRE/CREB	cAMP response element/cAMP response element binding protein
CRH	Corticotrophin releasing hormone
CV	Cone voltage
CYP11A1	Cholesterol side chain cleavage
CYP17	Cytochrome P450 17α-hydroxylase/17,20-lyase
CYP21	Cytochrome P450 21-hydroxylase
CYPB	Cytochrome P450 11β-hydroxylase
DHEA	Dehydroepiandrosterone
DIAPH1	Diaphanous-related homolog 1
DNA	Deoxyribonucleic acid
ESI	Electronspray ionization
FAD	Flavinadenine dinucleotide
FMN	Flavinmononucleotide
FRET	Fluorescent resonance energy transfer
GC	Gas chromatography
GDP	Guanine diphosphate
GR	Glucocorticoid receptor

GREs	Glucocorticoid response elements
GTP	Guanine triphosphate
HDL	High-density lipoprotein
H_E	H-line heterozygotes (*WT1/WT2*)
H_O	H-line homozygotes (*WT1/WT1*)
HPA axis	Hypothalamic-pituitary-adrenal axis
HSL	Hormone-sensitive lipase
IGFs	Insulin-like growth factors
IL	Interleukin
LDL	Low-density lipoprotein
L_E	L-line heterozygotes (*WT1/WT2*)
L_O	L-line homozygotes (*WT1/WT1*)
LOD	Limit of detection
LOQ	Limit of quantification
MRM	Multiple reaction monitoring
NADPH	Nicotinamide adenine dinucleotide phosphate
NOL	Number of lambs
P4	Progesterone
P5	Pregnenolone
PCR	Polymerase chain reaction
PFP	Pentafluorophenyl
POMC	Proopiomelanocortin
POR	P450 oxidoreductase
PVN	paraventricular nucleus
RNA	Ribonucleic acid
ROCK1	Rho-associated coiled-coil containing kinase 1
RSD	Residual standard deviation
SNP	Single nucleotide polymorphism
SRB1	Scavenger receptor B1
StAR	Steroid acute regulatory protein
TGFβ	Transforming growth factor-β
TMS	Trimethylsilyl
TNFα	Tumor necrosis factor-α
UPLC	Ultra performance liquid chromatography
VP	Vassopressin

WT1	CYP17 wild type 1 (Genbank accession nr. L40335)
WT2	CYP17 wild type 2 (Genbank accession nr. AF251388)
βFGFs	β-fibroblast growth factors

CHAPTER 1

Introduction

The sentient body is constantly coping with the onslaught from various stressors. These stressors may be of a real (physical) or perceived (psychological) nature and pose an intrinsic or extrinsic threat to homeostasis (Mormède et al., 2007). Such deviations in homeostasis activate adaptation mechanisms that counteract such stressors in their response(s). The adaptation mechanisms on the forefront of most stress-related conditions are the hypothalamic-pituitary-adrenal axis (HPA) and autonomic nervous system. However, the interpretation of the response of these mechanisms is far from straight forward. Interpretation of the stress, adaptation and emotional state of an animal on a psychophysiological and physiopathological level is based on the assessment of measurable indices of behaviour, biology, production traits and pathology (Dantzer and Mormède, 1983; Mormède et al., 2007). The measurement of cortisol (corticosterone in birds, mice and rats) for instance is a standard method for measuring stress in animals, since this is the main active hormone produced by the activation of the HPA axis (Mormède et al., 2007). Cortisol is a glucocorticoid hormone that is produced in the adrenal gland and act on gluconeogenic enzymes in the liver to transform proteins to energetic metabolites. This reflects the purpose of the adaptation mechanisms, which is to regulate and maintain energy fluxes or supply the defence mechanisms with metabolic fuels.

In the stimulation of the HPA axis, it is not only the magnitude of cortisol production that is important to counteract the stressor stimulus, but also the timeframe over which stress is incurred (Smith and Dobson, 2002; Mormède et al., 2007). This concept is best understood by Selye's (1946) definition of stress as the 'disease of adaptation'. In the acute phase the stress response counteracts stressors within its capacity at a harmless adaptive level (Ewbank, 1985). However, in the chronic phase the stress response is stretched beyond its limits to the point where it is non-adaptive and causes damage to the animal's health.

This study is concerned with the acute phase response of the HPA axis that ultimately results in the secretion of cortisol from the adrenal gland in the South African Merino. More specifically, this study investigates the synthesis of cortisol within the adrenal gland. The study set out to determine the relative contributions of two isoforms of a key steroidogenic enzyme, namely 17α-hydroxylase/17,20-lyase (CYP17), in cortisol biosynthesis. Two genetic sequences for ovine CYP17 have previously been published on Genbank (Genbank accession nrs. L40335 and AF251388) and it was shown by Storbeck et al. (2008a) that both sequences are present in the South

African Merino. The activities of these CYP17 isoforms and their relative contributions to cortisol production have not yet been compared to date. The two sequences will from hereon be referred to as *wild type 1* (*WT1*, L40335) and *wild type 2* (*WT2*, AF251388), while their expressed CYP17 isoforms will be written in non-cursive text (WT1 and WT2).

For the purpose of this study, the Biochemistry Department at Stellenbosch University collaborated with the Institute for Animal Production from the Western Cape Department of Agriculture. A project on selection responses to divergent selection for ewe multiple rearing ability was undertaken at the Institute for Animal Production since 1986, which received funding from both the South African wool industry and government. Two distinct Merino flocks were established that showed a marked divergent response in overall reproduction in the lines selected. The annual rate of genetic improvement in total weight of lambs weaned over three lambing opportunities, in the line selected for multiple rearing ability (H-line), was 1.8% of the overall phenotypic mean (Cloete et al., 2004). A corresponding decline of 1.2% of the overall mean was found in the line selected against multiple rearing ability (L-line). Genetic selection responses were symmetric, resulting in a cumulative difference of 21.2 kg, of lambs weaned over three lambing opportunities between the two lines in ewe progeny born in 1995. This line difference was supported by responses in behaviour conducive to an improved reproduction rate (Cloete and Scholtz, 1998). Moreover, slaughter data suggested a probability that L-line animals were more susceptible to stress than H-line contemporaries (Cloete et al., 2005a). These results, together with other distinct differences between the lines (Hoffman et al., 2003), suggest that differences between the populations could be detected on a genetic level and that the two different CYP17 isoforms could be involved in the differences in stress susceptibility between the two lines.

Chapter 2 highlights the importance of a functional HPA axis in sheep breeding. The necessity to include stress response characteristics in selection criteria to improve robustness is explored. An overview of the HPA axis is presented with a description of the actions of the glucocorticoids (cortisol and corticosterone). The production of cortisol, and other steroid hormones within the adrenal gland, is described in Chapter 3. An overview of the regulation of a glucocorticoid-specific acute phase response from the adrenal gland is also presented. Hereafter, the role of CYP17 in the production of cortisol, as well as its characteristics and regulation, is discussed in detail in Chapter 4.

Following the literature review in Chapters 2 to 4, are the chapters describing the experimental procedures, results and discussions of this study. In Chapter 5 an investigation of the presence of the

two *CYP17* genetic sequences in a South African Merino population is described using the *CYP17* genotyping test developed by Storbeck et al. (2008a) for the South African Angora goat. It was found that 14.4 % of the population contained only the *WT1* sequence (homozygous *WT1/WT1*) and 78.3 % of the population contained both *WT1* and *WT2* sequences (heterozygous *WT1/WT2*). However, no homozygous *WT2* sheep were identified and it was suggested that the genotyping test might not be sensitive enough, that there was a genetic anomaly or that the genotype was lethal.

A comparison of the *CYP17* sequences revealed that they encode two distinct CYP17 isoforms (WT1 and WT2, respectively) that differ by two amino acid residues. The activities of these isoforms were compared *in vitro* by expression in non-steroidogenic COS-1 cells. Site-directed mutagenesis revealed that both amino acid differences of the isoforms were collectively responsible for the small differences in the conversion assays. Kinetic parameters, K_m and V_{max}, were determined for both CYP17 isoforms, but were not significantly different. However, some significant differences were observed in time-dependent substrate conversion assays, but these differences were less pronounced in the presence of redox partner, cytochrome b_5. It was predicted that WT1 would be more advantageous for the production of cortisol. However, this hypothesis had to be investigated in a system where CYP17 is expressed in the presence of all the other enzymes involved in adrenal steroidogenesis, especially the two steroidogenic enzymes that compete with CYP17 for the substrates, namely 3β-hydroxysteroid dehydrogenase and cytochrome P450 21-hydroxylase.

The COS-1 expression system is not suitable for the expression of more than three enzymes (due to transfection limitations and bias). Therefore it was decided to use primary culture preparations from the adrenal glands of adult homozygous *WT1/WT1* and heterozygous *WT1/WT2* sheep. However, such a system would produce a complex mixture of steroids that presents a challenge for their detection and quantification. An ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) method was therefore developed for the accurate detection of the major steroids (13 in total) produced in the sheep adrenal. Chapter 6 presents the data pertaining to the development of this method and also reports on its validation. The method was used to analyse plasma steroids in the blood of sheep prior to and 60 minutes after insulin treatment, as well as primary cultures of adult adrenal cells under endogenous and adrenocorticotrophic hormone (ACTH) stimulated conditions after 48 hours. The plasma results showed a glucocorticoid-specific response to stimulation of the HPA axis by insulin induced hypoglycaemia. The results from adult adrenal cultures showed a general increase in all steroid metabolites that is likely to result from the

expressional upregulation of all steroidogenic enzymes, which is typical of a chronic response (usually after 24 hours of ACTH stimulation).

The relative contribution of each CYP17 isoform on adrenal steroidogenesis as a whole is presented in Chapter 7. Preparations of primary cultures from the adrenal glands of adult homozygous *WT1/WT1* and heterozygous *WT1/WT2* sheep were cultured over three days, establishing basal endogenous steroid levels, and then with the addition of pregnenolone, the precursor of adrenal steroid hormones. In addition, different stimulators of the HPA axis were added together with pregnenolone, namely ACTH, cyclic adenosine monophosphate (cAMP), forskolin and cholera toxin. Numerous differences were observed in the production of steroid metabolites between the CYP17 isoforms, including a higher cortisol production for homozygous *WT1/WT1* sheep compared to heterozygous *WT1/WT2* sheep. These results indicated that the small catalytic differences of the CYP17 isoforms translated to significant differences in cortisol production.

Furthermore, the production of various steroids was different in sheep that belonged to contrasting selection lines, derived from a divergent selection program. These sheep were either selected for (H-line) or against (L-line) the ability of a ewe to rear multiple offspring. The H- and L-lines were previously shown to have various differences in production and reproduction traits, including lamb output (Cloete et al., 2004), behavioural stress responses, meat quality (Cloete et al., 2005a), wool characteristics (Cloete et al., 2004), lamb survival and mothering ability (Cloete and Scholtz, 1998; Cloete et al., 2005b). All the sheep that were used in this study were obtained from this breeding program. It was interesting to observe that the most pronounced differences in cortisol production within the adult adrenal cells were due to selection line differences. This indicates that the selection program has also indirectly been successful in selection for cortisol production in the adrenal gland in response to HPA axis stimuli.

As previously mentioned, it is not necessarily the quantity of cortisol that is produced, but also the duration of cortisol output that determines the efficacy of the cortisol response. Chapter 8 explores how the observed *in vitro* differences in the amount of cortisol produced would translate to the *in vivo* level. The activity of the HPA axis as a whole was assessed by measuring the blood glucose and cortisol responses to insulin induced hypoglycaemia over 2 hours. The glucose levels in the H-line, for both homozygous *WT1/WT1* and heterozygous *WT1/WT2* sheep, returned to baseline levels after 2 hours, which indicated an adequate HPA axis response. The glucose levels of the L-line, however, did not recover after 2 hours for either *CYP17* genotype. With regard to cortisol production, the H-line and homozygous *WT1/WT1* sheep were able to produce more cortisol (at

equal duration) than the L-line and heterozygous *WT1/WT2* sheep, respectively. However, when these two groups were subdivided into four groups (according to selection line X *CYP17* genotype), the cortisol production was found to be the same in all groups (H-line homozygote *WT1*, H-line heterozygote *WT1/WT2* and L-line homozygote *WT1*), except for the L-line heterozygous *WT1/WT2* sheep that had a lower cortisol response. These results suggested that the *CYP17* genotype would have a small effect in animals with superior HPA axis activity, while it would have a large effect in animals with impaired HPA axis activity. This statement is supported by the study of Storbeck et al. (2008a), which showed that the *CYP17* genotype contributes to hypocortisolism in the South African Angora goat – a species known to have an impaired HPA axis and makes it vulnerable to cold stress.

Furthermore, the study explored the effects that the *CYP17* genotype could potentially have on stressful behaviour, feed intake and reproduction traits. For accurate statistical analyses, a large sample size with known *CYP17* genotypes were required (n > 400). A new *CYP17* genotyping test was designed and validated with the results from the previous genotyping test. More than 550 sheep were genotyped, but the homozygous *WT2* genotype remained undetected and it was suggested that this is most likely due to a genetic anomaly.

The effect of the CYP17 genotype on various measureable indices of reproductive performance, behavioural stress and feed intake was investigated. It was found that the *CYP17* genotype did not influence the parameters in which reproductive performance and feed intake was measured. However, the *CYP17* genotype had a profound influence on behavioural stress responses, where three parameters of a flock-isolation test was affected, namely the number of bleats uttered, the urinating frequency and the average distance from a human operator. Furthermore, the total cortisol response during the hypoglycaemic test was negatively correlated with the frequency that sheep defecated during the behavioural test. These results suggest that the behavioural stress test may potentially be used for indirect selection for *CYP17* genotypes and HPA axis function.

Chapter 9 presents an overview of the results from this study and reiterate the main conclusions drawn from these results. Recommendations for further research are also included in this chapter.

CHAPTER 2

The importance of the hypothalamic-pituitary-adrenal axis in stress management for the South African Merino

2.1 Merino farming in South Africa

Sheep farming accounts for one of the largest groups of commercial ruminant livestock in South Africa with sheep numbers estimated at 21.5 million sheep, while cattle and goat numbers are estimated at 13.7 and 2 million, respectively (Abstract of Agricultural Statistics, 2011). The sheep industry is important for South African agriculture, since 80 % of farmed land in South Africa is suitable only for sheep and goat farming due to relative arid climates and relatively poor soils (Livestock Development Strategy for South Africa, 2006). Furthermore, Merino and Merino-type breeds constitutes 52.4 % of the national sheep flock, while Merinos are also the most extensively studied sheep breed world-wide (Lynch et al., 1992; Schoeman et al., 2010; Abstract of Agricultural Statistics, 2011). The Merino breed had a major impact on the sheep populations of all the major sheep producing countries in the past century and Merinos contribute up to a third of all main breed types in some way (Mason, 1969; Lynch et al., 1992).

The South African income from animal products contributes up to 50.6 % of the total agricultural income and is derived from poultry (36.3 %), beef and veal (21.8 %), milk (14.6 %), small stock products (12.4 %) and eggs (10.5 %) (Abstract of Agricultural Statistics, 2011). The contribution of sheep derived products amounts to an annual gross turnover of approximately 5 billion ZAR, which is derived 72.4 % from meat and 27.6 % from wool (Abstract of Agricultural Statistics, 2011). Although South Africa exports 77% of the produced wool, it imports 28% of its mutton (Cape Wools SA, 2008). Moreover, mutton prices have shown a larger increment over the past two decades compared to meat derived from other livestock species (Abstract of Agricultural Statistics, 2011), which is indicative of a healthy local demand for the product. South Africa can therefore improve its production of mutton to meet this demand, while the production of wool would further contribute to the South African economy.

2.2 Current breeding strategies

Livestock recording and evaluation programmes, such as the National Small Stock Improvement Scheme in South Africa, have been developed to assist sheep farmers to select genetically superior

animals (Livestock Development Strategy for South Africa, 2006; Agricultural Research Council, 2009). The improved levels of production in such animals are expected to assist in the fight against rising input costs. A diagrammatic summary of this strategy is shown in Figure 1.1. The strategy proposes the genetic improvement of income per animal by increasing the total weight of lamb weaned, growth rate, as well as quantity and quality of fibre produced (Olivier, 1999). Genetic selection based on all these parameters is aimed at improving production traits (e.g. fibre diameter, meat quality) and fitness traits (e.g. lamb survival, pathogen resistance). Genetic selection is also a preferable alternative to improve fitness and production compared to some husbandry procedures, which increase the cost per animal and may be detrimental to the animals' welfare (such as mulesing) (Lynch et al., 1992).

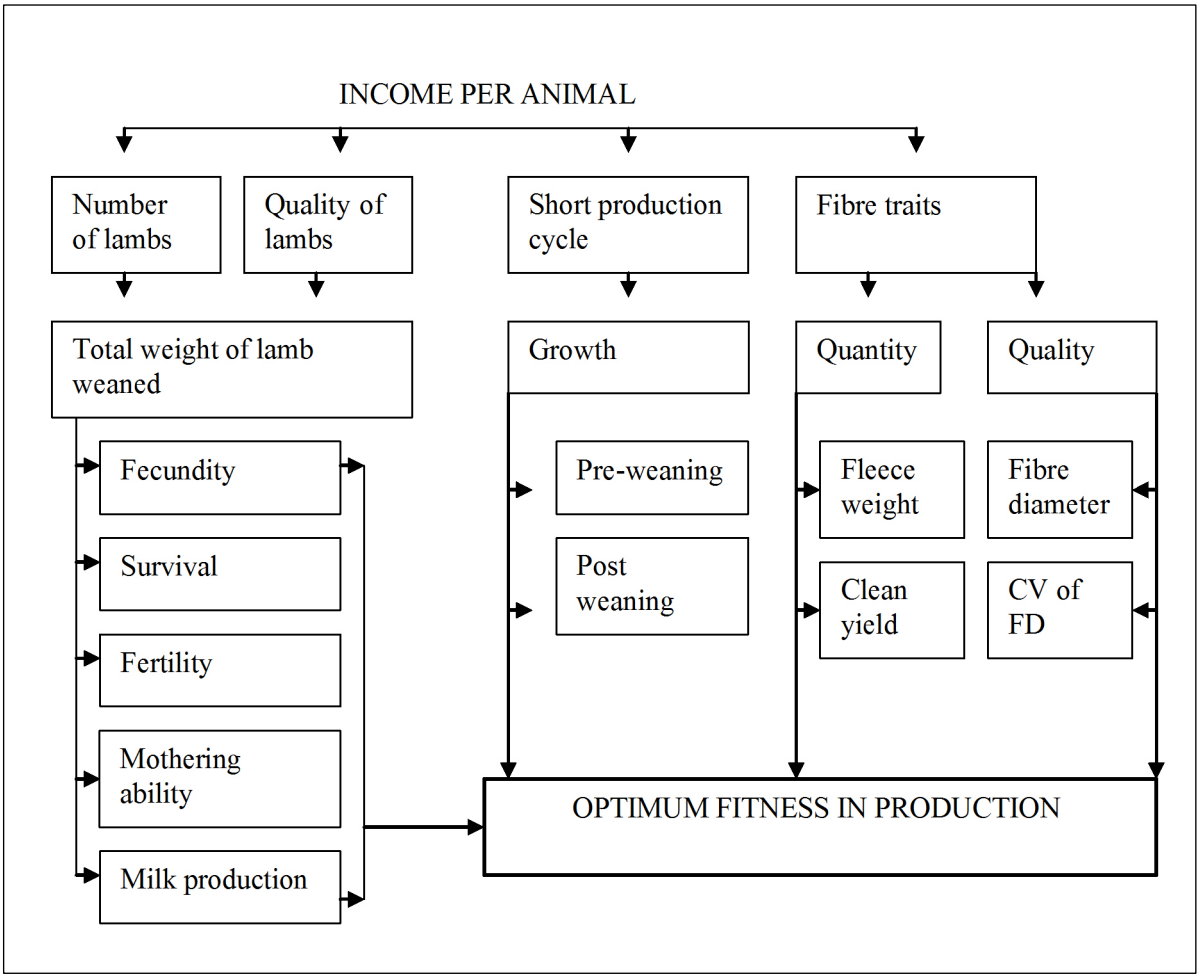

Figure 1.1. Summary of the strategy proposed by the National Small stock Improvement Scheme for the genetic improvement of cost per animal. CV: coefficient of variation; FD: fibre diameter. Adapted by SWP Cloete from Olivier (1999).

2.3 Robustness as breeding goal

It is a difficult task to improve production traits in a commercial practise by means of genetic progress if the environment in which the animals are raised does not support the full expression of their genetic potential (Mormède et al., 2011). It is thus important to include robustness-related traits in breeding objectives to such an extent that selection balances genetic change in production potential with the genetic change in environmental sensitivity (Knap and Rauw, 2009). Robustness is best described as an ability to combine a high production potential with resilience to stressors, which allows for the unproblematic expression of a high production potential in a wide variety of environments (Beilharz, 1998; Knap and Rauw, 2009). Such objectives are particularly necessary in South Africa, where animals are often raised in adverse production environments (Cloete and Olivier, 2010). These extreme environments, along with climate change and economic pressure, increase the importance of considering robustness traits in the development of sustainable breeding goals.

There are a number of examples where genetic selection based on production traits alone resulted in a reduction in robustness (Rauw et al., 1998; Star et al., 2008; Knap and Rauw, 2009; Siegel et al., 2009; Veerkamp et al., 2009). One example is the case of the South African Angora goat, where selection for lower fibre diameter resulted in hypocortisolism and a susceptibility to cold stress (Van Rensburg, 1971; Engelbrecht et al., 2000). The cause for hypoadrenocortisolism was found to be mainly due to the activities of two cytochrome P450 17α-hydroxylase/17,20-lyase (CYP17) isoforms (but three unique genotypes) in the adrenal gland (Storbeck et al., 2008a).

An efficient genetic selection program would incorporate quantitative traits for both production and robustness as selection objectives. Most quantitative traits are determined by an intricate network of interacting loci and environmental factors (Falconer and Makay, 1996). In the best of circumstances, the genetic variation of a quantitative trait is determined by a small number of major genes with moderate to large effects, together with a large number of minor genes with small effects, known as the oligogenic model (Mackay, 1996 and 2001). The identification of these loci (called quantitative trait loci or QTLs), or markers in linkage equilibrium with it, in the genome is fundamentally important for agriculture in terms of marker-assisted selection or marker-assisted management (Camp and Cox, 2002; Wu et al., 2007; DeRijk, 2009). These methods aid the speed and accuracy of estimating breeding values in genetic selection programs or to adapt management practices to better match the genotypes of livestock (e.g. feeding, pre-slaughter procedures and drug

therapy). This study focuses on the HPA axis as an indicator mechanism that adds to livestock robustness, since the HPA axis plays an integral role in adaptation to stressful situations.

2.4 Introduction to stress

The importance of the capacity of an animal to cope with stressors is perhaps better understood by defining stress and its consequences. Selye (1946) defined 'stress' as the disease of adaptation, where the mechanisms to cope with stressors become overextended and eventually breakdown. Ewbank (1985) extended this idea and define three phases that are seen as a continuum of responses to a stressor(s): stress, overstress and distress. Stress is when an animal copes with a stressor within its capacity at an adaptive and harmless level. Overstress is when the coping mechanism is extended, but still remains sufficient to counteract the stressor. Distress, however, is when a stressor stretches the coping mechanism beyond its limits to the point where the response is non-adaptive and results in damage to the animal's health, identified as the disease of adaptation.

The adaptive mechanisms for coping with stress lie within the nervous system, immune system, endocrine system and the interregulation between these systems (Mormède et al., 2007). For example an environmental factor that continually stimulates the HPA axis for numerous days will lead to an increase in glucocorticoid secretion from the adrenal cortex over an extended time period. These elevated glucocorticoid levels will eventually inhibit inflammatory processes and increase the animal's susceptibility to pathogens (Lynch, 1992; Spraker et al., 1984). This study will focus on stress in terms of stimulation of the HPA axis only.

2.5 Stress in sheep

Sheep are frequently subjected to routine handling procedures. Some differences in ease of handling between breeds have been reported and Merinos have been reported to be one of the easiest to handle (Lynch et al., 1992). Kilgour (1976) suggested that three basic behaviours of sheep should be recognized for successful sheep handling: 1) their strong flight reaction; 2) the prevailing role of vision in social organization; and 3) their flocking-follower behaviour. It is thus understandable why sheep avoid isolation from the flock, which results in unpredictable fearful behaviour and sometimes leads to injury during routine procedures (Degabrielle and Fell, 2001; Da Costa et al., 2004). Lynch et al. (1992) report that the separation of sheep from their flock, and the anxiety it causes, is likely to be the predominant source of suffering and is considered a potent stimulus of the HPA axis. Various studies have also shown that some husbandry practices, such as shearing,

crutching, drafting and transport, resulted in increased plasma cortisol levels (Grandin, 1997; Hargreaves and Hutson, 1990; Degabrielle and Fell, 2001; Da Costa et al., 2004). Cold stress and starvation are two other important factors that stimulate the HPA axis. Starvation and cold exposure are two of the four main, and often interrelated, factors responsible for most lamb deaths, along with difficult parturition and relatively low birth weight, according to the review of Alexander (1984).

Murphy et al. (1994) and Murphy (1999) have been able to correlate lamb survival with ewe temperament. These authors based their findings on Merino ewes selected for either high ('nervous') or low ('calm') reactivity to humans and flock-isolation. The mortality rate of lambs born to 'calm' ewes was half that of 'nervous' ewes. The authors proposed that the higher lamb survival could be ascribed to the display of superior maternal behaviour, in terms of grooming and bleating frequency, compared to the 'nervous' ewes (Murphy et al., 1994; Murphy, 1999). Furthermore, the degree to which maternal behaviour is displayed has been correlated with the concentrations of cortisol, progesterone and estradiol during the peripartum period (Pryce et al., 1988; Dwyer et al., 2004). However, Bickell et al. (2011) showed that the concentrations of progesterone and estradiol were similar in the two temperament lines from 4 days prior and 24 hours post parturition, which indicated that it was unlikely that these hormones contribute to the displayed maternal behaviour. The latter study also failed to support a hypothesis that 'calm' ewes and lambs coped better with the situation than their 'nervous' counterparts when they were subjected to a test involving a novel distraction during the early postnatal phase.

A similar concept of behavioural stress was investigated where a flock-isolation test was used to assess the stressful behaviour of Merino sheep that were divergently selected on the ability of a ewe to rear multiple offspring per birthing opportunity (Cloete et al., 2005a). The line selected for their ability to rear multiples (H-line) was previously shown to have a markedly higher lamb survival and improved maternal and offspring behaviour than the line selected against multiple rearing ability (L-line) (Cloete and Scholtz, 1998). Stressful behaviour were assessed during an arena test, which monitored the movement within the arena (number of lines crossed), distance from human operator (separating the sheep from its flock), number of bleats, number of urinating events and number of defecating events. The H-line allowed smaller distances between itself and a human, while the L-line defecated more frequently throughout the stress test (Cloete et al., 2005a). Furthermore the meat quality of these two lines was assessed at time of slaughter. It was found that at the same age and under the same management conditions the mean slaughter weight, dressing percentage, carcass

weight and vivid red meat colour was higher in the H-line than in the L-line. In relation to stress, the selection line difference in the pH of meat was the most important consideration. The collective observations from these studies demonstrate the complex relationship between stress, production (meat quality and ease of handling) and reproduction (lamb output, mothering ability and lamb survival). Incidentally, these results cannot be accepted as universal. A similar study involving an Australian Merino line selected for reproduction (the Fertility flock), and a random bred control line, failed to show conclusive evidence in behaviour in favour of ewes from the Fertility flock during contrived situations (Kilgour and Szantar-Coddington, 1995).

2.6 The hypothalamic-pituitary-adrenal axis

The HPA axis mediates stress responses in combination with the autonomic nervous system and behavioural adaptation (Manteuffel, 2002). An overview of the HPA axis is presented schematically in Figure 2.1. The hypothalamus receives neuronal input from various internal and external stimuli

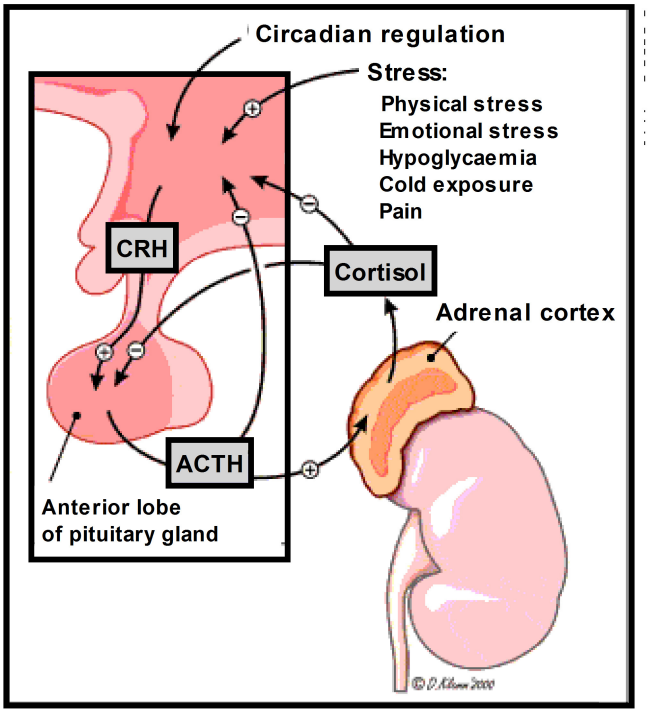

Figure 2.1. Overview of the hypothalamic-pituitary-adrenal axis. A stress stimulus is detected by the hypothalamus, which secretes corticotrophin releasing hormone (CRH) that in turn stimulates the release of adrenocorticotrophic hormone (ACTH) from the anterior pituitary into the bloodstream. The adrenal gland responds to ACTH by increasing its production and release of cortisol (glucocorticoid) into the bloodstream that counteracts the stressor. Cortisol also exerts a negative feedback effect on the hypothalamus and anterior pituitary. Reproduced from Kirk et al. (2000) (© David Klemm).

and conveys this signal to the anterior pituitary via corticotrophin releasing hormone (CRH) and vasopressin (VP). The synergistic action of CRH and VP stimulate the secretion of ACTH from the anterior pituitary gland. ACTH in turn stimulates the release of steroid hormones from the adrenal cortex (corticosteroids), of which glucocorticoids (cortisol and corticosterone) are the majority. The main active hormone in the HPA axis response is cortisol (such as sheep, cattle, pigs, mink, fox and fish) or corticosterone (such as birds, rats and mice) (Mormède et al., 2007). In fact, the measurement of cortisol has become the golden standard of evaluating stress in sheep (Mormède et al., 2011).

The assessment of HPA axis activity is a standard method of evaluating stress and welfare in animals (Mormède et al., 2007). Large differences in HPA axis activity have been found across species, breeds and individuals, which reflects the contribution of genetic factors and environmental influences. There is large variability within the system, which makes the genetic selection for superior HPA axis activity a promising tool in animal breeding (Mormède et al., 2011). Some of the main sources of variation arise from the pulsatile, diurnal and seasonal rhythms in secretion of corticosteroids, which is also influenced by physiological state, age, feed intake and environmental factors such as temperature and humidity (Mormède et al., 2007).

2.6.1 The hypothalamus

The hypothalamus controls the release of ACTH from the anterior pituitary gland by a neuronal structure known as the paraventricular nucleus (PVN) (Manteuffel, 2002). The small celled subdivisions of the PVN consist of specialized neurons that synthesize CRH and VP, which is released in the capillary bed of the median eminence where it reaches the pituitary via the hypothalamic-pituitary portal vessels (Figure 2.2) (Whitnall, 1993). The axons of the large celled part of the PVN extend into the posterior pituitary to release VP and oxytocin (Aguilera, 1998; Manteuffel, 2002). It has been proposed that VP maintains HPA axis activity during prolonged stimulation, whereas CRH seems to be mainly active during the acute stress response (Lightman, 1994; Bonaz and Rivest, 1998; Manteuffel, 2002). The PVN receives numerous inputs from the brain stem (neural inputs from the periphery), hypothalamic nuclei (metabolic and nyctohemeral inputs), limbic system (in relation to emotional state) and subfornical organ system (monitors blood plasma composition) (Manteuffel, 2002). The complexity of the various afferent and efferent pathways connected to the PVN explains why the HPA axis is sensitive to a wide range of external and internal stimuli to assess the homeostatic state of the sentient organism. This enables the

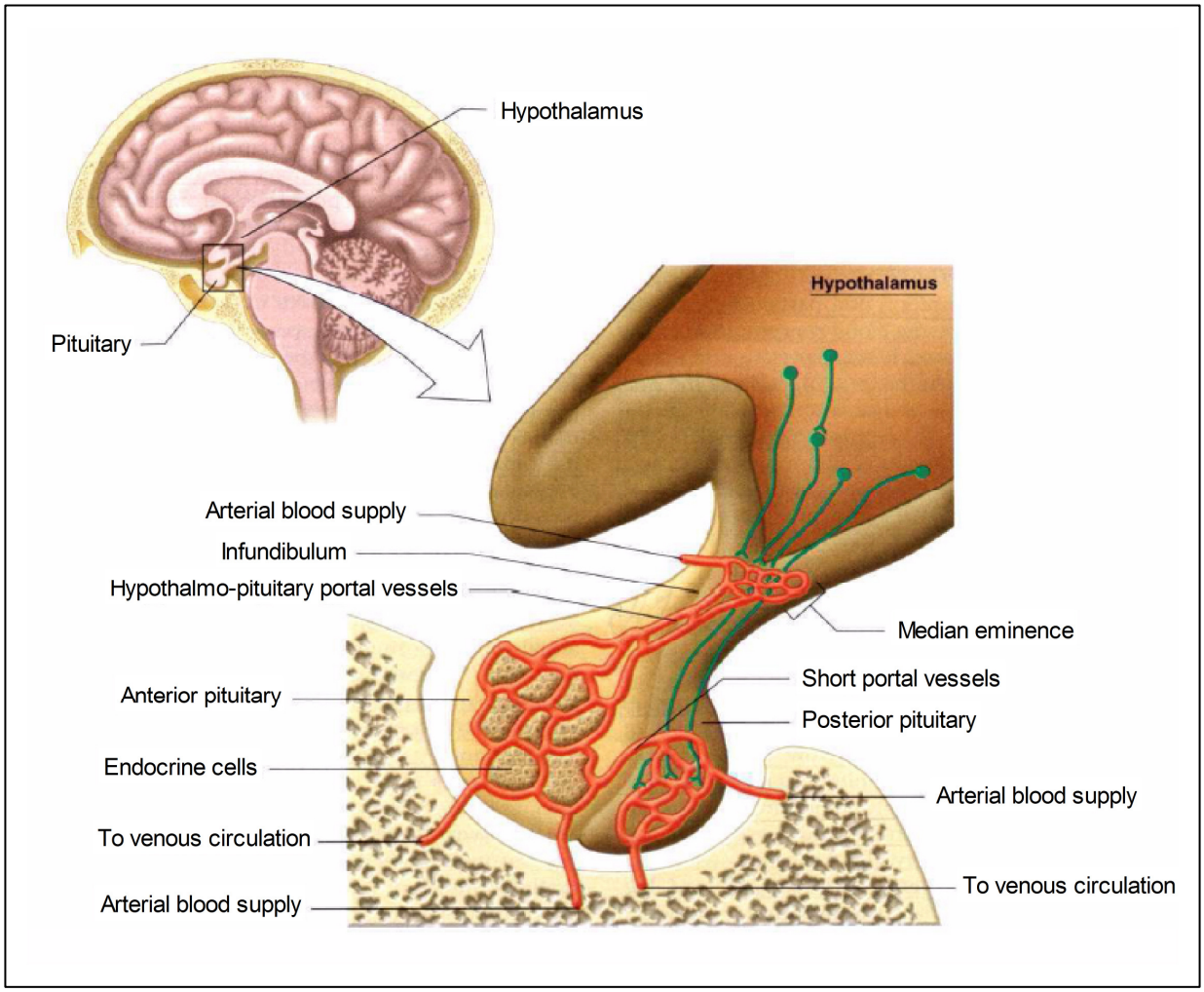

Figure 2.2. Anatomy of the pituitary gland. The top left illustration depicts the relation of the pituitary gland to the rest of the brain and the schematic enlargement shows its vascular and neuronal connections to the hypothalamus. Reproduced from Vander et al. (2004).

hypothalamus to communicate a state of stress (or normality) to the anterior and posterior pituitary as to which hormones to release for the maintenance of homeostasis.

Da Costa et al. (2004) studied emotional stress and its effects on the HPA axis of sheep. These researchers subjected sheep to flock-isolation and found that showing facial pictures of familiar sheep, compared to pictures of goats and inverted triangles, reduced the stress responses of these

sheep, in terms of behavioural (activity and protest vocalizations), autonomic (heart rate) and endocrine (cortisol and adrenaline) indices of stress. The mRNA expression of activity-dependent genes (c-*fos* and *zif*/268) was reduced in the PVN and the brain regions associated with fear (central and lateral amygdale), while their expression was increased in the brain regions dedicated to emotional control (orbitofrontal and cingluate cortex) and for processing faces (temporal and medial frontal cortices and basolateral amygdala). This study indicates the role of the PVN to translate

signals of emotional stress via the HPA axis to the adrenal gland for the release of cortisol. Furthermore, the emotional reactivity (temperament) of sheep has been successfully used as selection criterion in breeding programs to improve reproductive biology (Blache and Bickell, 2010). Selection for 'calm' ewes increased lamb survival and maternal behaviour (Murphy et al., 1994). The activity of the PVN has been shown to affect mother-young relationships of sheep, and the expression of c-*fos* in the PVN has been used as a marker for neuronal activity that correlated with the onset of maternal behaviour (Keller et al., 2004; Novak et al., 2011).

2.6.2 The pituitary

The hypothalamus is connected to the posterior pituitary by axons that extend down from the paraventricular and supraotic nuclei through the infundibulum (Figure 2.2) (Vander et al., 2004). These axons secrete VP and oxytocin, as previously mentioned, via exocytosis into the posterior pituitary capillaries, which drain directly into the main blood circulation (Aguilera, 1998; Manteuffel, 2002). VP facilitates the re-uptake of water in the kidney by increasing the permeability of the collecting ducts. Oxytocin increase contraction of smooth muscles in the mammary glands and uterus (Vander et al., 2004).

The hypothalamus is connected to the anterior pituitary by a special vascular system, the hypothalamic-pituitary portal system, which insures that blood flows directly from the hypothalamus to the anterior pituitary (Figure 2.2) (Vander et al., 2004). The first capillary bed at the base of the hypothalamus, the median eminence, converges to form the hypothalamic-pituitary portal vessels that pass through the infundibulum and lead to a second capillary bed in the anterior pituitary, the anterior pituitary capillaries (Figure 2.2). As mentioned previously, the CRH and VP are secreted by the PVN into the median eminence and reach the anterior pituitary via the hypothalamic-pituitary portal system.

In the anterior pituitary, CRH binds to corticotrophs, specialized secretory cells, to release ACTH (Vander et al., 2004). In addition to corticotrophs, the anterior pituitary also consists of four other types of secretory cells that are responsible for the production and secretion of different trophic hormones. Each secretory cell type responds to a specific hypophysiotrophic hormone secreted by various neurons in the hypothalamus into the median eminence. The secretory cells in the anterior pituitary consist of 20 % corticotrophs (secretes ACTH in response to CRH); 50 % somatotrophs (secretes growth hormone in response to growth hormone releasing hormone and growth hormone-inhibiting hormone); 20 % mammotrophs (secretes prolactin and regulated by prolactin-inhibiting

hormone); 5 % thyrotrophs (secretes thyroid stimulating hormone in response to thyrotrophin releasing hormone); and 5 % gonadotrophs (secrete follicle stimulating hormone and luteinizing hormone in response to gonadotropin releasing hormone). Various studies have shown that CRH inhibits the stimulation of gonadotrophs to release luteinizing hormone (for review see Rivier and Rivest, 1991). It is therefore not surprising that stress influences the reproductive endocrine axis in farm animals (Von Borell et al., 2007).

The binding of CRH to the CRH receptor of the corticotrophs activates adenylate cyclase and the accumulation of cyclic adenosine monophospate (cAMP) subsequently activates protein kinase A (Dunn and Berridge, 1990). This stimulation of adenylate cyclase by CRH is regulated by divalent ions and guanidine nucleotides, a common phenomenon observed for receptors coupled to adenylate cyclase (Chen et al., 1986). The synergistic action of CRH and VP is important in the physiological control of ACTH secretion. VP requires the presence of CRH to exert its full effect. VP has a weak ACTH-releasing activity *in vitro* for most species, and instead potentiates both CRH-stimulated ACTH release as well as CRH-induced accumulation of cAMP. VP act via a V1-like receptor that results in the stimulation of phosphatidylinositol hydrolysis and intracellular calcium ion fluxes that subsequently activates protein kinase C. Studies have shown that the relative potencies of CRH and VP are reversed in sheep, where CRH has a weak ACTH-releasing activity, but can potentiate the effects of VP on ACTH release (Familari et al., 1989; Owens and Nemeroff, 1992; Liu et al., 1994). Furthermore, AVP and ACTH secretion is stimulated by interleukin-6. The origins and significance (in terms of adreno-cortical regulation) of interleukin-6 (Mastorakos et al., 1994; Charmandari et al., 2005) will be discussed in another section.

ACTH is a polypeptide that is produced by the cleavage of the larger polypeptide proopiomelanocortin (POMC) (Stevens et al., 2010). Cleavage of POMC also yields endorphins (endogenous opioids) and lipotrophins (implicated in lipid metabolism) that can be secreted together with ACTH from corticotrophs in small quantities. ACTH enters the main blood circulation and reaches the adrenal gland where it primarily stimulates the secretion of glucocorticoids from the adrenal cortex, but also stimulates the secretion of mineralocorticoids and androgen precursors in some species (Vander et al., 2004). As stated previously, the main glucocorticoid product resulting from the stimulation of the HPA axis is cortisol in sheep, cattle, mink, fox and fish, and corticosterone in birds, mice and rats (Mormède et al., 2007).

2.6.3 The adrenal gland

In the adrenal cortex ACTH binds to the ACTH receptor on the outside of the cell membrane. This transmembrane ACTH receptor is associated on the inside of the cell membrane with the α subunit of a signal transducing G-protein, G_s (Vander et al., 2004). The G_s protein is a guanine triphosphate (GTP) switch protein and consists of three subunits, known as α, β and γ. When there is no ligand bound to the receptor, guanine diphosphate (GDP) is bound to the α subunit that is associated with the β and γ subunits. Binding of ACTH to the receptor induces a conformational change that cause the GDP in the α subunit to be replaced by GTP. The α subunit subsequently dissociates from the β and γ subunits to associate with adenylate cyclase, which is also bound to the inner cell membrane. This association increases the affinity of adenylate cyclase for adenosine triphosphate (ATP) to produce cAMP, a second messenger in various metabolic pathways. The cytosolic cAMP binds to and activates cAMP dependent protein kinases. The activated protein kinases can alter the catalytic activity of numerous enzymes by means of phosphorylation, including ribosomal phosphorylation, at specific serine and threonine residues. These responses lead to increased steroidogenesis and secretion of corticosteroids (of which the majority is glucocorticoids) in both acute (within minutes) and chronic (after a few hours) stimulation with ACTH (Miller and Auchus, 2011). The enzymes involved in adrenal steroidogenesis and their regulation will be discussed in another section.

The ACTH signal terminates when the levels of cytosolic cAMP decrease with the eventual conversion to AMP by phosphodiesterase (Vander et al., 2004). Furthermore, the hormonal response is limited by the slow GTPase activity of G_s that results in the dissociation of the α subunit from adenylate cyclase. The displacement of GDP with GTP in the α subunit also reduce the affinity of the receptor for the ligand, which may subsequently dissociate (Lodish et al., 2000).

2.7 Mechanism of action of glucocorticoids

The effectiveness of the HPA axis to counter stress will influence energy metabolism and food intake, immune responses, fertility and sexual libido, behaviour as well as the ability for learning in complex ways (Manteuffel, 2002). The final effectors of the HPA axis stress reponse are glucocorticoids. The mechanism of action and the main effects of glucocorticoids during stress will be discussed in the following paragraphs.

Glucocorticoids are lipophilic and can transverse the cell membrane. However, 90 % of glucocorticoids are transported in the blood, where they are bound to corticosteroid binding

globulin (CBG) (Smith and Hammond, 1992; Mihrshahi et al., 2006). The remaining 10 % of glucocorticoids are either free or bound to albumin. Free glucocorticoids readily diffuse across cell membranes and exert their effects via intracellular receptors known as glucocorticoid receptors (GR) (Duma et al., 2006). GR is a cytosolic protein that is expressed in almost all tissues types (Hantzis et al., 2002; Oakley et al., 1997). The GR is maintained in the cytoplasm as an inactive multi-protein complex, where it is bound to heat shock protein 90. Binding of the ligand to GR induces a conformational change that results in the dissociation of the multi-protein complex, followed by the translocation of GR into the nucleus. The GR is able to bind to DNA sequences, known as glucocorticoid response elements (GREs), where it can either transactivate or transrepress the transcription of responsive genes (Beato et al., 1996; Olefsky, 2001). There are different models for the molecular mechanisms by which the GR, as homodimers or GR monomer via protein-protein-interaction with other transcription factors, interact with different types of GREs (Newton, 2000), but is beyond the scope of this discussion.

This mechanism of action of the glucocorticoids allows for the regulation of the catabolic responses to stress, as well as non-stress related modulation of carbohydrate, protein and lipid metabolism. The effects of glucocorticoids on carbohydrate metabolism mostly involve the stimulation of gluconeogenesis and glycogen synthesis in the liver, while simultaneously increasing the substrate availability to these pathways by stimulation of lipolysis and the release of glycogenic amino acids from peripheral tissues. Glucocorticoids stimulate gluconeogenesis in the liver by activating key enzymes, such as glucose-6-phosphatase, phosphoenolpyruvate, tyrosine aminotransferase and gamma-glutamyltransferase (Barouki et al., 1982; Schmid et al., 1987; Orth et al., 1992; DuBios et al., 1995; Park et al., 2007). The availability of substrates for gluconeogenesis is increased by various mechanisms after exposure to increased glucocorticoids. Glucose uptake and utilization by peripheral tissues is limited by the action of glucocorticoids on glucose transport into the cells (Orth et al., 1992). The release of glycogenic amino acids from peripheral tissues is stimulated by glucocorticoids (Newton, 2000). The sensitivity of tissues to glucagons is increased by the permissive effect of glucocorticoids. The sensitivity to catecholamines in lipolysis (adipose tissue) and lactate production (muscle) is also enhanced by glucocorticoids. Lipolysis is therefore acutely activated in adipose tissue by glucocorticoids. The free fatty acids from the triacylglycerols provide the energy for the production of glucose from glycerol (Newton, 2000; Vander et al., 2004). Furthermore, glycogen synthesis in the liver is stimulated by the activation of glycogen synthase and the inactivation of glycogen phosphorylase by the action of glucocorticoids (Stalmans and Laloux, 1979).

Glucocorticoids also have a suppressive impact on immune function. Glucocorticoids are transported in the blood bound to CBG, as previously mentioned. CBG is a member of the serine protease inhibitor superfamily. CBG is cleaved by serine protease elastase, which accumulates at sites of inflammation, and thereby promotes the release of glucocorticoids (Pemberton et al., 1988; Rescher et al., 2006). Glucocorticoids are thus released at such sites of inflammation where they can exert anti-inflammatory effects. The innate immune response is altered by the action of glucocorticoids when it prevents the migration of leukocytes from blood circulation into extravascular fluids, decrease the number of circulating eosinophils and basophils, while increasing the blood counts of neutrophils, red blood cells and platelets (Kaliner, 1985; Reid and Perry, 1991; Newton, 2000; Kita et al., 2000). Glucocorticoids down-regulate the synthesis and secretion of pro-inflammatory cytokines, such as interleukin-6 and interleukin-1β (Angeli et al., 1999).The cytokine driven upregulation of some acute phase proteins are also enhanced by the action of glucocorticoids (Uhlar and Whitehead, 1999). The acquired immunity response is suppressed by the action of glucocorticoids where the number of circulating lymphocytes is decreased. Glucocorticoids also inhibit the production of anti-bodies and the activity of helper T-cells and cytotoxic T-cells.

Glucocorticoids have additional effects apart from energy metabolism and immunity. These include an increase in alertness and cognition, alteration in cardiovascular tone, increase in blood pressure, increase in respiratory rate and increase in bone resorption (Charmandari, 2005; Vander et al., 2004). Glucocorticoids inhibit the production and secretion of growth hormone and gonadotropin (Manteuffel, 2002; Chrousos et al., 2000), which subsequently inhibits growth and reproduction. An example is the disruption of preovulatory events that results in the impairment of follicular development (MacFarlane et al., 2000; Breen, 2005). Furthermore, the deposition of glycogen stores in the foetus closer to term is essential for neonatal survival (Whittle et al., 2001). The glycogen stores serve as energy source to sustain metabolism until the establishment of a suckling. In addition glucocorticoids are in part responsible for the onset of parturition (via a glucocorticoid-prostaglandin feed-forward loop), where increased glucocorticoids are either from maternal origin or result from the maturing foetal adrenal gland (Challis and Brooks, 1989; Whittle et al., 2001).

2.8 Regulation of HPA

One of the most pronounced features of the HPA axis is its nyctohemeral cycle, characterized by a diurnal (pulsatile) and circadian (24 hour rhythm) output (Weitzman et al., 1971). This nyctohemeral cycle is controlled by neuronal pacemakers in the PVN of the hypothalamus. This neuronal activity under resting conditions is not markedly altered by glucocorticoids. However, the

effects of glucocorticoids become apparent once the neurons are activated by neurotransmitter input (signals of stress) (Manteuffel, 2002).

Glucocorticoids exert a negative feedback on the HPA axis by acting on the pituitary, hypothalamus and higher levels in the central nervous system. This feedback action of glucocorticoids ensures the return of the HPA axis activity to basal levels after stimulation. The hippocampus (part of limbic system) and PVN are the two brain centres with the highest density of glucocorticoid and mineralocorticoid receptors and are considered to be the main regulators of glucocorticoid feedback in the brain (Meyer et al., 1998; Matthews, 1998). Only free corticoids are able to cross the blood-brain barrier and their concentrations thus determine the strength of the feedback, which ultimately inhibits CRH production and release (Manteuffel, 2002). Glucocorticoids also inhibit the production and release of ACTH from corticotrophs in the anterior pituitary. In addition, chronically elevated glucocorticoid levels can down-regulate the intracellular concentrations of their receptors.

2.9 Conclusion

The HPA axis is an important and complex stress-responsive neuroendocrine system. Large individual variations in the HPA axis activity have been described that have important physiopathological consequences (DeRijk, 2009). Glucocorticoids (cortisol and corticosterone) released by the adrenal gland exerts a wide range of effects, including effects on metabolism, inflammatory processes and the immune system. In terms of animal production, chronic exposure to cortisol (high basal cortisol levels) has negative effects on growth rate and feed efficiency, while it increases the fat/lean ratio of carcasses (Mormède et al., 2011). However, cortisol can also have beneficial effects. High cortisol in response to stressors can have positive effects on traits related to robustness and adaptation. For instance, animals that were able to mount a higher HPA axis response showed increased tolerance to heat stress (Nazifi et al., 2003; Michel et al., 2007) or an increased resistance to bacteria and parasites (Gross, 1976; Knap, 2009). It is suggested that the HPA axis activity should be included in selection criteria to improve robustness together with selection for production traits. Intense selection for fibre production and a reduced fibre diameter in the South African Angora, for instance, has resulted in hypocortisolism and increased susceptibility to cold stress (Van Rensburg, 1971; Engelbrecht et al., 2000; Storbeck et al., 2008a). Intense selection for lean tissue growth in French Large White pigs also resulted in reduced cortisol production that was associated with decreased piglet survival (Foury et al., 2007; Foury et al., 2009). Several sources of genetic polymorphism and candidate genes have been described in the HPA axis (DeRijk, 2009; Mormède et al., 2011). Individual variation in the production of cortisol

by the adrenal gland, bioavailability of hormones as well as receptor and post-receptor mechanisms may be targeted during selection. The integration of these sources of genetic variability allows for the development of a model for marker-assisted selection to improve animal robustness without the negative side effects on production (Mormède et al., 2011). Alternatively, various stress tests, such as the arena or isolation test, may be used to identify individuals with superior HPA axis for breeding purposes.

Some agricultural studies have attempted to identify phenotypic traits (such as litter size) that are associated with the glucocorticoid release from the adrenal gland (SanCristobal-Gaudy et al., 2001; Foury et al., 2007), but these traits are different among species and breeds. Some of these differences may be attributed to the inter- and intra-species differences in glucocorticoid production within the adrenal gland. As mentioned earlier, the main glucocorticoid produced in the adrenal gland is cortisol for most mammalian species, while corticosterone is the main glucocorticoid in birds and laboratory rodents. The pathway involved in the production of these glucocorticoids, namely adrenal steroidogenesis, will be discussed in the next chapter, with specific reference to the production of cortisol in the sheep adrenal gland.

CHAPTER 3

Adrenal Steroidogenesis

3.1 Introduction

In mammals, all steroid hormones are derived from cholesterol as a common precursor. The enzymes that facilitate the synthesis of steroid hormones are expressed in specialized cells of the adrenal gland, gonads and placenta during pregnancy. These cells are unable to store steroids and the regulation of steroid hormone availability therefore depends heavily on the activity of these steroidogenic enzymes and the availability of their substrates. The steroidogenic enzymes expressed in the adrenal cortex allows for the production of three classes of steroid hormones, namely glucocorticoids (cortisol and corticosterone), mineralocorticoids (aldosterone) and androgens (dehydroepiandrosterone and androstenedione). The regulation of glucocorticoid hormone production by the HPA axis and its physiological effects to counter stress were discussed in the previous chapter. The production of the mineralocorticoid, aldosterone, is mainly regulated by the angiotensin II pathway to maintain electrolyte concentrations in the extracellular fluids. Aldosterone stimulates the reabsorption of sodium from urine, saliva, gastric juices and sweat (Cho et al., 1998; Kim et al., 1998; Palmer, 2001). The adrenal androgens, dehydroepiandrosterone (DHEA) and androstenedione, serve as precursors of other reproductive steroid hormones, such as testosterone and estradiol, and are involved in sexual differentiation and protein anabolism (Miller and Auchus, 2011).

Furthermore, the adrenomedullary cells produce epinephrine and norepinephrine under the control of the sympathetic nervous system. The adrenal gland is central to various essential processes, namely electrolyte homeostasis, reproduction and stress responses mediated by both the HPA axis (involved in carbohydrate metabolism, lipid metabolism and immune responses) and the sympathetic nervous system (fight-or-flight responses).

3.2 Adrenal gland morphology

3.2.1 Anatomy and histology

Adrenal glands are present for most mammals, where one adrenal gland is located at the cranial end of each kidney (Vander et al., 2004). The adrenal gland consists of medullary cells that are

enveloped by cortical cells and the whole gland is encapsulated by a thin layer of connective tissue, as seen in Figure 3.1A. The primarily steroid-producing cortical cells and catecholamine-producing medullary cells (chromaffin cells) have two distinct embryological origins. The adrenal cortical cells originate from mesodermally derived foetal adrenal cells of the adrenal primordium, which forms from the condensation of celomic epithelium at the cranial end of the kidney during embryogenesis (Ehrhart-Bornstein et al., 1998). The cortical cells generally have a prolific system of smooth endoplasmic reticulum and numerous mitochondria of variable shape with tubulovesicular cristae. In Figure 3.2 a micrograph of a typical adrenocortical cell depicts the intracellular organization of the organelles.

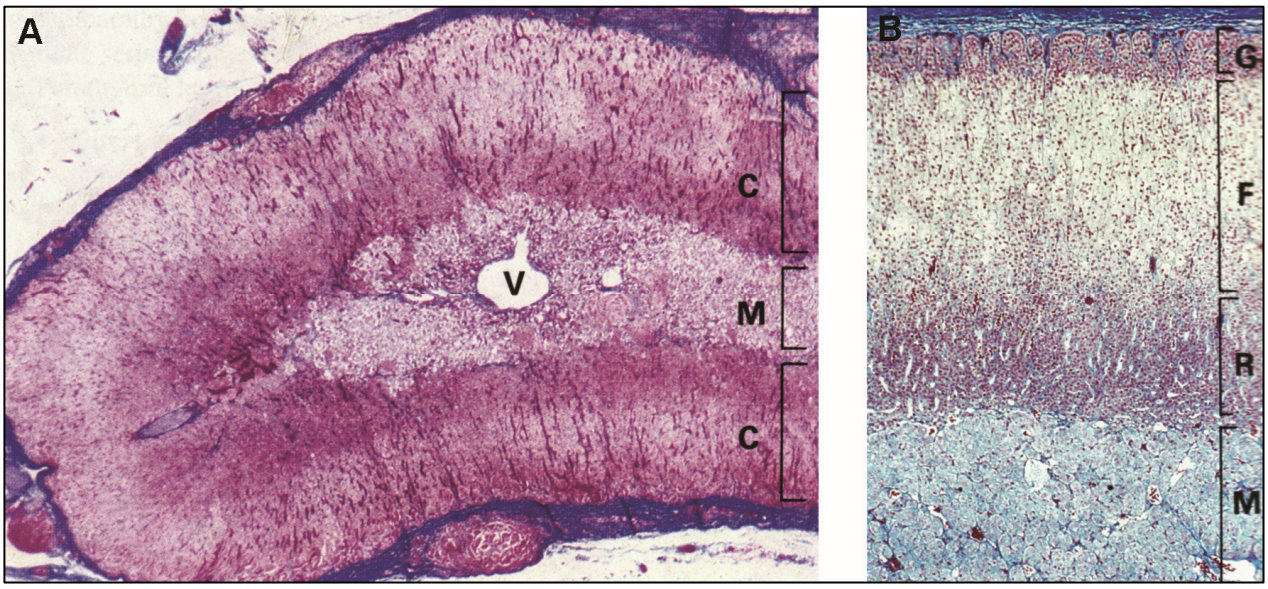

Figure 3.1. Cross-section of a human adrenal gland. **A:** The division between the medulla (M) and cortex (C) can be seen clearly in this case, as well as the surrounding capsule and central medullary vein (V). **B:** At higher magnification, the distinction between the three histological zones is observed, namely the zona glomerulosa (G), zona fasciculata (F), and the zona reticularis (R). More often the borders of the zones are less regular and more difficult to recognize than in this cross-section.

The adrenal cortex of most mammals consists of three morphologically different zones, as seen in Figure 3.1B. The different steroidogenic enzymes that are expressed in the cells of each zone will ultimately determine which steroid metabolites will be released by these zones. The three zones of the adrenal cortex, from the outer to inner zones, are the zona glomerulosa, zona fasciculata and zona reticularis (Ehrhart-Bornstein et al., 1998; Young et al., 2006). The zona glomerulosa is the outermost region of the adrenal cortex and makes up 15 % of the cortical cells (Ganong, 1995). This region consists of parenchymatous cells that are arranged in irregular small clusters that are separated by delicate trabeculae containing capillaries. Aldosterone is synthesised and secreted in this region (Hardy, 1981; Young et al., 2006). The glomerulosa tissue is capable of producing new

cortical cells and able to regenerate the other two zones if they are removed (Teebken and Scheumann, 2000). The second region, the zona fasciculata, contains larger cells that are polyhedral and arranged in radial cords (Hardy, 1981; Young et al., 2006). These cells make up 50 % of cortical cells and are usually packed with lipid droplets (Ganong, 1995). The steroid metabolites produced in this region are mostly the glucocorticoids, cortisol and corticosterone, with trace amounts of DHEA (Ehrhart-Bornstein et al., 1998). The cells of the third region, the zona reticularis, are loosely arranged in a network of branching cords and clusters that are separated by numerous capillaries (Young et al., 2006). These cells comprise 7 % of cortical cells and mostly produce the androgens, DHEA and androstenedione, with trace amounts of glucocorticoids (Ganong, 1995; Ehrhart-Bornstein et al., 1998). This zone only starts to develop slowly over the years after birth and in humans it becomes active in producing androgens at the age of 6 to 8 years in the event called “adrenarche” (Miller, 2009).

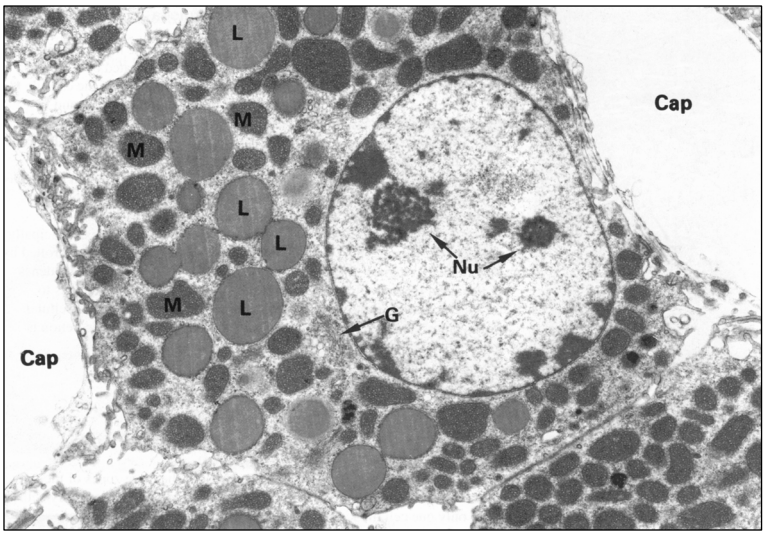

Figure 3.2. Micrograph of a steroid secreting adrenocortical cell. The steroid cell is intimately associated with the capillary (Cap) endothelium via microvillar channels. Numerous lipid droplets (L), which contain cholesterol esters, and mitochondria with variable shape are located in the abundant cytoplasm. A Golgi apparatus (G) can be seen adjacent to the nucleus and the cell is characterised by one or more prominent nucleoli (Nu) in the nucleus.

The adrenal gland generally consists of 72 % cortical cells and 28 % medullary cells, however, their distribution is not as clear-cut as a simple medulla enveloped by cortical cells (Ganong, 1995; Ehrhart-Bornstein et al., 1998). The medullary cells may radiate into the cortex or form islets, and the same can be observed with cortical cells within the medulla. This distribution varies across species and allows for extensive contact zones for paracrine interaction (Bornstein et al., 1991; Ehrhart-Bornstein et al., 1998).

The adrenomedullary chromaffin cells originate from the neural crest precursor cells that migrate into the embryonic adrenal gland to differentiate under the control of adrenocortical cells (Ehrhart-Bornstein et al., 1998). Chromaffin cells are generally characterized by their dense-cored catecholamine-containing vesicles. The main secretory products of chromaffin cells are the catecholamines, epinephrine and norepinephrine under sympathetic nervous control. However, various neurotransmitters, neuropeptides and proteins can also be contained in the chromaffin vesicles and released together with the catecholamines (Winkler et al., 1986).

3.2.2 Blood supply to the adrenal gland

The adrenal gland receives an abundant blood supply relative to its size (Young et al., 2006). It receives blood from the superior, middle and inferior suprarenal arteries that gathers in a plexus around the adrenal gland, just below the capsule. From this subcapsular arteriolar plexus, long and short cortical arteries supply blood to the medulla and cortex, respectively. The long cortical arteries form a network of capillaries that surrounds the medullary cells, before draining into the central medullary vein. The short cortical arteries supply blood to the adrenal cortex in an extensive network of thin-walled capillary sinusoids that extend through the zona fasciculata. These sinusoids end in a plexus in the zona reticularis, before collecting in venules that drain into the central medullary vein. The blood flow from these venules is regulated by smooth muscle contractions of the central medullary vein and subsequently serves as a regulatory mechanism of cortical blood flow (Ehrhart-Bornstein et al., 1998; Young et al., 2006).

3.3 Steroid hormone biosynthesis

3.3.1 Source of cholesterol

Cholesterol is the common precursor to active steroid hormones. All steroid hormone metabolites thus share the 4-ring cyclopentanophenanthrene structure, which can be observed in the structure of cholesterol in Figure 3.3. The supply, transport and storage of cholesterol is summarised diagrammatically in Figure 3.4. The majority of cholesterol supply to steroidogenesis originates from low-density lipoproteins (LDLs) in the plasma, derived from dietary cholesterol (Gwynne and Strauss, 1982), though this varies by species. Access to these lipoproteins is uncomplicated by the high degree to which adrenal tissue is vascularised (Jefcoate, 2002). Adrenal cells acquire circulating LDLs by LDL-receptor mediated endocytosis (Brown et al., 1979). These cholesterol

esters are subsequently hydrolysed within the endosome by lyposomal acid lipase to release cholesterol (Liu et al., 2000).

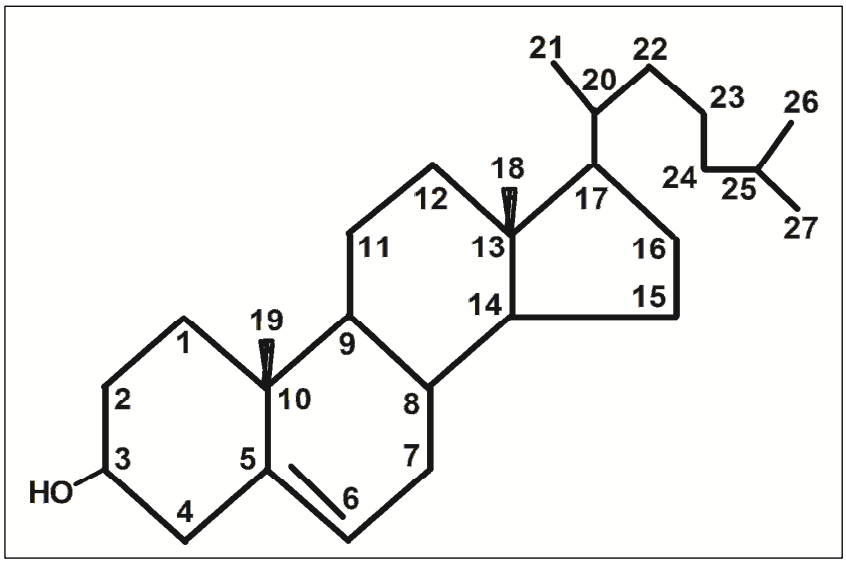

Figure 3.3. The structure of cholesterol that illustrates the cycloperhydropentanophenanthrene structure common to all steroid hormones. The carbon atoms (C) are numbered. Substituents and hydrogens are labelled according to their position behind (α) or in front (β) of the ring planar. Steroids are classified as "Δ^5" if there is a double bond between C-5 and C-6, while steroids are classified as "Δ^4" if there is a double bond between C-4 and C-5 (like most biologically active steroids). Adapted from Payne and Hales (2004).

In rodents the majority of cholesterol supply is obtained from high-density lipoproteins (HDLs) via scavenger receptor B1 (SRB1) in a two-step process (von Eckardstein et al., 2001). The HDLs first binds to the extracellular domain of SRB1, after which it is transferred to the plasma membrane. This action of SRB1 therefore alters the lipid composition of the cell membrane and increases the membrane fluidity (Connelly and Williams, 2004). This process takes place in specialized compartments of the plasma membrane, namely microvillar channels, of which the formation is regulated by ACTH and requires the expression of SRB1 (Connelly, 2009). The HDL-cholesterol esters are metabolised extrasomally (Sparrow and Pittman, 1990) by hormone-sensitive lipase (HSL) without the uptake and degradation of the entire HDL particle (von Eckardstein et al., 2001; Connelly and Williams, 2004; Kraemer et al., 2007). The cholesterol esters can be directed towards lipid droplets for storage, or can be converted to free cholesterol for utilization in steroidogenesis by HSL (Connelly, 2009; Miller and Auchus, 2011).

Furthermore the adrenal cells are capable of synthesising cholesterol *de novo* from acetate in the endoplasmic reticulum (Mason and Rainey, 1987). Cellular cholesterol, irrespective of its origin, can be esterified with fatty acids in the endoplasmic reticulum, where cholesterol esters accumulate

and bud off as lipid droplets. This esterification of cholesterol is catalysed by acyl-coenzyme A: cholesterol acyltransferase (ACAT) (Miller and Auchus, 2011). Cholesterol esters from lipid droplets are accessed and hydrolysed by cholesterol ester hydrolase and neutral cholesterol ester hydrolase (HSL), but the relative contributions of these two enzymes are not known (Kraemer, 2007).

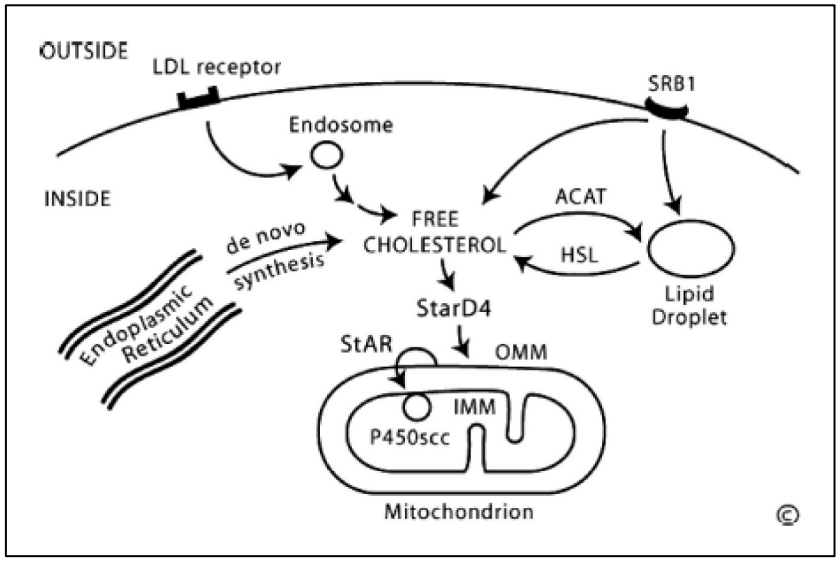

Figure 3.4. Schematic summary of the cholesterol economy in adrenocortical cells. LDL: low-density lipoprotein; SRB1: scavenger receptor B1; ACAT: acyl-coenzyme A: cholesterol acyltransferase; HSL: hormone-sensitive lipase; OMM: outer mitochondrial membrane; IMM: inner mitochondrial membrane; StAR: steroid acute regulatory protein; StarD4: StAR-related lipid transfer domain 4; P450scc: cytochrome P450 side chain cleavage. Reproduced from Miller and Auchus (2011) (© WL Miller).

The intracellular fate of cholesterol is largely regulated by sterol response element binding proteins (SREBPs) (Horton et al., 2002). These proteins belong to a group of transcription factors that regulate genes involved in the biosynthesis of cholesterol and fatty acids. The rate-limiting enzyme in cholesterol synthesis, known as 3-hydroxy-3-methylglutaryl co-enzyme A reductase, is activated by ACTH, while it is suppressed by adequate LDL concentrations. Cellular cholesterol is increased by the action of ACTH (within 3 minutes after ACTH treatment), where it also stimulates HSL, LDL uptake, transcription of LDL and SRB1 receptors, while it inhibits ACAT (Miller and Auchus, 2011).

Cellular cholesterol is virtually insoluble in aqueous solutions and is therefore transported through the cytoplasm by binding to proteins. Once cholesterol reaches the outer mitochondrial membrane, it is transported across to the inner mitochondrial membrane by the steroid acute regulatory protein (StAR). Ovine StAR has been cloned and identified (Juengel et al., 1995; Hogg et al., 2011). Once

cholesterol reaches the inner mitochondrial membrane, its conversion to pregnenolone is facilitated by cytochrome P450 cholesterol side-chain cleavage (CYP11A1). This is the first rate-limiting step in steroidogenesis and its regulation by multiple mechanisms makes it a finely tuned quantitative regulating step for steroidogenesis in terms of supplying pregnenolone to steroidogenesis. The type of steroid to be produced (qualitative regulation) is determined by the mechanism of the remaining steroidogenic enzymes and their cofactors (Miller and Auchus, 2011).

3.3.2 Overview of adrenal steroidogenesis pathway

Reactions that facilitate steroidogenesis are not confined to the adrenal gland, but also occur in other tissues such as the placenta, testes and ovaries. It is therefore a process that is not gland-specific, but is rather repeated in different glands by cell-type-specific expression of steroidogenic enzymes (Miller and Auchus, 2011). These steroidogenic enzymes belong to one of two major classes of proteins, known as the heme-containing cytochrome P450 proteins and the hydroxysteroid dehydrogenases. However, many of these enzymes, and the reactions they catalyse, are beyond the scope of this discussion and focus will be placed on the enzymes involved in sheep adrenal steroidogenesis only. A summary of the enzymes, the reactions they catalyse and the different tissues in which they are expressed, is depicted in Table 3.1.

The adrenal steroidogenesis pathway is summarized in Figure 3.5, and includes the structures of the steroids and expression of steroidogenic enzymes in the adrenal gland. As mentioned earlier, cholesterol is converted to pregnenolone by CYP11A1 in the mitochondria. Hereafter, pregnenolone moves to the endoplasmic reticulum, where it serves as substrate for either cytochrome P450 17α-hydroxylase/17,20-lyase (CYP17) or 3β-hydroxysteroid dehydrogenase/$\Delta^5 \rightarrow \Delta^4$ isomerase (3βHSD). Pregnenolone is hydroxylated at C-17 by CYP17 to yield 17-hydropregnenolone, which in turn acts as yet another substrate for CYP17. In this step, the bond-cleavage between C-17 and C-20 of 17-hydroxypregnenolone results in the formation of DHEA. The C-3 dehydrogenation of the Δ^5 steroids, namely pregnenolone, 17-hydroxypregnenolone and DHEA, by 3βHSD converts these metabolites to their Δ^4 isoforms, namely progesterone, 17-hydroxyprogesterone and androstenedione, respectively. Furthermore, CYP17 also mediates the hydroxylation of progesterone at C-17 to yield 17-hydroxyprogesterone, as well as the bond-cleavage between C-17 and C-20 of 17-hydroxyprogesterone to yield androstenedione. In some species such as the human, baboon and Angora goat, CYP17 has been reported to hydroxylate C-16

Table 3.1: Summary of enzymes involved in adrenal steroidogenesis, their tissue-specific expression, subcellular location and reactions catalysed.

Enzyme	Tissues-specific expression *(subcellular location)*	Substrate	Product
CYP11A1	Adrenal cortex, ovary, testis, placenta *(Mitochondria)*	Cholesterol	Pregnenolone
CYP17	Adrenal cortex (zona fasciculata & zona reticularis), Leydig cells, ovary (theca cells) *(Endoplasmic reticulum)*	Pregnenolone 17-hydroxypregnenolone Progesterone Progesterone 17-hydroxyprogesterone	17-hydroxypregnenolone Dehydroepiandrosterone 17-hydroxyprogesterone 16-hydroxyprogesterone Androstenedione
3βHSD	Adrenal cortex, Leydig cells, ovary *(Mitochondria & endoplasmic reticulum)*	Pregnenolone 17-hydroxypregnenolone Dehydroepiandrosterone	Progesterone 17-hydroxyprogesterone Androstenedione
CYP21	Adrenal cortex *(Endoplasmic reticulum)*	Progesterone 17-hydroxyprogesterone	Deoxycorticosterone 11-deoxycortisol
CYP11B	Adrenal cortex *(Mitochondria)*	Deoxycortisol Deoxycorticosterone 18-hydroxycorticosterone	Cortisol 18-hydroxycorticosterone Aldosterone

of progesterone, but not pregnenolone (Storbeck et al., 2008b). Progesterone and 17-hydroxyprogesterone then acts as substrates for cytochrome P450 21-hydroxylase (CYP21), which

hydroxylates these steroid metabolites at C-21 to respectively yield deoxycorticosterone and 11-deoxycortisol. A single enzyme, namely cytochrome P450 11β-hydroxylase (CYP11B), mediates the 11-hydroxylation of 11-deoxycortisol to cortisol in sheep, as well as all three steps required for the synthesis of aldosterone from deoxycorticosterone, namely the 11-hydroxylase, 18-hydroxylase and 18-methyl oxidase activities (Boon et al., 1997). In humans, these three steps are mediated by more than one enzyme (Miller and Auchus, 2011). The CYP11B enzyme is located in the mitochondrial membrane of all three adrenal zones. Kinetic studies have shown that CYP11B binds

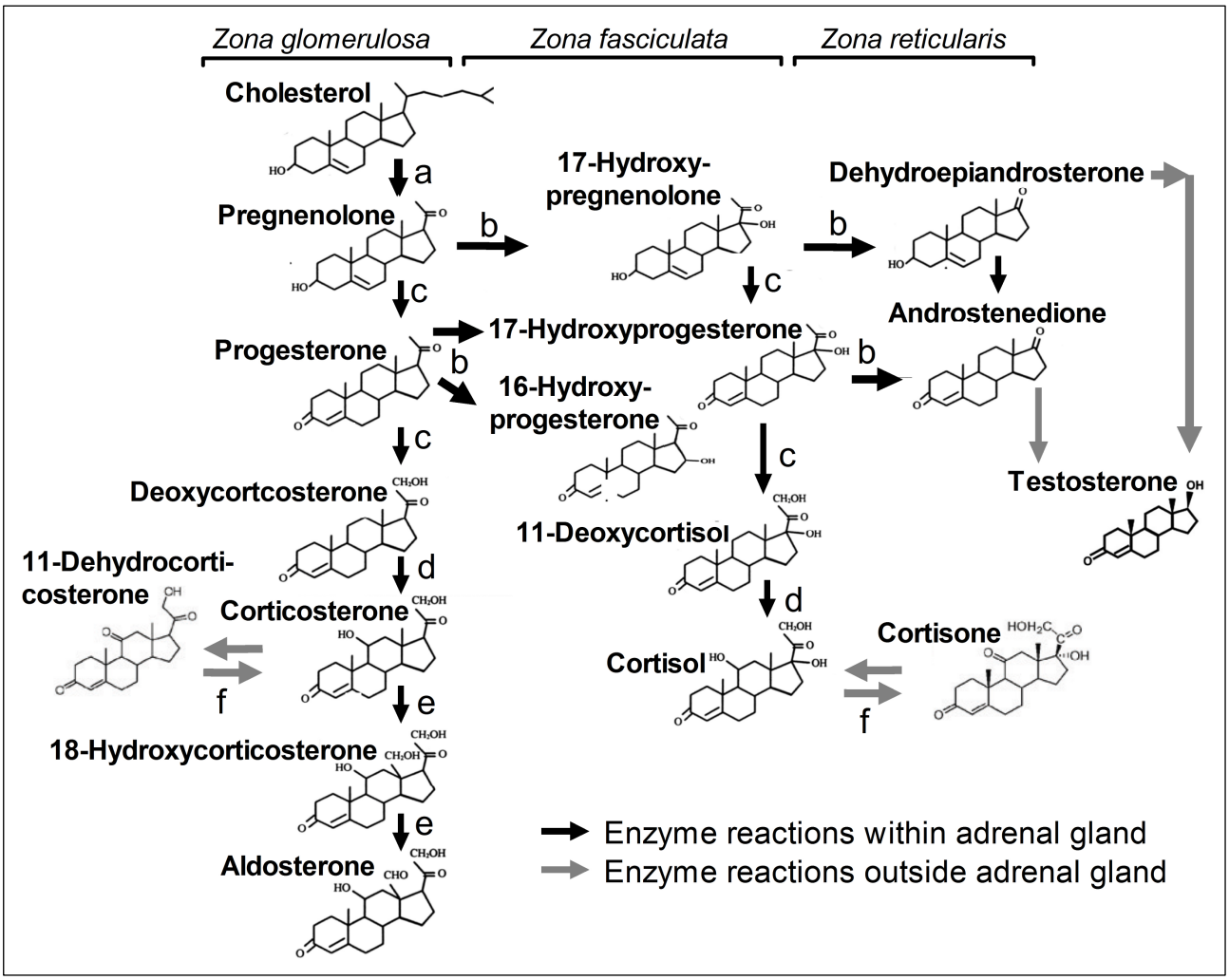

Figure 3.5. Schematic representation of adrenal steroidogenesis. Cholesterol is converted to pregnenolone, which may ultimately be converted to androgens (dehydroepiandrosterone or androstenedione), glucocorticoids (cortisol and corticosterone) or mineralocorticoids (aldosterone). Furthermore, the glucocorticoids may be converted to their inactive 11-oxo-derivatives in other tissues, such as adipose tissue. The steroidogenic enzymes involved are a: cytochrome P450 side chain cleavage; b: cytochrome P450 17α-hydroxylase/17,20-lyase; c: 3β-hydroxysteroid dehydrogenase; d: cytochrome P450 21-hydroxylase; e: cytochrome P450 11β-hydroxylase; and f: 11β- hydroxysteroid dehydrogenase types 1 and 2. Reactions mediated within the adrenal gland are depicted by black arrows, while grey arrows indicate reactions that are mediated in other tissues.

preferentially to deoxycorticosterone compared to corticosterone and 18-hydroxycorticosterone, and that these two latter intermediates are not released from the active site of the enzyme (Boon et al., 1997; Imai et al, 1998). Although CYP11B is expressed in all three zones of the adrenal cortex in sheep, cattle and pig species, the synthesis of aldosterone is only observed in the zona glomerulosa and the reason remains unknown (Boon et al., 1997; Lisurek and Bernhardt, 2004).

3.4 Importance of 11βHSD isoforms in glucocorticoid metabolism

An additional step that does not occur in the adrenal gland, but plays an important role in the metabolism of corticosterone and cortisol, is mediated by the isoforms of 11β-hydroxysteroid

dehydrogenase (11βHSD). These isoforms of 11βHSD mediate the interconversion between the active hormones, cortisol and corticosterone, and their inactive 11-oxo-derivatives, known as cortisone and 11-dehydrocorticosterone, respectively. Sheep 11βHSD types 1 (11βHSD1) and 2 (11βHSD) have been cloned and identified (Campbell and Yang, 1996; Simmons et al., 2010).

11βHSD1 is expressed in numerous tissues that are glucocorticoid responsive (e.g. pituitary gland, brain, lung, bone and eye), but it is most abundant in the adipose tissues and the liver. 11βHSD1 predominantly functions as an oxoreductase to convert cortisone (or 11-dehydrocorticosterone) to cortisol (or corticosterone) using NADPH as cofactor, but also mediates the oxidation of cortisol (or corticosterone) to cortisone (or 11-dehydrocorticosterone) using $NADP^+$ as cofactor. The interconversion is dependent on the availability of the cofactor, but can only function with high concentrations (micromolar) of glucocorticoids (Miller and Auchus, 2011; Quinkler and Stewart, 2003).

The other isoform, 11βHSD2, is predominantly found in target tissues of mineralocorticoids, such as the kidney, brain, colon, testis and placenta (Campbell and Yang, 1996). This isoform can only mediate the oxidation of cortisol (or corticosterone) to cortisone (or 11-dehydrocorticosterone) with NAD^+ as cofactor and functions with low concentrations of steroid (nanomolar). In these tissues 11βHSD2 “defends” the mineralocorticoid receptor from excess glucocorticoids (able to bind to this receptor) (Miller and Auchus, 2011; Quinkler and Stewart, 2003). Furthermore, the two isoforms play an important role during pregnancy in the regulation of active glucocorticoid concentrations (Miller and Auchus, 2011).

3.5 Regulation of adrenal steroidogenesis

The regulation of adrenal steroidogenesis is more complex than the conventional view that the circulating hormones, ACTH and angiotensin II, regulate steroid hormone secretion from the adrenocortical cells. Intraadrenal mechanisms are also in place, which influence the response of adrenocortical cells to ACTH stimulation. This study is primarily concerned with the HPA axis and will therefore place emphasis on the acute response of the adrenal to ACTH stimulation that ultimately results in the release of glucocorticoids, while briefly discussing intraadrenal mechanisms that influence this response.

The distinction between acute and chronic responses is made throughout the discussion, since ACTH stimulation regulates adrenal steroidogenesis at three levels (Ewbank, 1985; Miller and

Auchus, 2011). First, the acute response occurs within minutes and act mainly via StAR to increase cholesterol availability to the first committing step in steroidogenesis. In addition, blood flow to the adrenal gland is increased, CYP17 residues are phosphorylated (decrease 17,20-lyase activity relative to 17α-hydroxylation activity) and some cytoskeletal proteins are activated (facilitate interorganelle substrate delivery). Secondly, ACTH acts over hours to days via cAMP, while angiotensin II act via the calcium/calmodulin pathway, to increase the transcription of steroidogenic enzymes and their cofactors. Thirdly, the long-term exposure to ACTH over weeks to months promotes adrenal growth that results in adrenal cell hypertrophy and hyperplasia. This process is facilitated by intraadrenal interactions between adrenocortical cells, adrenomedullary cells, nerve fibres and immune cells via their secretory products (e.g. cytokines, growth factors and neurotransmitters).

3.5.1 Acute and chronic intracellular responses to ACTH

Steroidogenic cells can only store cholesterol, but not steroid hormones or their intermediary metabolites. The quantitative output of steroid hormones is therefore primarily controlled by the availability of cholesterol (and pregnenolone) to the enzymes in the steroidogenesis pathway (Miller and Auchus, 2011). Most of the acute regulating factors respond to ACTH stimulation in a way that increases the delivery of cholesterol to the inner mitochondrial membrane for its conversion to pregnenolone by CYP11A1. The transport of cholesterol from the outer to inner mitochondrial membrane by StAR is considered to be the main regulator of the acute ACTH response (Miller and Auchus, 2011). However, the exclusive regulation of cholesterol availability in the acute response would result in a general increase of all the steroid hormones and it is well-known that ACTH stimulation specifically increases glucocorticoid output in adrenocortical cells. Therefore, in the acute response, the basal expression of CYP11A1, CYP17, CYP21 and CYP11B in the zona fasciculata needs to be in favour of glucocorticoid production, and/or other non-transcriptional regulating factors must be involved in eliciting a glucocorticoid specific response. For instance the cytoskeleton (Li et al., 2010) and serine/threonine phosphorylation of CYP17 (Kempna et al., 2010) has been implicated in the non-transcriptional regulation of a glucocorticoid-specific response to ACTH. The regulation of cholesterol availability and non-transcriptional regulators of a glucocorticoid-specific response will be discussed in the following sections.

Long-term ACTH stimulation will ultimately lead to an increase in steroidogenic enzyme expression (Payne and Hales, 2004). Various mechanisms are in place for the expressional

upregulation of these enzymes, but these mechanisms are not the same for each steroidogenic enzyme. The expressional control of CYP17 will be discussed as an example of the chronic response in Chapter 4. The expressional upregulation in response to ACTH stimulation is mostly mediated by activated protein kinases – such as ribosomal phosphorylation – or by cAMP that can act directly via the cAMP response element (CRE)/CRE binding protein (CREB) system (Payne and Hales, 2004).

As mentioned earlier, the chronic response occurs hours to days after ACTH stimulation. Numerous studies have demonstrated the time-dependent increase in expression of steroidogenic enzymes hours after ACTH or cAMP stimulation. For example, Kempna et al. (2010) observed an increase in CYP17 mRNA in H295R cells twenty-four hours after cAMP stimulation, but no significant change within the first three hours. Generally the increases in mRNA expression is measured 24 hours after adding stimulation or inhibition agents to the experimental cells (Payne and Hales, 2004; Sirianni et al., 2005; Xing et al., 2011; Miller and Auchus, 2011).

The expressional regulation of steroidogenic enzymes by ACTH is also important for increases in cortisol that is required for the onset of parturition. In sheep it is well known that the cortisol concentrations increase concomitantly with an increase in ACTH in the last 10 to 15 days of gestation (Challis and Brooks, 1989). It has been demonstrated that the expression of CYP11A1, CYP17 and CYP21 is increased 2 to 3-fold in the foetal adrenal and is essential for the increase in adrenal steroidogenesis that precedes parturition (Phillips et al., 1994; McMillen et al., 1995). From the discussion it is clear that chronic and acute ACTH stimulation has various effects on steroidogenesis that will be discussed in the following sections.

3.5.2 Regulation of StAR and cholesterol availability

The intracellular concentration of cholesterol in adrenocortical cells is increased within three minutes of ACTH stimulation and peaks after 10 to 15 minutes (Young et al., 2006). ACTH increases the uptake of cholesterol esters from circulating lipoproteins (by increasing transcription of LDL receptors and SRB1 for microvillar channel formation) and increase cholesterol synthesis (by inhibiting ACAT and stimulating the activity of HSL and 3-hydroxy-3-methylglutaryl coenzyme A reductase) (Connelly and Williams, 2004; Kraemer, 2007; Miller and Auchus, 2011). The intracellular cholesterol is then transported by cytoplasmic StAR-like proteins to the outer

mitochondrial membrane, where StAR transports cholesterol from the outer to inner mitochondrial membrane for utilization in the steroidogenesis pathway.

From the observation that the mitochondrial transport of cholesterol is constrained by inhibitors of protein synthesis, it was concluded that this process is mediated by a short-lived protein species, later identified as StAR (Clark et al., 1994; Stocco and Clark; 1996). StAR is expressed as a 37-kDa protein with a mitochondrial leader sequence that directs it towards the mitochondria. This leader sequence is cleaved off upon entry into the mitochondrion to yield a 30-kDa intramitochondrial protein (Miller and Auchus, 2011). The 37-kDa cytoplasmic precursor has a short half-life, while its intramitochondrial 30-kDa form has a longer half-life and is active when associated with the outer mitochondrial membrane (Bose et al., 2002). The interaction between StAR and the outer mitochondrial membrane involves a conformational change that is required for the binding and discharge of cholesterol (Bose et al., 1999; Baker et al., 2005). Each StAR molecule appears to be recycled before the inactivation event and can thereby move hundreds of cholesterol molecules (Artemenko et al., 2001). The mode of action for StAR is complex and remains incompletely understood (Miller and Auchus, 2011).

ACTH stimulation results in the protein kinase dependent phosphorylation of the 37-kDa cytoplasmic precursor to yield a phosphorylated 30-kDa intramitochondrial StAR protein upon mitochondrial entry (Jefcoate, 2002). ACTH stimulation also results in the increase in transcription of StAR. The conversion of 37-kDa precursor to 30-kDa intramitochondrial protein reaches steady-state between formation and processing after 5 minutes, which is consistent with the time frame of ACTH stimulation of CYP11A1 activity (Tuckey et al., 2002; Jefcoate, 2002).

3.5.3 Cytoskeletal regulation of steroidogenesis

It has been suggested that the cytoskeleton is involved in the regulation of steroidogenesis, primarily where it may be involved in the spatial placing of mitochondria close to the endoplasmic reticulum (Sewer and Li, 2008; Li et al., 2010). This allows for efficient substrate delivery in steroidogenesis where the reactions involved are spatially distributed between the mitochondria and endoplasmic reticulum. Attention was initially drawn to the involvement of the cytoskeleton in the regulation of steroidogenesis when changes in adrenal cell morphology were observed on stimulation with ACTH (Voorhees et al., 1984). The stimulation of adrenocortical cells with ACTH was reported to result in the “rounding” of adrenal cells within 5 minutes and it correlated with

changes in steroidogenic output. This morphological change in adrenal cells was explained by the cAMP-dependent dephosphorylation of paxillin, a focal adhesion protein (Han and Rubin, 1996; Whitehouse et al., 2002). The organelles are subsequently clustered that brings the mitochondria in close proximity to the endoplasmic reticulum, where CYP17, CYP21 and 3βHSD are located and cholesterol is available from *de novo* synthesis.

Furthermore, various studies on cytoskeletal protein polymerization have shown that microfilaments and microtubules are implicated in the regulation of steroidogenesis (Sackett and Wolff, 1986; Rainey et al., 1984; Rainey et al., 1985; Denkova et al., 1992; Shiver et al., 1992; Hall and Almahbobi, 1997; Lee et al., 2001; Li et al., 2010). Lipid droplets, for example, have been shown to move along microtubule tracts in Y1 mouse adrenal cells (Nan et al., 2006). Treatment of H295R cells (immortalized human adrenocortical cells) with colchicine or nocodazole promoted microtubule depolymerisation, leading to decreased mitochondrial movement and increased DHEA production (Li et al., 2010). In contrast, stimulation of H295R cells with paclitaxel increased microtubule stabilization, leading to increased mitochondrial movement and cortisol production, but decreased DHEA production (Li et al., 2010). The stimulation of mitochondrial movement upon ACTH/cAMP stimulation was shown to be dependent on a Rho-protein (RhoA) and its effector protein, namely diaphanous-related homolog 1 (DIAPH1). Stimulation with ACTH temporarily increased the concentration of ser-188 phosphorylated RhoA (via protein kinase A) and GTP-bound RhoA (active form), which promoted interaction with DIAPH1 to ultimately increase mitochondrial movement (Li et al., 2010). It was therefore suggested that ACTH regulates cortisol production by increasing the interorganelle substrate transfer via dynamic mitochondrial trafficking.

3.5.4 Serine/threonine phosphorylation of CYP17

The 17,20-lyase activity of CYP17 may be altered by phosphorylation of its serine and/or threonine residues. This mechanism remains poorly understood and will be discussed in greater detail in Chapter 4. In summary, the serine and threonine residues of CYP17 can be phosphorylated to alter the 17,20-lyase activity relative to the 17α-hydroxylase activity. Kempna et al. (2010) suggested a mechanism where serine or threonine residues of CYP17 are selectively phosphorylated by two distinct intracellular signalling pathways. Stimulation of H295R cells with cAMP showed no alteration in CYP17 expression within the first three hours, but threonine phosphorylation of CYP17 resulted in a decrease in 17,20-lyase activity relative to 17α-hydroxylase activity. After 24 hours of cAMP stimulation, there was an increase in CYP17 expression and a simultaneous increase

in 17,20-lyase activity. In contrast, when H295R cells were subjected to starvation conditions (serum-deprived growth medium) the serine residues of CYP17 was selectively phosphorylated and resulted in an increase in 17,20-lyase activity relative to 17α-hydroxylase activity after 24 hours, without an increase in CYP17 expression. These results indicate that the threonine phosphorylation of CYP17 may be implicated in the acute response to ACTH/cAMP stimulation to selectively increase glucocorticoid production (by the inhibition of 17,20-lyase activity).

3.5.5 Neuroendocrine and vascular regulation

The adrenocortical nervous innervation appears to play a fine tuning role in the functions of the adrenal cortex (Ehrhart-Bornstein et al., 1998). The adrenal medulla is under sympathetic nervous control, while the adrenal cortex receives direct innervation, which is at least partly derived from the splanchnic nerve. The integrity of the sympathetic innervation is required for maintaining the diurnal steroidogenic output (Ottenweller and Meier, 1982; Dijkstra et al., 1996; Muglia et al., 1997), while the splanchnic nerve supply was found to enhance the ACTH-stimulated glucocorticoid response (Edwards and Jones, 1987). The neurotransmitters from nerves that innervate the adrenal cortex have various actions, including the modulation of the actions of humoral stimuli on the adrenal cortex, or having direct effects on growth and steroidogenesis (Ehrhart-Bornstein et al., 1998).

The adrenal innervation also plays a role in regulation of vasculature. Splanchnic nerve and ACTH stimulation increases the blood flow to the adrenal gland and enhances the access to cholesterol and oxygen (cosubstrate of all cytochromes P450 and some cofactors) (Young et al., 2006). The regulation of blood flow to the adrenal gland is complex and involves various local and humoral mediators (Ehrhart-Bornstein et al., 1998). Nearly each adrenocortical cell is adjacent to an endothelial cell, which facilitates the exchange of secretory products (Hinson and Kapas, 1998).

3.5.6 Interaction between adrenocortical and adrenomedullary cells

The interdispersion of cortical and medullary cells allows for complex regulatory circuits (Ehrhart-Bornstein et al., 1998). Further study is still required to elucidate how the secretory products of the adrenomedullary cells are involved in the regulation of adrenocortical activity. In summary, various adrenomedullary secretions, such as catecholamines and a whole series of neurotransmitters, may interact with adrenocortical cells by addition, potentiation or antagonism of their effects. In return the secretory products of the adrenal cortex, namely steroid hormones and cytokines, influence the

expression of proteins, catecholamines and neuropeptides in adrenomedullary cells. There is increasing evidence that the colocalization of medullary and cortical cells is a prerequisite for paracrine interactions within the adrenal gland. Gap junctions have been suggested to play a more important role in communication between these cell types than previously thought and the number of gap junctions increases rapidly with ACTH stimulation. (Colomer et al., 2009; Ehrhart-Bornstein et al., 1998)

Cytokines directly influence adrenocortical function, and are derived from either adrenal cells themselves (primarily cortical cells) or from immune cells that regularly infiltrate the adrenal gland. The localization (adrenal zona) of cytokine producing cells, as well as the type of cytokine produced, varies across species (Ehrhart-Bornstein et al., 1998). Generally cytokines like interleukin-1 (IL-1), IL-2 and IL-6 stimulate steroidogenesis (production of glucocorticoids with anti-inflammatory actions), while tumor necrosis factor-α (TNFα) and interferon-γ exert a regulatory influence on adrenal growth. Both the immune system and endocrine system play a crucial role, and interact at different levels, in the adaptive HPA axis response to deviations in homeostasis (from stress or disease). It has been suggested that the acute steroidogenic response is regulated at the level of the hypothalamus, while long-term regulation is mediated at the level of the adrenal by the locally produced cytokines, IL-1, IL-6 and TNFα (Chrousos, 1995).

Furthermore, adrenal cells produce growth factors that locally mediate the development and maintenance of the adrenal cortex (Ehrhart-Bornstein et al., 1998). These growth factors include transforming growth factor-β (TGFβ), insulin-like growth factors (IGFs) and β-fibroblast growth factors (βFGF), that mediate a variety of stimulatory and inhibitory effects on the growth and differentiation of the adrenal. These actions of the growth factors may well be the mechanism by which systemic factors, like ACTH, mediate their growth-regulating effects and contribute to their acute and chronic effects on steroidogenesis.

3.5.7 Renin-angiotensin system

Although the renin-angiotensin system is not necessarily activated in the HPA axis stress response, it may influence the adrenocortical response to ACTH and is therefore discussed briefly in this section. The renin-angiotensin system plays an important role in the regulation of adrenal steroidogenesis along with plasma concentrations of ACTH, Na^+ and K^+ (Ehrhart-Bronstein et al., 1998). A low Na^+ diet will increase both aldosterone and adrenal renin levels. In rats it was shown

that a high K^+ intake stimulates aldosterone and adrenal renin, while plasma renin activity was reduced (Mulrow, 1998). Specific angiotensin receptors are located predominantly in the zona glomerulosa and angiotensin II stimulates the production of aldosterone (mineralocorticoid) via the phosphatidyl inositol/ITP/intracellular calcium pathway (Ehrhart-Bornstein et al., 1998).

Bird et al. (1996) observed that stimulation of human adrenocortical cells with angiotensin II resulted not only in an increase in aldosterone production, but also in an increase in cortisol production. Another study by Bird et al. (1992) showed that angiotensin II stimulation of ovine adrenal cells suppressed CYP17 expression and inhibited the cortisol response (fasciculata-type function) to ACTH stimulation. Galtier et al. (1996) also showed that the induction of CYP17 by ACTH was inhibited by angiotensin II in bovine zona glomerulosa cells.

3.6 Conclusion

The adrenal gland is a highly specialized organ that is regulated by various mechanisms in a complex manner. From this perspective, large individual differences are expected in cortisol production, which further adds to individual variation within the HPA axis. Adrenal steroidogenesis entails the conversion of cholesterol to active steroid hormones. The quantitative steroidogenic output is dependent on the amount of cholesterol available to the first rate-limiting step: the conversion of cholesterol to pregnenolone by CYP11A1. The qualitative steroidogenic output is dependent on the activity of the other steroidogenic enzymes. CYP17 is the only steroidogenic enzyme that redirects steroid hormone synthesis away from aldosterone synthesis, towards cortisol and DHEA synthesis. The activity and regulation of CYP17 is therefore important in determining the qualitative steroidogenic output. The characteristics and regulation of this enzyme will be discussed in the following chapter.

CHAPTER 4

Cytochrome P450 17α-hydroxylase/17,20-lyase

4.1 Introduction

Cytochrome P450 17α-hydroxylase/17,20-lyase (CYP17) is a microsomal enzyme of the P450 super-family that is predominantly expressed in the adrenal gland and gonads where it mediates two types of reactions, namely 17α-hydroxylase and 17,20-lyase reactions. Originally these activities were thought to be mediated by two different enzymes and that the adrenal enzymes were different from the gonadal enzymes. This hypothesis was concluded from clinical observations that serum cortisol concentrations (reflecting 17α-hydroxylase activity) remained constant throughout life, whereas serum DHEA concentrations (reflecting 17,20-lyase activity) increased with age. The abrupt increase in serum DHEA at ages eight to ten years in humans, compared to low serum DHEA concentrations in early childhood, suggested that the 17,20-lyase activity was dissociated from the 17α-hydroxylase activity (Apter et al., 1979; Orentreich et al., 1984). This hypothesis supported the observation that some patients appeared to lack 17,20-lyase activity while the 17α-hydroxylase activity was normal (Zachmann et al., 1972). However, in 1981 and 1984 Nakajin et al. reported that one protein, isolated from pig testis, was responsible for both 17α-hydroxylase and 17,20-lyase activities (Nakajin et al., 1981a, 1981b, 1984). These reports were received with scepticism and the controversial hypothesis that one enzyme mediates both reactions was only accepted in 1986 when cDNA of bovine CYP17 was cloned and expressed in COS-1 cells. Both 17α-hydroxylase and 17,20-lyase activities were observed in cells transfected with vectors expressing the CYP17 cDNA, proving that CYP17 mediates both reactions (Zuber et al., 1986). Furthermore it was shown that the hypothesis of two tissue-specific isoforms was incorrect and that human CYP17 is encoded by a single gene on chromosome 10q24.3, which is expressed in both the adrenal gland and gonads (Matteson et al., 1986; Chung et al., 1987; Fan et al., 1992). The *CYP17* gene has also been found to be structurally related to the CYP21 gene (Picardo-Leonard and Miller, 1987; Miller and Auchus, 2011).

The importance of CYP17 in steroidogenesis is clear when considering its location at four branch points in the pathway, where it competes with 3βHSD and CYP21 for substrates (Figure 3.5). CYP17 is the only enzyme that redirects steroidogenesis away from mineralocorticoid (aldosterone) synthesis, towards glucocorticoid (cortisol) and androgen (DHEA) synthesis. The activity of CYP17

is regulated in several ways: 1) abundance of CYP17 by means of transcriptional control; 2) abundance of redox-partner; 3) abundance of cytochrome b_5; and 4) by serine/threonine phosphorylation of CYP17. The mechanism of action of CYP17 and its interaction with its cofactors thus plays a central role in the qualitative regulation of the steroidogenic output.

4.2 Cytochrome P450 enzymes

Most of the enzymes that mediate steroidogenesis are either cytochrome P450 enzymes or hydroxysteroid dehydrogenase (HSD) enzymes. These enzymes are functionally unidirectional and as a consequence the flux through steroidogenesis is not directed back to precursor steroids with the accumulation of steroid products (Miller and Auchus, 2011). The hydroxysteroid dehydrogenase enzymes are mechanistically reversible under certain *in vitro* conditions, but under normal *in vivo* conditions they mediate only one of either oxidation or reduction reactions (Agarwal and Auchus, 2005). Conversely, the cytochrome P450 enzymes mediate carbon-carbon bond cleavage reactions and hydroxylations that are mechanistically and physiologically irreversible (Hall, 1986).

Cytochrome P450 enzymes are membrane-bound proteins that belong to a superfamily of heme-containing proteins. These enzymes consist of approximately 500 amino acids and are found in animals, plants, fungi and bacteria (Gonzalez, 1988). The “P450” annotation is derived from “pigment 450”, since these enzymes absorb light maximally at 450 nm when the reduced form of the enzyme is complexed to carbon monoxide *in vitro*. The cytochrome P450 enzymes mediate hydroxylation and carbon-bond cleavage reactions (Miller and Auchus, 2011). They are monooxygenase enzymes that use nicotinamide adenine dinucleotide phosphate (NADPH) as electron donor for the reduction of molecular oxygen as follows:

$$RH + O_2 + NADPH + H^+ \rightarrow ROH + H_2O + NADP^+$$

In this reaction molecular oxygen is activated by the heme center of a cytochrome P450 enzyme that transfers one oxygen atom to the substrate (RH), while the other oxygen atom is reduced to a water molecule (Payne and Hales, 2004). The electron transfer from NADPH is mediated by two distinct electron transfer systems for cytochrome P450 enzymes that are bound to the mitochondria and endoplasmic reticulum. The mitochondrial bound cytochrome P450 enzymes are termed “type 1” and include the steroidogenic enzymes CYP11A, CYP11B1 and CYP11B2. These enzymes receive its electrons from NADPH via two proteins, namely a flavoprotein termed ferrodoxin reductase and an iron-sulphur protein termed ferrodoxin. The endoplasmic reticulum bound

cytochrome P450 enzymes are termed “type 2” and include the steroidogenic enzymes CYP17, CYP21 and CYP19. These enzymes receive its electrons from NADPH via only one protein, namely the two flavin containing P450 oxidoreductase (POR) (Miller, 2005). There are six cytochrome P450 enzymes in total that are involved in steroidogenesis, where these enzymes have multiple substrates and mediate numerous oxidation reactions (Miller and Auchus, 2011).

4.3 Catalytic activity

The CYP17 protein consists of 507 amino acids in the rat (Fevold et al., 1989) and mouse (Youngblood et al., 1991); 508 amino acids in the human (Chung et al., 1987); and 509 amino acids in the goat (Storbeck et al., 2007) and sheep (Swart et al., 2003) with a molecular mass of approximately 57 kDa. Three important domains are identified, namely the heme-binding site, substrate-binding site and redox-partner binding site. More than 40 mutations in the introns and exons of the human *CYP17* gene have been identified that result in 17α-hydroxylase/17,20-lyase deficiency (Yanase, 1995; Auchus, 2001; Costa-Santos et al., 2004a,b). The disease is generally characterized by the presence of homozygous or compound heterozygous mutations, but rare missense mutations in the redox-partner binding domain results in isolated 17,20-lyase deficiency (Geller et al., 1997).

CYP17 is associated with the endoplasmic reticulum and therefore obtains electrons from NADPH via POR. The electrons are transferred to flavinadenine dinucleotide (FAD, the first flavin in the POR protein), followed sequentially by transfer to flavinmononucleotide (FMN, the second flavin in POR) and the substrate (Miller, 2005; Payne and Hales, 2004). The 17α-hydroxylation and 17,20-lyase activities of CYP17 are two mixed-function oxidase reactions that each requires one molecule of oxygen and one molecule of NADPH. The hydroxylation and side chain cleavage reactions are both catalysed in a common active site and do not follow sequentially on one another, since it is a two-step process with the release of hydroxylated intermediate (Auchus and Miller, 1999; Soucy and Luu-The, 2000). However, Yamazaki et al. (1998) have suggested that the hydroxylated intermediate is not necessarily released from the active site. The hydroxylation mechanism of CYP17 is believed to proceed when the heme center of the cytochrome P450 enzyme has formed an iron-oxygen complex upon activation of molecular oxygen (Atkinson and Ingold, 1993; Auchus and Miller, 1999). The exact 17,20-lyase mechanism of CYP17, however, remains unknown, despite considerable study (Miller and Auchus, 2011). The same iron-oxygen complex as well as an iron-peroxide complex has both been suggested to be involved in the 17,20-lyase mechanism (Lee-Robichaud et al., 1995; Lee-Robichaud et al., 1997; Auchus and Miller, 1999).

The 17α-hydroxylase activity towards pregnenolone and progesterone is generally very similar. However, the 17,20-lyase activity and its preferential utilization of either 17-hydroxypregnenolone (Δ^5) or 17-hydroxyprogesterone (Δ^4) vary greatly among species. In the human and baboon the 17,20-lyase activity towards 17-hydroxypregnenolone can be 50 to 100-fold higher than 17-hydroxyprogesterone (Auchus and Miller, 1998; Miller and Auchus, 2011). Consequently the majority of sex steroids are derived from DHEA rather than from A4. This species-dependent variation in 17,20-lyase activity is attributed to the differences among these species in their augmentation by the accessory protein, cytochrome b_5. The 17,20-lyase activity can be increased 10-fold by the presence of cytochrome b_5, however, the 17,20-lyase activity never quite reaches the rate of the 17α-hydroxylation reactions (Miller and Auchus, 2011). CYP17 can also mediate the 16α-hydroxylation of pregnenolone and progesterone, but does not mediate hepatic 16α-hydroxylation (Miller and Auchus, 2011; Lachance et al., 1990). Human CYP17 mediates the 16α-hydroxylation of progesterone but not pregnenolone (Swart et al., 1993), due to the presence of an alanine instead of leucine at residue position 105 (Qiao et al., 2010; Miller and Auchus, 2011). The 16α-hydroxylation of progesterone has also been reported for baboon and goat CYP17 (Storbeck et al., 2007; Storbeck et al., 2008b), but has not been investigated for ovine CYP17 to date. Interestingly, porcine CYP17 converts approximately 10 % of pregnenolone to its $\Delta^{5,16}$ andiene product in the presence of cytochrome b_5, which results in the characteristic "boar taint" (Nakajin et al., 1985).

4.4 Interaction of CYP17 with POR

POR serves as a reductase for numerous non-P450 enzymes, including cytochrome b_5 (Enoch and Strittmatter, 1979), fatty acid elongase (Ilan et al., 1981), squalene monooxygenase (Ono and Bloch, 1975) and heme oxygenase (Wilks et al., 1995). POR is the only electron transfer protein for all microsomal cytochrome P450 enzymes, to which two electrons are transferred one by one from NADPH (Yamano et al., 1989; Wang et al., 1997). The mechanism of electron transport by POR is summarized in Figure 4.1.

The structure of POR is butterfly-shaped, where the N-terminus tethers the protein to the endoplasmic reticulum and joins the two lobes with an α-helical connecting domain. Each lobe contains a different flavin, namely FAD and FMN. A disordered "hinge" of 25 residues forms between the FMN domain and the connecting domain that allows substantial movement of the FMN domain relative to the FAD domain. When NADPH interacts with POR, the donated electron pair received by the FAD moiety induces a conformational change that permits the isoalloxazine rings of

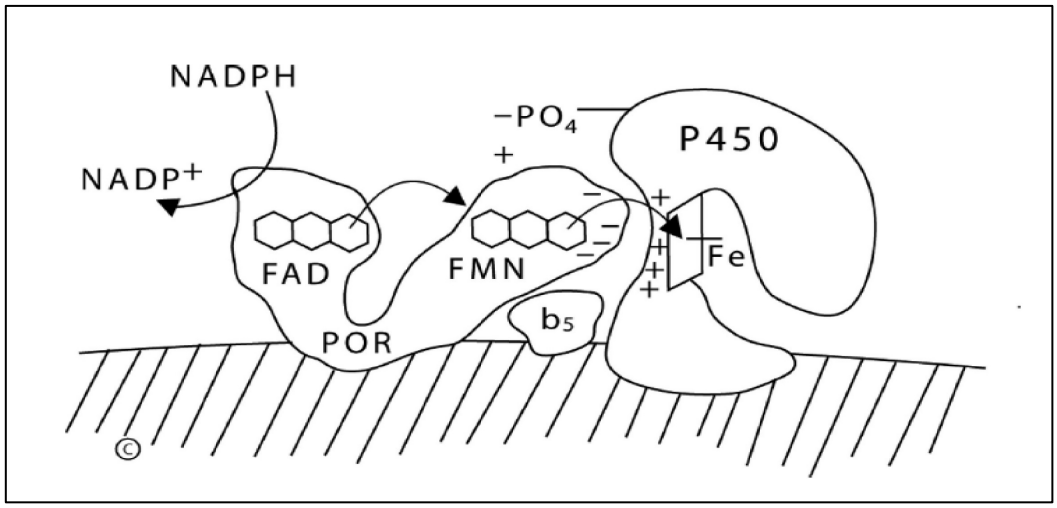

Figure 4.1. Schematic representation of the electron transport from nicotinamide adenine dinucleotide phosphate (NADPH) to a microsomal P450 enzyme. This electron transport is facilitated by the interactions with P450 oxidoreductase (POR) and cytochrome b_5 (b_5). POR contains two flavins that facilitate the electron transport process, namely flavinadenine dinucleotide (FAD) and flavinmononucleotide (FMN). Reproduced from Miller and Auchus (2011) (© WL Miller)

FAD and FMN to come into close proximity of each other, and the electrons are transferred to the FMN moiety (Ellis et al., 2009). A second conformational change is induced that returns POR to its original orientation, permitting the FMN domain to bind to the cytochrome P450 enzyme and electrons are transferred to its heme moiety. It has been suggested that the "hinge" region also accommodates significant reorientation of the FMN domain for docking to the cytochrome P450 enzyme (Miller, 2005; Auchus et al., 1998). The interaction between POR and the cytochrome P450 is coordinated by negative charges on the surface of the FMN domain, mostly contains acidic residues, and positive charges of the redox-partner binding site of the cytochrome P450 enzyme, mostly contains basic residues (Miller and Auchus, 2011). The distance between the FMN moiety of POR and the heme group on the opposite side of the cytochrome P450 is too far (18 Å) for the electrons to "jump", and instead the polypeptide is used as conduit (Sevrioukova et al., 1999). In humans the interaction between CYP17 and POR is facilitated by the allosteric action of cytochrome b_5 and the serine phosphorylation of CYP17 (Miller and Auchus, 2011).

The supply of electrons to microsomal cytochrome P450 enzymes is limited by POR. Indeed it was found that the 17,20-lyase activity of CYP17 is increased, relative to the hydroxylase activity, with an increase in POR concentrations (Yanagibashi and Hall, 1986; Lin et al., 1993). This effect of POR has profound implications for steroidogenesis, since the availability of reducing equivalents is a crucial regulating factor for the 17,20-lyase activity of CYP17. For example testicular microsomes have 3- to 4-fold higher POR to CYP17 ratios than adrenal microsomes and consequently showed an increased 17,20-lyase activity relative to hydroxylase activity (Yanagibashi and Hall, 1986).

4.5 Interaction with cytochrome b_5

Cytochrome b_5 is a small (12-17 kDa) hemoprotein that may augment the activity of various cytochrome P450 enzymes. It may occur as a soluble protein in red blood cells where it is expressed without its membrane-anchoring C-terminal, but is membrane-bound in other tissues such as the liver (Miller and Auchus, 2011). Cytochrome b_5 is expressed in the gonads and adrenal glands, where it can interact with CYP17. The expression of cytochrome b_5 in the adrenal gland is confined to the zona reticularis, although this confinement may vary by species. For this reason, the zona reticularis produce mainly androgens (DHEA and androstenedione), since cytochrome b_5 augments the 17,20-lyase activity of CYP17 that is expressed in this zone. CYP17 is also expressed in the zona fasciculata, but the expression of cytochrome b_5 is relatively low, and this zone thus mainly produces glucocorticoids (cortisol). Neither CYP17 nor cytochrome b_5 is expressed in the zona glomerulosa. Subsequently this zone is the main site for mineralocorticoid production (aldosterone).

The mechanism of action for cytochrome b_5 was presumed to involve the electron transport of a second electron from cytochrome b_5 to cytochrome P450 (Bridges et al., 1998). However, the reduction potential is unfavourable for electron transfer from cytochrome b_5 to the one-electron-reduced cytochrome P450. Instead it was suggested by Bridges et al. (1998) that cytochrome b_5 acts via an allosteric mechanism to promote the interaction of POR with CYP17 for efficient electron transport (Auchus et al., 1998; Lee-Robichaud et al., 1995) (Figure 4.1). However, further evidence is required, since it was only shown that it is not essential for cytochrome b_5 to be redox active to influence lyase activity (Bridges et al., 1998). Cytochrome b_5 thus plays a central role in the electron transport from NADPH to CYP17 and is the principal regulator of 17,20-lyase activity (Miller and Auchus, 2011).

Cytochrome b_5 consists of two domains, namely a heme-liganding (core 1) domain and a structural (core 2) domain. The C-terminal extends from the core 2 domain and forms a helix that anchors cytochrome b_5 to the membrane (Falzone et al., 1996). This helix of cytochrome b_5 is required to stimulate the 17,20-lyase activity of CYP17 in humans (Lee-Robichaud et al., 1997). The heme extends towards the periphery of cytochrome b_5, while the entire surface of cytochrome b_5 is dominated by residues with a negative charge (Falzone et el., 1996). Key residues with a positive charge have been identified in CYP17 that is important for its interaction with cytochrome b_5. Furthermore E48 and E49 have been identified as two residues that are required for high 17,20-lyase activity (Naffin-Olivos and Auchus, 2006). The exact molecular details of how cytochrome b_5 augments the interaction between POR and CYP17 are yet to be determined.

4.6 CYP17 gene expression and regulation

The human (Picado-Leonard and Miller, 1987; Kagimoto et al., 1989), rat (Fevold et al., 1989), mouse (Youngblood et al., 1991) and pig (Conley et al., 1992; Zhang et al., 1992) *CYP17* genes are encoded by a single gene that has been mapped to chromosome 10q24.3 in humans (Matteson et al., 1986; Fan et al., 1992) and chromosome 19 at 46 cM in mice (Youngblood et al., 1991). The *CYP17* gene is approximately 6 kb in length with 8 exons and the location of intron-exon boundaries are highly conserved among species (Youngblood and Payne, 1992). The first 550 base pairs in the 5' upstream region have high homology in these species and include the nonconsensus TATA box (Payne and Hales, 2004). A duplication of the *CYP17* gene has been reported by Storbeck et al. (2008a) in the case of the South African Angora and Boer goats. These breeds express two CYP17 isoforms that are encoded by two different *CYP17* alleles, and three distinct genotypes. This is the result of a duplication of the *CYP17* gene. The two CYP17 isoforms showed differences in catalytic activity and subsequently there was a difference in cortisol production among Angora goats with different *CYP17* genotypes. The *CYP17* genotypes were found to contribute to the vulnerability of the Angora goats to cold stress (Storbeck, et al. 2008). Furthermore, bovine CYP17 was previously thought to be the product of a single gene (Bhasker et al., 1989), but three paralogous copies of the *CYP17A1* gene has been identified in the bovine genome, of which two copies might be silenced by epigenetic modification (Vanselow and Fürbass, 2011). These recent studies (Storbeck et al., 2008; Vanselow and Fürbass, 2011) have shown that the genetic architecture of the *CYP17* gene, and its regulation, may be more complicated than initially considered. In ovine species, two genetic sequences for ovine CYP17 have been independently deposited on Genbank, however, their activities and effects on the steroidogenic output has not been compared to date (Swart et al., 2003; Storbeck et al., 2008a).

The expression of CYP17 in the gonads is generally confined to the Leydig cells in the testis and theca cells in the ovary. As mentioned previously the expression of CYP17 in adult adrenal cells are restricted to the zona reticularis and fasciculata (Miller and Auchus, 2011). In pregnancies at term, CYP17 is also expressed in the ovine foetal adrenal cells and placenta (Challis and Brooks, 1989; Connor et al., 2009). This expression of CYP17 during gestation plays an important role in the production of glucocorticoids, which are required for development of the foetal organs (Liggins, 1969; Liggins, 1994) and also trigger the parturition cascade (Challis et al., 2000). It is therefore anticipated that there are various factors that regulate the expression of CYP17.

Chronic exposure to pituitary trophic hormones (ACTH for adrenal cells and LH for thecal and Leydig cells) induce the upregulation of CYP17 expression via the cAMP-dependent protein kinase A signalling pathway. The protein kinase A activations results in the phosphorylation of various transcription factors that will upregulate the transcription of the steroidogenic enzymes (Stocco et al., 2005; Manna et al., 2003). In humans, the adrenal expression of *CYP17A1* has been found to be regulated by numerous transcription factors, including SF1, SP1, SP3, CTF2, CTF5, GATA4 and GATA6 (Rodriguez et al., 1997; Lin et al., 2001; Sewer et al., 2002; Jimenez et al., 2003; Flück and Miller, 2004; Sewer and Jagarlapudi, 2009). It was also demonstrated the transcription of *CYP17A1* may be regulated by the SREBP family of factors, which are involved in the synthesis of cholesterol and other sterols (Ozbay et al., 2006). In the case of *CYP17* transcriptional upregulation, but not all P450 enzymes involved in steroidogenesis, the cAMP can act directly as activator via the CRE/CREB system (Waterman, 1994). The cAMP responsive sequences may differ across species and are located at various positions upstream from the *CYP17* gene (Waterman and Keeney, 1996; Payne and Hales, 2004). Furthermore, it was shown that angiotensin II and TGFβ suppress *CYP17* gene expression in ovine adrenal cells (Ehrhart-Bornstein et al., 1998).

4.7 Serine/threonine phosphorylation van CYP17

CYP17 is not only regulated by the availability of electrons from its redox partner and the allosteric mechanism of cytochrome b_5, but also regulated by serine/threonine phosphorylation. Certain serine and threonine residues may be phosphorylated, which consequently increase the 17,20-lyase activity (Zhang et al., 1995). The augmentation of the 17,20-lyase activity by serine/threonine phosphorylation is independent of cytochrome b_5 and is neither additive nor cooperative (Pandey et al., 2003; Pandey and Miller, 2005). It was suggested that the serine/threonine phosphorylation increase the velocity of the reaction, rather than the affinity of the enzyme for the substrate. It was proposed that the negative charge of the phosphorylated residues in CYP17 promotes electrostatic interactions of CYP17 with POR (Pandey and Miller, 2005).

When a protein is activated by phosphorylation, such as CYP17, there is generally equilibrium between phosphorylation by a kinase and dephosphorylation by a phosphatase (Virshup, 2000). The dephosphorylation of human CYP17 has been shown to involve protein phosphatase 2A (PP2A), which in turn is inhibited by phosphoprotein SET (Pandey et al., 2003). The identification of the involved kinase(s) has, however, been a more difficult task. Wang et al. (2010) drew attention to the contrasting results obtained from *in vitro* studies and a whole-cell milieu. To date no kinase could be identified that is capable of augmenting the 17,20-lyase activity with subsequent phosphorylation

of serine and/or threonine residues of CYP17 *in vitro*. However, the 17,20-lyase activity has been shown to be influenced by two intracellular signalling proteins in a whole-cell milieu, namely protein kinase A (Zhang et al., 1995; Wang et al., 2010; Kempna et al., 2010) and rho-associated coiled-coil containing kinase 1 (ROCK1) (Tee et al., 2008). Differences between *in vitro* and *in vivo* results have led to conclusions such as the involvement of the ROCK/Rho signalling pathway upstream of the relevant kinase that phosphorylated CYP17 (Tee et al., 2008). This conclusion of Tee et al. (2008) was based on the observation that ROCK1 increases 17,20-lyase activity *in vivo*, but not *in vitro*, despite its capability to phosphorylate CYP17 *in vitro*. It is therefore unknown whether ROCK1 truly act upstream of the kinase that phosphorylates CYP17, or whether the observations of Tee et al. (2008) resulted from the experimental setup (*in vivo* vs. *in vitro*).

Interestingly, Rho proteins belong to a family of GTPase switch proteins that are involved in the transduction of various intracellular and extracellular signals, including vesicular trafficking, cytokinesis, cell migration and phagocytosis (Raftopoulou and Hall, 2004; Jaffe and Hall, 2005; Hall, 2005; Ridley, 2006). Li et al. (2010) investigated the role of Rho proteins in the regulation of steroidogenesis in terms of mitochondrial movement in H295R cells. The researchers observed an increase in active Rho proteins upon ACTH/cAMP stimulation, which acted via diaphanous-related homolog 1 (DIAPH1) to increase the mitochondrial movement and ultimately increase the cortisol production relative to DHEA production. They also observed an increase in mitochondrial movement and cortisol production under starvation conditions (cells devoid of serum). They proposed that ACTH signalling promotes interorganelle substrate delivery by stimulating the microtubule-dependent movement of mitochondria. Considering that the first (CYP11A1: cholesterol to pregnenolone) and last step (CYP11B: deoxycortisol to cortisol) in cortisol synthesis is occurs in the mitochondrion, while intermediary steps occur in the endoplasmic reticulum, the placement of mitochondria in close proximity of the endoplasmic reticulum will therefore aid in substrate delivery.

The mechanism of serine/threonine phosphorylation of CYP17 and the extent of it regulatory role in adrenal steroidogenesis remains poorly understood. In the study of Kempna et al. (2010), an interesting observation was made that CYP17 was phosphorylated exclusively at either threonine or serine residues when H295R cells were stimulated by cAMP or under starvation conditions (cells were devoid of serum), respectively. Serine phosphorylation of CYP17 under starvation conditions resulted in an increased 17,20-lyase activity. In contrast, the 17,20-lyase activity decreased upon threonine phosphorylation during the first three hours of cAMP stimulation. After 24 hours of

cAMP stimulation the *CYP17* gene expression increased markedly and consequently high levels of DHEA were detected. The researchers suggested that different signalling pathways may be involved in the differential phosphorylation of CYP17. If the observations from Li et al. (2010) are compared to the observations of Kempna et al. (2010), it would seem likely that short (0 – 3 hours) exposure to ACTH/cAMP stimulation involves the action of Rho-proteins via protein kinase A to phosphorylate CYP17 at threonine residues (via ROCK1) and facilitate mitochondrial movement to the endoplasmic reticulum (via DIAPH1), to ultimately increase the cortisol production relative to DHEA production. Further research in this field is, however, still required.

4.8 Physiological importance and CYP17 related disorders

The proper functioning of CYP17 is essential for the production of glucocorticoids and androgens. Various enzymatic defects in CYP17 activity has been reported since the first description of 17α-hydroxylase/17,20-lyase deficiency in 1966 by Biglieri et al. (Miller and Auchus, 2011). Computational modelling of human CYP17 allows for predictions of the effects that mutations may have (partial or complete) on one or both of these CYP17 activities (Auchus and Miller, 1999). These CYP17 defects are more commonly grouped according to 17α-hydroxylase deficiency, isolated 17,20-lyase deficiency and complete CYP17 deficiency, while the clinical manifestations are characterized by a deficiency in cortisol, but excessive secretion of precursor steroids.

A 17α-hydroxylase deficiency results in low cortisol production, overproduction of ACTH and the stimulation of steps to increase CYP17 activity. Patients experience mild symptoms of glucocorticoid deficiency, since corticosterone, which also displays glucocorticoid activity, is overproduced. This situation is thus similar to adrenal steroidogenesis in rats and mice, where CYP17 is not normally expressed (Voutilainen et al., 1986; Van Weerden et al., 1992; Pelletier et al., 2001). Furthermore 11-deoxycorticosterone is overproduced in the zona reticularis that causes sodium retention, hypertension and hypokalemia with suppressed plasma rennin activity and suppressed aldosterone production from the zona glomerulosa. Patient treatment with glucocorticoids results in the decrease of 11-deoxycorticosterone and increase in aldosterone production to normal levels (Scaroni et al., 1986).

Isolated 17,20-lyase deficiencies are very rare and are likely to result from impaired electron transport (Gupta et al., 2001; Geller et al., 1999). Patients have normal 17-hydroxycorticosteroids with reduced C19-steroids, which results in genital ambiguity (Geller et al., 1997) and/or failure to manifest adrenarche (Van den Akker et al., 2002).

Complete CYP17 deficiency is when the 17α-hydroxylase/17,20-lyase activity is absent and both adrenal and gonadal sex steroids cannot be synthesised. Consequently glucocorticoid deficiency symptoms accompany genital abnormalities (Miller and Auchus, 2011). Females are in general phenotypically normal, but do not undergo adrenarche and puberty (Biglieri et al., 1966). In males the external genitalia are absent or incompletely developed (New and Suvannakul, 1970).

Deficiency in 17α-hydroxylase/17,20-lyase is also a form of congenital adrenal hyperplasia, which is characterised by reduced or absent cortisol and adrenal androgen production (Patocs et al., 2005). Furthermore CYP17 dysfunction has been associated with numerous clinical conditions, including rheumatoid arthritis (Huang et al., 1999), endometrial cancer (McKean-Cowdin et al., 2001), polycystic ovary syndrome (Qin and Rosenfield, 1998; Strauss, 2003) and prostate cancer (Lunn et al., 1999). Interestingly a study of Blair and Mellon (2004) showed that mice, homozygous for the *CYP17* gene deletion, died by embryonic day 7. This suggests that the steroid products of CYP17 are essential for embryonic development in this species.

4.9 Conclusion

The CYP17 enzyme catalyses two distinct reactions on one single active site. However, its specific activities are independently regulated. The 17α-hydroxylase activity is relatively constant and regulated by gene expression, while the 17,20-lyase activity varies significantly and depends on the amount of cofactors present or by serine/threonine phosphorylation. ACTH/cAMP stimulation results in increases of CYP17 expression and serine/threonine phosphorylation to increase the glucocorticoid and androgen release from the adrenal gland. Functional expression of CYP17 in the adrenal cortex is essential for the production of cortisol. Various mutations in the CYP17 protein has been identified and can have mild to serious health implications. Two functional alleles for sheep CYP17 have been reported, but it is unknown whether their expressed proteins have different catalytic properties. These mutations add to the genetic variation in the functioning of the HPA axis. The implications of these mutations for cortisol production in the South African Merino will be investigated in the following chapter.

CHAPTER 5

In vitro comparison of the activities of two ovine CYP17 isoforms

5.1 Introduction

The preceding chapters provide a comprehensive discussion of the role of CYP17 in adrenal steroidogenesis. The aim of the current study was to compare the activities of the expressed proteins from the two *CYP17* alleles that were identified in the South African Merino population, namely *WT1* (Genbank accession nr. L40335) and *WT2* (Genbank accession nr. AF251388). The two nucleotide differences between these genetic sequences were originally thought to be an artefact of the polymerase chain reaction (PCR) (Swart et al., 2003), but Storbeck et al. (2008a) confirmed these sequences to be two distinct *CYP17* alleles (Storbeck et al., 2008a). The subsequent differences in the amino acid sequence of the CYP17 isoforms could ultimately result in differences in substrate affinity, reaction rate, interactions with POR and cytochrome b_5, or may result in no difference whatsoever. The catalytic activity of the CYP17 isoforms was therefore compared to investigate whether one isoform would produce more precursors of cortisol than the other isoform.

For the purpose of comparing the interaction between cytochrome b_5 and each CYP17 isoform, an experimental setup was required where the concentration of cytochrome b_5 could be controlled relative to the CYP17 expression. Furthermore, since the interaction between cytochrome b_5 and CYP17 is only possible if these two enzymes are expressed in the same membrane (endoplasmic reticulum), a whole cell milieu was suggested. The use of a COS-1 cell (immortalized African Green Monkey kidney cells) expression system was therefore proposed in the present study. Storbeck et al. (2007) successfully compared the activity of goat CYP17 isoforms, and their interactions with cytochrome b_5, by co-transfections of cytochrome b_5 and goat CYP17 plasmid constructs in COS-1 cells. Moreover, Goosen et al. (2010) showed that enzymes are transcribed at a similar level for co-transfections in COS-1 cells and that differences observed in steroid metabolism is due to catalytic differences, rather than inherent differences in the level of transcription and translation. This study provides evidence that it is not necessary to determine the level of enzyme expression with techniques such as Western blot analyses, which requires higher concentrations of recombinant proteins than the concentrations that are present in COS-1 cells (Goosen et al., 2010).

The effect of each amino acid difference could subsequently be investigated by establishing mutant plasmid constructs of CYP17 with site-directed mutagenesis. The activities of the CYP17 plasmid

constructs should also be assessed for all possible substrates of CYP17, namely pregnenolone, 17-hydroxypregnenolone, progesterone and 17-hydroxyprogesterone. The enzyme activity towards these substrates would show if one CYP17 isoform produces more cortisol precursors than the other.

The experimental approach of the present study was to first investigate the frequency distribution of the *CYP17* genotypes in a South African Merino population. For this purpose the genomic DNA from Merinos was subjected to the CYP17 genotyping test that was developed by Storbeck et al. (2008a). Secondly, the kinetic constants, V_{max} and K_m values, were determined for each CYP17 isoform with pregnenolone and progesterone as substrates. Thirdly, the activities of the CYP17 isoforms and mutant constructs were compared with time-dependent substrate conversions, by co-transfections with cytochrome b_5 in non-steroidogenic COS-1 cells. For this purpose the present study also includes the cloning of ovine cytochrome b_5 from liver tissue.

5.2 Materials and Methods

5.2.1 CYP17 genotyping with real-time PCR

Genomic DNA was isolated from blood of South African Merino sheep at the Elsenburg Research Farm (Elsenburg, Western Cape, South Africa) with the DNA isolation kit for mammalian blood (Roche, Mannheim, Germany). Blood was collected from the jugular vein of 145 adult sheep or from the heart chamber of 36 deceased lambs (lamb mortalities within 3 days postpartum of the 2008 lambing season). Genomic DNA was genotyped using the real-time PCR method developed by Storbeck et al. (2008a) for Angora goats, which was also shown to be a suitable *CYP17* genotyping test for Merino sheep (Storbeck et al., 2008a). The primers and hybridisation probes (TibMolBio, Berlin, Germany) were the same as previously reported (Storbeck et al., 2008a): LCLP, 5'-CCTGAAGGCCATACAAA-3'; LCRP, 5'-GGATACTGTCAGGGTGTG-3'; fluorescein-labelled *CYP17* sensor probe, 5'-TTCTGAGCAAGGAAATTCTGTTAGA-FL; LC640-labelled *CYP17* anchor probe, 640-TATTCCCTGCGCTGAAGGTGAGGA-3'. Real-time PCR was carried out using a LightCycler® 1.5 instrument. Amplification reactions (20µl) contained 2 µl LightCycler® FastStart DNA Master HybProbe Master Mix (Roche Applied Science, Mannheim, Germany), 3 mM MgCl2, 0.5 µM of each *CYP17* primer, 0.2 µM fluorescein-labelled *CYP17* sensor probe, 0.2 µM LC640-labelled *CYP17* anchor probe and 10 to 100 ng genomic DNA. Following an initial denaturation at 95°C for 10 min to activate the FastStart Taq DNA polymerase, the 35-cycle amplification profile consisted of heating to 95°C with a 8 s hold, cooling to 52°C with a 8 s hold and heating to 72°C with a 10 s hold. The transition rate between all steps was 20°C s^{-1}.

Data were acquired in single mode during the 52°C phase using LightCycler® software (version 3.5). Following amplification, melting-curve analysis was performed as follows: denaturation at 95°C with a 20 s hold, cooling to 40°C with a 20 s hold and heating at 0.2°C s^{-1} to 85°C with continuous data acquisition. The sensor probe had a one nucleotide mismatch with *WT1* and two nucleotide mismatches with *WT2*, which enabled genotyping with melting curve analysis as primer-probes dissociated at 58°C and 54°C, respectively. A no-template control (negative control) was included in each assay.

5.2.2 Cloning of ovine cytochrome b_5

Liver tissue was obtained from Merino sheep at a local abattoir, flash frozen and stored in liquid nitrogen. Total RNA was isolated from the liver homogenate using TRI Reagent (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. The cDNA was prepared from the isolated RNA using AMV Reverse Transcriptase and Oligo (dT) primers according to the manufacturer's instructions (Promega, Madison, WI, USA). Ovine cytochrome b_5 cDNA was amplified using PWO DNA Polymerase (Roche Applied Science, Mannheim, Germany) and subjected to sequence analysis. PCR amplification of cDNA consisted of 37 cycles of repeated denaturation (40 s at 94°C), annealing (45 s at 52°C), and enzymatic chain extension (1 min at 72°C). The primers used for amplification were: CYB5TOPO (Sense) 5′-CACCCTCGCTGAGTTAAGAAATG-3′ and CYB5RP (Antisense) 5′-CTCCCTGGACCAAAGCAG-3′. The PCR amplicon was cloned into a pcDNA3.2/V5/GW/D-TOPO® mammalian expression vector according to the manufacturer's instructions. Plasmid constructs were screened by restriction digest analyses with Acs I (Roche Applied Science, Mannheim, Germany) and positive clones were subsequently subjected to direct sequence analysis. Direct sequence analyses were performed on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, California) by the Central Analytical Facility of Stellenbosch University. Sequencing results were analysed with BioEdit Sequence Alignment Editor (version 7.0.5.2 © 1997-2007, T. Hall) software.

5.2.3 Site-directed mutagenesis

Site-directed mutagenesis was performed using the Genetailor site-directed mutagenesis kit (Invitrogen, Carlsbad, California, USA). The mammalian expression vector containing the *WT2* cDNA insert was used as template. The primers that were used to construct mutant G201S were LP210 (5'-TCCCGGCTGCTGCAGAGGTTCTACCTGGAGAT-3') and RP210 (5'-GAACCTCTGCAGCAGCCGGGACATGAAGAG-3'); while the primers used to construct mutant

N464Y were LP464 (5'-GATGGCATCCTGGAGGTTCTGAGCAAGGAAGT-3') and RP464 (5'-CAGAACCTCCAGGATGCCATCATTGACATT-3'). The integrity of the mutant constructs G210S and N464Y were confirmed with direct sequence analyses (ABI Prism 3100 Genetic Analyzer, Applied Biosystems, Foster City, California). All plasmid constructs consisted of the same type of mammalian expression vector (pcDNA3.2/V5/GW/D-TOPO®, Roche Applied Science, Mannheim, Germany).

5.2.4 Enzyme activity assay of ovine CYP17 isoforms in COS-1 cells

COS-1 cells were cultured at 37ºC and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM), containing 4 mM L-glutamine and 25 mM glucose (Sigma-Aldrich, St. Louis, MO, USA), and supplemented with 10% foetal calf serum, 1% penicillin-streptomycin (Gibco-Invitrogen, Grand Island, NY, USA) and 0.12% $NaHCO_3$. COS-1 cells were seeded 24 hours prior to transfection into 12-well dishes at a concentration of 1 x 10^5 cells mL^{-1}. The CYP17 (previously cloned by and obtained from Swart et al., 2003, and Storbeck et al., 2008a) and cytochrome b_5 plasmid constructs were transiently transfected into COS-1 cells, using Mirus TransIT®-LT1 transfection reagent (Mirus Bio Corporation, Madison, WI, USA) according to manufacturer's instructions. All transfection experiments were conducted in triplicate. Where the activities of the two CYP17 isoforms were compared, the same transfection combinations for both CYP17 plasmid constructs were done in one experiment. All transfections were performed using a total of 0.5 µg plasmid DNA. In co-transfections of CYP17 and cytochrome b_5, the total plasmid DNA content was kept constant by transfection with 0.25 µg CYP17 vector and 0.25 µg cytochrome b_5 or mammalian expression vector pCI-neo (Promega, Madison, Wisconsin), containing no insert. Positive control transfection reactions for ovine CYP17 and cytochrome b_5 were performed using Angora goat *CYP17* (GenBank accession nr. EF524063) and Angora goat cytochrome b_5 (GenBank accession nr. EF524066), respectively. Negative control transfection reactions were performed using pCI-neo (Promega, Madison, Wisconsin) containing no insert. Enzyme activities were assayed 72 h after transfection, using pregnenolone (1 µM), 17-hydroxypregnenolone (0.5 µM) or progesterone (1 µM) as substrates and 500 µL samples were collected over a 7 hour period for analysis by ultra-performance liquid chromatography (UPLC). In the determination of kinetic constants, [7-^{3}H]-pregnenolone or [1,2,6,7-^{3}H]-progesterone (PerkinElmer Life Sciences, Boston, MA, USA) was added to the varying substrate concentrations (0.25 to 12 µM for each substrate) and 50 µL samples were collected at specific time intervals for analysis by high performance liquid chromatography (HPLC).

On completion of each enzyme assay, the cells were washed with and collected in 0.1 M phosphate buffer, pH 7.4. The cells were subsequently disrupted using a Heat Systems W-10 Sonicator™ (Ultrasonics Inc, Plainview, NY, USA) and the protein concentration of the cell lysate was determined by the BCA method (Pierce Chemical, Rockford, Illinois) according to the manufacturer's instructions.

5.2.5 Liquid-liquid extraction of steroid metabolites

Steroid metabolites were extracted from the culture media by liquid-liquid extraction, using a 10:1 volume of dichloromethane to culture medium. Samples were vortexed for 20 min and centrifuged at 500 x g for 5 min. The water phase was aspirated and the organic phase transferred to a clean glass tube. The organic phase was evaporated under nitrogen gas and the steroids were redissolved in 100 µL methanol.

5.2.6 Steroid separation and quantification by HPLC

Chromatography was performed on a SpectraSYSTEM P4000 high performance liquid chromatograph (Thermo Separation™ Products, San Jose, CA, USA) coupled to a SpectraSYSTEM AS3000 automatic injector (Thermo Separation™ Products, San Jose, CA, USA) and a Flo-One liquid scintillation spectrophotometer (Radiomatic, Tampa, FL, USA). The ratio of scintillant to column effluent was 3:1. Steroid metabolites were separated on a Phenomenex C_{12} (60 mm×150 mm, 4 µm) column at a flow rate of 1 mL min^{-1}. The mobile phases for pregnenolone separation from the other Δ^5 steroid metabolites consisted of solvent A (65% methanol: 35% water) and solvent B (methanol). An isocratic flow consisting of 100 % A for 2 minutes was applied, followed by a 9 minute linear gradient to 100% B and isocratic elution with 100 % solvent B for 1 min. A 3 minute linear gradient returned the column to 100% A. The total run time per sample was 16 minutes and the injection volume was 90 µL. The mobile phases for progesterone separation from the other Δ^4 steroid metabolites consisted of solvent A (75% methanol: 25% water) and solvent B (methanol). An isocratic flow of 100% A was applied for 2 minutes, followed by a 9 minute linear gradient to 100% solvent B and isocratic elution with 100% solvent B for 1 minute. A 3 minute linear gradient returned the column to 100% solvent A. The total run time per sample was 14 minutes and the injection volume was 90 µL.

5.2.7 UPLC quantification of steroids

The UPLC-coupled atmospheric pressure chemical ionization (APCI) mass spectrometry (MS) assay was developed by Storbeck et al. (2008b). Chromatography was performed on an ACQUITY ultra performance liquid chromatograph (Waters, Milford, MA, USA). The substrates pregnenolone and progesterone were separated from their respective metabolites on a Waters UPLC BEH C18 (2.1 mm x 50 mm, 1.7 µm) column at 50°C flow rate of 0.4 ml min^{-1} as previously described (Storbeck et al., 2008b). The UPLC was connected to an API Quattro Micro tandem mass spectrometer (Waters, Milford, USA) as previously described (Storbeck et al., 2008b).

5.2.8 Statistical analysis

GraphPad Prism (version 5) software (GraphPad Software, San Diego, California) was used to analyse data. The frequency distribution of the *CYP17* genotype in two populations was compared with Fischer's exact t-test. Steroid concentrations were calculated from the percentage of total steroid detected per sample. Steroid metabolism assays were analysed with two-tailed paired t-tests for each steroid metabolite over time with either the CYP17 isoform or cytochrome b_5 as factor (e.g. pregnenolone concentrations for WT1 vs. WT2 over time in the absence of cytochrome b_5). Kinetic constants (K_m and V values) were determined by non-linear regression (GraphPad Software, San Diego, California) and direct linear plots (Eisenthal and Cornish-Bowden, 1974). Comparisons of substrate conversion for CYP17 isoform and mutant constructs were analysed with one-way ANOVA and Bonferroni's multi-comparison post-test for each substrate, respectively. Graphs represent the mean and standard error of the mean as calculated from triplicate experiments. A 95% confidence interval was used in all cases to determine statistical significance.

5.3 Results

5.3.1 Cloning of cytochrome b_5 *cloning*

Cytochrome b_5 is expressed in the liver and adrenal gland (as well as in other tissues) as a membrane-bound hemoprotein (Miller and Auchus, 2011). The reverse transcription PCR of cytochrome b_5 mRNA from sheep liver yielded a single 465 bp product that was subsequently sequenced and cloned. Sequence alignment of ovine cytochrome b_5 (*Ovis aries*, Genbank accession no. GQ471028) with Angora goat cytochrome b_5 (*Capra hircus*, Genbank accession no. EF524066) showed one nucleotide difference in the open reading frame at nucleotide position 177 (99.7% sequence similarity). However, both GTC and GCC codons of sheep and goat cytochrome b_5,

respectively, translated to alanine at residue position 59 in the predicted 134 residue protein (100 % identity).

5.3.2 Ovine CYP17 sequence alignment and genotyping

The two CYP17 isoform sequences have previously been cloned and inserted into a suitable mammalian expression vector (Swart et al., 2003; Storbeck et al., 2008a). A relative DNA copy number determination by Storbeck et al. (2008a) indicated that a single *CYP17* gene is present in South African Merinos, and that the two sequences - independently deposited on Genbank - are in fact two alleles of this gene and not a PCR artefact (Storbeck et al., 2008a). Sequence alignment of *WT1* and *WT2* cDNA sequences showed two nucleotide differences in the 1530 bp open reading frame (99.8 % sequence similarity), resulting in two amino acid differences in the predicted 509 residue protein (99.6 % homologous). The first single nucleotide polymorphism (SNP) at position 628 of *WT1* encoded for codon AGC that translated to S210, while codon GGC of *WT2* translated to G210. The second SNP at position 1390 of *WT1* encoded for codon TAC that translated to Y464, while codon AAC of *WT2* translated to N464.

The two SNP differences permitted allelic discrimination with melting curve analysis, from real-time PCR and hybridization probes, at nucleotide position 628. A melting curve peak at 58 ^{o}C indicated the presence of the *WT1* allele, whereas a melting curve peak at 54 ^{o}C indicated the presence of the *WT2* allele as shown in Figure 5.1. The accuracy of the genotyping test was confirmed with direct sequence analysis. Interestingly, no homozygous *WT2* sheep were detected. The possibility of a lethal homozygous *WT2* genotype was investigated by genotyping the genomic DNA samples from blood of early lamb mortalities (n = 36) during the 2008 lambing season. However, no individual sheep with a homozygous *WT2* genotype was detected in this group either.

The *CYP17* frequency distribution in 209 adult sheep was found to be 85.6 % heterozygous *WT1*/*WT2* and 14.4 % homozygous *WT1*/*WT1*. The *CYP17* frequency distribution was the same among adult sheep and early lamb mortalities ($P > 0.05$).

5.3.3 Kinetic analysis of ovine CYP17 isoforms

The activities of both CYP17 isoforms, expressed in COS-1 cells, were assayed with pregnenolone and progesterone as substrates. Pregnenolone was converted to 17-hydroxypregnenolone and DHEA, whereas progesterone was converted to 17-hydroxyprogesterone and low levels of 16-

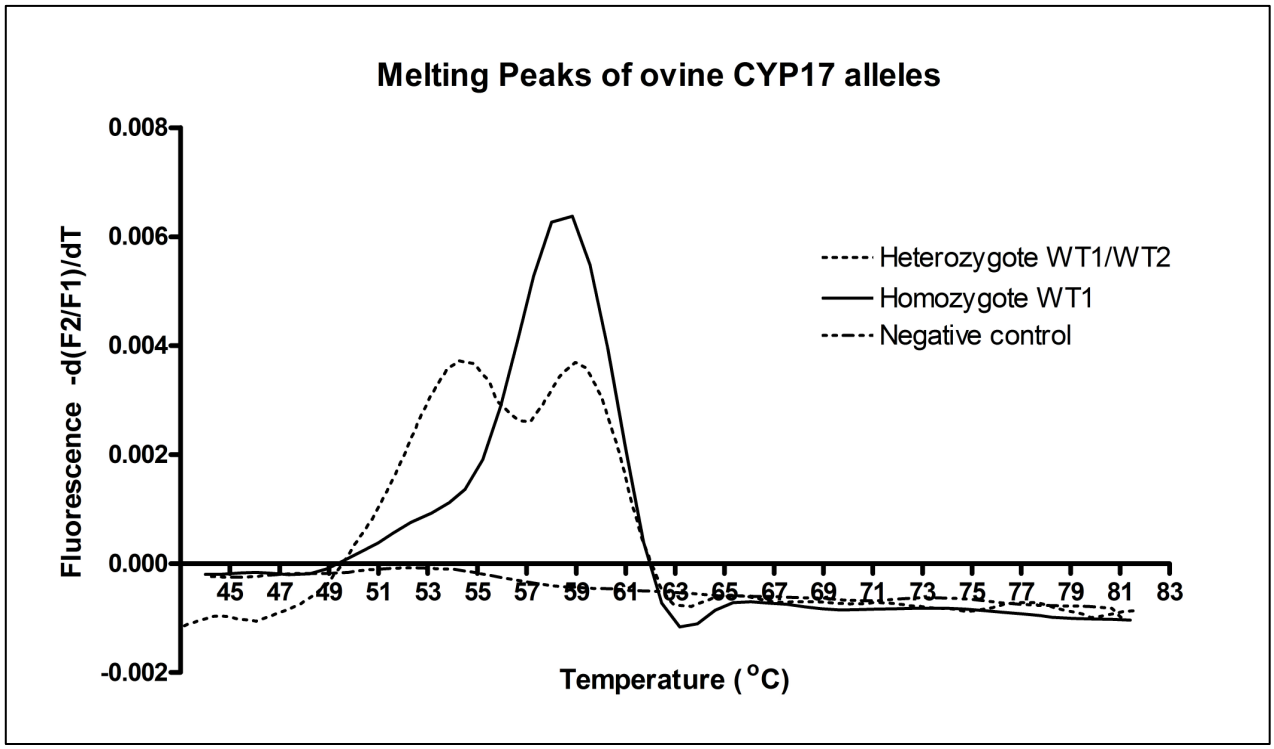

Figure 5.1. Typical melting curve analysis from real time PCR with hybridization probes. A peak at 58 °C indicates the presence of the *WT1* allele, whereas a peak at 54 °C indicates the presence of the *WT2* allele in a DNA sample.

hydroxyprogesterone and androstenedione (< 2%) over 7 hours. This is the first report that ovine CYP17 is able to mediate the 16α-hydroxylation of progesterone.

K_m and V values for each CYP17 isoform were determined for both pregnenolone and progesterone metabolism by non-linear regression (Table 5.1). These results coincided with the results obtained with the direct linear plot method (Eisenthal and Cornish-Bowden, 1974). No significant difference was observed between the CYP17 isoforms for either the K_m or V values (WT1 vs. WT2) for both pregnenolone and progesterone metabolism ($P > 0.05$).

Table 5.1. Summary of kinetic constant determinations of pregnenolone and progesterone metabolism. Initial reaction rates were determined by linear regression from at least four time points for each substrate concentration. K_m (μM) and V values are expressed as mean ± SEM of three replicate experiments. The R^2-values of all linear and non-linear regression lines were at least 0.95 and 0.98, respectively. A two-tailed unpaired t-test was done to compare the kinetic constants for each steroid substrate.

	Pregnenolone		Progesterone	
	K_m (μM)	$V^{\dagger}$	K_m (μM)	$V^{\dagger}$
WT1	1.7 ± 0.1	27.8 ± 0.8	1.6 ± 0.1	41.7 ± 1.4
WT2	1.9 ± 0.2	28.1 ± 0.8	1.8 ± 0.2	44.0 ± 2.3

†(nmol.h^{-1}.mg total protein^{-1})

The difference in pregnenolone metabolism over 7 hours between the two CYP17 isoforms in the absence of cytochrome b_5 is depicted in Figure 5.2. WT1 converted pregnenolone to 17-hydroxypregnenolone at a higher rate ($P < 0.05$) than WT2. Conversion of pregnenolone reached 50% after ~3 h for WT1 and ~3 h 40 min for WT2. Since the same amount of DHEA was produced for both CYP17 isoforms ($P > 0.05$) and more 17-hydroxypregnenolone was produced by WT1 ($P < 0.01$), WT1 would produce more cortisol precursors than WT2 in the absence of cytochrome b_5.

The difference in pregnenolone metabolism between the two CYP17 isoforms in the presence of cytochrome b_5 is depicted in Figure 5.3. WT1 converted pregnenolone to 17-hydroxypregnenolone at a higher rate ($P < 0.01$) than WT2. Conversion of pregnenolone was at 50% after ~2 h 8 min for WT1 and ~2 h 15 min for WT2. However, WT1 also produced more DHEA ($P < 0.05$) than WT2. As a result, the CYP17 isoforms produced the same amount of 17-hydroxypregnenolone ($P > 0.05$) available for cortisol production. 17-hydroxypregnenolone concentrations reached a plateau after 3 h at 25-36%, where its production from pregnenolone was matched by its conversion to DHEA.

The difference in 17-hydroxypregnenolone metabolism between the two CYP17 isoforms was investigated at 0.5 µM, since this concentration is closer to the plateau concentration observed in pregnenolone metabolism than 1 µM. In the absence of cytochrome b_5 (Figure 5.4), WT1 converted 17-hydroxypregnenolone at a higher rate ($P < 0.05$) than WT2. Conversion of 17-hydroxypregnenolone reached 50% after ~2 h 38 min for WT1 and after ~3 h 37 min for WT2. In the presence of cytochrome b_5 (Figure 5.5), however, there was no difference ($P > 0.05$) in 17-hydroxypregnenolone metabolism, where both CYP17 isoforms reached 50% conversion after ~1 h 20 min. Cytochrome b_5 thus enhanced the 17,20-lyase activity ($P < 0.05$) to such an extent that no difference was observed between the CYP17 isoforms.

The difference in progesterone metabolism between the two CYP17 isoforms in the absence of cytochrome b_5 is depicted in Figure 5.6. WT1 converted progesterone at a higher rate ($P < 0.01$) than WT2, where 50 % progesterone conversion was reached after ~1 h 30 min for WT1 as compared to ~2 h 20 min for WT2. More 17-hydroxyprogesterone ($P < 0.01$) was produced by WT1. The increased 17-hydroxyprogesterone produced by WT1 would potentially be available for cortisol production, which indicated that the presence of this isoform would lead to higher cortisol production than would be the case for WT2. Overall, very little androstenedione production was observed (< 3 %), as was also reported by Swart et al. (2003). However, statistical analysis still showed that more androstenedione ($P < 0.05$) was produced in the presence of WT1 than WT2. An equal amount of 16-hydroxyprogesterone ($P > 0.05$) was produced by both CYP17 isoforms.

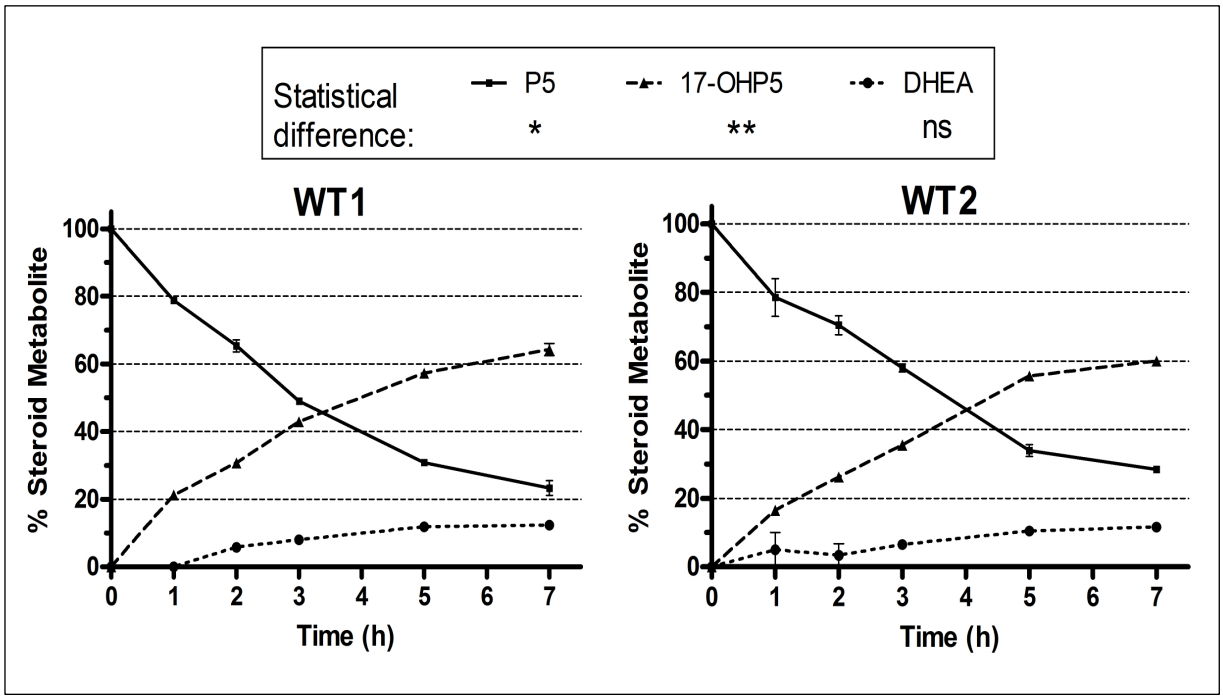

Figure 5.2. Conversion of 1 µM pregnenolone over 7 hours by WT1 (left) and WT2 (right), expressed in COS-1 cells in the absence of cytochrome b_5. A two-tailed paired t-test was done for each steroid metabolite (e.g. pregnenolone concentrations over time for WT1 vs. WT2). ns: P-value > 0.05; *P-value < 0.05; **P-value < 0.01; P5: pregnenolone; 17-OHP5: 17-hydroxypregnenolone; DHEA: dehydroepiandrosterone.

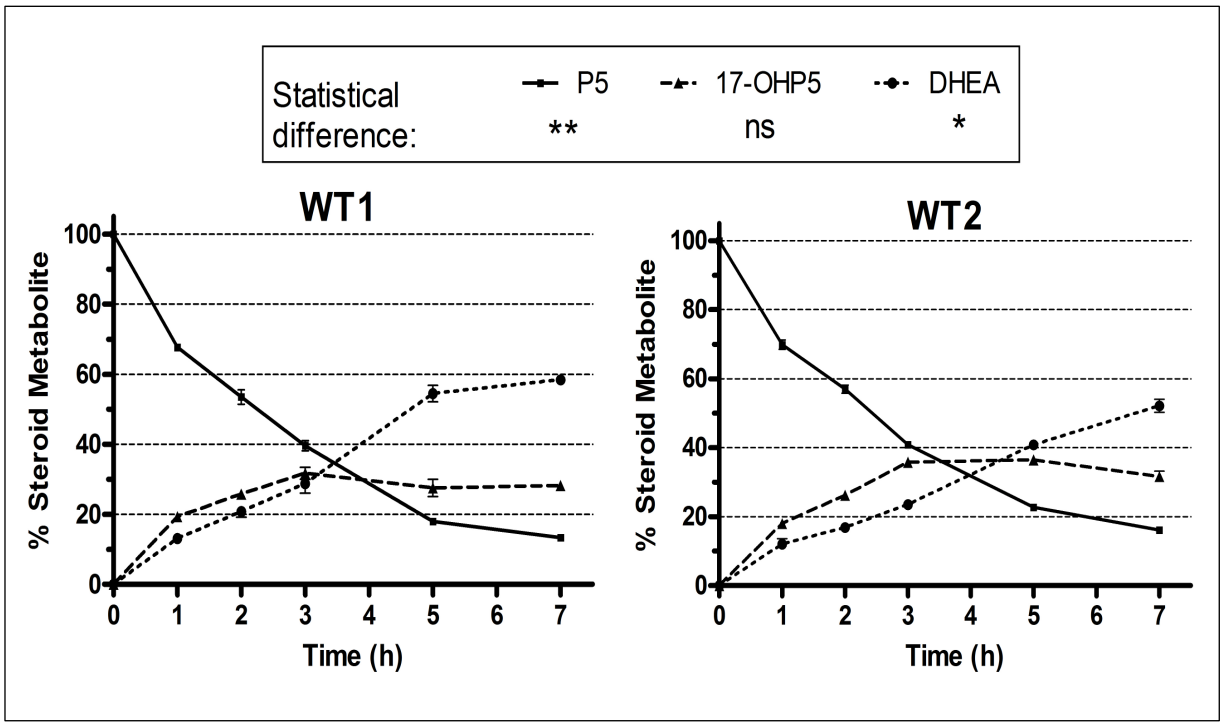

Figure 5.3. Conversion of 1 µM pregnenolone over 7 hours by WT1 (left) and WT2 (right), expressed in COS-1 cells in the presence of cytochrome b_5. A two-tailed paired t-test was done for each steroid metabolite. ns: P-value > 0.05; *P-value < 0.05; **P-value < 0.01; P5: pregnenolone; 17-OHP5: 17-hydroxypregnenolone; DHEA: dehydroepiandrosterone.

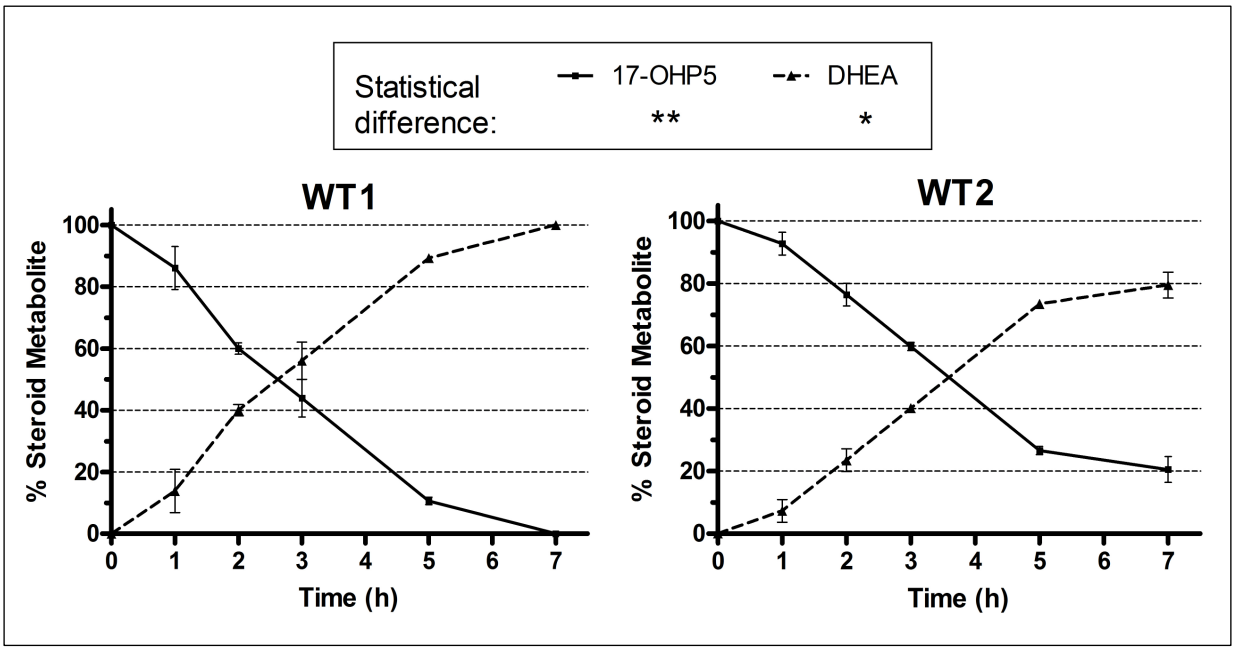

Figure 5.4. Conversion of 0.5 µM 17-hydroxypregnenolone over 7 hours by WT1 (left) and WT2 (right), expressed in COS-1 cells in the absence of cytochrome b_5. A two-tailed paired t-test was done for each steroid metabolite. *P-value < 0.05; **P-value < 0.01; 17-OHP5: 17-hydroxypregnenolone; DHEA: dehydroepiandrosterone.

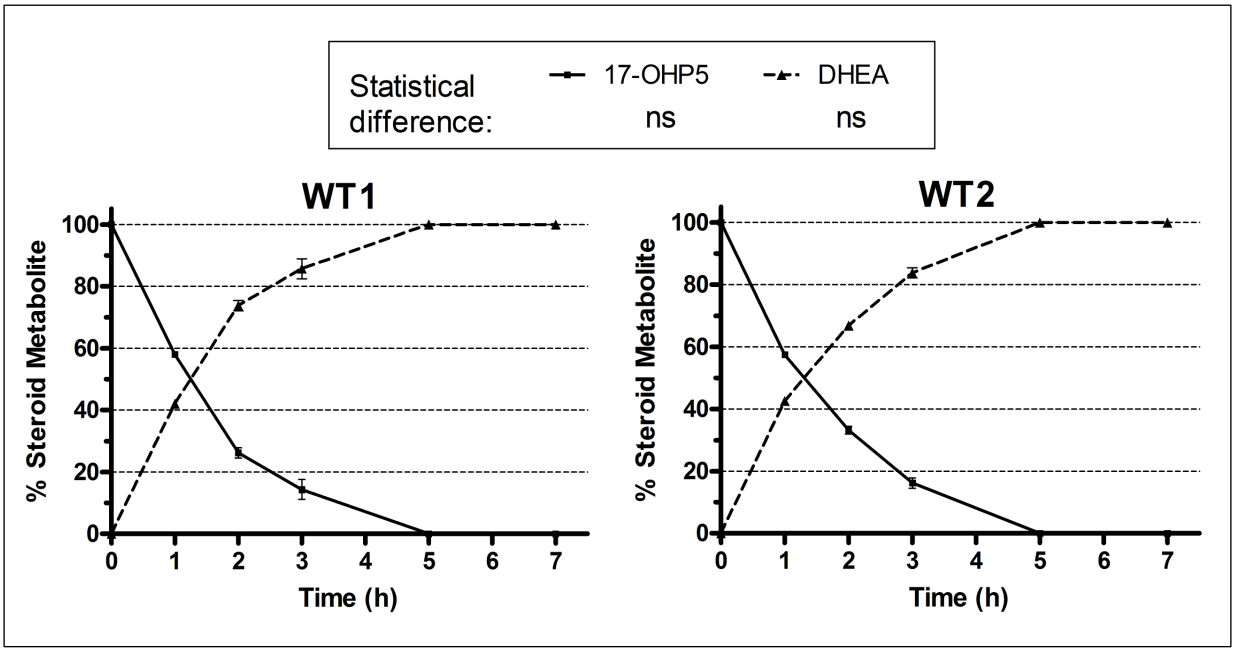

Figure 5.5. Conversion of 0.5 µM 17-hydroxypregnenolone over 7 hours by WT1 (left) and WT2 (right), expressed in COS-1 cells in the presence of cytochrome b_5. A two-tailed paired t-test was done for each steroid metabolite. ns: P-value > 0.05; 17-OHP5: 17-hydroxypregnenolone; DHEA: dehydroepiandrosterone.

Cytochrome b_5 enhanced the production of androstenedione ($P < 0.05$) and 16-hydroxyprogesterone ($P < 0.001$) for both CYP17 isoforms (Figure 5.7). As a result, progesterone conversion was the same for both CYP17 isoforms, where 50% progesterone conversion was reached after ~1 h in both instances. WT1 produced more 17-hydroxyprogesterone ($P < 0.01$), which would potentially be available for cortisol production than WT2. An equal quantity of androstenedione ($P > 0.05$) was

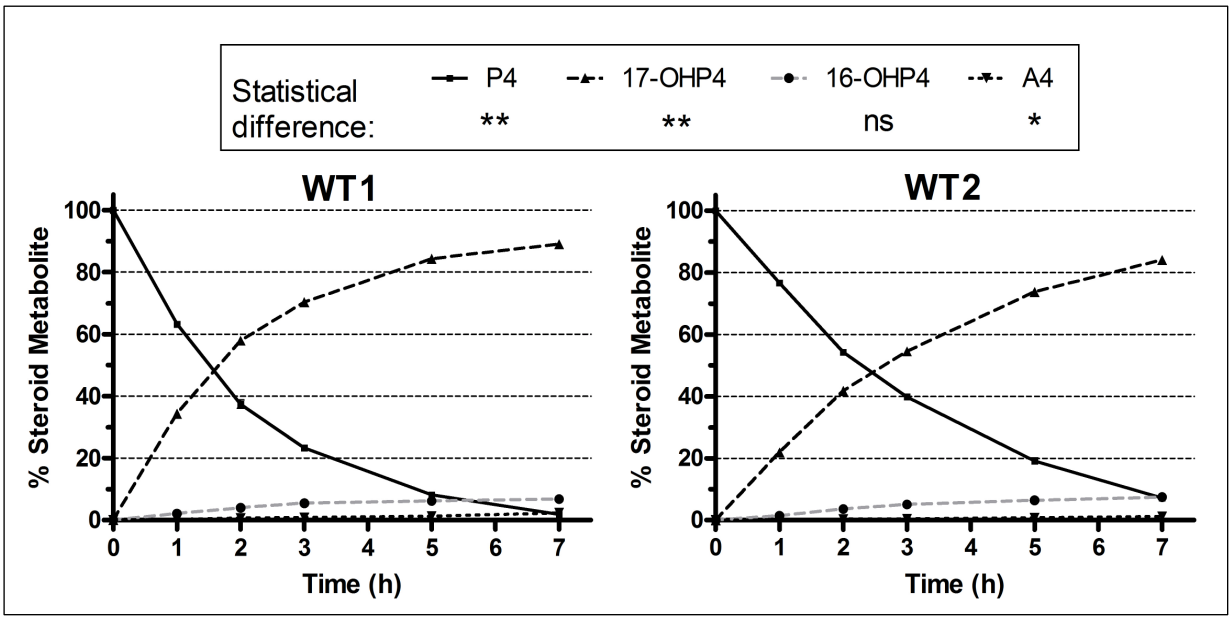

Figure 5.6. Conversion of 1 µM progesterone over 7 hours by WT1 (left) and WT2 (right), expressed in COS-1 cells in the absence of cytochrome b_5. A two-tailed paired t-test was done for each steroid metabolite. ns: P-value > 0.05; *P-value < 0.05; **P-value < 0.01; P4: progesterone; 17-OHP4: 17-hydroxyprogesterone; 16-OHP4: 16-hydroxyprogesterone; A4: androstenedione.

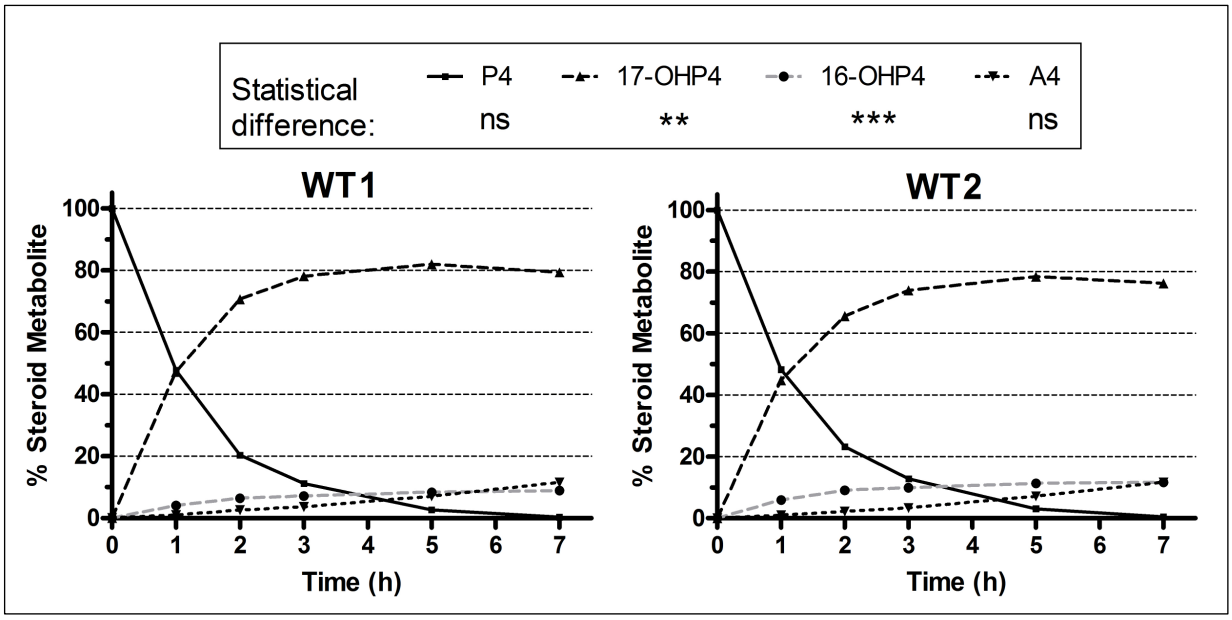

Figure 5.7. Conversion of 1 µM progesterone over 7 hours by WT1 (left) and WT2 (right), expressed in COS-1 cells in the presence of cytochrome *b*5. A two-tailed paired t-test was done for each steroid metabolite. ns: P-value > 0.05; **P-value < 0.01; ***P-value < 0.001; P4: progesterone; 17-OHP4: 17-hydroxyprogesterone; 16-OHP4: 16-hydroxyprogesterone; A4: androstenedione.

produced for both CYP17 isoforms, while WT2 produced more 16-hydroxyprogesterone ($P < 0.01$) than WT1.

Interestingly, the 17-hydroxyprogesterone/16-hydroxyprogesterone-ratio of WT1 was higher than WT2 in both the presence (WT1: 40.8 ± 0.6 vs. WT2: 33.7 ± 1.0) and absence (WT1: 29.4 ± 0.7 vs. WT2: 20.7 ± 0.3) of cytochrome b_5 ($P < 0.01$). In other words, given the same amount of

progesterone, WT1 would produce more 17-hydroxyprogesterone (precursor of cortisol) and less 16-hydroxyprogesterone (not a precursor of cortisol) than WT2.

In addition, the ratio of 17-hydroxypregnenolone/DHEA was 16- to 21-fold higher than the 17-hydroxyprogesterone/androstenedione ratio. This gives an indication that androgen synthesis is primarily mediated by the 17,20-lyase activity through the Δ^5 pathway rather than the Δ^4 pathway in this species, an observation consistent with previous studies (Mason et al., 1989; Auchus et al., 1998; Lee-Robichaud, 1995, Swart et al., 2003; Miller and Auchus, 2011). The removal of 17-hydroxypregnenolone to DHEA by CYP17 is therefore a more important consideration for cortisol production relative to the removal of 17-hydroxyprogesterone to androstenedione by CYP17 in the Δ^4 pathway (Miller and Auchus, 2011).

5.3.4 Site-directed mutagenesis

The effect of amino acid substitutions on the activity of the two CYP17 isoforms was investigated by means of expression in COS-1 cells. Figure 5.8 depicts the results of pregnenolone metabolism, where no significant difference ($P > 0.05$) was observed in the absence (Figure 5.8A) or presence (Figure 5.8B) of cytochrome b_5 between the CYP17 isoforms and mutant constructs. After four

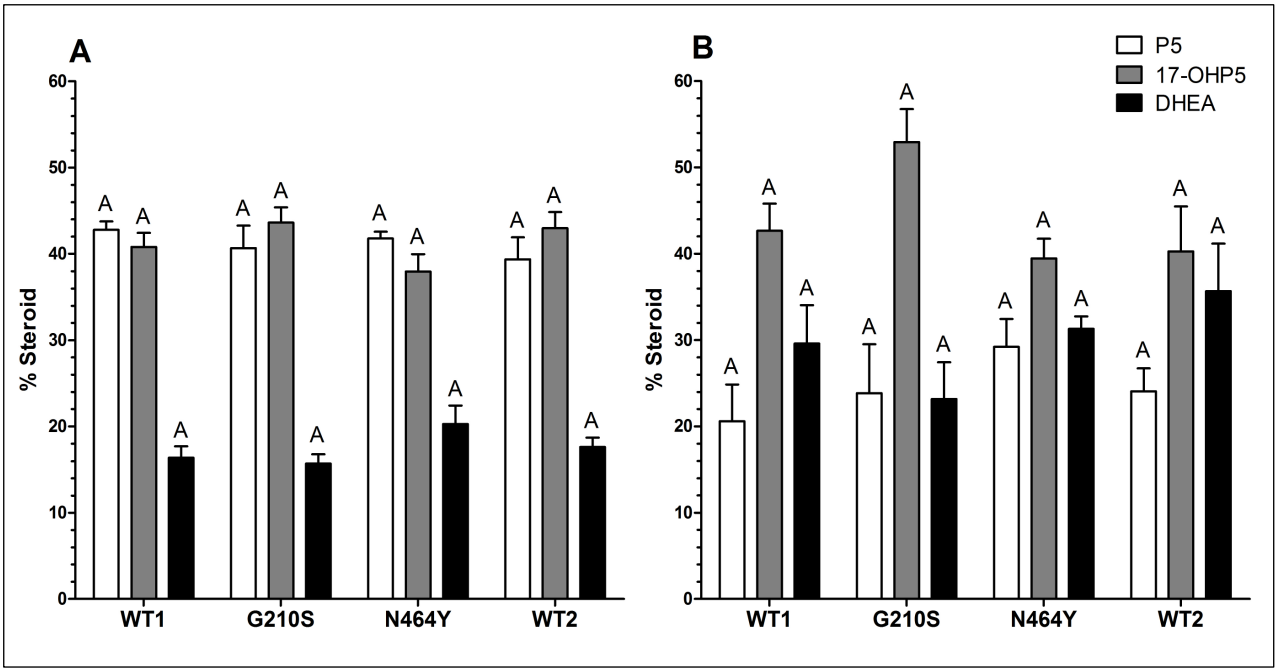

Figure 5.8. Conversion of 1 µM pregnenolone after 4 hours in the absence (A) or presence (B) of cytochrome b_5 by CYP17 isoforms (WT1 and WT2) and mutant constructs (G210S and N464Y). A one-way ANOVA was done for each steroid metabolite with Bonferroni's multiple comparison post-test. P5: pregnenolone; 17-OHP5: 17-hydroxypregnenolone; DHEA: dehydroepiandrosterone.

hours, approximately 59 % pregnenolone was converted to ~41 % 17-hydroxypregnenolone and ~8 % DHEA.

After four hours, 17-hydroxypregnenolone metabolism in the absence of cytochrome b_5 (Figure 5.9A) was higher for WT1 (~62 %; $P < 0.05$) than for mutant construct G210S (~44 %), while no significant difference was observed compared to WT2 (~56 %) and N464Y (~46 %). In the presence of cytochrome b_5 (Figure 5.9B), 17-hydroxypregnenolone metabolism after three hours was the same ($P > 0.05$) for all constructs (~68 %).

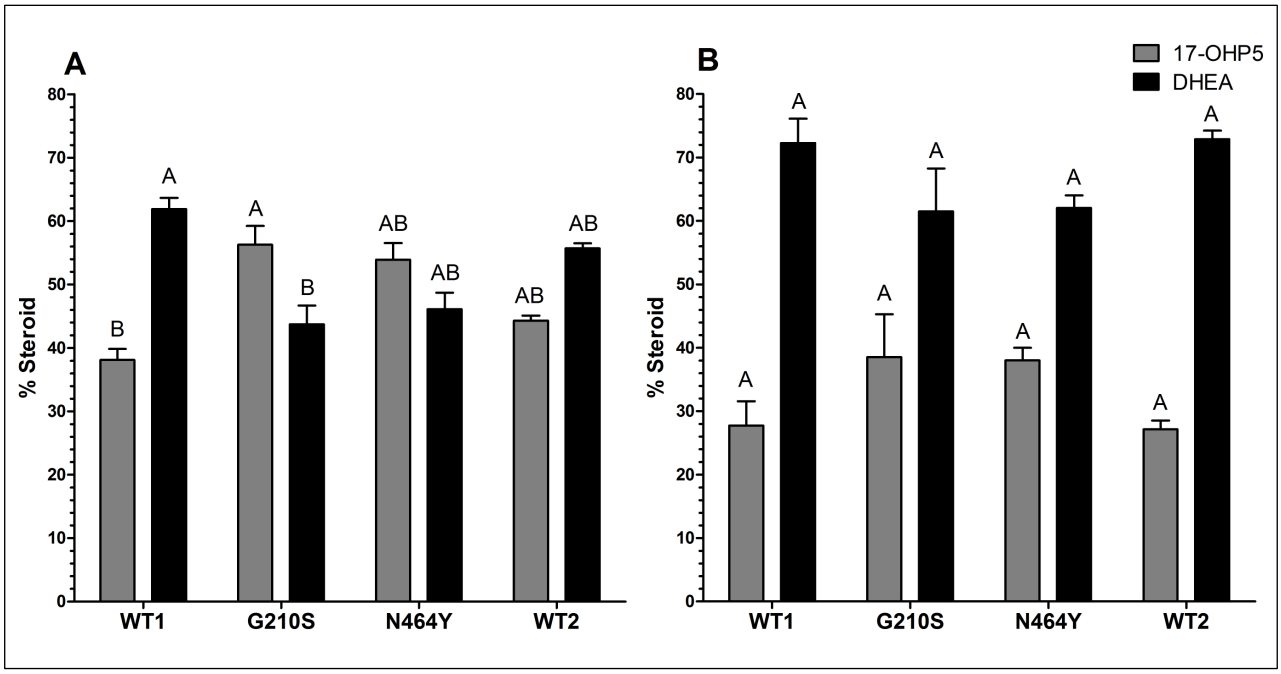

Figure 5.9. Conversion of 1 µM 17-hydroxypregnenolone after 4 hours in the absence (A) or 3 hours in the presence (B) of cytochrome b_5 by CYP17 isoforms (WT1 and WT2) and mutant constructs (G210S and N464Y). A one-way ANOVA was done for each steroid metabolite with Bonferroni's multiple comparison post-test. 17-OHP5: 17-hydroxypregnenolone; DHEA: dehydroepiandrosterone.

After two hours, approximately 60 % of the progesterone was converted to ~57 % 17-hydroxyprogesterone and small quantities (< 2 %) of androstenedione and 16-hydroxyprogesterone in the absence of cytochrome b_5 (Figure 5.10A). Progesterone metabolism was the same ($P > 0.05$) for all constructs, with the exception in 16-hydroxyprogesterone production, where WT1 produced larger quantities ($P < 0.01$) of 16-hydroxyprogesterone (~2.0 %) than WT2 (~1.2 %) and N464Y (~1.4 %). In the presence of cytochrome b_5, the production of 17-hydroxyprogesterone (Figure 5.10B) was the same ($P > 0.05$) for all constructs (~56 %), while these constructs showed distinct differences ($P < 0.01$) in the remainder of progesterone metabolism. After one hour, WT2 metabolized less progesterone (~63 %; $P < 0.05$) than WT1 (~68 %) and N464Y (~68 %), while G210S metabolized intermediary amounts of progesterone (~66 %). However, equal amounts of 17-

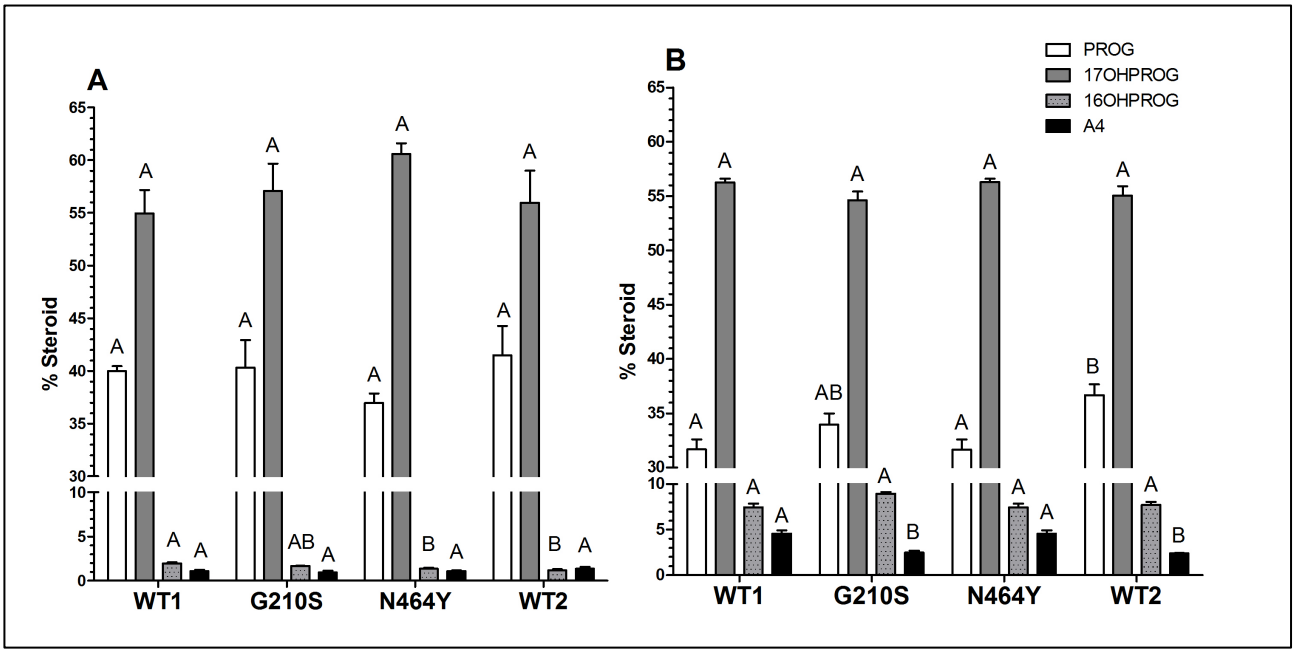

Figure 5.10. Conversion of 1 µM progesterone after 2 hours in the absence (A) or after 1 hour in the presence (B) of cytochrome b_5 by CYP17 isoforms (WT1 and WT2) and mutant constructs (G210S and N464Y). A one-way ANOVA was done for each steroid metabolite with Bonferroni's multiple comparison post-test. P4: progesterone; 17-OHP4: 17-hydroxyprogesterone; 16-OHP4: 16-hydroxyprogesterone; A4: Androstenedione.

hydroxyprogesterone (~56 %) and 16-hydroxyprogesterone (~8 %) were produced (P <0.001). Furthermore, the production of androstenedione by WT1 and N464Y (~4.6 %) was higher (P < 0.0001) compared to WT2 and G210S (~2.4 %).

From the results of site-directed mutagenesis, it can be concluded that both amino acid differences of WT1 and WT2 are collectively responsible for the catalytic differences or similarities observed between these two CYP17 isoforms.

5.4 Discussion

The *CYP17* genotyping method described in this chapter was developed by Storbeck et al. (2008) to establish the *CYP17* genotypes of Angora and Boer goats (Storbeck et al., 2008a). This study showed that the method was also suitable for ovine *CYP17* genotyping, since it was able to detect heterozygous *WT1*/*WT2* sheep. Storbeck et al. (2008a) reported that the two genetic sequences of CYP17 deposited on Genbank are not PCR artefacts, but two alleles of one *CYP17* gene as confirmed with a relative DNA copy number determination. In contrast the Angora and Boer goats had two *CYP17* alleles, but three genotypes because of a duplication of the *CYP17* gene. In the present study the same *CYP17* genotyping method was used to genotype 145 genomic DNA samples from South African Merinos. Only two *CYP17* genotypes were identified, namely homozygous *WT1/WT1* and heterozygous *WT1*/*WT2* sheep, but no homozygous *WT2* sheep. The possibility of a lethal homozygous *WT2* genotype was investigated by genotyping DNA from lambs

that died within 3 days of parturition. No homozygous *WT2* genotypes were detected in this group either. It is possible that this genotype is lethal at earlier stages of development, or that there is a genetic anomaly in the *CYP17* gene that was not detected in the relative copy number determination from the small sample (less than five sheep) studied by Storbeck et al. (2008a). Another consideration is to develop a *CYP17* genotyping test that is specific for ovine *CYP17*, which may be more sensitive than the current method (one or two nucleotide mismatches for ovine species) that was originally designed for caprine species (perfect nucleotide match or one mismatch). However, the accuracy of the current method was confirmed with direct sequence analysis and genotyping of plasmid vectors containing the appropriate cDNA insert (data not shown).

Expression of both CYP17 isoforms in COS-1 cells showed that ovine CYP17 is capable of mediating the 17α-hydroxylation of pregnenolone and progesterone; the 16α-hydroxylation of progesterone; and cleave the C-17/C-20 bond of 17-hydroxypregnenolone and 17-hydroxyprogesterone in the absence of cytochrome b_5. Determination of kinetic constants revealed that ovine CYP17 has similar affinities towards pregnenolone and progesterone, but catalyses progesterone at a slightly higher catalytic rate than pregnenolone. In the presence of cytochrome b_5 the hydroxylation of pregnenolone and progesterone appeared to increase, however, it is unlikely that cytochrome b_5 directly enhances the hydroxylation reaction and such a case has never been reported previously (Miller and Auchus, 2011). Instead cytochrome b_5 enhances the 17,20-lyase activity of CYP17 that ultimately eliminates any effects of product inhibition on the hydroxylation reaction. The 17,20-lyase activity towards 17-hydroxypregnenolone was roughly 25-fold higher than towards 17-hydroxyprogesterone. This observation is consistent with previous studies where it was suggested that the majority of precursors for the synthesis of sex steroids are derived from DHEA rather than androstenedione (Mason et al., 1989; Swart et al., 2003; Storbeck et al., 2007; Miller and Auchus, 2011).

Auchus and Miller (1999) constructed a molecular model for human CYP17 in 1999, to gain insights into its dual activities and predict the effects of various mutations in the CYP17 protein. The latter study was used as a basis to investigate the two amino acid differences in the ovine CYP17 isoforms. These amino acid differences are located at residue positions 210 and 464, respectively. The model indicated that the residue at position 210 is located in the F-helix, a core helix that forms part of the F-G loop. The residues that line the cleft in this F-G loop may serve to form an access pathway for the entry of the steroid into the substrate binding pocket (Auchus and Miller, 1999). The S210 residue is present in human, bovine and ovine WT1 CYP17, while G210 is present in porcine and ovine WT2 CYP17. The model indicated that the second residue, at position

464, is located in β-sheet 3. This is a core sheet that includes a turn, at residue positions 482 and 483, which forms the top of the substrate binding pocket (Auchus and Miller, 1999). The Y464 residue is present in ovine WT1 CYP17 only; the N464 residue is present in ovine WT2 and bovine CYP17; while the D464 residue is present in human and porcine CYP17.

The importance of an amino acid mutation at residue positions 210 and 464 is likely to be reflected in the catalytic activity of CYP17. However, WT1 and WT2 showed similar activities, with some differences indicated by the steroid conversion assays. WT1 showed a tendency towards a higher catalytic activity in all cases except the 16α-hydroxylation of progesterone, where WT2 had a higher catalytic activity. These differences were only observed in the time-dependent steroid assays, but were not reflected in the kinetic constant determinations and site-directed mutagenesis assays. Furthermore, cytochrome b_5 corrects the small catalytic differences between the CYP17 isoforms at the 17,20-lyase activities of the Δ^4 and Δ^5 pathways, while accentuating the differences at the 17α- and/or 16α-hydroxylation of pregnenolone and progesterone.

Another way in which the CYP17 isoforms may be different is in the regulation of CYP17 by serine/threonine phosphorylation. This mechanism is still not fully understood, but has been proposed to increase the 17,20-lyase activity relative to the 17α-hydroxylase activity by phosphorylation of certain serine and threonine residues of CYP17 (Zhang et al., 1995). As previously mentioned, WT1 contains a serine at residue position 210, whereas WT2 contains a glycine. However, it is unknown whether the S210 of WT1 is one of the residues that may be phosphorylated by this mechanism. Kempna et al. (2010) proposed a model whereby serine residues are exclusively phosphorylated to increase the 17,20-lyase activity (in response to cAMP stimulation), while threonine residues are selectively phosphorylated via another messenger pathway to inhibit 17,20-lyase activity (in response to starvation conditions). Pandey and Miller (2005) demonstrated that the serine/threonine phosphorylation of CYP17 acts to increase the velocity of the 17,20-lyase reaction, but does not increase the affinity for its substrate. They proposed that the negatively charged phosphate groups affect the electrostatic interactions between CYP17 and POR to increase electron transport. Furthermore, Pandey et al. (2003) showed that the increase in 17,20-lyase activity is not dependent on cytochrome b_5, nor is it cooperative or additive (Pandey and Miller, 2005).

In the present study, it was observed that 17-hydroxypregnenolone was converted to DHEA by WT1 (with S210) at a higher rate than WT2 (with G210), in the absence of cytochrome b_5. With cytochrome b_5, being present, however, the CYP17 isoforms had equal conversion rates. These

results may have suggested that S210 is more advantageous for electron transport from POR to CYP17, perhaps by the phosphorylation mechanism. However, the site-directed mutagenesis results did not support this statement. Additional experimental procedures are required to investigate whether the serine/threonine phosphorylation of CYP17 is different between the ovine CYP17 isoforms.

5.5 Conclusion

WT1 and WT2 produced equal amounts of 17-hydroxypregenolone, while WT1 produced more 17-hydroxyprogesterone than WT2 in the presence of cytochrome b_5. It is therefore concluded that WT1 produce more cortisol precursors than WT2. Whether these small differences in the activity of the CYP17 isoforms would manifest on the *in vivo* level to produce different quantities of cortisol, however, remains unknown. The competition of CYP21 and 3βHSD with CYP17 for substrates may intensify or mask these small catalytic differences and should also be considered. The current COS-1 expression system does not support such an experimental design, since results become less accurate when transfecting cells with more than three plasmid constructs. An experimental setup, that would closely resemble *in vivo* conditions, is the preparation of primary cultures from the adrenal glands of homozygous *WT1/WT1* and heterozygous *WT1/WT2* sheep. The complex mixture of steroids (13 steroids), however, presented a significant challenge for analyses as it is a relatively complex mixture. The structural similarity of these steroids severely complicates separation, which makes it difficult to determine the percentage distribution of steroid metabolites (as was done in the present study). Furthermore, absolute quantification requires the use of various expensive internal standards (deuterated steroids). The following chapter (Chapter 6) therefore presents the development of a UPLC tandem mass spectrometry method for the detection of all the adrenal steroids (100 %). This method enabled the detection of all steroids produced by the adrenocortical cells of primary cultures (Chapter 7), which could be expressed as percentage of total steroid produced (as was done in this chapter) without the use of internal standards.

CHAPTER 6

Simultaneous quantification of adrenal steroids using ultra performance liquid chromatography tandem mass spectrometry

6.1 Introduction

The analysis of endogenous steroids in humans and livestock has become the focus of endocrinologists and physiologists that investigate these important hormones. The quantification of these steroids is imperative to our understanding of reproductive fitness and stress management. In mammals, stress responses are mediated by the HPA axis in combination with the autonomic nervous system and behavioural adaptation (Mormède et al., 2007; Manteuffel, 2002). Stimulation of the HPA axis results in the secretion of adrenocorticotrophic hormone (ACTH) from the pituitary gland, which in turn stimulates the release of glucocorticoids, such as cortisol, from the adrenal cortex (Bush and Ferguson, 1953; McDonald and Reich, 1959). To date, the majority of animal HPA response studies have focussed on the detection of only one or two glucocorticoids, mainly cortisol (sheep, cows, pigs, fish) or corticosterone (birds, rats, mice), as well as total glucocorticoid concentration (Dantzer and Mormède, 1983; Hargreaves and Hutson, 1990; Apple et al., 1993; Engelbrecht et al., 2000; Smith and Dobson, 2002; Okeudo and Moss, 2005; Prunier et al., 2005; Storbeck et al., 2008a). The change in some adrenal steroid hormones (usually end products) and the ratios between the different metabolites in response to ACTH stimulation has been the focus of many investigations, but inadequate analytical methods have hampered comprehensive analyses, which would be more informative than the quantification of the single end products. Xing et al. (2011) recently demonstrated that, while the production of the glucocorticoids (cortisol and corticosterone) was stimulated by ACTH in human adrenals, the production of androgens and adrenal androgen precursors were also significantly up-regulated. Another aspect of glucocorticoid production that is often overlooked is the interconversion of the active glucocorticoids, cortisol and corticosterone, to their inactive 11-oxo-derivatives, cortisone and 11-dehydrocorticosterone, respectively, by 11βHSD (type 1 and 2) (Mormède et al., 2011).

The accurate detection and quantification of endogenous steroid hormones from experimental subjects remains a challenging task, due to structural homology and the low concentrations at which such hormones are normally present in biological fluids (Soldin and Soldin, 2009; Makin and Gower, 2010; Rauh, 2010). Routine quantification of endogenous steroid hormones is based predominantly on immunoassay methods, which are easily accessible to hospitals and laboratories

(Soldin and Soldin, 2009; Kushnir et al., 2011). However, immunoassays are steroid specific, hampered by cross-reactivity and prone to the overestimation of true steroid concentrations (Middle, 1998; Dorgan et al., 2002; Marks, 2002; Valdes and Jortani, 2002; Minutti et al., 2004; Etter et al., 2006; Soldin and Soldin, 2009). Gas chromatography linked to mass spectrometry (GC-MS) is considered the golden standard in steroid analysis and can quantify a large number of steroids at great sensitivity (65 steroids in 50 min: Ha et al., 2009). However, sample preparations involved in GC-MS can be laborious and complex, thus limiting the throughput (Ha et al., 2009; Rauh, 2010; Shackleton, 2010; Kushnir et al., 2011).

The development of modern HPLC resulted in significant improvements in resolution and selectivity of steroids over the traditionally used thin layer chromatography. Expensive radiolabeled tracers and radio-active flow detection are, however, required to quantify steroids that do not absorb in the UV-region, such as the Δ^5 steroids (pregnenolone, 17-hydroxypregnenolone and DHEA) (Makin and Gower, 2010; Rauh, 2010; Shackleton, 2010). In addition, conventional HPLC methods require relatively long run times, in excess of 50 minutes, to achieve the desired resolution (Miksik et al., 1999; Nithipatikom et al., 2005). Coupling MS to HPLC has made MS available as a universal detection alternative for liquid chromatography. Run times can be significantly reduced as a number of precursor and product ions can be detected simultaneously and selectively (Hauser, 2008; Soldin and Soldin, 2009; Rauh, 2010; Shackleton, 2010). Further developments in the field led to the incorporation of UPLC-MS/MS as an analytical tool which allows for smaller sample volumes together with higher throughput while achieving good resolution in shorter run times.

Various studies have been successful in achieving the quantification of 7 to 21 endogenous steroids in less than 18 minutes with UPLC-MS/MS (Guo et al., 2006; Carvalho et al., 2008; Janzen et al., 2008; Storbeck et al., 2008b; Cho et al., 2009; Simersky et al., 2009; Xing et al., 2011). However, most of these methods require additional derivatisation steps to achieve the required sensitivity for quantification (Xing et al., 2011). Even though recent developments in UPLC-MS/MS has significantly increased the number of steroids that can be separated and quantified in a single chromatographic separation (Holst et al., 2007; Soldin and Soldin, 2009; Kushnir et al., 2011), the quantification of the total steroid output of a complex endocrine gland, such as the adrenal gland, in a single analytical step has not been achieved.

The need for the development of a LC-MS/MS method to quantify all the adrenal steroids was recently demonstrated by the study of Xing et al. (2011), where steroid metabolites produced by adrenal cells were analysed. Three different LC-MS/MS methods had to be utilised: 1) an analysis

of underivatised steroid metabolites; 2) an analysis requiring derivatisation of keto-steroids with hydroxylamine; and 3) an analysis requiring the derivatisation of estrone and estradiol with dansyl chloride. In addition, an enzyme immunoassay was performed for the detection and quantification of cortisol, corticosterone and 11-hydroxyandrostenedione.

The aim of this study was to develop a sensitive and rapid UPLC-MS/MS method, for the detection of all the major endogenous adrenal steroids, without derivatisation, from sheep plasma and adrenal primary cells. These steroids included: pregnenolone, 17-hydroxypregnenolone, DHEA, progesterone, 17-hydroxyprogesterone, 16-hydroxyprogesterone, androstenedione, 11-deoxycortisol, cortisol, deoxycorticosterone, corticosterone, 18-hydroxycorticosterone, and aldosterone. Furthermore, the application of this method for quantification of adrenal steroids in plasma was tested, and additionally included the steroids 11-dehydrocorticosterone, cortisone, and testosterone. A pentafluorophenyl (PFP) stationary phase with trimethylsilyl (TMS) end capping was used as opposed to the more popular C_{18} columns (Rauh, 2010; Shackleton, 2010; Kushnir et al., 2011). The method was validated by investigating the change in steroid hormone production of South African Merino sheep in response to HPA axis stimulation.

6.2 Materials and Methods

6.2.1 Chemicals

Testosterone was purchased from The British Drug House Ltd. (Poole, England); 11-dihydrocorticosterone was from Merck (Darmstadt, Germany), and (9,11,12,12-d_4)-cortisol was purchased from Cambridge Isotope Laboratories Inc. (Andover, MA, USA). All other steroids as well as analytical grade methanol, isopropanol and dichloromethane were purchased from (Sigma-Aldrich, St. Louis, MO, USA). Formic acid and ethyl acetate, also analytical grade, were obtained from Merck (Darmstadt, Germany). Analytical grade water was prepared using the MilliQ purification system from Millipore (Billerica, MA, USA).

6.2.2 Standard solutions and quality-control samples

Steroids (2 mg mL^{-1}) and the internal standard (9,11,12,12-d_4)-cortisol (50 µg mL^{-1}) were dissolved in ethanol and stored at -20 ºC. Prior to use, d_4-cortisol was diluted in methanol to either 1000 ng mL^{-1} for the calibration curves or 100 ng mL^{-1} for quality control samples. Six quality control samples for method validation were prepared in low serum culture medium (see section 2.5.1) and steroid-free foetal calf serum (Highveld Biological, Lyndhurst, South Africa). Samples were spiked

with all of the 16 steroids and with the internal standard d_4-cortisol at a concentration of 100 ng mL^{-1} each. In addition, non-spiked low serum culture medium and steroid-free serum samples were prepared in triplicate as controls.

6.2.3 Method validation

Calibration standards for construction of calibration curves (n = 8), limits of detection (LOD) and limits of quantification (LOQ) (n = 13), were prepared at concentrations ranging from 0.009 ng mL^{-1} to 10000 ng mL^{-1}, containing all of the 16 steroids and the d_4-cortisol internal standard at a fixed concentration of 100 ng mL^{-1}. Calibration curves were generated by performing least-squares regression analysis on peak area ratios relative to the internal standard at different concentrations, within the sensitivity range of each steroid. The LOD and LOQ were defined as the lowest steroid concentration with a signal-to-noise (S/N) ratio larger than 3 and 10, respectively. Precisions were expressed as coefficients of variation (% RSD) and accuracy as percentage recovery, which were both determined from the quality control samples. In addition, the instrumental precision was calculated from the average coefficient of variation of all of the steroids in the quality control sample (100 ng mL^{-1}) for intra-day precision (quality control sample analysed six times within the same day) and inter-day precision (quality control sample analysed twice per day for four consecutive days).

6.2.4 UPLC-MS/MS instrument setup

UPLC–MS/MS was performed using a Waters Acquity UPLC (Milford, MA, USA) coupled to a Waters Xevo triple quadrupole mass spectrometer (Milford, MA, USA). All instruments were controlled by MassLynx Software 4.0 and the integration and quantification were performed using TargetLynx. The settings on the instrument were optimized for maximum sensitivity for all steroids with the optimal settings and fragments defined after injection of the pure standards (1 µg mL^{-1} in methanol). All steroids were analysed in multiple reaction monitoring (MRM) mode using an electrospray in the positive ionization mode (ESI+).

The following settings were used: capillary voltage of 2.8 kV, cone voltage 15–35 V (Table 6.1), collision energy 4–32 eV (Table 6.1), source temperature 100 ºC, desolvation temperature 500 ºC, desolvation gas 1000 L h^{-1}, cone gas 50 L h^{-1} and dwell time 0.002 sec. During analyses the samples were kept at 10 ºC.

Steroids were separated on a Kinetex PFP column (50 mm × 2.1 mm, 2.6 µm; Phenomenex, USA) at 45 ºC and a flow rate of 0.4 mL min^{-1} using mobile phases that consisted of solvent A (1% formic acid in water) and solvent B (49% methanol: 49% acetonitrile: 2% isopropanol). A linear gradient from 85% A to 60% B in 7.5 min, followed by a linear gradient to 100% in 0.1 min and an isocratic elution with 100% solvent B for 0.9 min was applied. The total run time was 10.5 min and the injection volume was 5 µL.

6.2.5 Assay procedures

6.2.5.1 Preparation of adrenal culture and extraction of steroids

The adrenal gland of a Merino ram (2 years of age) was used for the preparation of adrenal primary cells as previously described (Basset et al., 2004; Xing et al., 2011). In summary, after the adrenal gland was obtained at a local abattoir, it was minced and digested with culture medium containing 1 mg mL^{-1} collagenase D (Roche, Mannheim, Germany) for 1 hour at 37 ºC. The resulting cell suspension was filtered through a 100 µm mesh nylon filter, before collecting cells by centrifugation at 600 x g and resuspension in culture medium. Digestion and mechanical dispersion was carried out twice before seeding the collective cell suspension into 10 cm tissue culture dishes. After twenty-four hours the cells were trypsinised, counted and replated into 12-well tissue culture plates at a concentration of 300 000 live cells per well (cell viability > 75 %). The cells were cultured for four days in culture medium consisting of DMEM/F12 (Sigma-Aldrich, St. Louis, MO, USA) containing penicillin/streptomycin, gentamycin, kanamycin and 10% foetal calf serum (Gibco-Invitrogen, Grand Island, NY, USA). After day four, kanamycin was omitted from the culture medium to allow for more rapid cell growth. Twenty-four hours prior to the steroid assay, the foetal calf serum concentration in the culture medium was reduced to 0.1 %. The steroid assay was conducted after day six, when cells were confluent.

Culture medium containing 100 µM pregnenolone, with and without 1 µM ACTH, was added to the cells in triplicate and samples (400 µL) were removed from the medium after forty-eight hours. A liquid-liquid extraction of the steroids was performed by vortexing the samples in a 1:10 ratio in dichloromethane for twenty minutes, centrifugation at 500 x g for five minutes, followed by the aspiration of the aqueous phase. The dichloromethane phase was dried under gaseous nitrogen at 45 ºC and the dried residue dissolved in 200 µL methanol. Samples were stored at -20 ºC prior to UPLC-MS/MS analysis.

6.2.5.2 Plasma collection and extraction of steroids

Ten Merino rams (2-6 years of age) were injected intravenously with human insulin at a dose of 0.1 IU kg^{-1} body weight. Blood samples were collected from the jugular vein immediately prior to insulin administration and sixty minutes thereafter. Blood was collected in EDTA-treated collection tubes and immediately placed on ice. Representative plasma samples from each animal were obtained the same day of the test by centrifugation (2 500 x g; 10 min; 4 ºC), transferred to 1.5 mL microcentrifuge tubes and stored at -20 ºC. Ethics approval was obtained from the Departmental Ethics Committee for Research on Animals (DECRA ref: R08/21) of the Western Cape Department of Agriculture.

For liquid-liquid and solid phase extraction, plasma samples were thawed on ice. Internal standard was added (10 µL of 1000 ng mL^{-1} d_4-cortisol) to 500 µL plasma diluted with 500 µL water. After the addition of 1 mL acetonitrile and 4 mL ethyl acetate, samples were vortexed for ten minutes and centrifuged (500 × g; 10 min; 22 ºC). The aqueous phase was subsequently flash frozen using liquid nitrogen, after which the unfrozen organic phase was transferred to a test tube. The samples were subsequently dried under gaseous nitrogen at 45 ºC, redissolved in 200 µL methanol after which 800 µL water was added. Solid phase extraction was performed on 60 mg mL^{-1} strata-X, 33 µm polymeric reverse phase cartridges (Phenomenex, USA). The cartridges were preconditioned with 1 mL methanol, equilibrated with 1 mL water, and the sample (1 mL) was applied. The column was washed with 1mL 80% methanol (in water) and the steroids eluted with 1 mL methanol. The eluate was evaporated under gaseous nitrogen at 45 ºC, and the resulting dried steroid-containing residue dissolved in 100 µL methanol and stored at -20 ºC prior to UPLC-MS/MS analysis.

6.2.6 Statistical analysis

The data obtained in the conversion assays performed in the adrenal cells was normalised by expressing each steroid concentration as the fraction of the total sum of detected steroids (set equal to 100 µM). Steroid concentrations that were below the LOQ were set equal to zero. Plasma steroid concentrations that were obtained from LC-MS/MS analysis (in ng μL^{-1} representative of 500 µL plasma) were transformed to obtain the true physiological concentration (in ng mL^{-1}), by dividing by five and multiplying by a thousand. GraphPad Prism 5.03 was used in the statistical analysis of the data obtained from assays in adrenal cells (unpaired two-tailed Student's t-test) and the sheep plasma samples (paired two-tailed Student's t-test), to assess the treatment effect for each steroid metabolite, respectively (GraphPad Software, Inc., San Diego, CA, USA). Results were considered significant if $P < 0.05$.

6.3 Results and discussion

6.3.1 UPLC-MS/MS steroid detection

Various solvents, solvent gradients and columns were explored in the development of a suitable method. The use of ammonium formate (10mM) in the hydrophilic solvent A was considered, based on the method developed by Carvalho et al. (2008) to separate seven C-21 steroids. In ammonium formate DHEA exhibited a high abundance of protonated molecules due to the addition of water [M + $3H_2O$ + H^+]. However, the ionization efficiency of the steroid metabolites in the Δ^5 steroidogenic pathway (pregnenolone, 17-hydroxypregnenolone and DHEA) was substantially lower when using 5-10 mM ammonium formate (pH 2, 3 or 9) with or without 1% formic acid. The ionization efficiency of these three steroid metabolites was optimal in 1% formic acid, which was subsequently selected as solvent A.

Most steroids exhibited mass spectra with a high abundance of protonated molecules ([M + H^+]) in 1 % formic acid (listed in Table 6.1). DHEA and 11-dehydrocorticosterone exhibited a high abundance of protonated molecules with the loss of a water molecule ([M – H_2O + H^+]), whereas 17-hydroxypregnenolone exhibited a high abundance of protonated molecules due to the loss of two water molecules ([M – $2H_2O$ + H^+]). The ionization efficiency for all steroids in the mobile phase was tested in positive ESI mode. Although the detection limits for pregnenolone, 17-hydroxypregnenolone and DHEA were the highest of all the steroids (see Table 6.2), detection limits were adequate for quantification throughout (data not shown). The cone voltage and collision energy was optimized for each steroid (Table 6.1) and the resulting MRM transitions are depicted with chromatograms in Figure 6.1.

Due to the structural similarity of the steroids, many of the product ions have the same size, e.g. 97.0, 108.9 and 121.0 *m/z* (Table 6.1) and it was therefore necessary to separate these groups of steroids to avoid cross-talk. Five groups of steroids presented difficulty in separation: a) aldosterone and cortisone; b) 18-hydroxycorticosterone and cortisol; c) cortisol and cortisone; d) corticosterone and 11-deoxycortisol; and e) 17-hydroxyprogesterone, 16-hydroxyprogesterone, and deoxycorticosterone. The latter two groups (d and e) included isomers. For the separation of these steroids numerous columns with different stationary phases were tested, namely phenyl rings (Acquity UPLC, BEH Phenyl, 1.7 µm, 100 X 2.1 mm), C_{12} with TMS endcapping (Phenomenex, Synergi Max-RP, 80 Å, 4 µm, 150 X 4.6 mm), C_{18} with TMS endcapping (Phenomenex, Kinetex C18, 80 Å, 2.6 µm, 100 X 4.6 mm; Waters UPLC, BEH C_{18}, 1.7 µm, 2.1 X 100 mm) and polar embedded C_{18} with TMS endcapping (Phenomenex, Synergi Fusion-RP, 80 Å, 4 µm, 250 X 4.6

mm) (data not shown). The chosen column with a pentafluorophenyl (PFP) stationary phase, however, showed superior separation of all 16 steroids, excluding the internal standard (elution times depicted in Table 6.1). In particular the PFP column yielded a higher resolution between corticosterone and 11-deoxycortisol (Figure 6.1), as well as 18-hydroxycorticosterone and cortisol (Figure 6.1), than the other columns tested (data not shown).

Table 6.1. Optimal MRM transitions and parameters for MS/MS analysis of steroids. CV: Cone voltage; CE: Collision energy

Steroids (trivial name)	**Retention time[a] (min)**	**Precursor ion *m/z* (CV)**	**Product ion *m/z* (CE)**
Aldosterone	2.79	361.4 (30)	97.0 (32), 315.1 (20), 343.2 (18)
18-Hydroxycorticosterone	2.83	363.2 (30)	147.0 (22), 251.2 (20), 269.2 (15)
Cortisol	3.01	363.0 (30)	121.0 (20)
d_4-Cortisol	3.01	367.0 (35)	121.0 (25)
Cortisone	3.23	361.2 (30)	163.0 (30)
11-Dehydrocorticosterone	3.76	345.3 (30)	121.0 (20), 301.2 (25)
Corticosterone	3.96	347.0 (30)	121.0 (15), 329.1 (15)
11-Deoxycortisol	4.19	347.0 (30)	97.0 (15), 108.9 (15)
16-Hydroxyprogesterone	4.36	331.2 (30)	97.0 (15), 108.9 (15)
Testosterone	4.90	289.2 (30)	97.2 (22), 109.0 (22)
Dehydroepiandrosterone	5.01	271.2 (30)	243.0 (15), 253.2 (15)
Androstenedione	5.08	287.2 (30)	96.9 (15), 108.8 (15)
17-Hydroxypregnenolone	5.09	297.2 (30)	165.6 (25), 256.0 (10), 297.2 (4)
Deoxycorticosterone	5.15	331.2 (30)	97.0 (15), 108.9 (15)
17-Hydroxyprogesterone	5.32	331.2 (30)	97.0 (15), 108.9 (15)
Pregnenolone	6.34	317.2 (16)	159.1 (18), 281.2 (12)
Progesterone	6.49	315.2 (30)	96.9 (15), 297.2 (15)

[a] Retention times and chromatograms of each MRM function can be seen in Figure 6.1.

Table 6.2. Method validation results for LC-MS/MS analysis

Steroid (trivial name)	LOD[a] (ng mL^{-1})	LOQ[b] (ng mL^{-1})	Calibration range (ng mL^{-1})	Linearity (r^2)	Culture medium		Plasma	
					RSD[c] (%)	Recovery[c] (%)	RSD[c] (%)	Recovery[c] (%)
Aldosterone	0.1	1.0	1.0-10000	0.998	7.7	136.2	8.9	112.2
18-Hydroxycorticosterone	0.1	0.9	0.9-9000	0.996	9.3	130.8	9.2	111.9
Cortisol	0.1	0.1	0.1-9000	0.999	5.4	127.6	9.7	112.0
Cortisone	0.09	0.09	0.09-9000	0.999	4.6	131.5	8.7	105.0
11-Dehydrocorticosterone	0.09	0.9	0.9-9000	0.999	9.9	128.9	6.8	110.5
Corticosterone	1.0	9.0	9.0-9000	0.999	8.0	137.0	8.9	119.6
11-Deoxycortisol	0.09	0.09	0.09-9000	0.999	8.0	129.7	9.5	104.6
16-Hydroxyprogesterone	0.009	0.009	0.009-10000	0.998	7.8	133.6	9.8	116.4
Testosterone	0.09	0.1	0.1-2000	0.998	7.4	140.4	8.4	128.3
Dehydroepiandrosterone	0.9	10	10-9000	0.993	9.9	118.9	8.1	96.0
Androstenedione	0.1	0.9	0.9-10000	0.999	6.9	134.9	5.2	114.0
17-Hydroxypregnenolone	1.0	10	10-10000	0.994	6.5	124.4	9.8	105.0
Deoxycorticosterone	0.09	0.09	0.09-9000	0.998	9.4	138.4	3.8	109.6
17-Hydroxyprogesterone	0.09	0.09	0.09-10000	0.997	7.1	112.9	6.6	109.9
Pregnenolone	0.1	1.0	1.0-9000	0.994	9.0	125.6	12.3	80.6
Progesterone	0.01	1.0	1.0-10000	0.996	9.2	142.0	7.7	96.3

[a]Limit of detection
[b]Limit of quantification
[c]Residual standard deviation

The composition of the organic solvent B, was optimised for separation on a Kinetex PFP column (50 mm × 2.1 mm, 2.6 um; Phenomenex, USA) at 45 ºC. Solvents considered included 100 % methanol, 100 % acetonitrile, 50 % methanol: 50 % acetonitrile and 55 % methanol in 5 mM ammonium formate (data not shown). The best resolution for the majority of steroids was achieved using a solvent containing 49 % methanol: 49 % acetonitrile: 2 % isopropanol (solvent B). Steroids were subsequently separated at a flow rate of 0.4 mL min^{-1} using a linear gradient from 85% A to 60% B in 7,5 min, followed by a linear gradient to 100% in 0.1 min and an isocratic elution with 100% solvent B for 0.9 min. The total run time was 10.5 min and the injection volume was 5 µL.

All steroids mentioned were separated to baseline within 10.5 minutes (including equilibration), with the exception of 18-hydroxycorticosterone and cortisol, of which the peaks were resolved to at least 30 % above baseline.

The 16 steroids that were analysed included the 13 steroids known to be produced in the sheep adrenal gland, as well as the inactive 11-oxo-derivatives of cortisol and corticosterone, namely cortisone and 11-dehydrocorticosterone, respectively. The inclusion of these two steroids is important for stress related studies. Since the measurement of cortisol is considered the golden standard for measuring stress in sheep, d_4-cortisol was chosen as internal standard. Testosterone was also included in the analysis as only male animals were used in the *in vivo* study and a high concentration of testosterone is therefore expected. The inclusion of testosterone in the method development was done to ensure that there is no cross-talk with the other steroids of interest.

6.3.2 Method validation

Method validation requires the evaluation of linearity, LOD and LOQ from calibration samples, as well as the evaluation of precision and accuracy from quality control samples. Excellent linearity was obtained from calibration samples with r^2 values > 0.993. This linearity was maintained for an extensive calibration range up to 4 orders of magnitude and is shown in Table 6.2. Although concentrations as high as 10000 ng mL^{-1} are not physiologically relevant, they can occur under *in vitro* conditions and it was therefore necessary to establish these linearity ranges to facilitate in the designing of *in vitro* experimental setups. LOD and LOQ results are shown in Table 6.2. 16-Hydroxyprogesterone had the lowest detection and quantification limit at 0.009 ng mL^{-1}, while 17-hydroxyprogesterone had the highest LOQ of 10 ng mL^{-1}.

Accuracy was measured as the percentage steroid recovery from 6 quality control samples of either culture medium (containing 0.1 % foetal calf serum) for the liquid-liquid extraction procedure or steroid-free foetal calf serum for the solid-phase-extraction procedure. No steroids were detected in non-spiked samples of low serum culture medium or steroid-free serum.

The steroid recovery from culture medium with liquid-liquid extraction was generally higher than the steroid recovery from serum samples (more complex matrix) using SPE in combination with liquid-liquid extraction. Steroid recoveries from culture medium ranged between 112.9 % (17-hydroxyprogesterone) and 142.0 % (progesterone), with precisions (% RSD) ranging between 4.6 (cortisone) and 9.9 (DHEA). Steroid recoveries from serum samples ranged between 80.6 %

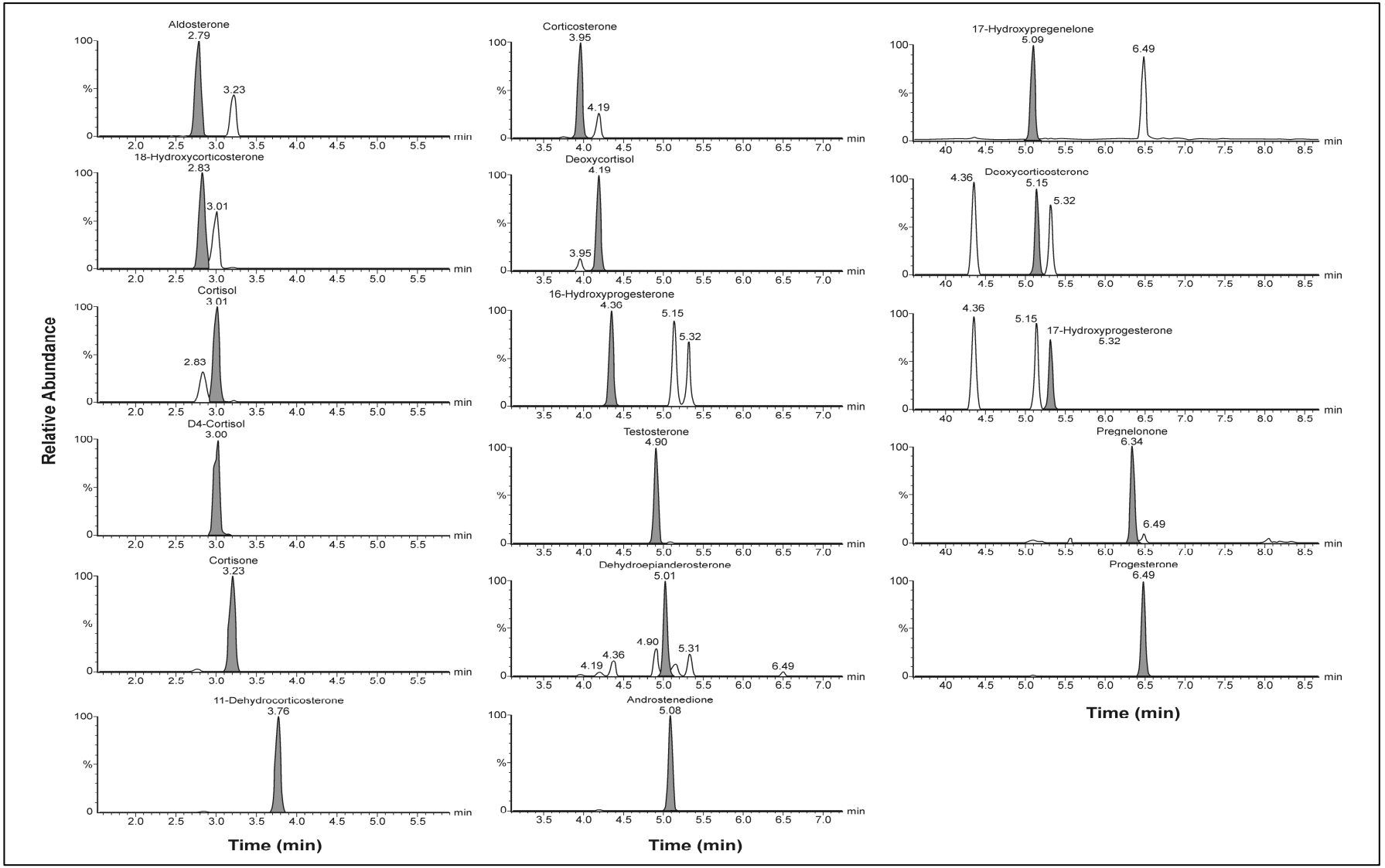

Figure 6.1. Multiple reaction monitoring (MRM) chromatograms of the steroids separated on a Phenomenex Kinetex PFP column (50mm × 2.1 mm, 2.6 µm) at 45 ºC. The MRM transitions and settings are listed in Table 6.1.

(pregnenolone) and 128.3 % (testosterone), with precisions ranging between 3.8 % (deoxycorticosterone) and 12.3 % (pregnenolone). The difference found in recoveries may result from both a matrix effect and the more elaborate extraction procedure followed in the preparation of serum samples.

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The low ionization efficiencies of pregnenolone, 17-hydroxypregnenolone and DHEA may contribute to the relatively high LOD and LOQ, and the subsequent lower linearity, precision and accuracy obtained for these compounds. The instrument precision was found to be 4.8 % intra-day, but increased to 9.8 % inter-day (over 4 days). This may be the result of drift in the signal of the mass spectrometer and the source that could become contaminated during the day. For this reason, a new standard dilution series was prepared for each day, which was analysed daily at the commencement and cessation of each sample list.

6.3.3 Quantification and analyses of steroids in adrenal cells and plasma

The validated method was used to analyse the production of steroid hormones in sheep adrenal primary cultures upon addition of 100 µM pregnenolone in the absence or presence of 1 µM ACTH. The predominant steroids detected in the primary cultures after forty-eight hours of incubation with 100 µM pregnenolone were progesterone (19.39 ± 0.27 x 10^3 ng mL^{-1}), 17-hydroxyprogesterone (3.93 ± 0.18 x 10^3 ng mL^{-1}); pregnenolone (3.81 ± 0.20 x 10^3 ng mL^{-1}), corticosterone (2.86 ± 0.04 x 10^3 ng mL^{-1}) and 17-hydroxypregnenolone (1.25 ± 0.04 x 10^3 ng mL^{-1}) (Table 6.3). The other

Table 6.3. Steroid hormones produced in sheep adrenal primary cultures after forty-eight hours with 100 µM pregnenolone treatment, with or without 1 µM ACTH. Data represents the mean and standard error of the mean of triplicate values with *P*-values reported by Student's t-tests. Note that concentrations were calculated from the fraction of total steroid added as substrate (100 µM).

	Pregnenolone (ng mL^{-1})	**Pregnenolone +ACTH** (ng mL^{-1})	**Fold change**	***P*-value**
Pregnenolone	3.81 ± 0.20 $x10^3$	3.36 ± 0.42 $x10^3$	0.88 ± 0.12	0.3866
17-Hydroxypregnenolone	1.25 ± 0.04 $x10^3$	1.16 ± 0.02 $x10^3$	0.95 ± 0.04	0.3291
Dehydroepiandrosterone	276.89 ± 30.36	288.72 ± 35.24	1.04 ± 0.17	0.8117
Progesterone	19.39 ± 0.27 $x10^3$	18.00 ± 0.35 $x10^3$	0.98 ± 0.02	0.4209
17-Hydroxyprogesterone	3.93 ± 0.18 $x10^3$	4.56 ± 0.06 $x10^3$	1.16 ± 0.05 *	0.0289
16-Hydroxyprogesterone	347.14 ± 25.54	422.87 ± 6.32	1.22 ± 0.09 *	0.0451
Androstenedione	37.22 ± 0.66	42.23 ± 2.19	1.13 ± 0.06	0.0936
Deoxycorticosterone	299.18 ± 32.01	305.25 ± 37.87	1.02 ± 0.17	0.9085
Corticosterone	2.86 ± 0.04 $x10^3$	2.98 ± 0.09 $x10^3$	1.04 ± 0.04	0.3030
18-Hydroxycorticosterone	16.43 ± 1.27	24.25 ± 2.40	1.48 ± 0.19 *	0.0451
Aldosterone	1.15 ± 0.54	0.53 ± 0.25	0.47 ± 0.31	0.3634
11-Deoxycortisol	402.02 ± 29.55	400.63 ± 38.35	1.00 ± 0.12	0.9785
Cortisol	471.05 ± 7.80	576.65 ± 11.23	1.22 ± 0.03 **	0.0015

*$P < 0.05$; **$P < 0.01$

adrenal steroids were also present at low, but detectable concentrations, while cortisone, 11-dehydrocorticosterone and testosterone were not detected since these steroids are not synthesised in the sheep adrenal. As expected, the addition of 1 µM ACTH resulted in a 1.22-fold increase in cortisol production ($P < 0.01$). Moreover, the addition of ACTH also resulted in significant increases ($P < 0.05$) in the production of 17-hydroxyprogesterone (1.16-fold), 16-hydroxyprogesterone (1.22-fold) and 18-hydroxycorticosterone (1.48-fold). Progesterone, 17-hydroxyprogesterone, pregnenolone, corticosterone and 16-hydroxyprogesterone remained the predominant steroids during the ACTH treatment. 17-Hydroxyprogesterone is a precursor of cortisol, which explains the concomitant concentration increases in these two steroids. In addition, the 16-hydroxyprogesterone (not a precursor of cortisol) concentration also increased, which suggest that the hydroxylase activity of CYP17 towards progesterone (produces 16- and 17-hydroxyprogesterone) was increased by ACTH addition.

Table 6.4. Steroid hormones produced in sheep plasma immediately prior to insulin administration and 60 minutes thereafter. Data represents the mean and standard error of the mean of 10 sheep with *P*-values reported by Student's t-tests.

	Pre-insulin (ng mL^{-1})	**Post-insulin** (ng mL^{-1})	**Fold change**	***P*-value**
Adrenal steroids				
Pregnenolone	-	-	-	-
17-Hydroxypregnenolone	-	-	-	-
Dehydroepiandrosterone	-	-	-	-
Progesterone	0.16 ± 0.06	0.10 ± 0.03	0.63 ± 0.30	0.1289
17-Hydroxyprogesterone	0.07 ± 0.01	0.05 ± 0.02	0.71 ± 0.30	0.3859
16-Hydroxyprogesterone	0.117 ± 0.026	0.106 ± 0.019	0.91 ± 0.26	0.5957
Androstenedione	0.17 ± 0.03	0.14 ± 0.02	0.82 ± 0.19	0.3611
Deoxycorticosterone	0.18 ± 0.10	0.09 ± 0.02	0.50 ± 0.3	0.2621
Corticosterone	0.27 ± 0.10	0.49 ± 0.18	1.81 ± 0.95	0.3537
18-Hydroxycorticosterone	-	-	-	-
Aldosterone	0.13 ± 0.03	0.17 ± 0.03	1.31 ± 0.38	0.2563
11-Deoxycortisol	0.05 ± 0.01	0.14 ± 0.06	2.80 ± 1.32	0.2048
Cortisol	11.64 ± 2.20	21.12 ± 3.24	1.81 ± 0.44 *	0.0205
Other steroids				
Cortisone	1.40 ± 0.12	1.90 ± 0.26	1.36 ± 0.22 *	0.0499
11-Dehydrocorticosterone	0.10 ± 0.02	0.13 ± 0.02	1.30 ± 0.33	0.2110
Testosterone	3.44 ± 0.94	1.71 ± 0.42	0.50 ± 0.19	0.0551

*$P < 0.05$

From the foregoing, it is evident that the UPLC-MS/MS method allowed accurate analysis of adrenal steroidogenesis products in a primary culture system. The potential of the method to measure plasma steroids was also investigated in sheep administered insulin. The steroid concentrations in the plasma were significantly lower than in the adrenal cell cultures with the predominant steroid hormones under basal conditions (pre insulin injection) being cortisol (11.64 ± 2.20 ng mL^{-1}), testosterone (3.44 ± 0.94 ng mL^{-1}) and cortisone (1.40 ± 0.12 ng mL^{-1}) (Table 6.4). Androstenedione (0.17 ± 0.03 ng mL^{-1}), aldosterone (0.13 ± 0.03 ng mL^{-1}), 16-hydroxyprogesterone (0.117 ± 0.026 ng mL^{-1}), 11-dehydrocorticosterone (0.10 ± 0.02 ng mL^{-1}), 17-hydroxyprogesterone (0.07 ± 0.01 ng mL^{-1}), and 11-deoxycortisol (0.05 ± 0.01 ng mL^{-1}) were all present at low, but

detectable concentrations. Pregnenolone, 17-hydroxypregnenolone, DHEA and 18-hydroxycorticosterone concentrations were below the limits of detection.

Insulin administration resulted in a significant ($P < 0.05$) increase in cortisol concentrations (1.81-fold). The cortisol response to insulin-induced hypoglycaemia is consistent with response data from previous studies on Merinos, such as Engelbrecht et al., 2000, as well as Smith and Dobson, 2002. Interestingly, a 1.36-fold increase in cortisone concentrations (the inactive 11-oxo-derivative of cortisol) was also observed. However, the cortisol:cortisone ratio also increased 1.34-fold from 8.3 to 11.1 in response to insulin administration. This indicates that the stress response not only resulted in a higher production of cortisol in the adrenal gland, but also increased the fraction of active glucocorticoids relative to inactive glucocorticoids. The measurement of both cortisol and cortisone would thus seem to be a more accurate approach than the measurement of cortisol alone in stress-related studies.

Although 100 µM pregnenolone was added to the primary adrenal cultures, which is approximately 3 orders of magnitude higher than concentrations normally encountered *in vivo*, a similar cortisol specific response was observed in these adrenocortical cells. The addition of 100 µM pregnenolone was done to ensure that this substrate is not depleted within 48 hours and impede downstream catalytic reactions.

6.4 Conclusion

The method developed in this study is sensitive and specific for the quantification of the steroids involved in the HPA axis stress response. The limit of quantification and linearity across a wide concentration range makes this method suitable for both *in vivo* and *in vitro* studies. Analysis of 16 steroids from a 5 µL injection volume was done in 10.5 minutes per run without derivatisation, and is considered progress in the quest for detecting multiple steroids in a single analysis by UPLC-MS/MS in comparison with other current methods. This method is a simple alternative to labour intensive GC-MS and the less accurate immunoassay methods.

Investigation of the plasma steroid responses to insulin induced hypoglycaemia showed a cortisol and cortisone specific response. Not only was the production of cortisol in the adrenal gland increased, but the ratio of active glucocorticoids relative to inactive glucocorticoids was increased outside the adrenal gland. It is suggested that cortisol should be monitored together with cortisone in stress-related studies, since the interconversion between these two steroids by 11βHSD may affect conclusions drawn based on the analysis of cortisol alone. Investigation of the adrenocortical

cell response to ACTH stimulation showed a cortisol specific response that is thought to be mediated by an increase in CYP17 activity towards progesterone, as reflected by the concomitant increases in 16- and 17-hydroxyprogesterone. It was therefore important to investigate the relative contributions of the two ovine CYP17 isoforms towards cortisol production of sheep. This study is described in the next chapter.

CHAPTER 7

Relative contribution of two ovine CYP17 isoforms in the steroidogenic output of adult adrenocortical cells

7.1 Introduction

The two CYP17 isoforms from Chapter 5 showed little difference in catalytic activity under *in vitro* conditions, and it is unclear whether these small differences would affect cortisol production or alter the total steroidogenic output (see Table 5.1). The substrate competition between CYP17 and 3βHSD needs to be considered, as well as the modulating effect of cytochrome b_5. The use of primary culture preparations of adrenocortical cells allows an *in vitro* experimental setup that closely resembles the *in vivo* conditions of the adrenal gland. The current study thus investigated the relative contribution of the two CYP17 isoforms on the cortisol production, and general steroidogenic output, from adrenocortical cells of either homozygous *WT1/WT1* or heterozygous *WT1/WT2* sheep. Furthermore, the animals that were used in this study belonged to one of two selection lines, with either high (H-line) or low (L-line) fitness traits. The H-line exhibited superior reproductive performances relative to the L-line (Cloete et al., 2004), while lamb survival was also reported to respond to selection (Cloete et al., 2009). Moreover, the L-line was characterised by behavioural responses indicative of a wilder nature (Cloete et al., 2005a, 2010), which may result in stress under normal husbandry practices. It is proposed that the HPA axis function of the H-line is superior to that of the L-line, and that some of these line differences may be explained by differences in adrenal steroidogenesis. The *CYP17* genotype was therefore investigated in the current study as contributor to HPA axis function due to its potential effects on cortisol production.

The steroidogenic output from primary cultures of adult adrenocortical (AA) cells could be assessed either under endogenous conditions (Xing et al., 2011) or by the addition of a common precursor, such as pregnenolone (Engelbrecht et al., 2000). As mentioned in Sections 3.3.1 and 3.5.1, the supply of cholesterol to CYP11A1, and it subsequent conversion to pregnenolone, is the primary regulating mechanisms of the quantitative steroidogenic output. Therefore the addition of an excess pregnenolone to AA cells is proposed, rather than endogenous conditions, to reduce variation in the experimental system, since the aim is to ascertain differences in steroidogenesis among *CYP17* genotypes. Moreover, pregnenolone was chosen rather than cholesterol, since cholesterol is virtually insoluble in aqueous solutions and renders it unsuitable as substrate in such experiments.

An additional goal of these experiments with AA cells was to compare the steroidogenic responses of unstimulated and ACTH stimulated cells. ACTH was added to AA cells to stimulate adrenal steroidogenesis and distinguish stimulated from basal secretion. The comparison of the glucocorticoid responses between the *CYP17* genotypes may therefore reveal whether the activity of the CYP17 isoforms is regulated differently, or whether there are differences in the intracellular ACTH responses of the selection lines. Furthermore, the intracellular signal transduction pathways may be different between the selection lines. For this reason it is suggested that compounds are added to AA cells, which influence the cAMP second messenger pathway. As previously mentioned in Section 2.6.3, ACTH binds to an ACTH receptor on the outer surface of the adrenal cell membrane, which results in the activation of adenylate cyclase to produce cAMP from ATP via G_s (a G-protein on the inner surface of the cell membrane). The cAMP acts as second messenger to ultimately increase glucocorticoid secretion (Miller and Auchus, 2011). Cholera toxin and forskolin are compounds that prolong the production of cAMP.

Cholera toxin is produced by the bacterium, *Vibrio cholerae*, which inhibits the GTPase activity of G_s. The A_1 peptide of this 87-kD multimeric protein catalyses the ADP-ribosylation of Arg^{210} in the α subunit of G_s, using NAD^+ as substrate (Garrett and Grisham, 1995). This irreversible ADP-ribosylation of G_s subsequently prevents it from dissociating from adenylate cyclase (Gilman, 1987). As a consequence the activation of adenylate cyclase is prolonged and the elevated levels of cAMP cause epithelial cells to secrete high volumes of fluid. This results in the characteristic symptom of diarrhoea in cholera victims and will lead to death if fluids are not replenished.

Forskolin was isolated form the root of an Indian plant, *Coleus forskohlii*, and has been used as therapeutic agent for abdominal colic, heart diseases, respiratory disorder and other diseases (Tatee et al., 1996). Forskolin prolongs the adenylate cyclase activity by directly binding to adenylate cyclase (Seamon and Daly, 1981, 1984; Nelson and Seamon, 1985, 1986; Bender and Neer, 1983). However, the association of G_s with adenylate cyclase is required for the full expression of the effect of forskolin (Darfler et al., 1982; Schimmer et al., 1987; Juska and de Foresta, 1995).

Against this background, steroid metabolism was studied in primary cultures of adult adrenocortical cells, obtained from H- and L-line rams of different CYP17 genotypes, in the presence or absence of ACTH. Stimulation by using either cholera toxin or forskolin was investigated simultaneously, to investigate possible differences in the production of intracellular cAMP.

7.2 Material and Methods

7.2.1 Animals

The South African Merino sheep that were used in this study, originated from the breeding program at the Elsenburg Research Farm, situated at lattitude 33° 51‘ S and longitude 18° 50‘ E at an elevation of 177 m. The breeding program commenced in 1986 from the same base population, where sheep were divergently selected on maternal ranking values for number of lambs weaned per mating opportunity (Cloete et. al., 2004, 2009). This divergent selection resulted in significant differences in reproduction (Cloete et al., 2004), lamb survival (Cloete and Scholtz, 1998; Cloete et al., 2009) and their responses to flock separation (Cloete et al., 2005a; 2010), where the H-line outperformed the L-line. The *CYP17* genotype of numerous sheep from this breeding program was previously determined (Chapter 4). The adrenal glands of 8 sexually mature rams were collected at a local abattoir during routine slaughter schedules for the preparation of primary cultures. The experiment was structured according to a 2 x 2 statistical design, with *CYP17* genotype (*WT1/WT1* vs. *WT1/WT2*) and selection line (H-line vs. L-line) as factors. Each of the four groups were represented by only two individuals (separate primary cultures), due to practical and logistical constraints.

7.2.2 Preparation of primary cultures from adult adrenal cells

The adrenal glands from Merino rams were used for the preparation of primary cultures of adult adrenal (AA) cells as previously described (Basset et al., 2004; Xing et al., 2011). Ethanol (70 % v/v) was used to rinse the adrenal glands after collection at the abattoir and after excess fat was removed at the laboratory. The glands were stored in ice cold EBS (Earl’s balanced salts, Sigma-Aldrich, St. Louis, MO, USA) and kept on ice for no more than five hours. The adrenals were then sliced with the use of a microtome, minced with scissors and digested with culture medium containing 1 mg mL^{-1} collagenase D (Roche, Mannheim, Germany), 0.1 mg mL^{-1} DNase I (Roche, Mannheim, Germany), penicillin/streptomycin and gentamycin for one hour at 37 ºC. The resulting cell suspension was filtered through a 100 µm mesh nylon filter, before collecting the cells by centrifugation at 600 x *g* and resuspending the cells in culture medium. The remainder of undigested adrenal tissue was subjected to a second round of digestion, filtration and resuspension, after which the collective cell suspension from an individual sheep was seeded into 10 cm tissue culture dishes. After 24 hours the cells were trypsinised, counted and replated into 12-well tissue culture plates at a concentration of 300 000 cells per well (cell viability > 75 %). The cells were cultured for four days in culture medium consisting of DMEM/F12 (Sigma-Aldrich, St. Louis, MO,

USA) containing penicillin/streptomycin, gentamycin, kanamycin and 10% foetal calf serum (Gibco-Invitrogen, Grand Island, NY, USA). After day four, kanamycin was omitted from the culture medium to allow for more rapid cell growth. Twenty-four hours prior to the steroid metabolism assay, the foetal calf serum concentration in the culture medium was reduced to 0.1 %. The steroid assay was conducted after day six, when the cells were confluent.

7.2.3 Steroid metabolism assay

The steroidogenic output of AA cells was investigated in control and stimulated AA cells. No steroid was added to the growth medium of control AA cells and therefore represented basal conditions. The growth medium of all the stimulated AA cells contained 100 µM pregnenolone. In addition to pregnenolone, AA cells were stimulated by the addition of 1 µM ACTH, 10 µM forskolin or 100 µM cholera toxin. The substrate additions are shown in Table 7.1. Assays were performed in triplicate for individual sheep, where each group (four groups in total: H-line *CYP17* homozygous, H-line *CYP17* heterozygous, L-line *CYP17* homozygous or L-line *CYP17* heterozygous) was represented by two individuals. Substrate media sample aliquots (400 µL) were removed at six, twelve, twenty-four, forty-eight and seventy-two hours after initial addition of media. A (9,11,12,12-d_4)-cortisol internal standard was added to all 48 hour samples to measure the true concentrations of steroids in the media. A liquid-liquid extraction of the steroids in the respective media was performed. Samples were vortexed for twenty minutes in a 1:10 ratio of medium:dichloromethane, centrifuged at 500 x *g* for five minutes and the aqueous phase was aspirated. The dichloromethane phase was dried under gaseous nitrogen at 45 ºC and the dried residue dissolved in 200 µL methanol. Samples were stored at -20 ºC prior to analysis. The UPLC-

Table 7.1. Summary of the additions to substrate media for the study of steroid metabolism in adult adrenal cells. The "X" indicates the compounds that were added to the various substrate media.

Description of substrate media	Growth Medium (0.1 % serum)	Pregnenolone (100 µM)	ACTH (1 µM)	Forskolin (10 µM)	Cholera toxin (100 µM)
Basal conditions	X				
Substrate addition	X	X			
ACTH stimulation	X	X	X		
Forskolin stimulation	X	X		X	
Cholera toxin stimulation	X	X			X

MS/MS method described in Chapter 6 was used to analyse all thirteen steroids that have previously been shown to be produced in the sheep adrenal gland.

7.2.4 Statistical analysis

GraphPad Prism 5.03 was used for all statistical analysis (GraphPad Software, Inc., San Diego, CA, USA). The UPLC-MS/MS data obtained for pregnenolone conversion in the AA cells was normalised by expressing each steroid concentration as the fraction of the total sum of detected steroids (set equal to 100 µM). Comparisons of steroid biosynthesis in AA cells were made for each steroid metabolite over 72 hours for the selection lines (H- vs. L-line) and *CYP17* genotype (homozygous *WT1/WT1* vs. heterozygous *WT1/WT2*) with a paired t-test, while the selection line x *CYP17* genotype sub-groups (H_O, H_E, L_O and L_E) were compared with a repeated measures one-way ANOVA and Bonferroni's post-test. The effects of forskolin and cholera toxin on steroidogenesis, relative to the effects of ACTH, were analysed for each steroid metabolite with a one-way ANOVA and Dunnett's post-test (ACTH as control). Concentrations of steroid metabolites after 48 hours that were calculated with the use of an internal standard were compared to concentrations that were calculated without the use of an internal standard (normalised as fraction of total steroid detected, set equal to 100 µM) with unpaired t-tests. Results were considered to be significant if the *P*-value was lower than 0.05.

7.3 Results

The steroid output from the AA cells were assessed separately for each group of a 2 x 2 design (*CYP17* genotype x selection line group), namely H-line homozygous *WT1/WT1* (H_O), H-line heterozygous *WT1/WT2* (H_E), L-line homozygous *WT1/WT1* (L_O), and L-line heterozygous *WT1/WT2* (L_E). Under basal conditions, the AA cells produced low levels (< 0.3 µM) of endogenous steroids (Figure 7.1). These concentrations were close to their lower limits of quantification (LOQ) in the UPLC-MS/MS method. A large standard error of the mean was therefore present in the data for all the steroids measured under these conditions. The LOQ values are indicated by a shaded background in Figure 7.1. The concentrations of 17-hydroxypregnenolone, DHEA, androstenedione, corticosterone, 18-hydroxycorticosterone, and aldosterone were particularly close to the LOQ and accurate comparisons of the *CYP17* genotype groups were thus not possible. For this reason, no significant difference in the endogenous steroid production between the *CYP17* genotypes were observed, with the exception of corticosterone production, where the L_O group produced more corticosterone than the H_O group. The L_O group produced corticosterone at levels higher than the LOQ, while the production of corticosterone in the

H_O group remained low at levels equal and below the LOQ. Only two other significant differences ($P < 0.05$) in endogenous steroid levels were observed under basal conditions ($P < 0.05$), namely where the pregnenolone levels in the L_E group were higher than the L_O group (below LOQ) and 11-deoxycortisol levels in the H_E group were higher than the H_O group. A gradual accumulation of all steroids was observed as steroidogenesis continued over 72 hours at a basal rate, with peak concentrations observed after 48 hours.

The steroid concentrations at 48 hours were calculated by two different quantification methods. The one quantification method calculates the steroid concentration as the fraction of total steroid that was initially added to the AA cells (100 µM). This method therefore normalise the data and does not require the use of an internal standard for quantification. The other quantification method calculates the absolute steroid concentrations from the internal standard (deuterated cortisol) concentration, where an equal amount of internal standard was added to all the 48 hour samples prior to steroid extraction. The steroid concentrations that were calculated from each quantification method did not differ significantly ($P > 0.05$). This indicates that the use of an internal standard to quantify steroids is redundant in this study, since the detection of all the adrenal steroids is possible and may be expressed as the fraction of the total steroid content.

As seen in Figure 7.2, the addition of pregnenolone markedly increased the concentrations of all steroids ($P < 0.05$), except for aldosterone. The levels of 18-hydroxycorticosterone and aldosterone remained low throughout the different AA cell treatments, with the concentration of aldosterone consistently at its LOQ. For this reason, conclusions about the aldosterone responses could not be drawn. The aldosterone profiles were not included in Figures 7.1 to 7.5, but have been supplied in addendum A (Figure A.1).

The predominant steroids identified were pregnenolone, progesterone, 17-hydroxyprogesterone and 17-hydroxypregnenolone. It is interesting to note that corticosterone was present at a relatively high concentration (maximum at 3 – 12 µM), which was 5- to 26-fold higher than cortisol. This is in contrast to endogenous conditions, where equal amounts of corticosterone and cortisol were produced. The cortisol:corticosterone ratio of each group at 48 hours was unique ($P < 0.0001$), and remained constant for that group, irrespective of the stimulation applied ($P > 0.05$). The H_O group produced the most cortisol relative to corticosterone with an average cortisol:corticosterone ratio of 5.2 ± 0.7 (48 h), followed by the H_E group with a cortisol:corticosterone ratio of 9.6 ± 1.5, the L_O group with 17.4 ± 1.8, and lastly the L_E group produced the least cortisol with a cortisol:corticosterone ratio of 26.0 ± 2.1.

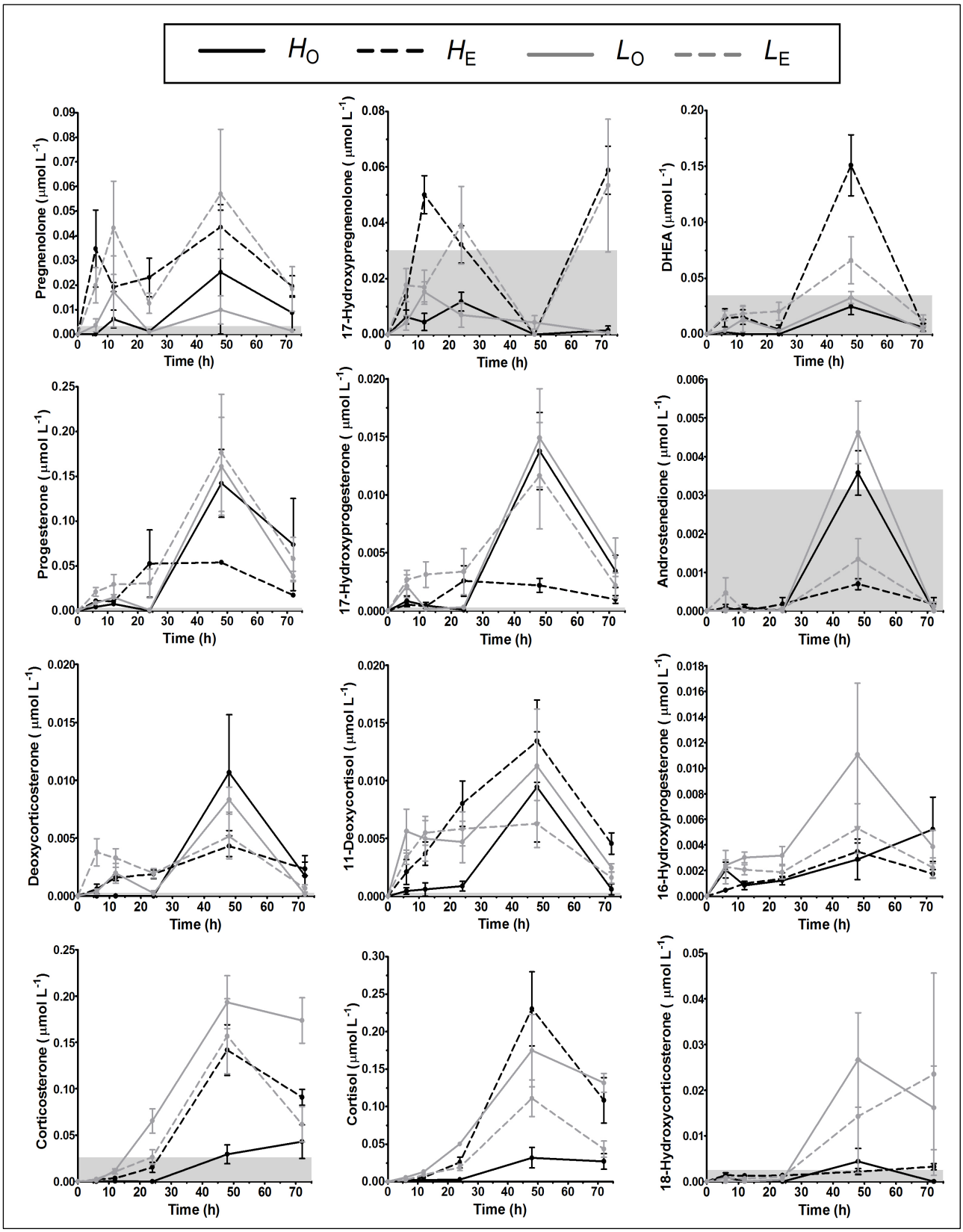

Figure 7.1. Steroidogenesis in sheep adrenocortical cells under basal conditions. The shaded background indicates the limit of quantification with the UPLC-MS/MS method. The H-line is represented by a black coloured line (H_O and H_E), the L-line represented by a grey coloured line (L_O and L_E), homozygous *WT1* groups are represented by a solid line (H_O and L_O), and heterozygous *WT1/WT2* groups by a broken line (H_E and L_E).

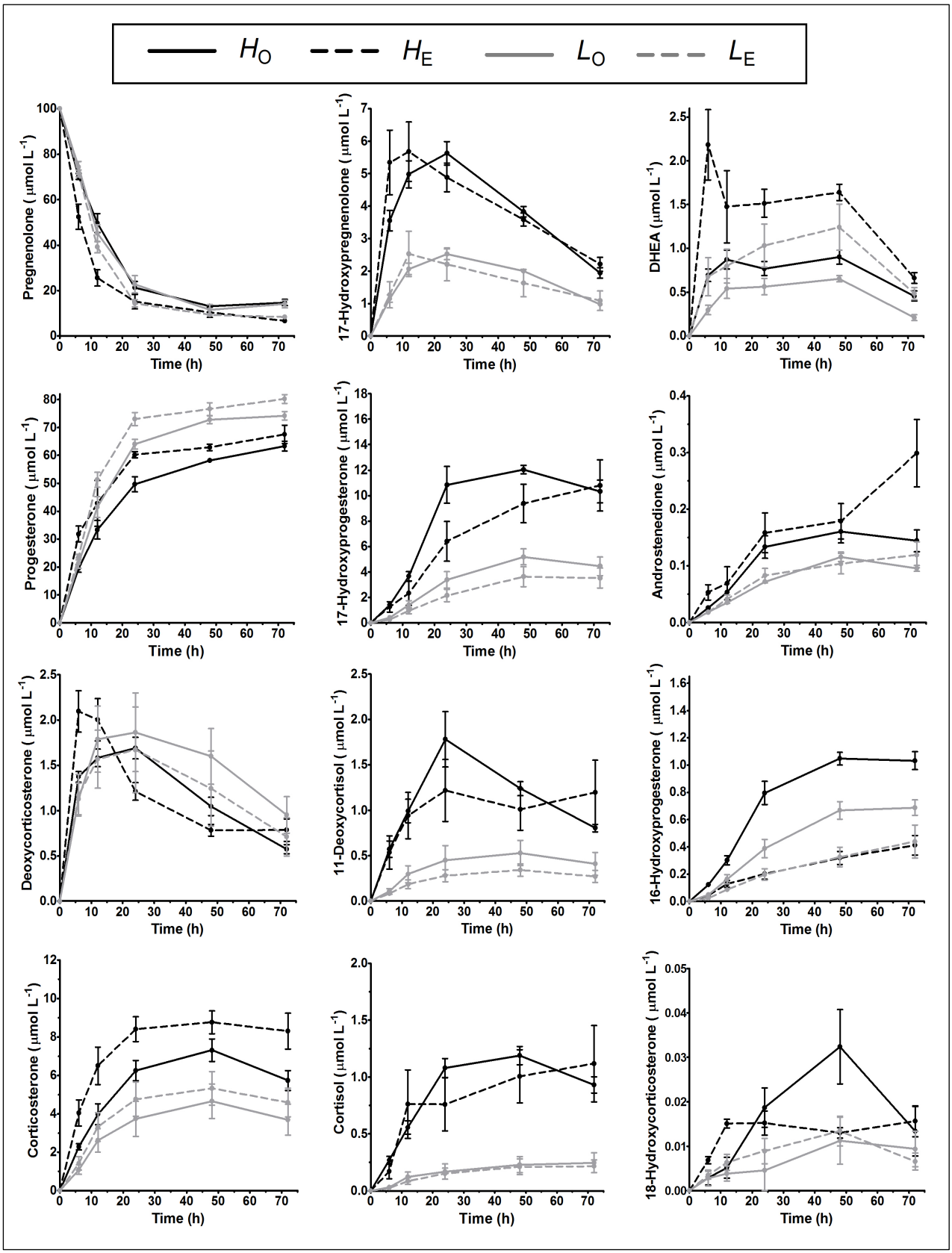

Figure 7.2. Steroidogenesis in sheep adrenocortical cells incubated over 72 hours with 100 µM pregnenolone. The H-line is represented by a black coloured line (H_O and H_E), the L-line represented by a grey coloured line (L_O and L_E), homozygous *WT1* groups are represented by a solid line (H_O and L_O), and heterozygous *WT1/WT2* groups by a broken line (H_E and L_E).

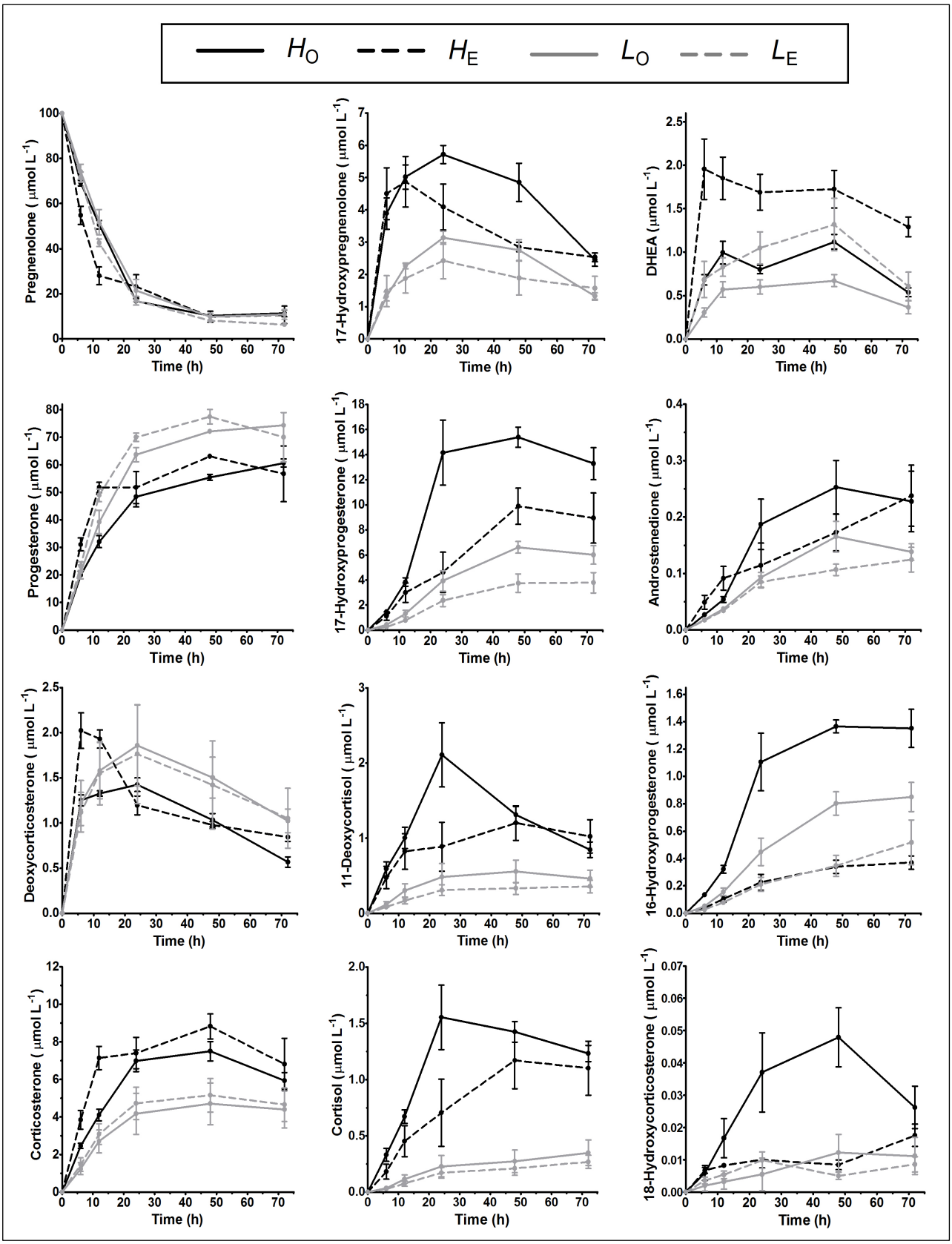

Figure 7.3. Steroidogenesis in sheep adrenocortical cells incubated over 72 hours with 100 µM pregnenolone and 1 µM ACTH. The H-line is represented by a black coloured line (H_O and H_E), the L-line represented by a grey coloured line (L_O and L_E), homozygous *WT1* groups are represented by a solid line (H_O and L_O), and heterozygous *WT1/WT2* groups by a broken line (H_E and L_E).

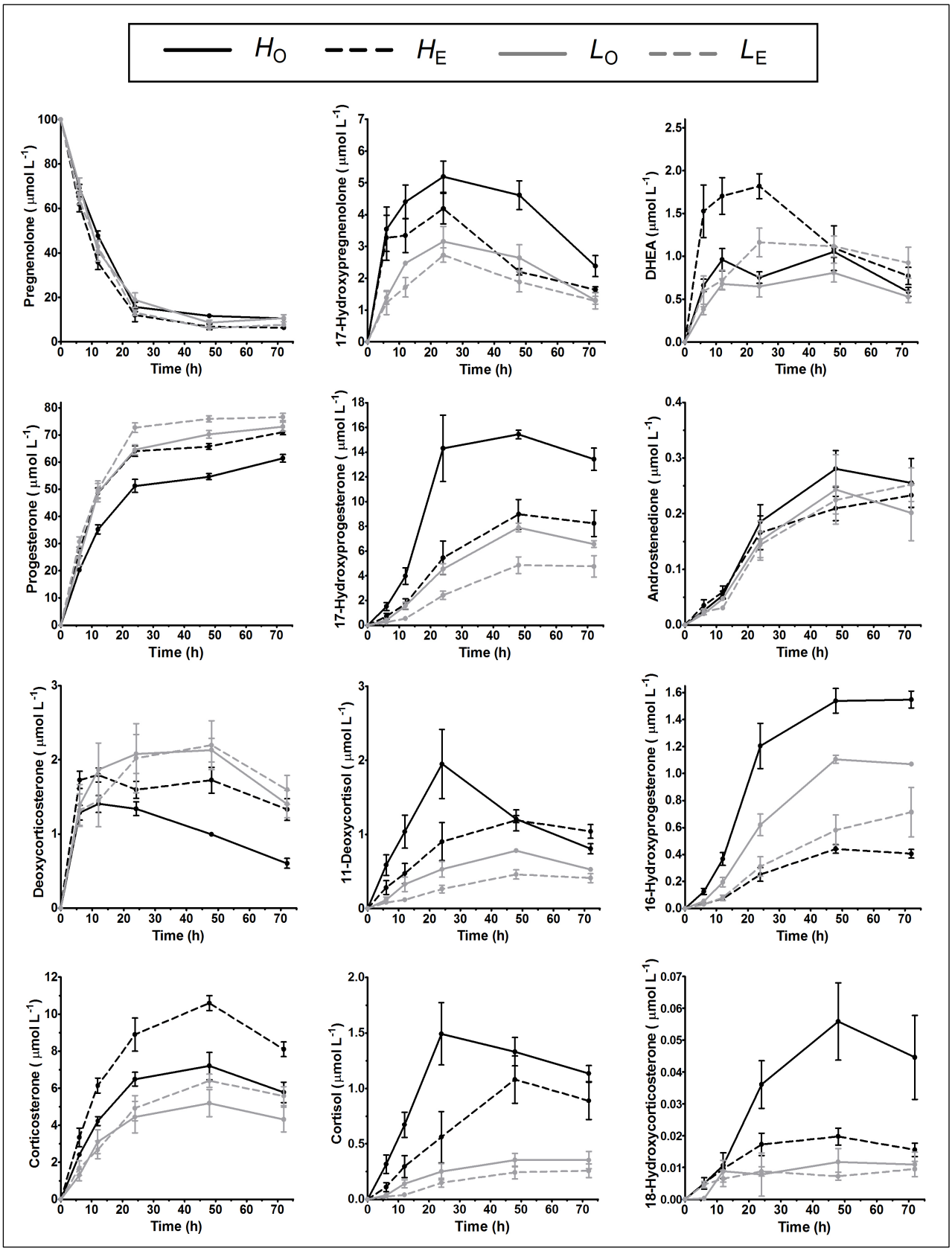

Figure 7.4. Steroidogenesis in sheep adrenocortical cells incubated over 72 hours with 100 µM pregnenolone and 10 µM Forskolin. The H-line is represented by a black coloured line (H_O and H_E), the L-line represented by a grey coloured line (L_O and L_E), homozygous *WT1* groups are represented by a solid line (H_O and L_O), and heterozygous *WT1/WT2* groups by a broken line (H_E and L_E).

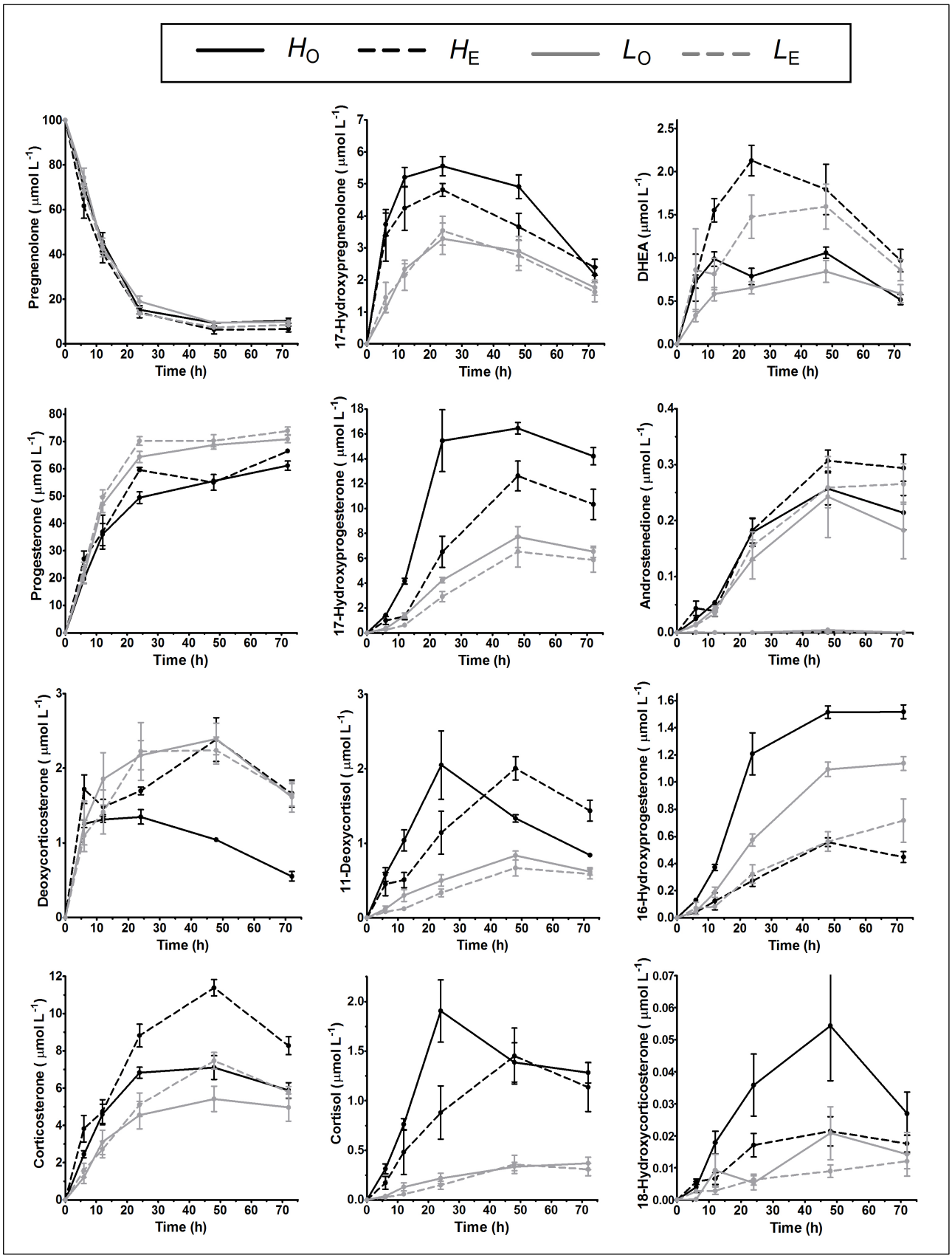

Figure 7.5. Steroidogenesis in sheep adrenocortical cells incubated over 72 hours with 100 µM pregnenolone and 100 µM cholera toxin. The H-line is represented by a black coloured line (H_O and H_E), the L-line represented by a grey coloured line (L_O and L_E), homozygous *WT1* groups are represented by a solid line (H_O and L_O), and heterozygous *WT1/WT2* groups by a broken line (H_E and L_E).

There were detectable differences in steroidogenesis among the four groups of AA cultures in the presence of 100 µM pregnenolone ($P < 0.05$), although the concentrations of progesterone, deoxycorticosterone, 18-hydroxycorticosterone and aldosterone were not significantly different among the groups ($P > 0.05$). At a first glance of Figure 7.2, it was clear that the H-line generally produced more 17-hydroxypregnenolone, 17-hydroxyprogesterone, 11-deoxycortisol, cortisol, corticosterone, DHEA and androstenedione than the L-line ($P < 0.05$). The only conclusive differences between *CYP17* genotypes were observed within the H-line, where the H_O group showed lower conversion of pregnenolone, lower production of DHEA and corticosterone, as well as higher production of 16-hydroxyprogesterone ($P < 0.05$). Cortisol production reached a maximum of 1.19 ± 0.08 µM for the H_O group, 1.12 ± 0.07 µM for the H_E group, 0.25 ± 0.09 µM for the L_O group, and 0.22 ± 0.06 µM for the L_E group.

Upon ACTH stimulation (Figure 7.3), steroidogenesis was markedly different among the four AA cultures for all steroid metabolites assayed, except for pregnenolone and deoxycorticosterone ($P > 0.05$). The H-line produced more 17-hydroxypregnenolone, 17-hydroxyprogesterone, 11-deoxycortisol, cortisol, corticosterone, 18-hydroxycorticosterone and DHEA than the L-line ($P < 0.05$). The only differences in *CYP17* genotypes were observed within the H-line, where the H_O group showed lower production of DHEA and higher production of 16-hydroxyprogesterone ($P < 0.05$). Cortisol production reached a maximum of 1.55 ± 0.29 µM for the H_O group, 1.17 ± 0.25 µM for the H_E group, 0.35 ± 0.11 µM for the L_O group, and 0.27 ± 0.06 µM for the L_E group.

Stimulation with forskolin (Figure 7.4), affected steroidogenesis differently amongst the four AA cultures for all steroid metabolites, except for androstenedione and aldosterone. The H-line produced more 17-hydroxypregnenolone, 17-hydroxyprogesterone, 11-deoxycortisol, cortisol, corticosterone, and 18-hydroxycorticosterone, while less progesterone and deoxycorticosterone was produced compared to the L-line ($P < 0.05$). The only differences in *CYP17* genotypes were observed within the H-line, where the H_O group showed lower conversion of pregnenolone, lower production of progesterone, corticosterone and DHEA, while producing more 17-hydroxyprogesterone and 16-hydroxyprogesterone ($P < 0.05$). Cortisol production reached a maximum of 1.49 ± 0.28 µM for the H_O group, 1.08 ± 0.21 µM for the H_E group, 0.36 ± 0.06 µM for the L_O group, and 0.26 ± 0.06 µM for the L_E group.

Upon stimulation with cholera toxin (Figure 7.5), steroidogenesis differed amongst the four AA cultures for all steroid metabolites, except for aldosterone. The H-line produced more 17-hydroxypregnenolone, 17-hydroxyprogesterone, 11-deoxycortisol, cortisol, corticosterone, and 18-

hydroxycorticosterone, while less progesterone and deoxycorticosterone was produced compared to the L-line ($P < 0.05$). The only differences in *CYP17* genotypes were observed within the H-line, where the H_O group showed lower conversion of pregnenolone, lower production of deoxycorticosterone, corticosterone and DHEA, while producing more 16-hydroxyprogesterone ($P < 0.05$). Cortisol production reached a maximum of 1.90 ± 0.31 µM for the H_O group, 1.45 ± 0.28 µM for the H_E group, 0.37 ± 0.06 µM for the L_O group, and 0.36 ± 0.09 µM for the L_E group.

There were no significant differences in the effects of forskolin, cholera toxin and ACTH on the production of cortisol ($P > 0.05$). The effect of forskolin was, however, significantly different ($P < 0.05$) compared to the effect of ACTH for the H_O group with respect to 17-hydroxypregnenolone and 16-hydroxyprogesterone; the H_E group with respect to 17-hydroxypregnenolone; the L_O group with respect to 17-hydroxyprogesterone, 16-hydroxyprogesterone, androstenedione, deoxycorticosterone and DHEA; as well as the L_E group with respect to 16-hydroxyprogesterone. The effect of cholera toxin was significantly different ($P < 0.05$) compared to the effect of ACTH for the H_O group with respect to 17-hydroxyprogesterone and 16-hydroxyprogesterone; the L_O group with respect to 16-hydroxyprogesterone, deoxycorticosterone, corticosterone and DHEA; as well as the L_E group with respect to 16-hydroxyprogesterone and androstenedione.

The morphology of the AA cells differed among treatments and the microscopic images are shown in Figure 7.6. AA cells that were treated with pregnenolone (Figure 7.6A) displayed similar normal cell morphology as expected from AA cells under basal conditions (no treatment, data not shown). Treatment of AA cells with ACTH (Figure 7.6B), however, resulted in a “round” cell morphology in certain cells, while treatment with forskolin and cholera toxin caused “rounding” of almost all the AA cells. Despite the altered cell morphology, these cells were still viable as reflected by their ability to convert pregnenolone to the various steroid metabolites. These cells also remained attached (signal of viability) to the tissue culture plate throughout the three day experiment.

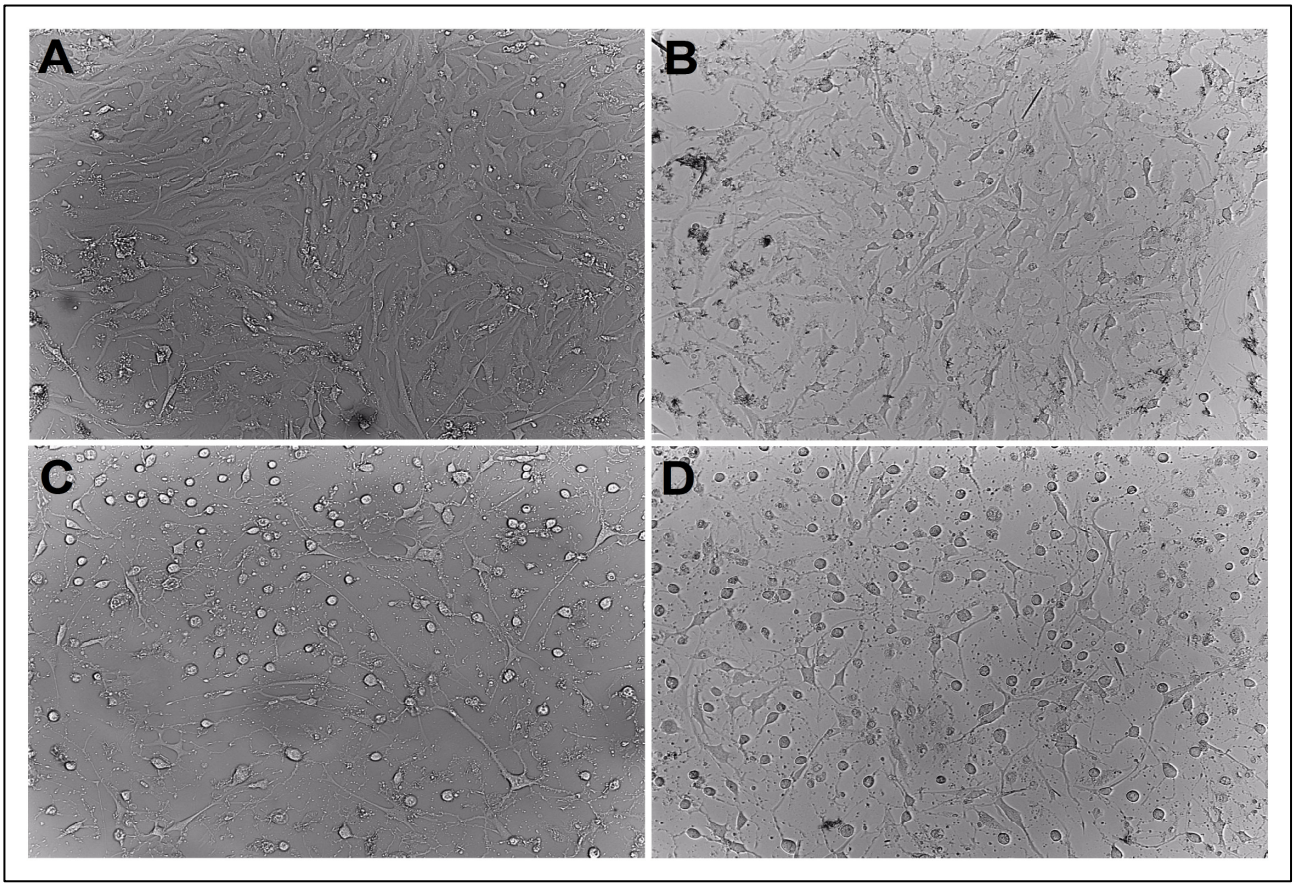

Figure 7.6. Microscope images of primary cultures of adult adrenal gland cells (AA cells). These images displays the appearance of AA cells with different substrates: A) 100 µM pregnenolone; B) 100 µM pregnenolone and 1 µM ACTH; C) 100 µM pregnenolone and 10 µM forskolin; and D) 100 µM pregnenolone and 100 µM cholera toxin. Images were obtained with CellOne software using an Olympus XR microscope at X10 enlargement.

7.4 Discussion

The results obtained in this study showed that the concentrations of pregnenolone and 16-hydroxyprogesterone are exclusively affected by the *CYP17* genotype, while the selection lines predominantly affected the concentrations of 17-hydroxypregnenolone, 11-deoxycortisol, cortisol and 18-hydroxycorticosterone. Moreover, the interaction between the *CYP17* genotype and selection lines affected the concentrations of DHEA, progesterone, 17-hydroxyprogesterone, deoxycorticosterone and corticosterone. These observations explain the unique cortisol:corticosterone ratio for each of the four AA cell groups. The cortisol:corticosterone ratio indicated that the H_O group produced more cortisol (~2-fold) relative to corticosterone when compared to the H_E group. It is noticeable, however, that the cortisol production was not significantly different among *CYP17* genotypes over 72 hours, but that it was significantly different within the H-line after 24 hours. The H_O group produced more cortisol after 24 hours than the H_E group when stimulated with ACTH, forskolin and cholera toxin, but not under basal conditions or with pregnenolone treatment (no ACTH, forskolin or cholera toxin). The cortisol concentrations

also started to decrease after this 24 hour peak. The current study therefore confirms that the presence of WT1 was more advantageous for cortisol production than WT2, as was suggested in Chapter 5. In all cases, it was clear that the H-line was able to elicit a higher total glucocorticoid response than the L-line. This glucocorticoid production difference was the most pronounced for cortisol. The contention that this is the result of differences in expression of steroidogenic enzymes is likely, but requires further investigation. Such a study would be possible, since the cDNAs from these AA cells were obtained, but remains to be analysed for the relative expression of the steroidogenic enzymes (data not yet available).

Interestingly, where 3βHSD and CYP17 are the first enzymes to compete for pregnenolone as common substrate, pregnenolone (100 µM) is primarily converted to progesterone (maximum at 50 – 80 µM) instead of 17-hydroxypregnenolone (maximum at 2 – 7 µM). Therefore it would be reasonable to assume that the competition between CYP17 and CYP21 for progesterone is the next important enzyme-competing step where a large quantity of steroid may be redirected towards cortisol production (50-80 % of total potential precursors of cortisol). However, the supply of 17-hydroxyprogesterone (maximum at 2 – 18 µM) from the conversion of 17-hydroxypregnenolone by 3βHSD should not be neglected and remains an important supply of precursor for cortisol production.

The homozygous *WT1/WT1* groups for both the H- and L-lines unexpectedly produced more 16-hydroxyprogesterone than the heterozygous *WT1/WT2* groups in the AA cells. This is in contrast to observations from Chapter 5, where WT1 produced less 16-hydroxyprogesterone than WT2. However, WT1 displayed a higher 17-hydroxyprogesterone/16-hydroxyprogesterone-ratio than WT2. The catalytic properties thus suggest that a higher expression of WT1 is required to obtain higher 16-hydroxyprogesterone concentrations compared to WT2. In the AA cells, the production of 16-hydroxyprogesterone was shown to be under the exclusive control of the *CYP17* genotype, while the concentrations of 17-hydroxyprogesterone were influenced by both the *CYP17* genotype and selection line. Furthermore, the concentrations of 16-hydroxyprogesterone were different when stimulated with forskolin and cholera toxin, compared to stimulation with ACTH, for the H_O, L_O and L_E groups. It is speculated that the higher 16-hydroxyprogesterone production in the homozygous *WT1/WT1* compared to heterozygous *WT1/WT2* sheep may result from a higher expression of the WT1 isoform. It might be possible that an unequal expression of the CYP17 isoforms could also result from either a duplication of the *WT1* sequence, or a difference in expressional control of the two isoforms, such as the case of bovine *CYP17A1* (Vanselow and

Fürbass, 2011). However, such statements cannot be supported by the present data and it is suggested that the cDNA obtained from these AA cells should be analysed for expressional bias.

This study investigated how the glucocorticoid production in the sheep adrenal gland is affected by the *CYP17* genotype, with particular emphasis on cortisol production. In Chapter 5, small catalytic differences were observed in the activity of the two CYP17 isoforms. It was predicted that the WT1 isoform would be more likely to support cortisol production than the WT2 isoform. However, it was unknown how these minor catalytic differences would affect steroidogenesis as a whole. The results from the present study provides evidence in support of the hypothesis that the small catalytic differences of the two CYP17 isoforms may translate to significant differences in cortisol production *in vitro*, where the presence of WT1 results in a higher cortisol production than WT2.

In addition to the *CYP17* genotype, the differences in adrenal steroidogenesis were investigated for sheep that are considered to have a superior (H-line) or inferior (L-line) ability to cope with stress (Cloete et al., 2005a). These sheep were subjected to an arena test, were their behavioural stress responses to isolation from their flock - a potent stimulus of the HPA axis - were monitored. The present study thus investigated if there were any differences in the HPA axis at the level of adrenal steroidogenesis between the H- and L-lines. It was found that the production of cortisol was significantly higher in the H-line, compared to the L-line. These differences in steroidogenesis are likely the result of genetic differences between the selection lines (Cloete et al., 2004; Cloete et al., 2005a). The results also reflect the genetic variability within adrenal steroidogenesis that may be exploited to improve cortisol responses to the HPA axis if these putative genetic factors could be identified. The current study identified *CYP17* as one such a factor.

The observation that there were no significant differences in the effects of ACTH, compared to forskolin and cholera toxin, on the production of cortisol, suggests that the intracellular signalling pathway is intact for each selection line x *CYP17* genotype group. In other words, it is unlikely that the variation in steroidogenic responses to ACTH stimulation among these groups stems from variation in the ACTH-stimulated production of intracellular cAMP via G_s. The Bonferroni's post-test, however, indicated differences in some steroid metabolite concentrations within each group for ACTH, forskolin and cholera toxin. This finding might imply that the intracellular responses of each group to intracellular cAMP may be different, rather than the production of cAMP. However, further research is required for such a conclusion.

Forskolin and cholera toxin was observed to have a "rounding" effect on the cellular morphology of the AA cells. Treatment with ACTH showed a similar effect, but to a lesser extent. This

phenomenon was previously observed by Voorhees et al. (1984) in Y1 mouse adrenal tumour cells. These researchers observed a "rounded" cell morphology of adrenal cells within 5 minutes of ACTH treatment and it correlated with changes in steroidogenic output (higher glucocorticoid production). This morphological change in adrenal cells was explained by the cAMP-dependent dephosphorylation of paxillin, a focal adhesion protein (Han and Rubin, 1996; Whitehouse et al., 2002). The organelles are subsequently clustered, which brings the mitochondria (where CYP11A1 is located) in close proximity to the endoplasmic reticulum, where CYP17, CYP21 and 3βHSD are located. Forskolin and cholera toxin causes the prolonged activation of adenylate cyclase and the higher production of cAMP consequently results in the higher degree of "roundedness" compared to ACTH treatment.

Although the differences in the intracellular cholesterol economy between the H- and L-line AA cells in response to ACTH stimulation (no pregnenolone added) would be interesting to investigate, the focus of the current study was to investigate the relative contributions of the CYP17 isoforms in steroidogenesis. The addition of pregnenolone as substrate in the current experimental setup was merely done to minimize variability within the system to reveal the true effects of the two CYP17 isoforms in the AA cells. Furthermore, the endogenous steroid concentrations were low and near the LOQ for the method employed, which made it difficult to accurately measure any differences that could result between the four different groups of AA cells. If such a study should be undertaken, a significantly higher injection volume is suggested for UPLC-MS/MS analysis, though would complicate subsequent separation and quantification.

The concentration of aldosterone was consistently low throughout all the AA cell treatments. The production of aldosterone is regulated primarily by angiotensin II and renin, and the results suggest that aldosterone levels are not increased upon ACTH stimulation. However, the concentrations of aldosterone in the samples were near the LOQ, which made conclusions about its regulation by ACTH impossible. It might be possible that the cells from the zona glomerulosa – the outer most zone of the adrenal cortex – were damaged in the preparative procedure of the AA cultures. This theory could explain why low concentrations of aldosterone, and 18-hydroxycorticosterone, were detected in the AA cultures, since CYP11B that mediates their production is expressed exclusively in the glomerulosa cells.

7.5 Conclusion

Both the *CYP17* genotype and selection line was responsible for qualitative differences in adrenal steroidogenesis. It is plausible to consider selection for the *CYP17* genotype to improve the

glucocorticoid response from the adrenal gland in Merino sheep. It is also evident that additional factors play an important role in steroidogenesis and is reflected in the marked differences between the H- and L-lines. The results suggest that glucocorticoid production responded to the divergent selection imposed on these lines, as reflected by the distinct differences in adrenal steroidogenesis of sheep from the H- and L-lines. It would be worth the effort to identify the putative genetic factors that influence adrenal steroidogenesis with the purpose of utilizing it as selection criteria in a breeding objective to improve the glucocorticoid response and ultimately robustness in sheep. It would also be interesting to investigate possible differences in the cholesterol economy between the selection lines, as well as compare their quantitative supply of pregnenolone to steroidogenesis on ACHT stimulation.

The H- and L-lines have shown distinct differences in their steroidogenic output, which prompts the question of how the HPA axis responses of these animals would compare to one another. The next chapter investigates the *in vivo* HPA axis responses of the H- and L-lines and the relative contribution of the *CYP17* genotype in these responses.

CHAPTER 8

Stress coping ability in a divergently selected Merino population: The potential of *CYP17* as genetic marker for robustness

8.1 Introduction

The proper functioning of the HPA axis is crucial in the adaptive response of an animal to various stressors. The effectiveness of the HPA axis response is dependent not only on the quantity of cortisol that is produced, but also on the time that is required to produce sufficient cortisol and the responsiveness of target tissues to circulating cortisol concentrations (Smith and Dobson, 2002; Mormède et al., 2007; Mormède et al., 2011). The efficiency at which the HPA axis produces and responds to cortisol will ultimately determine how quickly a stressor is counteracted and whether the animal is in a state of stress, distress or overstress (Selye, 1946; Ewbank, 1985). It is therefore proposed that the HPA axis response of animals be investigated in a time-dependent manner.

The measurement of glucocorticoids (cortisol in sheep) is the gold standard for measuring the stress responses in animals, since it reflects the functioning of the HPA axis (Mormède et al., 2007, 2011). Therefore it is suggested that the cortisol response to simulated stress be measured to assess the HPA axis function. Various stressful situations may be simulated for the measurement of the HPA axis responses, including the transport of animals, isolation from the flock, and administration of insulin, CRH or ACTH. The administration of insulin simulates hypoglycaemic stress and allows the measurement of glucose concomitantly with cortisol concentrations, which allows the monitoring of the both the response and recovery of the animal to the stress. An insulin-induced hypoglycaemic stress test is therefore proposed to monitor the time-dependent cortisol responses of sheep.

Although the isolation of a sheep from its flock is also a potent stimulus of the HPA axis (Lynch et al., 1992), the measurement of cortisol is an invasive approach that would compromise the stress responses of sheep under such circumstances. Instead the measurement of behavioural responses is suggested for the assessment of isolation stress. Since researchers have been able to correlated ewe temperament with lamb survival and maternal behaviour (Murphy et al., 1994; Murphy, 1999), Cloete et al. (2005a) assessed the behavioural responses to flock isolation (reflects temperament) of South African Merino sheep, which were divergently selected for (H-line) or against (L-line) the ability of a ewe to rear multiple offspring per birthing opportunity. The H-line was previously

shown to have a markedly higher lamb survival and maternal behaviour than the L-line (Cloete and Scholtz, 1998). Cloete et al. (2005a) observed that the H-line showed a superior stress coping ability than the L-line, where the H-line allowed smaller distances from humans, while the L-line lambs defecated more frequently throughout the stress test (Cloete et al., 2005a). Furthermore the meat quality of these two lines was assessed at time of slaughter (Hoffman et al., 2003; Cloete et al., 2005a). It was found that the mean slaughter weight, dressing percentage, carcass weight and vivid red meat colour was higher in the H-line than in the L-line (Cloete et al., 2005a). In relation to stress, the selection line difference in the pH of meat was the most important consideration. The collective observations from these studies demonstrate the complex relationship between stress, production (meat quality and ease of handling) and reproduction (mothering ability and lamb survival). Cloete et al. (2005a) proposed that divergent selection, for and against the ability of ewes to rear multiples, may have resulted in differences in the functioning of the HPA axis in the H- and L-line animals. However, the activity of the HPA axis of the H- and L-line animals has not been assessed on a physiological level to date (e.g. measurement of cortisol response to stress).

The present study thus set out to investigate the functionality of the HPA axis in the H- and L-lines by assessing their cortisol response to insulin induced hypoglycaemic stress. In addition, the relative contribution of the *CYP17* genotype was investigated within each selection line for both the cortisol responses to hypoglycaemic stress and behavioural responses to flock isolation (arena test). An ovine specific *CYP17* genotyping test was developed to analyse genomic DNA from the blood of sheep in the breeding program. Furthermore, correlations among the cortisol response and various measurable indices of reproduction and arena stress test performance were investigated.

8.2 Materials and methods

8.2.1 CYP17 genotyping

8.2.1.1 Genomic DNA isolation from blood samples

Blood samples were obtained from sheep of the previously mentioned breeding program (Chapter 7). Blood samples were collected from the jugular vein in EDTA treated tubes, without prior knowledge of the selection line to which the individual sheep belong to. Blood samples were stored at –20^{o}C for downstream analysis. The NucleoSpin Blood Core Kit from (Macherey-Nagel, Düran, Germany) was used to isolate genomic DNA from the blood samples. All isolations were performed by the DNA Sequencing Laboratory of the Central Analytical Facility at Stellenbosch University, Stellenbosch, South Africa. The protocol was optimised for DNA yield (all chemicals included with

kit, except ethanol). Briefly, 400 µL blood, 400 µL buffer BQ1 and 25 µL proteinase K was mixed and incubated at 60 ^{o}C overnight. After the addition of 400 µL ethanol, the contents were mixed, then transferred to the binding plate and overlayed with 150 µL buffer B5 before applying the vacuum. The binding plate was then washed with 600 µL buffer BW and two separate 900 µL buffer B5 applications in three respective vacuum steps. The silica membrane was dried for 15 minutes under vacuum, before eluting the DNA in two sequential vacuum steps with 100 µL elution buffer that was pre-heated to 70 ^{o}C. DNA yields were typically between 2 (in blood samples stored for 6 years) to 50 ng.µL^{-1} (blood samples stored for less than a year) with 260nm/280nm ratios within the acceptable range of 1.8 to 2.2. Blood samples from sheep born from 1995 to 2010 were analysed.

8.2.1.2 CYP17 genotyping with real time polymerase chain reaction

The Assays-by-Design^{SM} service for SNP genotyping assays (part number: 4332077) from Applied Biosystems (California, USA) was used to develop an ovine *CYP17* genotyping method. This real time PCR method utilized TaqMan® MGB probes with FAM™ and VIC® dye-labels (Applied Biosystems and Roche Molecular Systems Inc., California, USA). The sequence of the VIC®-labelled probe was 5'-CTTCCTTGCTCAGAACC-3' and its fluorescence indicated the presence of the *WT1* allele. The sequence of the FAM™-labelled probe was 5'-TCCTTGCCCAGAACC-3' and indicated the presence of the *WT2* allele. The unlabelled primer sequence of the forward primer was 5'-CCTGAAGGCCATACAAAATGTCAAT-3' and the unlabelled reverse primer sequence was 5'-GCGCAGGGAATATGTCTAACAGAA-3'. Genomic DNA was used as template for reactions, which were performed in duplicate in 96-well plates (25 µL final volume/well) according to the manufacturer's instructions. In each assay a negative (no-template) and two positive controls were included in duplicate. A positive control for each allele consisted of plasmid vectors that contained the cDNA insert of either *WT1* or *WT2*. The *CYP17* genotypes of 562 genomic DNA samples were analysed.

Furthermore, the presence of the SNP at nucleotide position 628 was investigated with direct sequence analysis. The ACS forward primer designed by Storbeck et al. (2007) was used in the analyses: 5'-GAGATCCTGTCAGACAACCA-3'. An ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, California) by the Central Analytical Facility of Stellenbosch University to perform direct sequence analyses. Sequencing results were analysed with BioEdit Sequence Alignment Editor (version 7.0.5.2 © 1997-2007, T. Hall) software.

8.2.2 Stress tests

8.2.2.1 Insulin induced hypoglycaemic stress

This stress test was performed on thirty-eight adult breeding rams (2-6 years of age) from the breeding program (17 H- and 21 L-line sheep) at the Elsenburg Research Farm, situated at lattitude 33° 51‘ S and longitude 18° 50‘ E at an elevation of 177 m. Sheep were kept in separate pens, but maintained visual contact with the members of the flock, and given *ad libitum* access to water and ground alfalfa hay for 1-2 weeks. The feed intake was weighed during the housing period to measure daily feed intakes and was normalized per body weight. Human insulin (Actrapid® HM, Novo Nordisk, Johannesburg, South Africa) was diluted to 1 IU mL^{-1} 1% NaCl solution 2 hours prior to the stress test and administered intravenously to the left jugular vein at a dosage of 0.1 IU kg^{-1} body weight. Blood samples were collected from the right jugular vein of each animal in EDTA treated collection tubes (Becton, Dickinson and Company, UK) that were immediately placed on ice. The first blood collection was done directly prior to insulin administration, followed by blood collections at 15, 30, 60, 90 and 120 min post insulin administration. Representative plasma samples from each animal were obtained the same day of the test by means of centrifugation (2 500 x g; 10 min; 4 ^{o}C), after which plasma samples were transferred to 1.5 mL microcentrifuge tubes and stored at 4 ^{o}C until analysis followed the next day. Plasma glucose and cortisol levels were determined with radio-immunoassay by the PathCare Reference Laboratory (PathCare Park, N1 City, Goodwood, Cape Town, South Africa). Cortisol and glucose measurements of sheep that did not reach a hypoglycaemic state (defined as < 2 mmol plasma glucose L^{-1}) were excluded from the data. Ethics approval for this stress test was obtained from the Departmental Ethics Committee for Research on Animals (DECRA reference R08/21) of the Western Cape Department of Agriculture.

8.2.2.2 Animals and breeding program

All the animals in the current study belonged to the South African Merino breeding program that was undertaken in 1986, where sheep have been divergently selected for their ability to rear multiple offspring (Cloete and Durand, 1994; Cloete et al., 2004). The selection lines were derived from the same base population and selection within each line was based on maternal ranking values for number of lambs weaned per lambing opportunity (Cloete and Durand, 1994). Number of lambs weaned per mating in the line selected in the upward direction (H-line) has been proved to be near to double that of the line selected in the downward direction (L-line) (Cloete and Scholtz, 1998). Furthermore, these lines have also showed marked differences in lambing behaviour (Cloete and

Scholtz, 1998; Cloete et al., 2003; Cloete et al., 2005b). Reproductive performance of breeding ewes from 1996 to 2008 was recorded as: number of lambs born per mating opportunity (NLB); number of lambs weaned per lambing opportunity (NLW); and total weight weaned per lambing opportunity (TWW) (H_E n = 221 H_O n = 75; L_E n = 50; L_O n = 22). Refer to Cloete and Durand (1994), as well as Cloete et al. (2004; 2009), for more details about the breeding program.

8.2.2.3 Arena stress test

In this isolation stress test, sheep entered a 10.6 m X 4.0 m arena (marked out in 18 squares) one-by-one (H_E n = 260; H_O n = 74; L_E n = 53; L_O n = 13). The arena was surrounded by wooden panels to prevent escape, but still allowing visual contact of six sheep on the opposite side of the arena, behind a split-pole fence, where a human operator was situated on a chair. The operator remained motionless, while the behaviour of the sheep was assessed for 3 minutes according to the following parameters: number of bleats, number of defecation events, number of urinating events, average distance from human operator (meters); and movement as number of the boundaries between squares that were crossed (crosses). For more details about this arena test and its recordings, refer to Cloete et al. (2005a).

8.2.3 Statistical analysis

GraphPad Prism (version 4) software (GraphPad Software, San Diego, California) was used for all statistical analysis. For the hypoglycaemic stress test, plasma glucose and cortisol responses over time were analysed for the selection lines (H- vs. L-line) and *CYP17* genotype (homozygous *WT1/WT1* vs. heterozygous *WT1/WT2*) with a paired t-test, while the selection line x *CYP17* genotype sub-groups (H_O, H_E, L_O and L_E) were compared with a repeated measures one-way ANOVA and Bonferroni's post-test. The peak cortisol concentrations at 60 minutes post insulin treatment, as well as the area under the curve of the normalized cortisol response (cortisol concentration divided by the glucose concentration), were compared with a two-way ANOVA and Bonferroni's post-test. Fisher's exact test was used to analyse the *CYP17* genotype frequencies (H- vs. L-line). The average feed intakes of the *CYP17* genotype X selection line sub-groups were compared with a two-way ANOVA and Bonferroni's post-test.

The interaction effects of *CYP17* genotype and selection line was tested with a two-way analysis of variance and a Bonferroni's post-test for each parameter in the insulin tolerance test (parameters: cortisol production 60 minutes post insulin administration, and area under the curve for total cortisol produced over 2 hours post insulin administration), arena test (parameters: average distance

between the sheep and the human operator, movement in arena depicted by number of squares crossed, number of bleats, number of urinating events, and number of defecating events), as well as reproductive performance assessment (parameters: number of lambs born per lambing opportunity, number of lambs weaned per lambing opportunity, total weight weaned per lambing opportunity). The relationship between mean cortisol production and the means of each reproduction or behaviour trait recorded was investigated with a correlation test between the means (taking into account the standard error of the mean and number of observations) of each *CYP17* genotype x selection line (H_E: H-line heterozygotes *WT1/WT2*; H_O: H-line homozygotes *WT1/WT1*; L_E: L-line heterozygotes *WT1/WT2*; and L_O: L-line homozygotes *WT1/WT1*).

8.3 Results

8.3.1 CYP17 genotyping results

The positive controls indicated that the TaqMan assay was suitable for the detection of both *WT1* and *WT2* alleles, as shown in Figure 8.1 (areas D and A, respectively). The VIC-specific fluorescence of the homozygous *WT1/WT1* genomic DNA samples corresponded with the fluorescence area D, where the *WT1* positive controls were also detected. Area D (Figure 8.1) was located between VIC-fluorescence of 2.3 to 3.2 and FAM-fluorescence below 1.2 (as indicated by the negative controls in area E). The positive control for *WT2* (area A, Figure 8.1) showed FAM-fluorescence of 3.6 to 4.8 and a VIC-fluorescence below 1.7. However, no homozygous *WT2/WT2* genotypes were detected in the 562 genomic samples that were analysed. The heterozygous *WT1/WT2* samples therefore showed fluorescence between these values, and interestingly formed two distinct groups of heterozygote animals. These two heterozygous groups consistently fell in either area B or C of Figure 8.1, even when these samples were re-analysed. Moreover, when 10 samples from area B (Figure 8.1) were analysed with the previous *CYP17* genotyping method (LightCycler real time PCR with FRET hybridisation probes), these samples showed melting curve peaks with approximately equal height (data not shown). In contrast, 10 samples from area C (Figure 8.1) showed skewed melting curve peaks with the LightCycler genotyping method, with a higher peak associated with the *WT1* melting temperature (data not shown). Furthermore, direct sequence analysis showed unequal double peaks in the sequence chromatogram from a sample in area B of the Taqman assay (Figure 8.2), where the higher peak was associated with the presence of adenine at nucleotide position 628 (*WT1*). In contrast, the double peaks of the sequence chromatogram from a sample in area C of the Taqman assay showed peaks of similar height (Figure

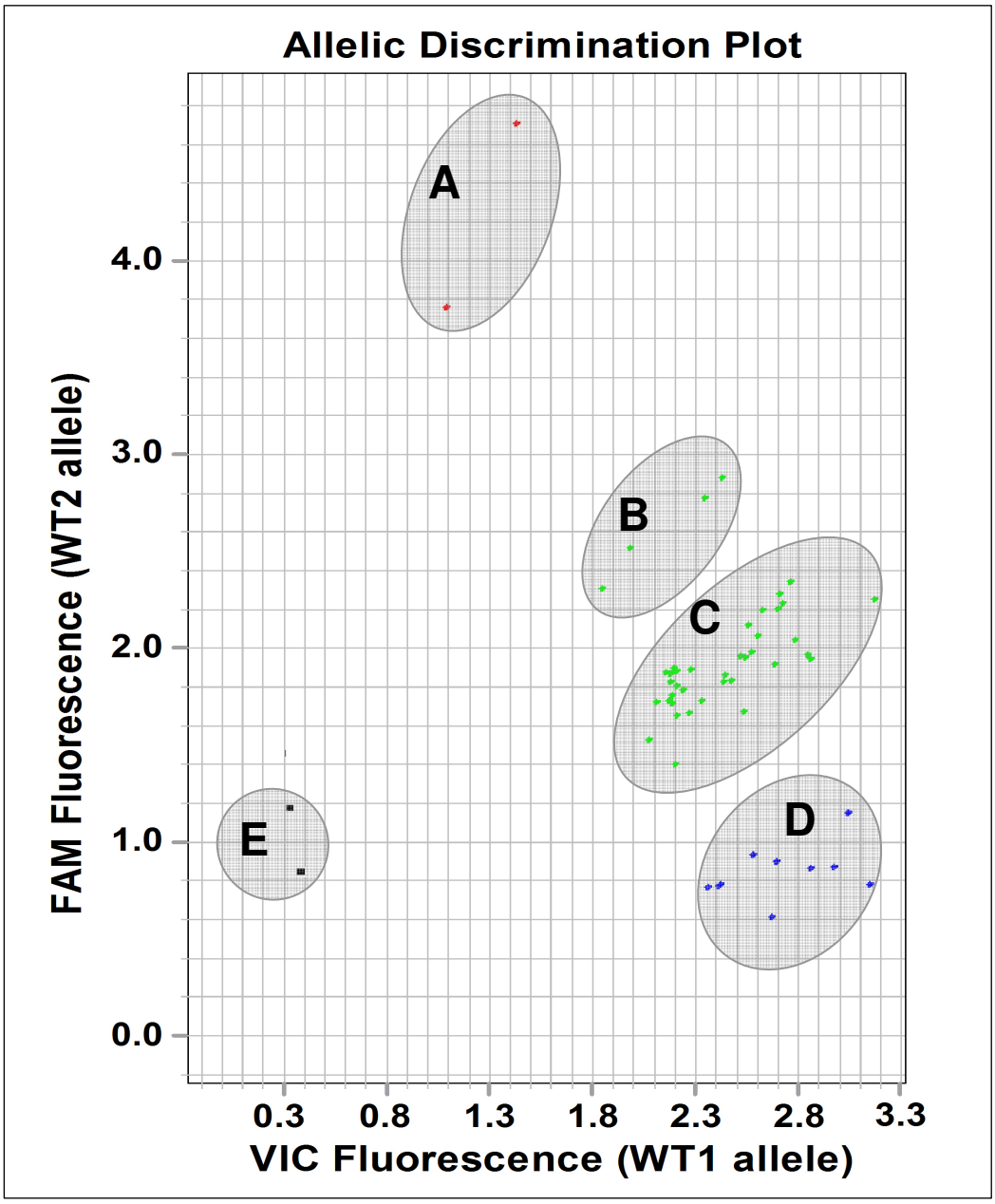

Figure 8.1. Typical allelic discrimination plot from the TaqMan genotyping assay. The VIC fluorescent dye (x-axis) indicates the presence of the WT1 allele, and area D therefore represents homozygous *WT1* samples and positive controls for *WT1*. The FAM fluorescent dye (y-axis) indicates the presence of the *WT2* allele, and area A therefore represents the positive controls for *WT2*. Area E represents the no-template negative controls. Heterozygous *WT1/WT2* samples were collectively detected in areas B (equal LightCycler melting peaks) and C (unequal LightCycler melting peaks).

8.3). For the purpose of the present study, however, the two heterozygous *WT1/WT2* groups were treated as one collective group.

From the analyses of 562 samples with the TaqMan assay, it was observed that 78.3 % of the population were heterozygous *WT1/WT2* and 21.7 % were homozygous *WT1/WT1*. Within the heterozygous group, 61.6 % were detected in area C of Figure 8.1, while 16.7 % were detected in area B. The same *CYP17* frequency distribution was observed in both the H- and L-lines (Fisher's exact test: $P > 0.05$).

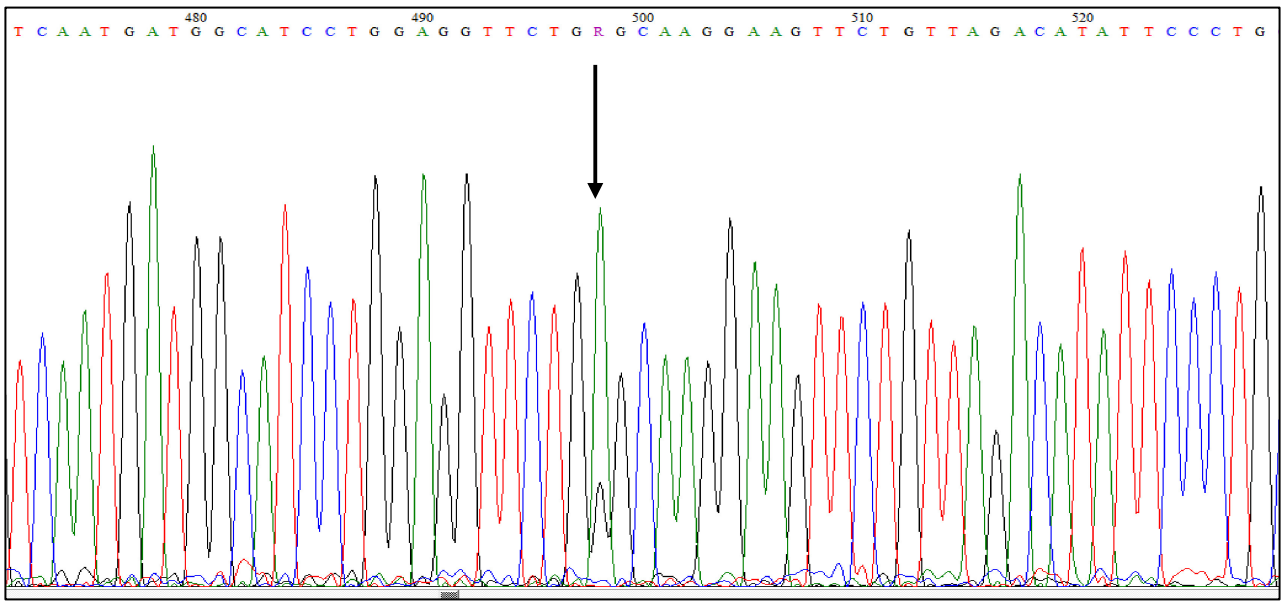

Figure 8.2. A segment from a direct sequence chromatogram depicting the unequal peak heights at nucleotide position 628 (indicated by the arrow), where the higher peak is associated with the presence of adenine (*WT1*). The ACS forward primer from Storbeck et al. (2007) was used to confirm the presence of this single nucleotide polymorphism in the South African Merino. BioEdit Sequence Alignment Editor (version 7.0.5.2 © 1997-2007, T. Hall) software was used in the analysis.

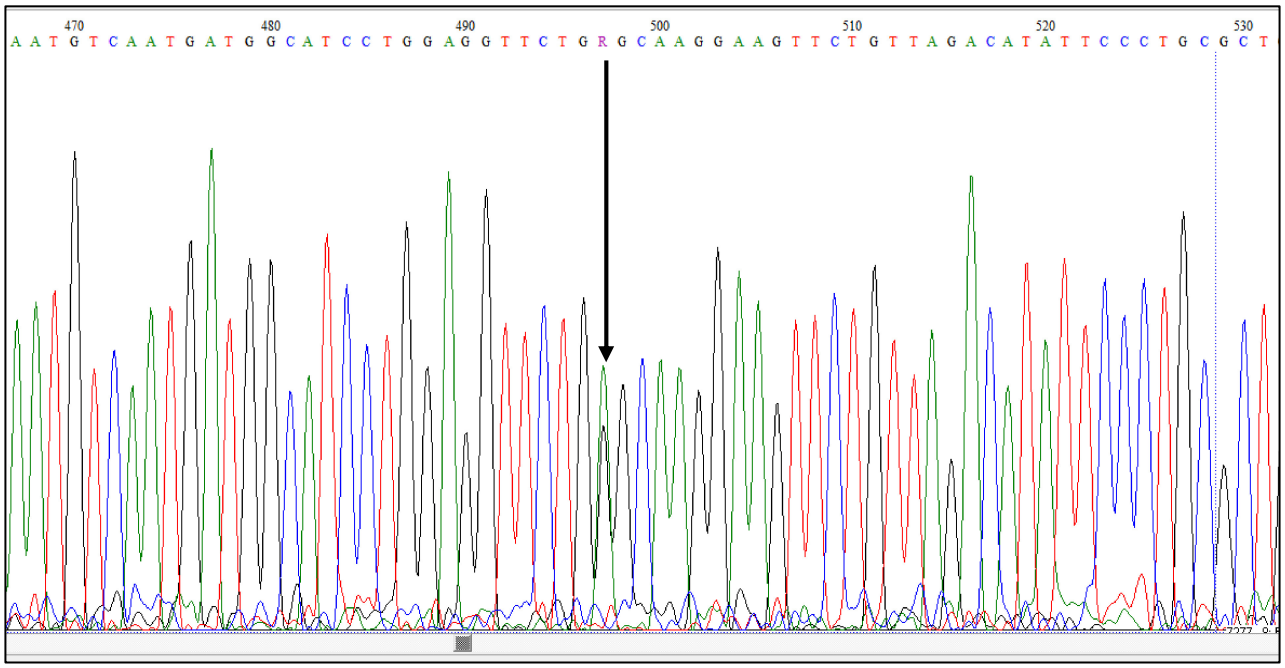

Figure 8.3. A segment from a direct sequence chromatogram depicting the similar peak heights at nucleotide position 268 (indicated by the arrow). The ACS forward primer from Storbeck et al. (2007) was used to confirm the presence of this single nucleotide polymorphism in the South African Merino. BioEdit Sequence Alignment Editor (version 7.0.5.2 © 1997-2007, T. Hall) software was used in the analysis.

8.3.2 Insulin induced hypoglycaemic stress test

The plasma glucose and cortisol responses of the rams were first grouped according their selection lines, namely the H- and L-lines (Figure 8.4). Glucose levels of the H-line reached hypoglycaemia (minimum: 1.82 ± 0.07 mmol glucose L^{-1}) quicker at 30 min and returned to baseline levels after 2 hours, while L-line glucose levels reached a hypoglycaemic state (minimum: 1.77 ± 0.11 mmol glucose L^{-1}) at 60 min and remained below the baseline after 2 hours. The H-line produced a higher cortisol peak (116.4 ± 7.35 nmol cortisol L^{-1}) at 60 min than the L-line (87.79 ± 12.03 nmol cortisol L^{-1}). However, a two-tailed paired t-test showed that the H-line did not have a higher cortisol response throughout the two hours than the L-line ($P = 0.0967$). The glucose response of the H-line, however, had a faster recovery rate (paired t-test: $P = 0.0003$) than the L-line and the glucose concentrations of the H-line was higher 90 and 120 minutes post insulin administration.

When the rams were grouped according to their *CYP17* genotype (Figure 8.5), the cortisol response of the homozygous *WT1/WT1* group was higher (paired t-test: $P = 0.0416$; maximum: 120.95 ± 14.62 nmol cortisol L^{-1}) compared to the heterozygous *WT1/WT2* group (maximum: 93.07 ± 8.74 nmol cortisol L^{-1}) (Figure 8.5B). The glucose responses, however, was not different between these two groups (paired t-test: $P = 0.7497$; minima: 1.81 ± 0.07 mmol glucose L^{-1} for the *WT1/WT2* genotype vs. 1.91 ± 0.19 mmol glucose L^{-1} for the *WT1/WT1* genotype) (Figure 8.5A).

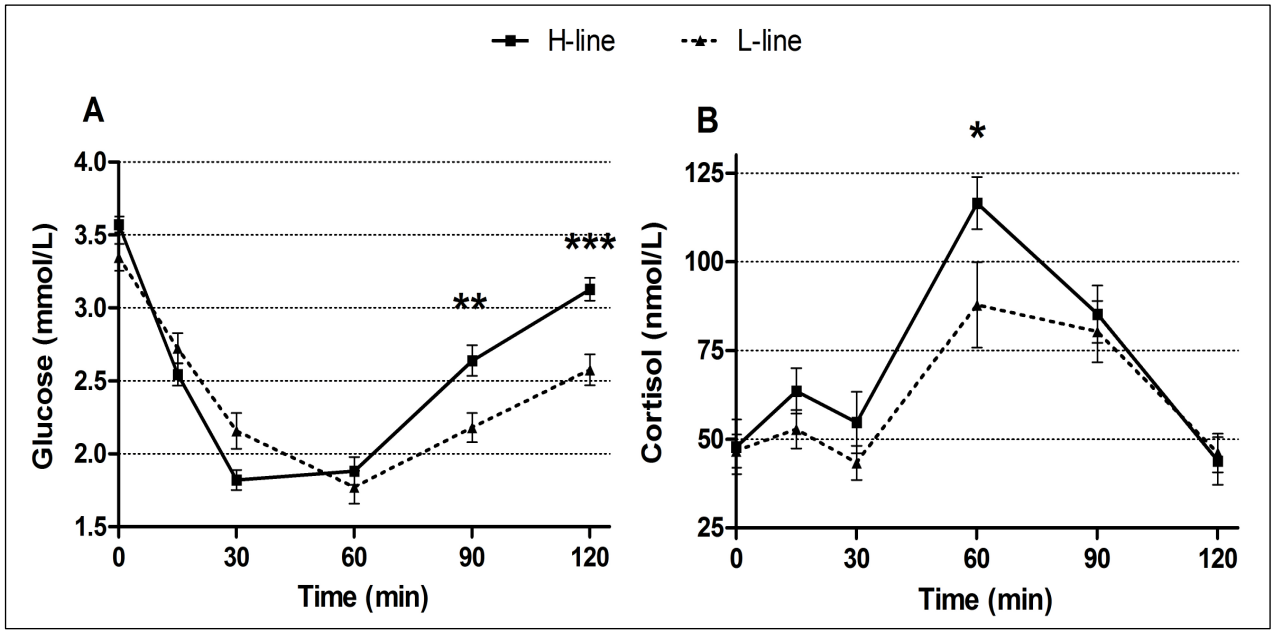

Figure 8.4. Plasma glucose (A) and cortisol (B) responses of the H- (solid line) and L-lines (broken line) to insulin induced hypoglycaemia. Results are representative of 18 H-line and 20 L-line Merino rams and expressed as mean ± SEM. *$P < 0.05$; **$P < 0.01$; ** $P < 0.001$.

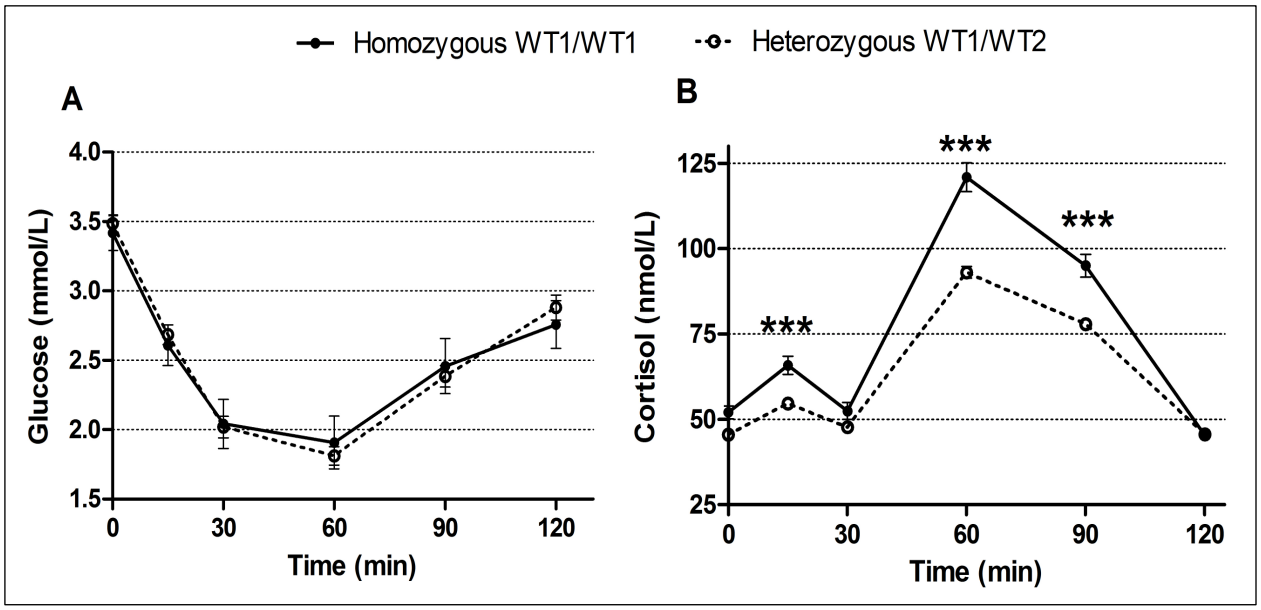

Figure 8.5. Plasma glucose (A) and cortisol (B) responses of homozygous *WT1* (solid line) and heterozygous *WT1/WT2* (broken line) sheep to insulin induced hypoglycaemia. Results are representative of 26 heterozygous *WT1/WT2* and 12 homozygous *WT1* Merino rams and expressed as mean ±SEM. ***$P < 0.001$.

The cortisol production of homozygous *WT1/WT1* sheep and H-line sheep seemed to have a common higher trend, which was the motivation for subdividing the H- and L-line sheep into their respective *CYP17* genotypes (Figure 8.6), namely H-line homozygous *WT1/WT1* (H_O), H-line heterozygous *WT1/WT2* (H_E), L-line homozygous *WT1/WT1* (L_O), and L-line heterozygous *WT1/WT2* (L_E) sheep. A repeated measures one-way ANOVA showed that the *CYP17* genotype and selection line are important factors influencing the cortisol response ($P = 0.0246$). Inspection of the graphs suggested that all sub-groups had an equally high cortisol production (Bonferroni: $P > 0.05$), except for the L_E group (72.83 ± 11.51 nmol cortisol L^{-1}) that showed a significantly lower (Bonferroni: $P < 0.05$) cortisol production than the L_O group (125.21 ± 26.57 mmol glucose L^{-1}) (Figure 8.6 C and D). Interestingly, the glucose responses (Figure 8.4 A and B) appeared to be distinct for each sub-grouping (one-way ANOVA: $P = 0.0485$), however, the only significant difference in glucose response was observed between the H_O (1.95 ± 0.11 nmol glucose L^{-1}) and L_O (1.72 ± 0.34 nmol glucose L^{-1}) groups. The glucose responses of the H_O and H_E groups returned to baseline (> 30 mmol L^{-1} glucose), while the L_O and L_E groups still remained below baseline after 2 hours. In this case, the glucose levels of the L_O group remained the lowest of all sub-groups (Figure 8.6B), despite its apparent largest cortisol response (Figure 8.6D).

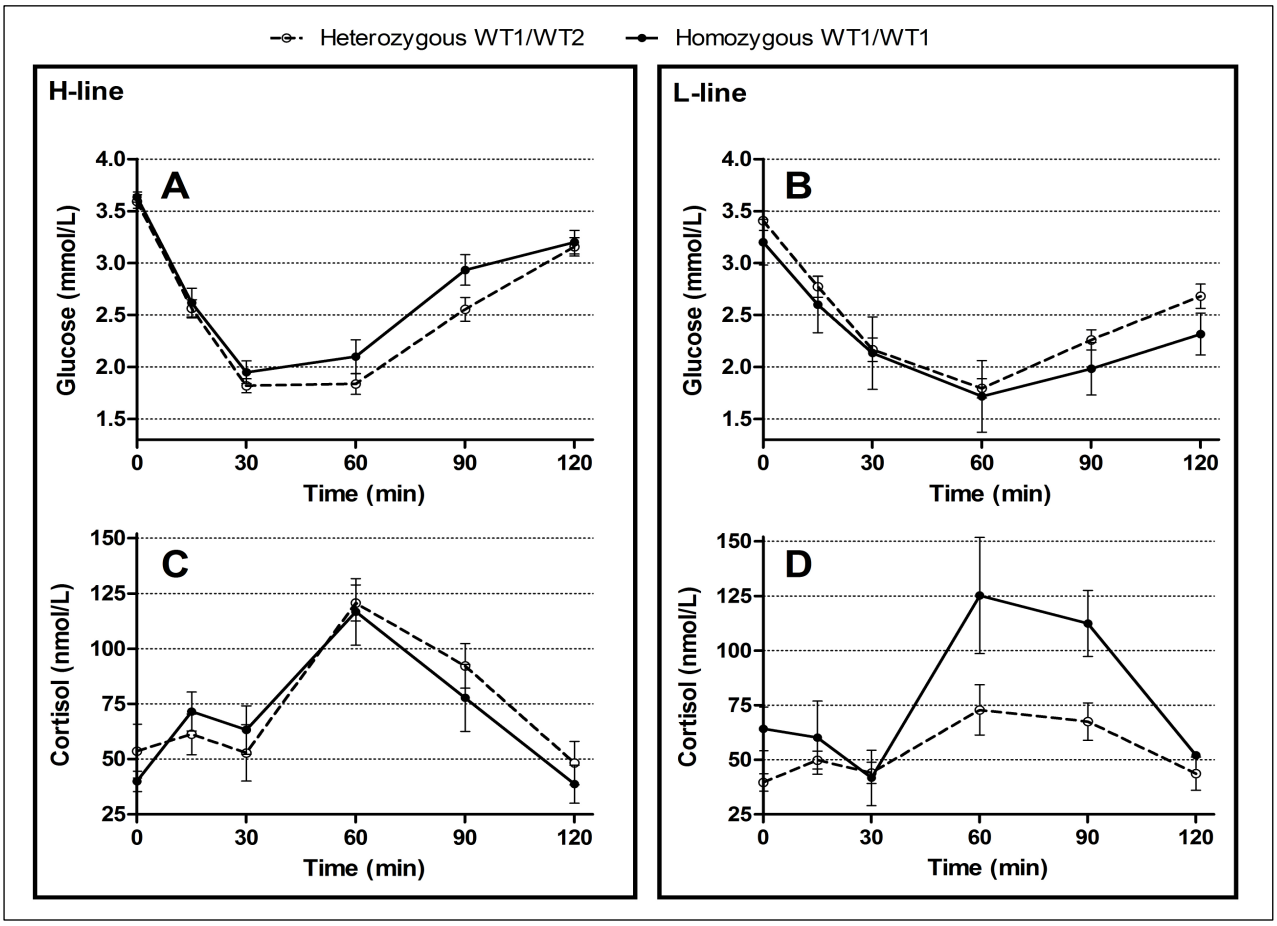

Figure 8.6. The plasma glucose (A and B) and cortisol (C and D) responses of the *CYP17* x breeding line subgroups to hypoglycaemic stress. Homozygous *WT1* groups are represented by solid lines, while heterozygous *WT1/WT2* sheep are represented by broken lines. The responses of H-line sheep (left: A and C) and L-line sheep (right: B and D), respectively, have been plotted on the same graph. Results are representative of 17 H-line (11 HE and 6 HO) and 21 L-line (15 LE and 6 LO) Merino rams and expressed as mean ±SEM.

The peak cortisol concentrations for the *CYP17* genotype x selection line sub-groups at 60 minutes post insulin treatment were compared with a two-way ANOVA, which indicated that neither the *CYP17* genotype ($P = 0.1268$) nor the selection line ($P = 0.3418$) were significant factors. However, the interaction between these factors approached significance ($P = 0.0524$). As mentioned previously, it is not only the magnitude of the cortisol response that is important, but also the duration of the cortisol output. For this reason the area under the curve (AUC) was calculated for the cortisol responses (cortisol concentration normalised by glucose concentration) and compared with a two-way ANOVA. These result indicated that total cortisol response was affected by the *CYP17* genotype ($P = 0.0378$), but not the selection line ($P = 0.1003$). The interaction between the selection line and the *CYP17* genotype, however, was significant ($P = 0.0226$). Differences in cortisol responses between the *CYP17* genotype were only found within the L-line (2751.5 ± 57.5 AUC for the *WT1/WT1* genotype vs. 1765.0 ± 179.0 AUC for the *WT1/WT2* genotype; Bonferroni: $P < 0.05$). In contrast, cortisol output was independent from *CYP17* genotype in the H-line

(respectively 2528.5 ± 225.5 AUC for the *WT1/WT1* genotype vs. 2610.5 ± 37.5 AUC for the *WT1/WT2* genotype; Bonferroni: $P > 0.05$).

The average daily feed intake (normalized for body weight) of sheep in the week prior to the hypoglycaemic stress test was not influenced by the *CYP17* genotype or selection line (Two-way ANOVA: *CYP17* genotype $P = 0.4864$, Selection line $P = 0.1828$, *CYP17* genotype X selection line $P = 0.360$). No significant difference observed in the average daily feed intakes among any of the groups (Bonferroni: $P > 0.05$; H_E: 30.88 ± 1.13 g feed kg^{-1} body weight, H_O: 31.34 ± 0.50 g feed kg^{-1} body weight, L_E: 34.17 ± 1.01 g feed kg^{-1} body weight, L_O: 31.78 ± 1.89 g feed kg^{-1} body weight).

8.3.3 Arena stress test

The arena test performance of sheep in the H_E (n = 260), H_O (n = 74), L_E (n = 53) and L_O (n = 13) subgroups were compared. As seen by the sample size, the L-line sheep were poorly represented compared to the H-line, due to the effects of downward selection on the birth rate and survival of L-line animals. The stressful behaviour of sheep was tested before one year of age (prior to exposure to various handling procedures) of lambs born from 2001 to 2008 of which the *CYP17* genotypes were known. The effect of the selection line and *CYP17* genotype, as well as their interaction, was assessed with a two-way ANOVA, followed by a Bonferroni's post-test, for each arena test

Table 8.1. Summary of the behavioural responses of sheep to the arena test that depicts the means (± standard error of the mean) and *P*-values from the two-way ANOVA with *CYP17* genotype (CG) and selection line (SL) as factors. Traits that were considered were the average distance from the human operator (ADIS), number of crosses (NCROSS), number of bleats uttered (NBL), number of urinating events (NUR) and number of defecating events (NDEF).

	H Line		L Line		*P*-values		
	WT1/WT1	*WT1/WT2*	*WT1/WT1*	*WT1/WT2*	SL	CG	SL x CG
Trait							
ADIS	3.65 ± 0.16	3.79 ± 0.99	3.05 ± 0.55	4.21 ± 0.28	0.7301	0.0192*	0.0646
NCROSS	18.70 ± 1.33	19.40 ± 0.75	15.46 ± 2.54	17.32 ± 1.30	0.1709	0.5096	0.7650
NBL	12.82 ± 1.23	17.50 ± 0.76	11.69 ± 3.11	18.42 ± 1.43	0.9574	0.0038**	0.6017
NUR	4.01 ± 0.78	1.79 ± 0.23	4.54 ± 2.45	2.49 ± 0.68	0.4466	0.0083**	0.9116
NDEF	1.16 ± 0.14	1.06 ± 0.07	0.77 ± 0.26	1.26 ± 0.13	0.6093	0.2852	0.1018

*$P < 0.05$; **$P < 0.01$

parameter (Table 8.1). It was found that the *CYP17* genotype ($P < 0.05$), but not the selection line or its interaction with the *CYP17* genotype ($P > 0.05$), had a significant effect on three arena test parameters, namely the number of bleats ($P = 0.0038$, *WT1/WT2*: 18.58 ± 0.69 bleats vs. *WT1/WT1*: 13.76 ± 1.15 bleats), number of urinating events ($P = 0.0083$; *WT1/WT2*: 1.45 ± 0.15 events vs. *WT1/WT1*: 3.45 ± 0.71 events) and the average distance allowed between the sheep and the human operator ($P = 0.0192$; *WT1/WT2*: 4.50 ± 0.17 meters vs. *WT1/WT1*: 4.26 ± 0.28 meters). The animals of the L_E group on average kept a longer distance (Bonferroni: $P < 0.05$; L_E: 4.21 ± 0.28 meters) from the human operator (signal of stress) compared to the L_O group (3.05 ± 0.55 meters) that showed a higher cortisol response to hypoglycaemia (better ability to cope with stress). The H_E group uttered more bleats (17.50 ± 0.76 bleats), but urinated less frequently (1.79 ± 0.23 events) during the arena test than the H_O group ($P < 0.05$; H_O: 12.82 ± 1.23 bleats, 4.01 ± 0.78 events). Although the psychological stress responses of these two H-line groups were different, their responses to physiological stress (insulin-induced hypoglycaemia) were the same.

Furthermore, the correlation between cortisol production and the arena test parameters were investigated for four sub-groups. When the peak cortisol concentrations were used in the analysis, there was no evident correlation ($P > 0.05$). However, when the area under the curve for the cortisol responses (cortisol concentration normalised by glucose concentration) was used in the analysis, a strong correlation was found with the defecating frequency (*P:* 0.0200; Pearson r: 0.98; R^2: 0.9603; n = 4). The L_E group that produced the lowest cortisol response in the hypoglycaemic test also defecated at the highest absolute frequency during the arena test. The average distance from the human operator also showed a certain degree of correlation, although it only approached significance (*P:* 0.0640; Pearson r: 0.9360; R^2: 0.8761; n = 4).

8.3.4 Reproductive performance

The reproductive performance of H_E (n = 221), H_O (n = 75), L_E (n = 50), and L_O (n = 22) ewes were compared (Table 8.2). The motivation for this investigation is that CYP17 also plays a central role in the production of reproductive hormones, the production of cortisol to initiate parturition, and it has been suggested that a high cortisol production is linked to litter size (SanCristobal-Gaudy et al., 2001; Mormède et al., 2007). The H- and L-line has been previously shown to have marked differences in the number of lambs born and weaned per lambing opportunity (Cloete and Scholtz, 1998). In the current study, the effect of the *CYP17* genotype, and its interaction with the selection line, was investigated by a two-way ANOVA and Bonferroni's post-test (Table 8.2).). It is clear

Table 8.2. Summary of reproductive performances, in terms of ewe reproduction and lamb output, that depicts the means (± standard error of the mean) and *P*-values from the two-way ANOVA with *CYP17* genotype (CG) and selection line (SL) as factors. Traits that were considered were the number of lambs born per joining (NLB), number of lambs weaned per lambing (NLW) and total weight of lamb weaned per lambing (TLWW).

Trait	H-Line		L-Line		*P*-values		
	WT1/WT1	*WT1/WT2*	*WT1/WT1*	*WT1/WT2*	SL	CG	SL x CG
NLB	1.14 ± 0.06	1.18 ± 0.04	0.73 ± 0.09	0.80 ± 0.06	< 0.0001***	0.4513	0.7851
NLW	0.97 ± 0.05	0.92 ± 0.03	0.54 ± 0.08	0.57 ± 0.05	< 0.0001***	0.8835	0.5664
TLWW	22.79 ± 1.18	20.93 ± 0.74	11.15 ± 1.72	12.30 ± 1.15	< 0.0001***	0.8117	0.3127

***$P < 0.001$

that the selection line is the only factor that affected reproductive output ($P < 0.01$), while the *CYP17* genotype, as well as the interaction between the two factors, had no effect ($P > 0.30$).

8.4 Discussion and conclusion

The TaqMan genotyping assay was an effective method for determining the *CYP17* genotype of sheep. Two heterozygous *WT1/WT2* groups were consistently detected, but the cause of the difference between these groups remains unknown. The data from the TaqMan assay corresponded with the LightCycler method used previously. Storbeck et al. (2008a) also observed two heterozygous *CYP17* genotypes, with either equal or unequal melting peak heights by using the same LightCycler method, for the South African Angora and Boer goats. They found that this phenomenon was the result of a *CYP17* duplication of the one allele, which gave rise to three genotypes (but two alleles): homozygous ACS- (one high melting peak: two ACS- copies); heterozygous ACS+/ACS- with unequal peaks (one ACS+ and two ACS- copies); and heterozygous ACS+/ACS- with equal peaks (two ACS+ and two ACS- copies). The homozygote of the other allele was never detected in the populations investigated. In addition, they also analysed heterozygous *WT1/WT2* samples from the South African Merino and found that sheep only have one *CYP17* copy (relative to the goat). However, less than five heterozygous sheep were analysed, which might not have been representative of both heterozygous groups observed in the current study. In an attempt to investigate whether there may indeed be a *CYP17* duplication, a relative DNA copy number determination was done for samples of both heterozygous groups with the TaqMan assay, using the $2^{-\Delta\Delta C}{}_{T}$ method (Livak and Schmittgen, 2001). However, the data were inconsistent and a conclusion could not be drawn, since twice the amount *WT2* was detected in the

"equal peak" (Figure 8.1 area B) compared to the "unequal peak" (Figure 8.1 area C) heterozygous group, while half the amount of *WT1* was detected in the homozygous *WT1/WT1* group compared to both heterozygous groups (data not shown). It seems likely that the missing homozygous *WT2* genotype may be the result of a genetic anomaly after all, but it cannot be conclusively proven at this stage. The recent study by Vanselow and Fürbass (2011), together with the study of Storbeck et al. (2008), has demonstrated that genetic anomalies of the *CYP17* gene (such as multiple copies) may be more common than initially considered. It is suggested that more sensitive methods, such as the fluorescent *in situ* hybridisation technique, should be utilised to establish the number of *CYP17* copies in the sheep genome. The present study therefore did not distinguish between the two heterozygous groups, and they were treated as one group throughout analyses. The reason why no homozygous *WT2* sheep were detected is most probably the result of a genetic anomaly, which may also explain the recurrence of the two heterozygous groups. However, this finding warrants further investigation and fluorescent *in situ* hybridisation is recommended to determine the number of *CYP17* copies in the ovine genome.

The breeding program was successful in improving the reproductive performance, in terms of lamb output, lamb survival and growth, in the H-line (Cloete and Scholtz, 1998; Cloete et al., 2003, 2004, 2009), but the *CYP17* genotype apparently does not contribute to these differences. It would, however, be interesting to investigate the influence of the *CYP17* genotype on maternal behaviour, since cortisol production has previously been linked to the degree of maternal behaviour displayed by ewes (Pryce et al., 1988; Dwyer et al., 2004). Such an investigation is suggested for future studies.

While the weekly feed intake of sheep seemed to not be affected by the *CYP17* genotype, the *CYP17* genotype had significant influences on behavioural responses to isolation stress. The homozygous *WT1/WT1* sheep uttered less bleats and approached humans more easily than heterozygous *WT1/WT2* sheep, which indicates that homozygous *WT1/WT1* sheep have a 'calmer' temperament. These significant parameters corresponded to certain parameters that showed significant differences in the H- and L-lines in the study of Cloete et al. (2005a). In the study by Kilgour and Szantar-Coddington (1995), it was also found that Merinos of the Fertility flock (that were selected for rearing ability) allowed smaller ($P < 0.05$) distances to the human operator and also bleated less often than Random control ewes. However, the homozygous *WT1/WT1* sheep in the present study also urinated more frequently than heterozygous *WT1/WT2* sheep, which indicates stressful behaviour. The higher urinating frequency observed for homozygous *WT1/WT1* (and L-line sheep) might be explained by their higher production of deoxycorticosterone (as seen in

Chapter 6) that is known to inhibit plasma renin activity and act via the renin-angiotensin system (Scaroni et al., 1986). The number of bleats uttered; the frequency of urinating events and the average distance from the human operator during the arena test therefore hold potential selection criteria to assist in the identification of Merinos belonging to the respective *CYP17* genotypes, after further development.

Furthermore, the total cortisol response to hypoglycaemia correlated (indirectly proportional) to the frequency of defecation during the arena test, while also showing some degree of correlation with the average distance from the human operator. These two parameters coincided with the observations by Cloete et al. (2005a) that the H-line displayed less stressful behaviour, in terms of a lower defecating frequency and also allowing smaller distances from the human operator than the L-line. These results suggest that these two parameters of the arena test may be utilised to select for superior HPA axis function in terms of total cortisol responses. The common selection criterion for both these objectives is based on the distance from to the human operator, while the number of lines crossed (agitated movement) appears to not be related to the *CYP17* genotype or cortisol production.

It is noteworthy that the arena test was performed on both male and female contemporaries, while the production of cortisol in response to insulin-induced hypoglycaemia was evaluated in only male contemporaries. The reason for using only male contemporaries in the last mentioned stress test was firstly due to the limitation of L-line animals available for experimentation and breeding purposes, due to the effects of downward selection on the birth rate and survival of L-line animals. The L-line ewes were therefore reserved for breeding purposes. Secondly, the evaluation of only male contemporaries was expected to minimize individual variation in cortisol responses. Turner et al. (2002) observed a higher cortisol response in adult Romney Marsh rams compared to ewes in response to insulin-induced hypoglycaemia, while the cortisol response to ACTH administration was the same. In contrast, Van Lier et al. (2003) observed a higher cortisol response to ACTH administration in Corriedale ewes compared to rams. These results indicate that some gender-specific differences in cortisol responses to various stimuli may exist in certain sheep breeds. In the present study it is assumed that the same trend in cortisol responses of rams that represent a certain group (selection line X *CYP17* genotype) would be observed in ewes of the same group, but some variation between male and female cortisol responses may be anticipated. This assumption should be kept in mind with interpretation of correlations with data from the arena test and reproductive performance.

The measurement of only cortisol, and not cortisone, in the hypoglycaemic stress test was done by radioimmunoassay, since the UPLC-MS/MS method was incompletely developed at that time. It is, however, suggested that future stress test measurements include the detection of cortisone concomitant with cortisol. From the results obtained by the hypoglycaemic test it was seen that the cortisol response to hypoglycaemia is greatly affected by the *CYP17* genotype, while the selection line affects the response of glucose to the cortisol output. This statement is also supported by the large standard errors of the means observed in the cortisol responses (Figure 8.4B) and small standard errors of the means observed in the glucose responses (Figure 8.4A) of the H- and L-lines. The opposite trend in standard errors of the means was observed for the cortisol and glucose responses when the data was grouped according to *CYP17* genotype compared to when the data was grouped according to selection line (Figure 8.4). This indicates that the *CYP17* genotype greatly affects the cortisol response, but not the glucose response. Furthermore, the initial low cortisol peak at 0 – 30 min post insulin administration is suggested to result from the sheep's fear for human interaction (Figures 8.4-8.6). The cortisol responses that followed the hypoglycaemic drop in glucose levels after 30 min (< 2.00 mmol glucose L^{-1}), however, were much higher than this initial cortisol peak.

The higher cortisol response observed for the homozygous *WT1/WT1* group confirms that the presence of the *WT1* allele is more advantageous for cortisol production than the *WT2* allele. This finding is consistent with the previous predictions for WT1 from *in vivo* studies. The H-line also showed an improved ability to cope with stressors compared to the L-line. The differences in the cortisol and glucose responses between the H- and L-line suggest that the *CYP17* genotype will have a small effect in animals with a proper functioning HPA axis, while it will have a large effect in animals with an impaired HPA axis. This statement is supported by the studies of Storbeck et al. (2008a), which showed that the *CYP17* genotype has a great effect on the cortisol production of Angora goats – animals that are known to have an impaired HPA axis (Storbeck et al., 2008a; Hough et al., 2010) – while cortisol production of Boer goats are largely independent of *CYP17* genotype (Engelbrecht et al., 2000).

It is therefore suggested that selection for *WT1* may be utilised to improve the cortisol response of animals with an impaired HPA axis, while it would not markedly improve the cortisol production of robust animals. Large individual variation was observed in the cortisol and glucose responses, which suggest that other putative genetic factors are involved that may potentially be used as additional markers for robustness.

CHAPTER 9

General discussion and conclusion

9.1 Summary of results and recommended future studies

The proper functioning of the HPA axis is crucial to counteract stressor stimuli in farmed livestock. In theory, animals with superior HPA axis activity will be able to adapt more efficiently to stress and is considered to be more robust. A properly functioning HPA axis facilitates a coordinated stress response that elicits a cortisol specific response from the adrenal gland in sheep. Both the magnitude and duration of the cortisol output is important factors that determine the efficacy of the cortisol response. The observations made in the present study showed that the time-dependent cortisol response is a more accurate reflection of the HPA function than the measurement of only the peak cortisol concentrations. Moreover, the measurement of cortisone and corticosterone concentrations, together with cortisol, is advised, since the ratio of these hormones relative to cortisol may be altered during a state of stress. The developed UPLC-MS/MS method could be used for the analysis of stress-related steroids or adrenal steroid profiles. It is also important to develop a method for the analyses of reproductive hormone profiles to possibly investigate correlations with reproductive traits.

The present study has shown that the divergent selection criteria, that shaped the H- and L-lines, also resulted in differences in the HPA axis responses. This statement is reflected by the higher cortisol response and more rapid glucose recovery profiles in the H-line, compared to the L-line. It was observed the *CYP17* genotype played an important role in the cortisol responses of these lines. The *CYP17* genotype had little effect on the cortisol response of the H-line, which arguably consists of sheep with good levels of robustness (see Cloete et al., 2004; Cloete et al., 2005). However, the *CYP17* genotype had a large effect on the cortisol response of the L-line, which consists of sheep with poor robustness (see Cloete et al., 2004; Cloete et al., 2005). It is therefore suggested that selection based on the *CYP17* genotype will only improve cortisol responses in animals with an impaired HPA axis function, while maintaining the cortisol responses of animals with sufficient HPA axis activity. This statement is supported by the observations of Storbeck et al. (2008), which showed that the *CYP17* genotype profoundly affects the cortisol response in Angora goats that are known to have an inefficient HPA axis activity (Storbeck et al., 2008; Hough et al., 2010). In contrast, cortisol production was independent of *CYP17* genotype in Boer goats, which can be considered to be a more robust breed (Engelbrecht et al., 2000).

It was interesting to observe, from the results obtained in studies with primary cultures of adrenocortical cells (Chapter 7), that the most prominent differences in cortisol production resulted from selection line differences. These results indicated that these selection lines are likely to have intraadrenal differences, other than the *CYP17* genotype. The identification of the intraadrenal genetic factors that contributes to these differences will prove useful to marker-assisted selection with robustness as objective in future.

The presence of *WT1* in the *CYP17* genotype was shown to be more advantageous for cortisol production than the presence of *WT2*. This was seen in studies on both the *in vitro* and *in vivo* level. The level of expression of WT1 relative to WT2, however, remains to be determined. The cDNA from the primary cultures are available for such a study, but has not been analysed to date due to time constraints. Furthermore, the reason why no homozygous *WT2* genotypes were detected needs to be established. The use of the fluorescent *in situ* hybridization technique is suggested to establish whether there are any *CYP17* copies in the ovine genome.

The *CYP17* genotype seemed to not influence the measurable indices of reproduction (lamb output, lamb survival and growth) and weekly feed intake. The maternal behaviour of ewes and the relationship with the *CYP17* genotype was not investigated, and is suggested for future studies. The *CYP17* genotype was found to influence behavioural responses of sheep subjected to a flock-separation test. The frequency at which sheep urinated and uttered bleats in the arena test, as well as the average distance from the human operator, was different between homozygotes *WT1* and heterozygotes *WT1/WT2*. Furthermore, the cortisol responses correlated with the frequency of defecation and potentially the distance from the human operator. These two parameters of the arena test may therefore be developed to identify animals with superior cortisol production. It is proposed that an easy-to-perform behavioural stress test is developed to identify animals homogeneous for *WT1* and/or the efficient production of cortisol to ultimately improve sheep robustness.

9.2 Discussion about the implications of CYP17 genotypes for the South African Merino

What is the physiological relevance of the *CYP17* genotype and its subsequent effects on cortisol production? From the present study it was demonstrated that, given the same stimulus, WT1 would produce more cortisol than WT2. This implies that, theoretically, the HPA axis will be activated for a shorter time to produce adequate amounts of cortisol in the presence of *WT1*, compared to the presence of *WT2*. As mentioned in section 2.7, the effectiveness of the HPA axis to counter stress will complexly influence energy metabolism, immune responses, behaviour, ability for learning, fertility and sexual libido (Manteuffel, 2002). The differentially HPA-mediated glucocorticoid

response from homozygous *WT1/WT1* or heterozygous *WT1/WT2* sheep may therefore have various implications for traits related to robustness and production. In the present study it was shown that certain parameters of energy metabolism and behaviour were affected by the *CYP17* genotype, while certain parameters of fertility were apparently unaffected. These parameters and traits may, however, not be the only factors that are influenced by the *CYP17* genotype and may require further investigation.

The effect of the *CYP17* genotype on cortisol production may be masked by other factors that influence the HPA axis activity. The H- and L-lines were shown to have different coping strategies to psychological and physiological stressors, which also implied that their sensitivity to the *CYP17* genotype would be different. These selection lines have provided evidence that genetic selection resulted in differences in HPA axis activity. The inferior HPA axis activity of the L-line may be related to some of the poor traits, related to production and robustness, observed in this group compared to the H-line.

Furthermore, the complexity of the regulation of the HPA axis was demonstrated by the correlations between *CYP17* genotype, cortisol production and behavioural responses to physiological and psychological stressors. For instance homozygous *WT1/WT1* sheep uttered less bleats and approached humans more easily than heterozygous *WT1/WT2* sheep, which indicates that homozygous *WT1/WT1* sheep have a better temperament. However, the homozygous *WT1/WT1* group also urinated more frequently than heterozygous *WT1/WT2* sheep, which indicates stressful behaviour. The higher urinating frequency observed for homozygous *WT1/WT1* and L-line sheep might be explained by their higher production of deoxycorticosterone that inhibits plasma renin activity and act via the renin-angiotensin system. Similarly, the same group may respond differently to other forms stress, such as the H_E and H_O group that had equal cortisol responses to insulin-induced hypoglycaemia (physiological stress), while the H_E group uttered more bleats (signal of stress) than the H_O group during the arena test (psychological stress).

This study provided a thorough investigation into the relative contributions of the two CYP17 isoforms on cortisol production in the sheep adrenal. It was found that WT1 is more advantageous for cortisol production than WT2, which was reflected both on an *in vitro* and *in vivo* level.

9.3 Publication and presentation of the work

The preliminary results for the hypoglycaemic stress test and *CYP17* genotype frequency distributions (LightCycler) was presented at the 18th conference of the Association for the

Advancement of Animal Breeding and Genetics (AAABG 2009, Adelaide, Australia) and was published in the proceedings (Addendum B; Van der Walt et al., 2009). The data from a comparison of the total cortisol responses (AUC of normalised cortisol per glucose concentrations) of the South African Angora goat (data from Storbeck et al., 2008a) and Merinos (the current study) were presented at the 9th World Congress on Genetics Applied to Livestock Production (WCGALP 2010, Leipzig, Germany) and published in the proceedings (Addendum C; Hough et al., 2010). Selected data has also been presented at local conferences, namely the South African Society of Animal Science (SASAS 2009, 2011) and the South African Society for Biochemistry and Molecular Biology (SASBMB 2010). The data obtained through this PhD will be submitted for publication in scientific journal in the ensuing months.

Reference List

Abstract of Agricultural Statistics (2011) Compiled by Directorate: Statistics and Economic Analysis of the Department of Agriculture, Forestry and Fisheries. Agricultural Information Services, Private Bag X144, Pretoria 0001, South Africa.

Agarwal AK and Auchus RJ (2005) Cellular redox state regulates hydroxysteroid dehydrogenase activity and intracellular hormone potency. Endocrinology 146: 2531-2538.

Agricultural Research Council (2009) Small Stock Improvement Scheme, ARC-Animal Improvement Institute, Private Bag X2, IRENE 0062. http://www.arc.agric.za/home.asp?pid=2753 (accessed date: 10 November 2011).

Aguilera G (1998) Corticotropin releasing hormone, receptor regulation and the stress response. Trends in Endocrinology and Metabolism 9: 329-336.

Alexander G (1984) Constraints to lambs' survival. In: Reproduction in sheep. Lindsay DR and Pearce DT (eds) Australian Academy of Science and the Australian Wool Corporation, Canberra, pp. 199-209.

Angeli A, Masera RG, Sartori ML, Fortunati N, Racca S, Dovio A, Staurenghi A and Frairia R (1999) Modulation by cytokines of glucocorticoid action. Annals of the New York Academy of Sciences 876: 210-220.

Apple JK, Minton JE, Parsons KM and Unruh JA (1993) Influence of repeated restraint and isolation stress and electrolyte administration on pituitary-adrenal secretions, electrolytes, and other blood constituents of sheep. Journal of Animal Science 71: 71-77.

Apter D, Pakarinen A, Hammond GL and Vihko R (1979) Adrenocortical function in puberty. Acta Paediatrica Scandinavica 68: 599-604.

Artemenko IP, Zhao D, Hales DB, Hales KH and Jefcoate CR (2001) Mitochondrial processing of newly synthesized steroidogenic acute regulatory protein (StAR), but not total StAR, mediates cholesterol transfer to cytochrome P450 side chain cleavage enzyme in adrenal cells. The Journal of Biological Chemistry 276: 46583-46596.

Atkinson JK and Ingold KU (1993) Cytochrome P450 hydroxylation of hydrocarbons: variation in the rate of oxygen rebound using cyclopropyl radical clocks including two new ultrafast probes. Biochemistry 32: 9209-9214.

Auchus RJ and Miller WL (1999) Molecular modeling of human P450c17 (17α-hydroxylase/17,20-lyase): Insights into reaction mechanisms and effects of mutations. Molecular Endocrinology 13: 1169-1182.

Auchus RJ, Lee TC and Miller WL (1998) Cytochrome b5 augments the 17,20-lyase activity of human P450c17 without direct electron transfer. The Journal of Biological Chemistry 273: 3158-3165.

Auchus, RJ (2001) The genetics, pathophysiology, and management of human deficiencies of P450c17. Endocrinology Metabolism Clinics of North America 30: 101-119.

Baker BY, Yaworsky DC and Miller WL (2005) A pH-dependent molten globule transition is required for activity of the steroidogenic acute regulatory protein, StAR. The Journal of Biological Chemistry 280: 41753-41760.

Barouki R, Chobert MN, Billon MC, Finidori J, Tsapis R and Hanoune J (1982) Glucocorticoid hormones increase the activity of gamma-glutamyltransferase in a highly differentiated hepatoma cell line. Biochimica et Biophysica Acta 721(1): 11-21.

Bassett MH, Suzuki T, Sasano H, De Vrie CJ, Jimenez PT, Carr BR and Rainey WE (2004) The orphan nuclear receptors NURR1 and NGFIB regulate adrenal aldosterone production. The Journal of Biological Chemistry 279: 37622-37630.

Beato M, Truss M and Chavez S (1996) Control of transcription by steroid hormones. Annals of the New York Academy of Sciences 784: 93-123.

Beilharz RG (1998) Environmental limit to genetic change. An alternative theorem of natural selection. Journal of Animal Breeding and Genetics 115: 433-437.

Bender JL and Neer EJ (1983) Properties of the adenylate cyclase catalytic unit from caudate nucleus. The Journal of Biological Chemistry 258: 2432-2439.

Bhasker CR, Adler BS, Dee A, John ME, Kagimoto M, Zuber MX, Ahlgren R, Wang XD, Simpson ER and Waterman MR (1989) Structural characterization of the bovine CYP17 (17α-hydroxylase) gene. Archives of Biochemistry and Biophysics 271: 479-487.

Bickell S, Poindron P, Nowak R, Ferguson D, Blackberry M and Blache D (2004) Maternal behaviour and peripartum levels of oestradiol and progesterone show little difference in Merino ewes selected for calm or nervous temperament under indoor housing conditions. Animal 5(4): 608-614.

Biglieri EG, Herron MA and Brust N (1966) 17-Hydroxylation deficiency in man. The Journal of Clinical Investigation 45: 1946-1954.

Bird IM, Magness RR, Mason JI and Rainey WE (1992) Angiotensin-II acts via the type 1 receptor to inhibit 17a-hydroxylase cytochrome P450 expression in ovine adrenocortical cells. Endocrinology 130: 3113-3121.

Bird IM, Pasquarette MM, Rainey WE and Mason JI (1996) Differential control of 17α-hydroxylase and 3β-hydroxysteroid dehydrogenase expression in human adrenocortical H295R cells. The Journal of Clinical Endocrinology and Metabolism 81(6): 2171-2178.

Blache D and Bickell SL (2010) Temperament and reproductive biology: emotional reactivity and reproduction in sheep Revista Brasileira de Zootecnia 39: 401-408.

Blair SR and Mellon SH (2004) Deletion of the mouse P450c17 gene causes early embryonic lethality. Molecular and Cellular Biology 34: 5383-5390.

Bonaz B and Rivest S (1998) Effect of chronic stress on CRF neuronal activity and expression of its type 1 receptor in the rat brain. American Journal of Physiology 44: 1438-1449.

Bornstein SR, Ehrhart-Bornstein M, Usadel H, Böckmann M and Scherbaum WA (1991) Morphological evidence for a close interaction of chromaffin cells with cortical cells within the adrenal gland. Cell Tissue Research 265: 1-9.

Bose HS, Lingappa VR and Miller WL (2002) Rapid regulation of steroidogenesis by mitochondrial protein import. Nature 417: 87-91.

Bose HS, Whittal RM, Baldwin MA and Miller WL (1999) The active form of the steroidogenic acute regulatory protein, StAR, appears to be a molten globule. Proceedings of the National Academy of Sciences of the United States of America 96: 7250-7255.

Breen KM, Billings HJ, Wagenmaker ER, Wessinger EW and Karsch FJ (2005) Endocrine basis for disruptive effects of cortisol on preovulatory events. Endocrinology 146: 2107-2115.

Bridges A, Gruenke L, Chang YT, Vakser IA, Loew G and Waskell L (1998) Identification of the binding site on cytochrome P450 2B4 for cytochrome b5 and cytochrome P450 reductase. The Journal of Biological Chemistry 273: 17036-17049.

Brown MS, Kovanen PT and Goldstein JL (1979) Receptormediated uptake of lipoprotein-cholesterol and its utilization for steroid synthesis in the adrenal cortex. Recent Progress in Hormone Research 35: 215-257.

Bush IE and Ferguson KH (1953) The secretion of the adrenal cortex in sheep. Journal of Endocrinology 10: 1-8.

Camp NJ and Cox A (2002) Methods in Molecular Biology, Vol 195. In: Quantitative trait loci: methods and protocols. Human Press Inc., New Jersey, USA.

Campbell LE and Yang MYK (1996) Ovine 11β-hydroxysteroid dehydrogenase type 2 gene predicts a protein distinct from that deduced by the cloned kidney cDNA at the C-terminus. Molecular and Cellular Endocrinology 119: 113-118.

Cape Wools SA (2008) Annual report 2008/2009. PO Box 2191, North End, Port Elizabeth 6056.

Carcangiu V, Vacca GM, Parmeggiani A, Mura MC, Pazzola M, Dettori ML and Bini PP (2008) The effect of shearing procedures on blood levels of growth hormone, cortisol and other stress haematochemical parameters in Sarda sheep. Animal 2: 606-612.

Carvalho VM, Nakamura OH and Vieira JGH (2008) Simultaneous quantitation of seven endogenous C21 adrenal steroids by liquid chromatography tandem mass spectrometry in human serum. Journal of Chromatography B 872: 154-161.

Challis G Jr. and Brooks AN (1989) Maturation and activation of hypothalamic-pituitary-adrenal function in foetal sheep. Endocrine Reviews 10: 182-204.

Challis G Jr., Matthews SG, Gibb W and Lye SJ (2000) Endocrine and paracrine regulation of birth at term and preterm. Endocrine Reviews 21: 514-550.

Charmandari E, Tsigos C and Chrousos G (2005) Endocrinology of the stress response. Annual Review of Physiology 67: 259-284.

Chen FM, Bilezikjian LM, Perrin MH, Rivier J and Vale W (1986) Corticotropin releasing factor receptor-mediated stimulation of adenylate cyclase activity in the rat brain. Brain Research 381: 49-57.

Cho H, Kim JD, Lee WY, Chung BC and Choi MH (2009) Quantitative metabolic profiling of 21 endogenous corticosteroids in urine by liquid chromatography-triple quadrupolemass spectrometry. Analytica Chimica Acta 632: 101-108.

Cho JH, Musch MW, Bookstein CM, McSwine RL, Rabenau K and Chang EB (1998) Aldosterone stimulates intestinal Na+ absorption in rats by increasing NHE3 expression of the proximal colon. American Journal of Physiology - Cell Physiology 274: C586-C594.

Chrousos GP (1995) The hypothalamic-pituitary-adrenal axis and immune-mediated inflammation. New England Journal of Medicine 332: 1351-1362.

Chrousos GP (2000) The role of stress and the hypothalamic-pituitary-adrenal axis in the pathogenesis of the metabolic syndrome: neuro-endocrine and target tissue-related causes. International Journal of Obesity and Related Metabolic Disorders 24(Supplementary 2): S50-55.

Chung BC, Picado-Leonard J, Haniu M, Bienkowski M, Hall PF, Shively J and Miller WL (1987) Cytochrome P450c17 (steroid 17α-hydroxylase/17,20 lyase): cloning of human adrenal and testis cDNAs indicates the same gene is expressed in both tissues. Proceedings of the National Academy of Sciences of the United States of America 84: 407-411.

Clark BJ, Wells J, King SR and Stocco DM (1994) The purification, cloning and expression of a novel luteinizing hormone-induced mitochondrial protein in MA-10 mouse Leydig tumor cells. Characterization of the steroidogenic acute regulatory protein (StAR). The Journal of Biological Chemistry 269: 28314-28322.

Cloete JJE, Cloete SWP and Hoffman LC (2005a) Behaviour of Merinos divergently selected for multiple rearing ability in response to external stimuli. Small Ruminant Research 60: 227-236.

Cloete SWP and Durand A (1994) A preliminary note on Merino sheep subjected to divergent selection on maternal values for lambs weaned per ewe joined. South African Journal of Animal Science 24: 27-9.

Cloete SWP and Olivier JJ (2010) South African Sheep and Wool Industry. In: The International Sheep and Wool Handbook. D.J. Cottle (ed) Nottingham University Press, Manor Farm, Thrumpton, Nottingham NG11 0AX, United Kingdom, pp. 95-112.

Cloete SWP and Scholtz AJ (1998) Lamb survival in relation to lambing and neonatal behaviour in medium wool Merino lines divergently selected for multiple rearing ability. Australian Journal of Experimental Agriculture 38: 801-811.

Cloete SWP, Gilmour AR, Olivier JJ and van Wyk JB (2004) Genetic and phenotypic trends and parameters in reproduction, greasy fleece weight and live weight in Merino lines divergently selected for multiple rearing ability. Australian Journal of Experimental Agriculture 44: 745-754.

Cloete SWP, Scholtz AJ, Cloete JJE and van Wyk JB (2005b) The ability of Merino ewes and lambs to reunite after separation, as affected by divergent selection for ewe multiple rearing capacity. Australian Journal of Experimental Agriculture 45: 1131-1137.

Cloete SWP, Van Wyk JB, Scholtz AJ, Gilmour AR (2003) Genetic and environmental parameters for lambing behaviour in Merino lines divergently selected for ewe maternal rearing ability. Proceedings of the New Zealand Society of Animal Production 63: 169-172.

Colomer C, Desarménien MG and Guérineau NC (2009) Revisiting the stimulus-secretion coupling in the adrenal medulla: role of gap junction-mediated intercellular communication. Molecular neurobiology 40(1): 87-100.

Conley AJ, Graham-Lorence SE, Kagimoto M, Lorence MC, Murry BA, Oka K, Sanders D and Mason IJ (1992) Nucleotide sequence of a cDNA encoding porcine testis 17α-hydroxylase cytochrome P-450. Biochimica et Biophysica Acta 1130: 75-77.

Connelly MA (2009) SRBI mediated HDL cholesteryl ester delivery in the adrenal gland. Molecular and Cellular Endocrinology 300: 83-88.

Connelly MA and Williams (2004) SR-BI and HDL cholesteryl ester metabolism. Endocrine Research 30(4): 698-703.

Connor KL, Bloomfield FH, Oliver MH, Harding JE and Challis JRG (2009) Effect of periconceptional undernutrition in sheep on late gestation expression of mRNA and protein from genes involved in foetal adrenal steroidogenesis and placental prostaglandin production. Reproductive Sciences 16(6): 573-583.

Costa-Santos M, Kater CE and Archus RJ (2004b) Two prevalent CYP17 mutations and genotype-phenotype correlations in 24 Brazilian patients with 17α-hydroxylase. The Journal of Clinical Endocrinology and Metabolism 89: 49-60.

Costa-Santos M, Kater CE, Dias EP and Auchus RJ (2004a) Two intronic mutations cause 17-hydroxylase deficiency by disrupting splice acceptor sites: direct demonstration of aberrant splicing and absent enzyme activity by expression of the entire CYP17 gene in HEK-293 cells. The Journal of Clinical Endocrinology and Metabolism 89: 43-48.

Da Costa AP, Leigh AE, Man M and Kendrick KM (2004) Face pictures reduce behavioural, autonomic, endocrine and neural indices of stress and fear in sheep. Proceedings of the Royal Society of London B 271: 2077-2084.

Dallman MF, Strack AM, Akana SF, Bradbury MJ, Hanson ES, Scribner KA and Smith M (1993) Feast and famine: critical role of glucocorticoids with insulin in daily energy flow. Frontiers in Neuroendocrinology 14: 303-47.

Dantzer R and Mormède P. (1983) Stress in farm animals: a need for reevaluation. Journal of Animal Science 57: 6-18.

Darfler FJ, Mahan LC, Koachman AM and Insel PA (1982) Stimulation by forskolin of intact S49 lymphoma cells involves the nucleotide regulatory protein of adenylate cyclase. The Journal of Biological Chemistry 257: 11901-11907.

Degabrielle R and Fell LR (2001) Changes in behaviour, cortisol and lymphocyte types during isolation and group confinement of sheep. Immunology and Cell Biology 79: 583-589.

Denkova R, Ivanov I and Dimitrova M (1992) Microtubules and regulation of granulosa cell steroidogenesis by porcine granulosa cell conditioned medium. Endocrine Regulations 26: 195-199.

DeRijk RH (2009) Single nucleotide polymorphisms related to HPA axis activity. Neuroimmunomodulation 16: 340-352.

Dijkstra I, Binnekade R and Tilders FJH (1996) Diurnal variation in resting levels of corticosterone is not mediated by variation in adrenal responsiveness to adrenocorticotropin but involves splanchnic nerve integrity. Endocrinology 137: 540-547.

Dorgan JF, Fears TR, McMahon RP, Aronson Friedman L, Patterson BH and Greenhut SF (2002) Measurement of steroid sex hormones in serum: A comparison of radioimmunoassay and mass spectrometry. Steroids 67: 151-158.

DuBois DC, Xu ZX, McKay L, Almon RR, Pyszcznski N and Jusko WJ (1995) Differential dynamics of receptor down-regulation and tyrosine aminotransferase induction following glucocorticoid treatment. The Journal of Steroid Biochemistry and Molecular Biology 54(5-6): 237-243.

Duma D, Jewell CM and Cidlowski JA (2006) Multiple glucocorticoid receptor isoforms and mechanisms of post-translational modification. The Journal of Steroid Biochemistry and Molecular Biology 102(1-5): 11-21.

Dunn AF and Berridge CW (1990) Physiological and behavioral responses to corticotropin-releasing factor administration: is CRF a mediator of anxiety or stress responses? Brain Research Reviews 15: 71-100.

Dwyer CM, Gilbert CL and Lawrence AB (2004) Prepartum plasma estradiol and postpartum cortisol, but not oxytocin, are associated with interindividual and breed differences in the expression of maternal behaviour in sheep. Hormones and Behavior 46: 529-543.

Edwards AV and Jones CT (1987) The effect of splanchnic nerve stimulation on adrenocortical activity in conscious calves. The Journal of Physiology 382: 385-396.

Eisenthal R and Cornish-Bowden A (1974) The direct linear plot. A new graphical procedure for estimating enzyme kinetic parameters. Biochemical Journal 139: 715-720.

Ellis J, Gutierrez A, Barsukov IL, Huang WC, Grossmann JG and Roberts GC (2009) Domain motion in cytochrome P450 reductase. Conformational equilibria revealed by NMR and small-angle x-ray scattering. The Journal of Biological Chemistry 284: 36628-36637.

Engelbrecht Y, Herselman T, Louw A and Swart P (2000) Adrenal function in Angora goats: a comparative study of adrenal steroidogenesis in Angora goats, Boer goats, and Merino sheep. Journal of Animal Science 78: 371-379.

Enoch HG and Strittmatter P (1979) Cytochrome b5 reduction by NADPH-cytochrome P450 reductase. The Journal of Biological Chemistry 254: 8976-8981.

Etter ML, Eichhorst J and Lehotay DC (2006) Clinical determination of 17-hydroxyprogesterone in serum by LC–MS/MS: Comparison to Coat-A-Count™ RIA method. Journal of Chromatography B 840: 69-74.

Ewbank R (1985) The behavioural needs of farm and laboratory animals. In: Animal experimentation: Improvements and alternatives. Marsh N and Haywood S (eds) FRAME: Fund for the replacement of animals in medical experiments, Nottingham, UK, pp. 31-35.

Falconer DS and Mackay TFC (1996) Introduction to quantitative genetics. Fourth ed., Longman, New York, USA.

Falzone CJ, Mayer MR, Whiteman EL, Moore CD and Lecomte JT (1996) Design challenges for hemoproteins: the solution structure of apocytochrome b5. Biochemistry 35: 6519-6526.

Familari M, Smith AI, Smith R and Funder JW (1989) Arginine vasopressin is a much more potent stimulus to ACTH release from ovine anterior pituitary cells than ovine corticotrophin-releasing factor: in vitro studies. Neuroendocrinology 50: 152-157.

Fan YS, Sasi R, Lee C, Winter JS, Waterman MR and Lin CC (1992) Localization of the human CYP17 gene (cytochrome P450(17α)) to 10q24.3 by fluorescence in situ hybridization and simultaneous chromosome banding. Genomics 14: 1110-1111.

Fevold HR, Lorence MC, McCarthy JL, Trant JM, Kagimoto M, Waterman MR and Mason JI (1989) Rat P450(17α) from testis: characterization of a full-length cDNA encoding a unique steroid hydroxylase capable of catalyzing both Δ^4- and Δ^5-steroid-17,20-lyase reactions. Molecular Endocrinology 3: 968-975.

Flück CE and Miller WL (2004) GATA-4 and GATA-6 modulate tissue-specific transcription of the human gene for P450c17 by direct interaction with Sp1. Molecular Endocrinology 18: 1144-1157.

Foury A, Geverink NA, Gil M, Gispert M, Hortos M, Font i Furnols M, Carrion D, Blott SC, Plastow GS and Mormede P (2007) Stress neuroendocrine profiles in five pig breeding lines and the relationship with carcass composition. Animal 1: 973-982.

Foury A, Tribout T, Bazin C, Billon Y, Bouffaud M, Gogue´ JM, Bidanel JP and Mormede P (2009) Estimation of genetic trends from 1977 to 2000 for stress-responsive systems in French Large White and Landrace pig populations using frozen semen. Animal 3: 1681-1687.

Galtier A, Liakos P, Keramidas M, Feige JJ, Chambaz EM and Defaye G (1996) ACTH, angiotensin II and TGF beta participate in the regulation of steroidogenesis in bovine adrenal glomerulosa cells. Endocrine Research 22(4): 604-612.

Ganong WF (1995) Review of medical physiology. Appleton and Lange, Norwalk, CT, USA.

Garrett RH and Grisham CM (1995) The molecular basis of hormone action. In: Molecular aspects of cell biology. Saunders College publishing and Harcourt Brace College publishers, USA, pp. 1185-1188.

Geller DH, Auchus RJ and Miller WL (1999) P450c17 mutations R347H and R358Q selectively disrupt 17,20-lyase activity by disrupting interactions with P450 oxidoreductase and cytochrome b5. Molecular Endocrinology 13: 167-175.

Geller DH, Auchus RJ, Mendonca BB and Miller WL (1997) The Genetic and functional basis of isolated 17,20-lyase deficiency. Nature Genetetics 17: 201-205.

Geller DH, Auchus RJ, Mendonça BB and Miller WL (1997) The genetic and functional basis of isolated 17,20 lyase deficiency. Nature Genetetics 17: 201-205.

Gilman AG (1987) G proteins: Transducers of receptor-generated signals. Annual Review of Biochemistry 56: 615-649.

Gonzalez FJ (1988) The molecular biology of cytochrome P450s. Pharmacological Reviews 40: 243-288.

Goosen P, Swart AC, Storbeck K and Swart P (2010) Hypocortisolism in the South African Angora goat: The role of 3βHSD. Molecular and Cellular Endocrinology 315: 182-187.

Grandin T (1997) Assessment of stress during handling and transport. Journal of Animal Science 75: 249-257.

Gross WB (1976) Plasma steroid tendency, social environment and *Eimeria necatrix* infection. Poultry Science 55: 1508–1512.

Guo T, Taylor RL, Singh RJ and Soldin SJ (2006) Simultaneous determination of 12 steroids by isotope dilution liquid chromatography-photospray ionization tandem mass spectrometry. Clinica Chimica Acta 372: 76-82.

Gupta MK, Geller DH and Auchus RJ (2001) Pitfalls in characterizing P450c17 mutations associated with isolated 17,20 lyase deficiency. The Journal of Clinical Endocrinology and Metabolism 86: 4416-4423.

Gwynne JT, Strauss JF 3rd (1982) The role of lipoproteins in steroidogenesis and cholesterol metabolism in steroidogenic glands. Endocrine Reviews 3: 299-329.

Ha YW, Moon JY, Jung HJ, Chunga BC and Choi MH (2009) Evaluation of plasma enzyme activities using gas chromatography-mass spectrometry based steroid signatures. Journal of Chromatography B 877: 4125-4132.

Hall A (2005) Rho GTPases and the control of cell behaviour. Biochemical Society Transactions 33: 891-895.

Hall PF (1986) Cytochromes P450 and the regulation of steroid synthesis. Steroids 48: 133-196.

Hall PF and Almahbobi G (1997) Roles of microfilaments and intermediate filaments in adrenal steroidogenesis. Microscopy Research and Techniques 36: 463-479.

Han JD and Rubin CS (1996) Regulation of cytoskeleton organization and paxillin dephosphorylation by cAMP. The Journal of Biological Chemistry 271: 29211-29215.

Hantzis V, Albiston A, Matsacos D, Wintour EM, Peers A, Koukoulas I, Myles K, Moritz K and Dodic M (2002) Effect of early glucocorticoid treatment on MR and GR in late gestation ovine kidney. Kidney International 61: 405-413.

Hardy RN (1981) Endocrine Physiology. Edward Arnold, London, U.K., pp. 113-137.

Hargreaves AL and Hutson GD (1990) The stress response in sheep during routine handling procedures. Applied Animal Behaviour Science 26: 83-90.

Hauser B, Deschner T and Boesch C (2008) Development of a liquid chromatography-tandem mass spectrometry method for the determination of 23 endogenous steroids in small quantities of primate urine. Journal of Chromatography B 862: 100-112.

Hinson JP and Kapas S (1998) The role of endothelial cell products in the regulation of adrenocortical function: actions of endothelin, nitric oxide, adrenomedullin and PAMP. Hormone and Metabolism Research 30: 334-340.

Hoffman LC, Schmidt D, Muller MM, Cloete JJE and Cloete SWP (2003) Sensory and objective mutton quality characteristics of SA Merino sheep selected for and against reproductive fitness. South African Journal of Animal Science 33(1): 52-64.

Hogg K, McNeilly AS and Duncan WC (2011) Prenatal androgen exposure leads to alterations in gene and protein expression in the ovine foetal ovary. Endocrinology 152(5): 2048-2059.

Holst JP, Soldin SJ, Tractenberg RE, Guo T, Kundra P, Verbalis JG and Jonklaas J (2007) Use of steroid profiles in determining the cause of adrenal insufficiency. Steroids 72: 71-84.

Horton JD, Goldstein JL and Brown MS (2002) SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver. The Journal of Clinical Investigation 109: 1125-1131.

Hough D, Storbeck K, Cloete SWP and Swart P (2010) Improving stress coping ability: comparison between the CYP17 genotype of Ovis aries and Capra hircus. Proceedings of the World Congress on Genetics Applied to Livestock Production 9: ID628.

Huang J, Ushiyama T, Inoue K, Mori K and Hukuda S (1999) Possible association of CYP17 gene polymorphisms with the onset of rheumatoid arthritis. Clinical and Experimantal Reumatology 17: 721-724.

Ilan Z, Ilan R and Cinti DL (1981) Evidence for a new physiological role of hepatic NADPH:ferricytochrome (P450) oxidoreductase. Direct electron input to the microsomal fatty acid chain elongation system. The Journal of Biological Chemistry 256: 10066-10072.

Imai T, Yamazaki T and Kominami S (1998) Kinetic studies on bovine cytochrome P45011B catalyzing successive reactions from 11-deoxycorticosterone to aldosterone. Biochemistry 37: 8097–8104.

Jaffe AB and Hall A (2005) Rho GTPases: biochemistry and biology. Annual Review of Cell and Developmental Biology 21: 247-269.

Janzen N, Sander S, Terhardt M, Peter M and Sander J (2008) Fast and direct quantification of adrenal steroids by tandem mass spectrometry in serum and dried blood spots. Journal of Chromatography B 861: 117-122.

Jefcoate C (2002) High-flux mitochondrial cholesterol trafficking, a specialized function of the adrenal cortex. The Journal of Clinical Investigation 110: 881-890.

Jimenez P, Saner K, Mayhew B and RaineyWE (2003) GATA-6 is expressed in the human adrenal and regulates transcription of genes required for adrenal androgen biosynthesis. Endocrinology 144: 4285-4288.

Juengel JL, Meberg BM, Turzillo AM, Nett TM and Niswender GD (1995) Hormonal regulation of messenger ribonucleic acid encoding steroidogenic acute regulatory protein in ovine corpora lutea. Endocrinology 136(12): 5423-5429.

Juska A and de Foresta B (1995) Analysis of effects of corticotropin, forskolin, and fluoride on activity of adenylate cyclase of bovine adrenal cortex. Biochimica et Biophysica Acta 1236: 289-298.

Kagimoto K, Waterman MR, Kagimoto M, Ferreira P, Simpson ER and Winter JS (1989) Identification of a common molecular basis for combined 17α-hydroxylase/17,20-lyase deficiency in two Mennonite families. Human Genetics 82: 285-286.

Kaliner M (1985) Mechanisms of glucocorticosteroid action in bronchial asthma. Journal of Allergy and Clinical Immunology 76: 321-329.

Katagiri M, Kagawa N and Waterman MR (1995) The role of cytochrome b5 in the biosynthesis of androgens by human P450c17. Archives of Biochemistry and Biophysics 317: 343-347.

Keller M, Meurisse M and Levy F (2004) Mapping the neural substrates involved in maternal responsiveness and lamb olfactory memory in parturient ewes using Fos imaging. Behavioral Neuroscience 118: 1271-1284.

Kempna P, Hirch A, Hofer G, Mullis PE and Flück CE (2010) Impact of differential P450c17 phosphorylation by cAMP stimulation and by starvation conditions on enzyme activities and androgen production in NCI-H295R Cells. Endocrinology 151(8): 3686–3696.

Kilgour R (1976) Sheep behaviour: its importance in farming systems, handling, transport and pre-slaughter treatment. Proceedings of sheep assembly and transport workshop, W.A. Department of Agriculture, pp. 64-84.

Kilgour RJ and Szantar-Coddington MR (1995) Arena behaviour of ewes selected for superior mothering ability differs from that of unselected ewes. Animal Reproduction Science 37: 133-141.

Kim GH, Masilamani S, Turner R, Mitchell C, Wade JB and Knepper MA (1998) The thiazide-sensitive Na-Cl cotransporter is an aldosterone-induced protein. Proceedings of the National Academy of Sciences of the United States of America 95: 14552-14557.

Kirk LF Jr, Hash RB, Katner HP and Jones T (2000) Cushing's disease: Clinical manifestations and diagnostic evaluation. American Family Physician 62(5): 1119-1127.

Kita H, Jorgensen RK, Reed CE, Dunnette SL, Swanson MC, Bartemes KR, Squillace D, Blomgren J, Bachman K and Gleich GL (2000) Mechanism of topical glucocorticoid treatment of hay fever: IL-5 and eosinophil activation during natural allergen exposure are suppressed, but IL-4, IL-6, and IgE antibody production are unaffected. Journal of Allergy and Clinical Immunology 106: 521-529.

Knap PW and Rauw WM (2009) Resource allocation theory applied to farm animal production. In: Selection for high production in pigs. WM Rauw (ed) CABI Publishing, Wallingford, UK, pp. 210-229.

Kraemer FB (2007) Adrenal cholesterol utilization. Molecular and Cellular Endocrinology 265-266:42-45.

Kushnir MM, Rockwood AL, Roberts WL, Yue B, Bergquist J and Meikle AW (2011) Liquid chromatography mass spectrometry for analysis of steroids in clinical laboratories. Clinical Biochemistry 44: 77-88.

Lee LJ, Chen JS, Ko TL and Wang SM (2001) Mechanism of colchicineinduced steroidogenesis in rat adrenocortical cells. Journal of Cellular Biochemistry 81: 162-171.

Lee-Robichaud P, Kaderbhai MA, Kaderbhai N, Wright JN and Akhtar M (1997) Interaction of human CYP17 (P-45017α, 17α-hydroxylase-17,20 lyase) with cytochrome b5: importance of the orientation of the hydrophobic domain of cytochrome b5. Biochemical Journal 321: 857-863.

Lee-Robichaud P, Wright JN, Akhtar ME and Akhtar M (1995) Modulation of the activity of human 17α-hydroxylase-17,20-lyase (CYP17) by cytochrome b5: endocrinological and mechanistic implications. Biochemical Journal 308: 901-908.

Li D and Sewer MB (2010) RhoA and DIAPH1 mediate adrenocorticotropin-stimulated cortisol biosynthesis by regulating mitochondrial trafficking. Endocrinology 151(9): 4313-4323.

Liggins GC (1969) Premature delivery of foetal lambs infused with glucocorticoids. Journal of Endocrinology 45: 515-523.

Liggins GC (1994) The role of cortisol in preparing the foetus for birth. Reproduction, Fertility and Development 6: 141-150.

Lightman SL (1994) How does the hypothalamus respond to stress? Neurosciences 6: 215-219.

Lin D, Black SM, Nagahama Y and Miller WL (1993) Steroid 17α-hydroxylase and 17,20-lyase activities of P450c17: contributions of serine 106 and P450 reductase. Endocrinology 132: 2498-2506.

Liu J, Heikkila P, Meng QH, Kahri AI, Tikkanen MJ and Voutilainen R (2000) Expression of low and high density lipoprotein receptor genes in human adrenals. European Journal of Endocrinology 142: 677-682.

Liu J, Robinson PJ, Funder JW and Engler D (1994) A comparative study of the role of adenylate cyclase in the release of adrenocorticotropin from the ovine and rat anterior pituitary. Molecular and Cellular Endocrinology 101: 173-181.

Livak KJ and Schmittgen TD (2001) Analyses of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C}{}_T$ method. Methods 25: 402-408.

Livestock Development Strategy for South Africa (2006) Compiled by Directorate: Statistics and Economic Analysis of the Department of Agriculture, Forestry and Fisheries. Agricultural Information Services, Private Bag X144, Pretoria 0001, South Africa.

Lodish H, Berk A, Zipursky SL, Matsudaira P, Baltimore D and Darnell J (2000) Molecular cell biology, 4th ed. WH Freeman and Company, New York, NY, pp. 862-871.

Lunn RM, Bell DA, Mohler JL and Taylor JA (1999) Prostate cancer risk and polymorphism in 17 hydroxylase (CYP17) and steroid reductase (SRD5A2). Carcinogenesis 20: 1727-1731.

Lynch JJ, Hinch GN and Adams DB (1992) The behaviour of sheep. In: Biological principles and implications for production. CSIRO Publications, Melbourne, Australia.

MacFarlane MS, Breen KM, Sakurai H, Adams BM and Adams TE (2000) Effect of duration of infusion of stress-like concentrations of cortisol on follicular development and the preovulatory surge of LH in sheep. Animal Reproduction Science 63: 167-175.

Mackay TFC (1996) The nature of quantitative genetic variation revisited: lessons from Drosophila bristles. Bio Essays 18: 113-121.

Mackay TFC (2001) Quantitative trait loci in Drosophila. Nature Reviews Genetics 2: 11-20.

Makin HLJ and Gower DB (2010) Steroid Analysis. In: General methods for the extraction, purification, and measurement of steroids by chromatography and mass spectrometry. Makin HLJ, Honour JW, Shackleton CHL and Griffiths WJ (eds) First ed., Springer, London, UK.

Manna PR , Wang X and Stocco DM (2003) Involvement of multiple transcription factors in the regulation of steroidogenic acute regulatory protein gene expression. Steroids 68: 1125-1134.

Manteuffel G (2002) Central nervous regulation of the hypothalamic–pituitary–adrenal axis and its impact on fertility, immunity, metabolism and animal welfare, a review. Archiv Tierzucht 45: 575-595.

Marks V (2002) False-positive immunoassay results: A multicenter survey of erroneous immunoassay results from assays of 74 analytes in 10 donors from 66 laboratories in seven countries. Clinical Chemistry 48: 2008-2016.

Mason IL (1969) A world dictionary of livestock breeds, types and varieties, 2nd edn, Commonwealth Agricultural Bureaux, Farnham Royal, UK.

Mason JI and Rainey WE (1987) Steroidogenesis in the human foetal adrenal: a role for cholesterol synthesized de novo. The Journal of Clinical Endocrinology and Metabolism 64: 140-147.

Matteson KJ, Picado-Leonard J, Chung BC, Mohandas TK and Miller WL (1986) Assignment of the gene for adrenal P450c17 (steroid 17α-hydroxylase/17,20 lyase) to human chromosome 10. The Journal of Clinical Endocrinology and Metabolism 63: 789-791.

Matteson KJ, Picado-Leonard J, Chung BC, Mohandas TK and Miller WL (1986) Assignment of the gene for adrenal P450c17 (17α-hydroxylase/17,20 lyase) to human chromosome 10. The Journal of Clinical Endocrinology and Metabolism 63: 789-791.

Matthews SG (1998) Dynamic changes in glucocorticoid and mineralocorticoid receptor mRNA in the developing guinea pig brain. Developmental Brain Research 107: 123-32.

McDonald IR and Reich M (1959) Corticosteroid secretion by the autotransplanted adrenal gland of the conscious sheep. The Journal of Physiology 147: 33-50.

McKean-Cowdin R, Feigelson HS, Pike MC, Coetzee GA, Kolonel LN and Henderson BE (2001) Risk of endometrial cancer and estrogen replacement therapy history by CYP17 genotype. Cancer Research 61: 848-849.

McMillen IC, Phillips ID, Ross JT, Robinson JS and Owens JA (1995) Chronic stress: The key to parturition? Reproduction, Fertility and Development 7: 499-507.

Meyer U, Kruhoffer M, Flugge G and Fuchs E (1998) Cloning of glucocorticoid receptor and mineralocorticoid receptor cDNA and gene expression in the central nervous system of the tree shrew (*Tupaia belangeri*). Molecular Brain Research 55: 243-53.

Michel V, Peinnequin A, Alonso A, Buguet A, Cespuglio R and Canini F (2007) Decreased heat tolerance is associated with hypothalamic-pituitary-adrenocortical axis impairment. Neuroscience 147: 522-531.

Michopoulos F, Lai L, Gika H, Theodoridis G and Wilson I (2009) UPLC-MS-based analysis of human plasma for metabonomics using solvent precipitation or solid phase extraction. Journal of Proteome Research 8: 8114-2121.

Middle J (1998) Standardization of steroid hormone assays. Annals of Clinical Biochemistry 35: 354-363.

Mihrshahi R, Lewis JG and Ali SO (2006) Hormonal effects on the secretion and glycoform profile of corticosteroid-binding globulin. The Journal of Steroid Biochemistry and Molecular Biology 101(4-5): 275-285.

Miksik I, Vylitova M, Pacha J and Deyl Z (1999) Separation and identification of corticosterone metabolites by liquid chromatography–electrospray ionization mass spectrometry. Journal of Chromatography B 726: 59-69.

Miller WL (2005) Regulation of steroidogenesis by electron transfer. Endocrinology 146: 2544–2550.

Miller WL (2009) Androgen synthesis in adrenarche. Reviews in Endocrine and Metabolic Disorders 10: 3–17.

Miller WL and Auchus RJ (2011) The molecular biology, biochemistry, and physiology of human steroidogenesis and its disorders. Endocrine Research 32(1): 81-151.

Minutti ZC, Lacey JM, Magera MJ, Hahn SH, McCann M, Schulze A, Cheillan D, Dorche C, Chace DH, Lymp JF, Zimmerman D, Rinaldo P and Matern D (2004) Steroid profiling by tandem mass spectrometry improves the positive predictive value of newborn screening for congenital adrenal hyperplasia. The Journal of Clinical Endocrinology and Metabolism 89: 3687-3693.

Mormède P, Andanson S, Aupérin B, Beerda B, Guémené D, Malmkivist J, Manteca X, Manteuffel G, Prunet P, Van Reenen CG, Richard S and Veissier I (2007) Exploration of the hypothalamic-pituitary-adrenal function as a tool to evaluate animal welfare. Physiology and Behavior 92: 317-339.

Mormède P, Foury A, Terenina E and Knap PW (2011) Breeding for robustness: the role of cortisol. Animal 5(5): 651-657.

Muglia LJ, Jacobson L, Weninger SC, Luedeke CE, Bae DS, Jeong KH and Majzoub JA (1997) Impaired diurnal adrenal rhythmicity restored by constant infusion of corticotropin-releasing hormone in corticotropin-releasing hormone-deficient mice. The Journal of Clinical Investigation 99: 2923-2929.

Mulrow PJ (1998) Renin-angiotensin system in the adrenal. Hormone and Metabolism Research 30: 346-349.

Murphy PM (1999) Maternal behaviour and rearing ability of Merino ewes can be improved by strategic feed supplementation during late pregnancy and selection for calm temperament. PhD thesis, The University of Western Australia.

Murphy PM, Purvis IW, Lindsay DR, LeNeindre P, Orgeur P and Poindron P (1994) Measures of temperament are highly repeatable in Merino sheep and some are related to maternal behaviour. Proceedings of the Australian Society of Animal Production 20: 247-250.

Naffin-Olivos JL and Auchus RJ (2006) Human cytochrome b5 requires residues E48 and E49 to stimulate the 17,20-lyase activity of cytochrome P450c17. Biochemistry 45: 755-762.

Nakajin S and Hall PF (1981b) Microsomal cytochrome P450 from neonatal pig testis. Purification and properties of a C21 steroid side-chain cleavage system (17α-hydroxylase-C17,20-lyase). The Journal of Biological Chemistry 256: 3871-3876.

Nakajin S, Shinoda M, Haniu M, Shively JE and Hall PF (1984) C21 steroid side-chain cleavage enzyme from porcine adrenal microsomes. Purification and characterization of the 17α-hydroxylase/C17,20 lyase cytochrome P450. The Journal of Biological Chemistry 259: 3971-3976.

Nakajin S, Shively JE, Yuan PM and Hall PF (1981aa) Microsomal cytochrome P450 from neonatal pig testis: two enzymatic activities (17α-hydroxylase and C17,20-lyase) associated with one protein. Biochemistry 20: 4037-4042.

Nakajin S, Takahashi M, Shinoda M and Hall PF (1985) Cytochrome b5 promotes the synthesis of Δ^{16}-C19 steroids by homogeneous cytochrome P450 C21 side-chain cleavage from pig testis. Biochemical and Biophysical Research Communications 132: 708-713.

Nan X, Potma EO and Xie XS (2006) Nonperturbative chemical imaging of organelle transport in living cells with coherent anti-strokes Raman scattering microscopy. Biophysical Journal 91: 728-735.

Nazifi S, Saeb M, Rowghani E and Kaveh K (2003) The influences of thermal stress on serum biochemical parameters of Iranian fat-tailed sheep and their correlation with triiodothyronine (T_3), thyroxine (T_4) and cortisol concentrations. Comparative Clinical Pathology 12: 135-139.

Nelson CA and Seamon KB (1985) Regulation of [^{3}H]forskolin binding to human platelet membranes by GppNHpp, NaF, and prostaglandin E_1. FEBS Letters 183: 349-352.

Nelson CA and Seamon KB (1986) Binding of [^{3}H] to human platelet membranes. The Journal of Biological Chemistry 261: 13469-13473.

New MI and Suvannakul L (1970) Male pseudohermaphroditism due to 17α-hydroxylase deficiency. The Journal of Clinical Investigation 49: 1930-1941.

Newton R (2000) Molecular mechanisms of glucocorticoid action: what is important? Thorax 55(7): 603-613.

Nithipatikom K, Holmes BB, Isbell MA, Hanke CJ, Gomez-Sanchez CE and Campbell WB (2005) Campbell, Measurement of steroid synthesis in zona glomerulosa cells by liquid chromatography–electrospray ionization–mass spectrometry: Inhibition by nitric oxide. Analytical Biochemistry 15: 203-210.

Novak R, Keller M and Lévy F (2011) Mother-young relationships in sheep: A model for a multidisciplinary approach of the study of attachment in mammals. Journal of Neuroendocrinology 23: 1042-1053.

Oakley RH, Webster JC, Sar M, Parker Jr CR and Cidlowski JA (1997) Expression and subcellular distribution of the beta-isoform of the human glucocorticoid receptor. Endocrinology 138(11): 5028-5038.

Okeudo NJ and Moss BW (2005) Serum cortisol concentration in different sex-types and slaughter weights, and its relationship with meat quality and intramuscular fatty acid profile. Pakistan Journal of Nutrition 4: 64-68.

Olefsky JM (2001) Nuclear receptor minireview series. The Journal of Biological Chemistry 276: 36836-36864.

Olivier JJ (1999) The South African Merino performance testing scheme In 'Rising to the challenge – Breeding for the 21st Century Customer'. Beef Industry and CRC for Premium Quality Wool Industry Symposia. Supplement to the Proceedings of the Association for the Advancement of Animal Breeding Genetics 13: 119-124.

Ono T and Bloch K (1975) Solubilization and partial characterization of rat liver squalene epoxidase. The Journal of Biological Chemistry 250: 1571-1579.

Orentreich N, Brind JL, Rizer RL and Vogelman JH (1984) Age changes and sex differences in serum dehydroepiandrosterone sulfate concentrations throughout adulthood. The Journal of Clinical Endocrinology and Metabolism 59: 551-555.

Orth DN, Kovacs WJ and DeBold CR (1992) The adrenal cortex. In: Wilson JD, Foster DW (eds) Textbook of Endocrinology. WB Saunders Company, Pensylvania, US, pp. 489-619.

Ottenweller JE and Meier AH (1982) Adrenal innervation may be an extrapituitary mechanism able to regulate adrenocortical rhythmicity in rats. Endocrinology 111: 1334-1338.

Owens MJ and Nemeroff CB (1992) Physiology and pharmacology of corticotropin-releasing factor. Phamacological Reviews 43: 425-436.

Ozbay T, Rowan A, Leon A, Patel P and Sewer MB (2006) Cyclic adenosine 5'-monophosphate-dependent sphingosine-1-phosphate biosynthesis induces human CYP17 gene transcription by activating cleavage of sterol regulatory element binding protein 1. Endocrinology 147: 1427-1437.

Palmer LG (2001) Intracellular pH as a regulator of Na+ transport. Journal of Membrane Biology 184: 305-311.

Pandey AV and Miller WL (2005) Regulation of 17,20 lyase activity by cytochrome b5 and by serine phosphorylation of P450c17. The Journal of Biological Chemistry 280: 13265-13271.

Pandey AV, Mellon SH and Miller WL (2003) Protein phosphatase 2A and phosphoprotein SET regulate androgen production by P450c17. The Journal of Biological Chemistry 278: 2837-2844.

Park MJ, Kong HJ, Kim HY, Kim HH, Kim JH and Cheong JH (2007) Transcriptional repression of the gluconeogenic gene PEPCK by the orphan nuclear receptor SHP through inhibitory interaction with C/EBPalpha. Biochemical Journal 402(3): 567-574.

Patocs A, Liko I, Varga I, Gergics P, Boros A, Futo L, Kun I, Bertalan R, Toth S, Pazmany T, Toth M, Szücs N, Horanyi J, Glaz E and Racz K (2005) Novel mutation of the CYP17 gene in two unrelated patients with combined 17α-hydroxylase/17,20-lyase deficiency: Demonstration of absent enzyme activity by expressing the mutant CYP17 gene and by three-dimensional modeling. The Journal of Steroid Biochemistry and Molecular Biology 97: 257-265.

Pelletier G, Li S, Luu-The V, Tremblay Y, Bèlanger A and Labrie F (2001) Immunoelectron microscopic localization of three key steroidogenic enzymes (cytochrome P450scc, 3β-hydroxysteroid dehydrogenase and cytochrome P450c17) in rat adrenal cortex and gonads. Journal of Endocrinology 171: 373-383.

Pemberton PA, Stein PE, Pepys MB, Potter JM and Carrell RW (1988) Hormone binding globulins undergo serpin conformational change in inflammation. Nature 336(6196): 257-258.

Phillips ID, Ross JT, Young IR and McMillen IC (1994) Adrenal steroidogenic enzyme expression in the hypothalamopituitary disconnected foetal sheep. Proceedins of the Australian Society of Medical Research 33: 150.

Picado-Leonard J and Miller WL (1987) Cloning and sequence of the human gene for P450c17 (steroid 17α-hydroxylase/17,20 lyase): similarity with the gene for P450c21. DNA 6: 439-448.

Prunier A, Mounier AM and Hay M (2005) Effects of castration, tooth resection, or tail docking on plasma metabolites and stress hormones in young pigs. Journal of Animal Science 83: 216-222.

Pryce CR, Abbott AH, Hodges JK and Martin RD (1988) Maternal behaviour is related to prepartum urinary estradiol levels in the red-bellied tamarin monkeys. Physiology and Behavior 44: 717-726.

Qiao J, Chen X, Zuo CL, Gu YY, Liu BL, Liang J, Lu YL, Tang JF, Wu YX, Chen MD, Chen JL, Wu WL and Song HD (2010) Identification of steroid biosynthetic defects in genotype-proven heterozygous individuals for 17α-hydroxylase/17,20-lyase deficiency. Clinical Endocrinology (Oxford) 72: 312-319.

Qin KN and Rosenfield RL (1998) Role of cytochrome P450c17 in polycystic ovary syndrome. Molecular and Cellular Endocrinology 145: 111-121.

Quinkler M and Stewart PM (2003) Hypertension and the cortisol-cortisone shuttle. Journal of Clinical Endocrinology and Metabolism 88(6): 2384-2392.

Raftopoulou M and Hall A (2004) Cell migration: Rho GTPases lead the way. Developmental Biology 265: 23-32.

Rainey WE, Kramer RE, Mason JI and Shay JW (1985) The effects of taxol, a microtubule-stabilizing drug, on steroidogenic cells. Journal of Cellular Physiology 123: 17-24.

Rainey WE, Shay JW and Mason JI (1984) The effect of cytochalasin D on steroid production and stress fiber organization in cultured bovine adrenocortical cells. Molecular and Cellular Endocrinology 35: 189-197.

Rauh M (2010) Steroid measurement with LC–MS/MS. Application examples in pediatrics. The Journal of Steroid Biochemistry and Molecular Biology 121: 520-527.

Rauw WM, Kanis E, Noordhuizen-Stassen EN and Grommers FJ (1998) Undesirable side effects of selection for high production efficiency in farm animals: a review. Livestock Production Science 56: 15-33.

Reid SD and Perry SF (1991) The effects and physiological consequences of raised levels of cortisol on rainbow trout (*Oncorhynchus mykiss*) erythrocyte beta-adrenoreceptors. Journal of Experimental Biology 158: 217-240.

Rescher U, Goebeler V, Wilbers A and Gerke V (2006) Proteolytic cleavage of annexin 1 by human leukocyte elastase. Biochimica et Biophysica Acta 1763(11): 1320-1324.

Ridley AJ (2006) Rho GTPases and actin dynamics in membrane protrusions and vesicle trafficking. Trends in Cellular Biology 16: 522-529.

Rivier C and Rivest S (1991) Effect of stress on the activity of the hypothalamic-pituitary-gonadal axis: peripheral and central mechanisms. Biology of Reproduction 45: 523-532.

Rodriguez H, Hum DW, Staels B and Miller WL (1997) Transcription of the human genes for cytochrome P450scc and P450c17 is regulated differently in human adrenal NCIH295 cells than in mouse adrenal Y1 cells. The Journal of Clinical Endocrinology and Metabolism 82: 365-371.

Sackett DL and Wolff J (1986) Cyclic AMP-independent stimulation of steroidogenesis in Y-1 adrenal tumor cells by antimitotic agents. Biochimica et Biophysica Acta 888: 163-170.

SanCristobal-Gaudy M, Bodin L, Elsen JM and Chevalet C (2001) Genetic components of litter size variability in sheep. Genetics Selection Evolution 33: 249-271.

Sanner BM, Meder U, Zidek W and Tepel M (2002) Effects of glucocorticoids on generation of reactive oxygen species in platelets. Steroids 67: 715-719.

Scaroni C, Opocher G and Mantero F (1986) Renin-angiotensin-aldosterone system: a long-term follow-up study in 17α-hydroxylase deficiency syndrome. Clinical and Experimental Hypertension A 8: 773-780.

Schimmer BP, Tsao J, Borenstein J and Endrenyi L (1987) Forskolin-resistance Y1 mutants harbour defects associated with the guanyl nucleotide-binding regulatory protein, G_s. The Journal of Biological Chemistry 262: 15521-15526.

Schmid E, Schmid W, Jantzen M, Mayer D, Jastorff B and Schutz G (1987) Transcription activation of the tyrosine aminotransferase gene by glucocorticoids and cAMP in primary hepatocytes. European Journal of Biochemistry 165(3): 499-506.

Schoeman SJ, Cloete SWP and Olivier JJ (2010) Returns on investment in sheep and goat breeding in South Africa. Livestock Science 130: 70-82.

Schwarz E, Liu A, Randall H, Haslip C, Keune F, Murray M, Longo N and Pasquali M (2009) Use of steroid profiling by UPLC-MS/MS as a second tier test in newborn screening for congenital adrenal hyperplasia: The Utah experience. Pediatric Research 66: 230-235.

Seamon K and Daly JW (1981) Activation of adenylate cyclase by the diterpene forskolin does not require the guanine nucleotide regulatory protein. The Journal of Biological Chemistry 256: 9799-9801.

Seamon KB, Vaillancourt R, Edwards M and Daly JW (1984) Binding of [^{3}H]forskolin to rat brain membranes. Proceedings of the National Academy of Sciences of the United States of America 81: 5081-5085.

Selye H (1946) The general adaptation syndrome and the diseases of adaptation. Journal of Clinical Endocrinology 6: 117-230.

Sevrioukova IF, Li H, Zhang H, Peterson JA and Poulos TL (1999) Structure of a cytochrome P450-redox partner electron-transfer complex. Proceedings of the National Academy of Sciences of the United States of America 96: 1863-1868.

Sewer MB and Jagarlapudi S (2009) Complex assembly on the human CYP17 promoter. Molecular and Cellular Endocrinology 300: 109-114.

Sewer MB and Li D (2008) Regulation of steroid hormone biosynthesis by the cytoskeleton. Lipids 43(12): 1109-115.

Shackleton C (2010) Clinical steroid mass spectrometry: A 45 year history culminating in HPLC-MS/MS becoming an essential tool for patient diagnosis. The Journal of Steroid Biochemistry and Molecular Biology 121: 481-490.

Shiver TM, Sackett DL, Knipling L and Wolff J (1992) Intermediate filaments and steroidogenesis in adrenal Y-1 cells: acrylamide stimulation of steroid production. Endocrinology 131: 201-207.

Siegel PB, Honaker CF and Rauw WM (2009) Selection for high production in poultry. In: Resource allocation theory applied to farm animal production (ed) WM Rauw. CABI Publishing, Wallingford, UK, pp. 230-242.

Simersky R, Novak O, Morris DA, Pouzar V and Strnad M (2009) Identification and quantification of several mammalian steroid hormones in plants by UPLC-MS/MS. Journal of Plant Growth Regulation 28: 125-136.

Simmons RM, Satterfield MC, Welsh TH Jr., Bazer FW and Spencer TE (2010) HSD11B1, HSD11B2, PTGS2, and NR3C1 expression in the peri-implantation ovine uterus: effects of pregnancy, progesterone and interferon tau. Biology of Reproduction 82: 35-43.

Sirianni R, Rehman KS, Carr BR, Parker CR Jr. and Rainey WE (2005) Corticotropin-releasing hormone directly stimulates cortisol and the cortisol biosynthetic pathway in human foetal adrenal cells. The Journal of Clinical Endocrinology and Metabolism 90: 279-285.

Smith CL and Hammond GL (1992) Hormonal regulation of corticosteroid-binding globulin biosynthesis in the male rat. Endocrinology 130(4): 2245-2251.

Smith RF and Dobson H (2002) Hormonal interactions within the hypothalamus and pituitary with respect to stress and reproduction in sheep. Domestic Animal Endocrinology 23: 75-85.

Soldin SJ and Soldin OP (2009) Steroid hormone analysis by tandem mass spectrometry. Clinical Chemistry 55(6): 1061-1066.

Soucy P and Luu-The V (2000) Conversion of pregnenolone to DHEA by human 17α-hydroxylase/17,20-lyase (P450c17): Evidence that DHEA is produced from the released intermediate 17α-hydroxypregnenolone. European Journal of Biochemistry 267: 3243-3247.

Sparrow CP and Pittman RC (1990) Cholesterol esters selectively taken up from high density lipoproteins are hydrolyzed extralysosomally. Biochimica et Biophysica Acta 1043: 203-210.

Spraker TR, Hibler CP, Schoonveld GG and Adney WS (1984) Pathologic changes and microorganisms found in Bighorn sheep during a stress-related die-off. Journal of Wildlife Diseases 20(40): 319-327.

Stalmans W and Laloux M (1979) Glucocorticoids and hepatic glycogen metabolism. In: Baxter JD, Rousseau GG (eds) Glucocorticoid hormone action. Springer-Verlag, New York, NY, pp. 518-533.

Star L, Ellen ED, Uitdehaag K and Brom FWA (2008) A plea to implement robustness into a breeding goal: poultry as an example. Journal of Agricultural and Environmental Ethics 21: 109-125.

Stevens A, Begum G, Cook A, Connor K, Rumball C, Oliver M, Challis J, Bloomfield F and White A (2010) Epigenetic changes in the hypothalamic proopiomelanocortin and glucocorticoid receptor genes in the ovine fetus after periconceptional undernutrition. Endocrinology 151: 3652-3664.

Stocco DM and Clark BJ (1996) Regulation of the acute production of steroids in steroidogenic cells. Endocrine Reviews 17: 221-244.

Stocco DM, Wang X, Jo Y and Manna PR (2005) Multiple signalling pathways regulating steroidogenesis and steroidogenic acute regulatory protein expression: more complicated than we thought. Molecular Endocrinology 19: 2647-2659.

Storbeck K, Kolar NW, Stander M, Swart AC, Prevoo D and Swart P (2008b) The development of an ultra performance liquid chromatography-coupled atmospheric pressure chemical ionization mass spectrometry assay for seven adrenal steroids. Analytical Biochemistry 37: 11-20.

Storbeck K, Swart AC, Slabbert JT and Swart P (2007) The identification of two CYP17 alleles in the South African Angora goat. Drug Metabolism Reviews 39: 467-480.

Storbeck K, Swart AC, Snyman MA and Swart P (2008a) Two CYP17 genes in the South African Angora goat (Capra hircus) – the identification of three genotypes that differ in copy number and steroidogenic output. FEBS Journal 275: 3934-3943.

Strauss JF (2003) Some new thoughts on the pathophysiology and genetics of polycystic ovary syndrome. Annals of the New York Academy of Sciences 997: 42-48.

Swart P, Lombard N, Swart AC, van der Merwe T, Murry BA, Nicol M and Mason JI (2003) Ovine steroid 17α-hydroxylase cytochrome P450: characteristics of the hydroxylase and lyase activities of the adrenal cortex enzyme. Archives of Biochemistry and Biophysics 409: 145-152.

Tatee T, Narita A, Narita K, Izumi G, Takahira T, Sakurai M, Fujita A, Hosono M, Yamashita K, Enomoto K and Shiozawa A (1996) Forskolin Derivates. I. Synthesis, and cardiovascular and adenylate cyclase-stimulating activities of water-soluble forskolins. Chemical and Pharmaceutical Bulletin 44(12): 2274-2279.

Taylor PJ, Cooper DP, Gordon RD and Stowasser M (2009) Measurement of aldosterone in human plasma by semiautomated HPLC-tandem mass spectrometry. Clinical Chemistry 55: 1155-1162.

Tee MK, Dong Q and Miller WL (2008) Pathways leading to phosphorylation of P450c17 and to the posttranslational regulation of androgen biosynthesis. Endocrinology 149: 2667-2677.

Teebken OE and Scheumann GF (2000) Differentiated corticosteroid production and regulation after selective transplantation of cultured and noncultured adrenocortical cells in the adrenalectomixed rat. Transplantation 70: 836-843.

Tuckey RC, Headlam MJ, Bose HS and Miller WL (2002) Transfer of cholesterol between phospholipid vesicles mediated by the steroidogenic acute regulatory protein (StAR). The Journal of Biological Chemistry 277: 47123-47128.

Turner AI, Canny BJ, Hobbs RJ, Bond JD, Clarke IJ and Tilbrook AJ (2002) Influence of sex and gonadal status of sheep on cortisol secretion in response to ACTH and on cortisol and LH secretion in response to stress : Importance of different stressors. Journal of Endocrinology 173: 113-122.

Uhlar CM, Whitehead AS (199) Serum amyloid A, the major vertebrate acute-phase reactant. European Journal of Biochemistry 265(2): 501-523.

Valdes R Jr. and Jortani S (2002) Unexpected suppression of immunoassay results by cross-reactivity: Now a demonstrated cause for concern. Clinical Chemistry 48: 405-406.

Van Den Akker EL, Koper JW, Boehmer AL, Themmen AP, Verhoef-Post M, Timmerman MA, Otten BJ, Drop SL and De Jong FH (2002) Differential inhibition of 17α-hydroxylase and 17,20 lyase activities by three novel missense CYP17 mutations identified in patients with P450c17 deficiency. The Journal of Clinical Endocrinology and Metabolism 87: 5714-5721.

Van der Walt D, Cloete SWP, Storbeck K and Swart P (2009) The role of cytochrome P450 17α-hydroxylase/ 17,20-lyase (CYP17) in the stress coping ability in a divergently selected Merino sheep population. Proceedings of the Association for the Advancement of Animal Breeding and Genetics 18: 100.

Van Rensburg SJ (1971) Reproductive physiology and endocrinology of normal and habitually aborting Angora goats. Onderstepoort Journal of Veterinary Research 38:1.

Van Weerden W, Bierings H, Steenbrugge G, Jong F and Schroder F (1992) Adrenal glands of mouse and rat do not synthesize androgens. Life Sciences 50: 857-861.

Vander A, Sherman J and Luciano D (2004) Human physiology, the mechanisms of body function, 9th ed. McGraw-Hill Companies Inc., New York, NY.

Vanselow J and Fürbass R (2011) The bovine genome contains three differentially methylated paralogous copies of the P450c17 en coding gene (*CYP17A1*). General and Comparative Endocrinology 170(3): 475-479.

Veerkamp RF, Windig JJ, Calus MPL, Ouweltjes W, de Haas Y and Beerda B (2009) Selection for high production in dairy cattle. In: Resource allocation theory applied to farm animal production (ed) WM Rauw. CABI Publishing, Wallingford, UK, pp. 243-260.

Virshup DM (2000) Protein phosphatase 2A: a panoply of enzymes. Current Opinions in Cellular Biology 12: 180-185.

Von Borell E, Dobson H and Prunier A (2007) Stress, behaviour and reproductive performance in female cattle and pigs. Hormones and Behavior 52: 130-138.

Von Eckardstein A, Nofer JR and Assmann G (2001) High density lipoproteins and arteriosclerosis role of cholesterol efflux and reverse cholesterol transport. Arteriosclerosis, Thrombosis, and Vascular Biology 21: 13-27.

Voorhees H, Aschenbrenner J, Carnes J and Mrotek J (1984) Rounding and steroidogenesis of enzyme- and ACTH-treated Y-1 mouse adrenal tumor cells. Cell Biology International Reports 8: 483-497.

Voutilainen R, Tapanainen J, Chung BC, Matteson KJ and Miller WL (1986) Hormonal regulation of P450scc (20,22-desmolase) and P450c17 (17α-hydroxylase/17,20-lyase) in cultured human granulosa cells. The Journal of Clinical Endocrinology and Metabolism 63: 202-207.

Wang M, Roberts DL, Paschke R, Shea TM, Masters BS and Kim JJ (1997) Three-dimensional structure of NADPH-cytochrome P450 reductase: prototype for FMN- and FAD containing enzymes. Proceedings of the National Academy of Sciences of the United States of America 94: 8411-8416.

Wang YH, Tee MK and Miller WL (2010) Human cytochrome P450c17: single step purification and phosphorylation of serine 258 by protein kinase A. Endocrinology 151: 1677-1684.

Waterman MR (1994) Biochemical diversity of cAMP-dependent transcription of steroid hydroxylase genes in the adrenal cortex. The Journal of Biological Chemistry 269: 27783-27786.

Waterman MR and Keeney DS (1996) Signal transduction pathways combining peptide hormones and steroidogenesis. Vitamans and Hormones 52: 129-148.

Weitzman ED, Fukushima D, Nogeire C, Roffwarg H, Gallagher TF and Hellman L (1971) Twenty-four hour pattern of the episodic secretion of cortisol in normal subjects. The Journal of Clinical Endocrinology and Metabolism 33: 14-22.

Whitehouse BJ, Gyles SL, Squires PE, Sayed SB, Burnes CJ, Persaud SJ and Jones PM (2002) Interdependence of steroidogenesis and shape changes in Y1 adrenocortical cells: studies with inhibitors of phosphoprotein phosphatases. Journal of Endocrinology 172: 583-593.

Whitnall MH (1993) Regulation of the hypothalamic corticotropin-releasing hormone neurosecretory system. Progress in Neurobiology 40: 573-629.

Whittle WL, Patel FA, Alfaidy N, Holloway AC, Fraser M, Gyomorey S, Lye SJ, Gibb W and Challis JRG (2001) Glucocorticoid regulation of human and ovine parturition: the relationship between foetal hypothalamic–pituitary–adrenal axis activation and intrauterine prostaglandin production. Biology of Reproduction 64: 1019-1034.

Wilks A, Black SM, Miller WL and Ortiz de Montellano PR (1995) Expression and characterization of truncated human heme oxygenase (hHO-1) and a fusion protein of hHO-1 with human cytochrome P450 reductase. Biochemistry 34: 4421-4427.

Winkler H, Apps DK and Fischer-Colbrie R (1986) The molecular function of adrenal chromaffin granules: established facts and unresolved topics. Neuroscience 18: 261-290.

Wu R, Ma C and Casella G (2007) Statistical genetics of quantitative traits. Springer Science and Business Media, New York, USA.

Xing Y, Edwards MA, Ahlem C, Kennedy M, Cohen A, Gomez-Sanchez CE and Rainey WE (2011) The effects of ACTH on steroid metabolomic profiles in human adrenal cells. Journal of Endocrinology 209: 327-335.

Yamano S, Aoyama T, McBride OW, Hardwick JP, Gelboin HV and Gonzalez FJ (1989) Human NADPH-P450 oxidoreductase: complementary DNA cloning, sequence, vaccinia virus-mediated expression, and localization of the CYPOR gene to chromosome 7. Molecular Pharmacology 36: 83-88.

Yamazaki T, Ohno T, Sakaki T, Akiyoshi-Shibata M, Yabusaki Y, Imai T and Kominami S (1998) Kinetic analysis of successive reactions catalyzed by bovine cytochrome P45017α,lyase. Biochemistry 37: 2800-2806.

Yanagibashi K and Hall PF (1986) Role of electron transport in the regulation of the lyase activity of C21 side chain cleavage P450 from porcine adrenal and testicular microsomes. The Journal of Biological Chemistry 261: 8429-8433.

Yanase T (1995) 17α-Hydroxylase/17,20-lyase defects. The Journal of Steroid Biochemistry and Molecular Biology 53: 153-157.

Yanase T, Simpson ER and Waterman MR (1991) 17α-Hydroxylase/17,20-lyase deficiency: from clinical investigation to molecular definition. Endocrine Reviews 12: 91-108.

Young B, Lowe JS, Stevens A and Heath JW (2006) Wheather's Functional Histology: A text and colour atlas. In: Organ systems. Ozols I and Whitehouse A (eds) 5th ed. Churchill Livingstone, Elsevier Ltd., London, U.K, pp. 331-341.

Youngblood GL and Payne AH (1992) Isolation and characterization of the mouse P450 17α-hydroxylase/C17–20-lyase gene (Cyp17): transcriptional regulation of the gene by cyclic adenosine 3',5'-monophosphate in MA-10 Leydig cells. Molecular Endocrinology 6: 927-934.

Youngblood GL, Sartorius C, Taylor BA and Payne AH (1991) Isolation, characterization, and chromosomal mapping of mouse P450 17α-hydroxylase/C17–20 lyase. Genomics 10: 270-275.

Zachmann M, Völlmin JA, Hamilton W and Prader A (1972) Steroid 17,20 desmolase deficiency: a new cause of male pseudohermaphroditism. Clinical Endocrinology (Oxf) 1: 369-385.

Zhang LH, Rodriguez H, Ohno S and Miller WL (1995) Serine phosphorylation of human P450c17 increases 17,20-lyase activity: implications for adrenarche and the polycystic ovary syndrome. Proceedings of the National Academy of Sciences of the United States of America 92: 10619-10623.

Zhang P, Nason TF, Han XG and Hall PF (1992) Gene for 17α-hydroxylase/C(17–20) lyase P-450: complete nucleotide sequence of the porcine gene and 5' upstream sequence of the rat gene. Biochimica et Biophysica Acta 1131: 345-348.

Zuber MX, Simpson ER and Waterman MR (1986) Expression of bovine 17α-hydroxylase cytochrome P450 cDNA in non-steroidogenic (COS-1) cells. Science 234: 1258-1261.

Addendum A

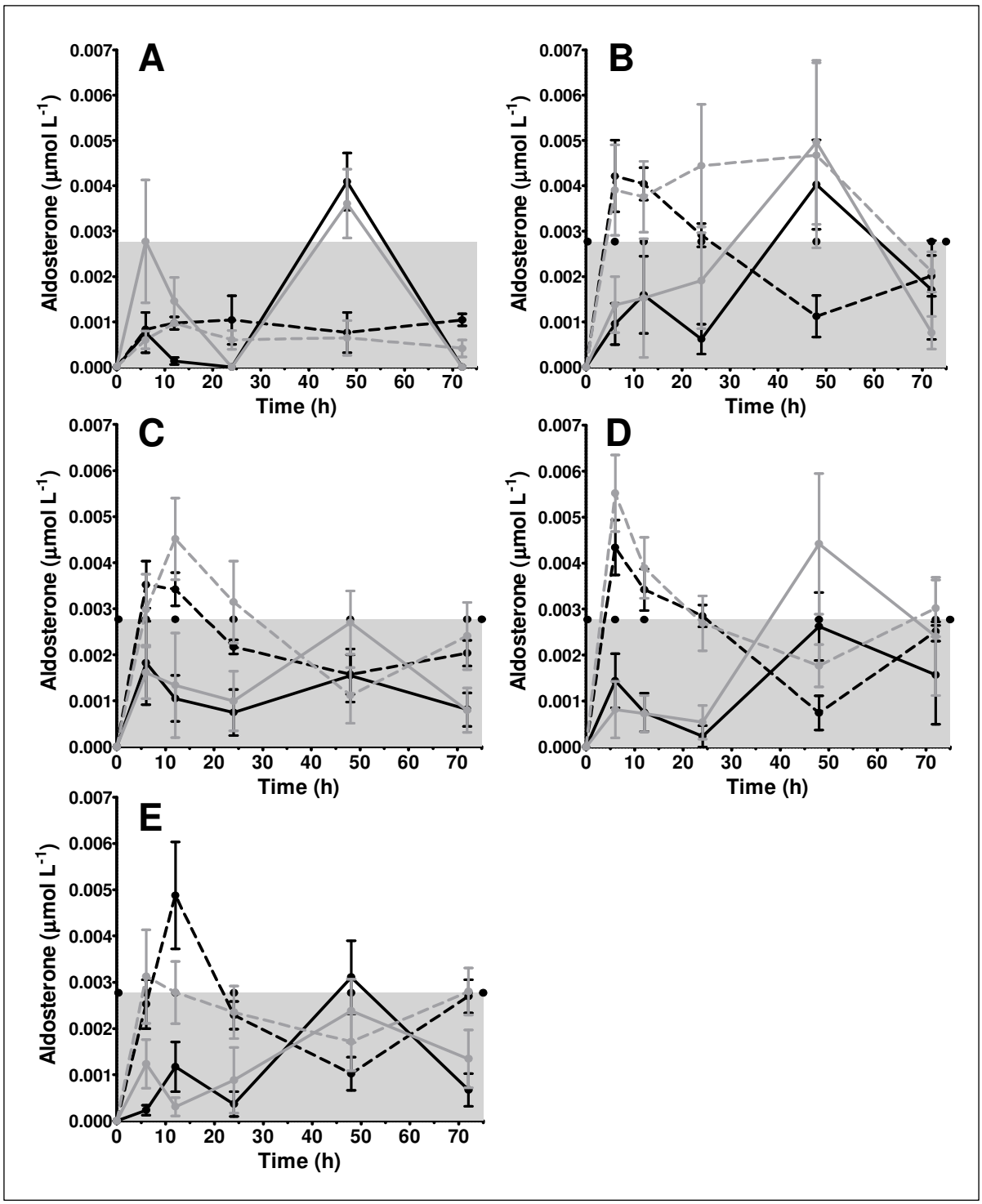

Figure A.1. Aldosterone concentrations in AA cells incubated over 72 hours (A) under basal conditions; (B) with 100 µM pregnenolone; (C) with100 µM pregnenolone and 1 µM ACTH; (D) with100 µM pregnenolone and 10 µM forskolin; and (D) with100 µM pregnenolone and 100 µM cholera toxin. The H-line is represented by a black coloured line (H_O and H_E), the L-line represented by a grey coloured line (L_O and L_E), homozygous *WT1* groups are represented by a solid line (H_O and L_O), and heterozygous *WT1/WT2* groups by a broken line (H_E and L_E).

Addendum B

Proceedings of the Association for the Advancement of Animal Breeding and Genetics 18: 100

THE ROLE OF CYTOCHROME P450 17α-HYDROXYLASE/17,20-LYASE (CYP17) IN THE STRESS COPING ABILITY OF A DIVERGENTLY SELECTED MERINO SHEEP POPULATION

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SUMMARY

South African Merino sheep were selected divergently from the same base population for their ability to rear multiples. Two distinct populations were formed over a period of more than 20 years of selection. Reproduction (and therefore presumably fitness) in the line selected in the upward direction (H-line) was substantially improved compared to the line selected in the downward direction (L-line). In the present study, it was demonstrated that the H-line was more stress-tolerant than the L-line in terms of their glucose and cortisol response when challenged with insulin. Sheep from the breeding program were genotyped according to one of two cytochrome P450 17α-hydroxylase/17-20 lyase (CYP17) alleles, as these genotypes were previously linked to the ability of Angora goats to cope with external stressors. However, no association was found between CYP17 genotype and selection line. The difference in insulin induced stress response between the H- and the L-line can therefore not be attributed to CYP17 genotype.

INTRODUCTION

Fitness of farm animals (defined as reproduction and survival) has long been identified as being of economic importance. Yet fitness traits have seldom been incorporated in selection programs for livestock (Goddard 2009). These fitness traits can be linked to genotypic markers that can be identified within livestock breeding programmes. These genetic targets can then be recorded and included in selection criteria to ultimately improve livestock fitness. In this study we look at cytochrome P450 17α-hydroxylase/17-20 lyase (CYP17) genotype as a possible genetic target to link to stress coping ability, a fitness trait.

CYP17 plays a critical role in the production of mineralocorticoids, glucocorticoids and androgens by the adrenal cortex in mammals (Vander *et al.* 2001). These steroid hormones are involved in fitness, since they play a vital role in the control of water and mineral balance, stress management and reproduction, respectively. CYP17 catalyses two distinct reactions, namely: a 17α-hydroxylation and a C17-C20 lyase reaction (Nakajin *et al.* 1981). This dual enzymatic activity places CYP17 at key branch points in the biosynthesis of adrenal steroid hormones.

Cortisol and corticosterone are the glucocorticoid hormones produced in the adrenal gland, which play an important role in stress management (Vander *et al.* 2001). As in humans, cortisol is by far the main glucocorticoid in sheep that counters a stress stimulus. Cortisol production is stimulated when the adrenal gland receives a "stress-signal" through the hypothalamus-pituitary-adrenal axis, via adrenocorticotropic hormone. The decreased ability of an animal to produce cortisol will lead to a reduced ability to counteract stress associated with the environment. Such an example was observed by Engelbrecht and Swart (2000) in Angora goats. These animals had a decreased ability to produce cortisol compared to Merino sheep and Boer goats, and accordingly exhibited a reduced ability to cope with insulin-induced stress.

Stress has been shown to reduce fitness, as reflected by growth, reproduction and survival of farm animals. Divergent selection for number of lambs weaned in Merino sheep, an example of a composite fitness trait, has resulted in marked differences in responses between the lines in this

trait (Cloete *et al*. 2004). Differences between the lines in survival of lambs and behavioural adaptations conducive to lamb survival were also observed (Cloete and Scholtz 1998).

Two CYP17 alleles have previously been identified in Merino sheep (Genbank accession no. L40335/WT1 and AF251388/WT2) and confirmed by Storbeck *et al.* (2008). However, it has not been established in this species whether a specific CYP17 genotype would enhance cortisol production, and thus stress coping ability, relative to the other. In this study, we investigated whether the observed divergence in fitness (as reflected by number of lambs weaned) observed in a Merino selection experiment can be related to the genotypic composition of ovine CYP17.

MATERIALS AND METHODS

Breeding program. A Merino sheep breeding program has been undertaken since 1986 in which sheep have been divergently selected for their ability to rear multiples (alternatively defined as number of lambs weaned per mating). The selection lines were derived from the same base population and selection within each line based on maternal ranking values for number of lambs weaned per lambing opportunity (Cloete *et al*. 2004). Number of lambs weaned per mating in the line selected in the upward direction (H-line) has been proved to be near to double that of the line selected in the downward direction (L-line) (Cloete *et al.* 2004).

Stress test. Stress coping ability was tested on 24 rams from this breeding program (13 H-line and 11 L-line sheep), housed at the Elsenburg Research farm near Stellenbosch, South Africa. These rams were injected intravenously with human insulin (Actrapid® HM, Novo Nordisk, Johannesburg, South Africa) after which 6 blood samples of each animal were collected over a 2 hour period and placed on ice. Blood samples were centrifuged at 2 500x*g* for 10 minutes (4^{o}C) to acquire representative plasma samples from each animal. Plasma glucose and cortisol levels were determined by PathCare Reference Laboratory (PathCare Park, N1 City, Goodwood, Cape Town, South Africa). Ethics approval for this stress test was obtained from the Departmental Ethics Committee for Research on Animals (DECRA reference R08/21).

Genomic DNA isolation. Blood samples of both H- (n=105) and L-line (n=31) sheep were collected in EDTA treated collection tubes (BD Vacutainer® Blood Collection Tubes; Pronto™ Quick Release Holder and Eclipse™ Blood Collection Needles). Blood samples were also acquired from the heart chamber of 36 lambs that had died during the 2008 lambing season. Genomic DNA was isolated using the Wizard® Genomic DNA isolation kit (Promega, Madison, Wisconsin) according to the instructions provided by the manufacturer.

CYP17 genotyping with real time polymerase chain reaction (RT-PCR). All 172 sheep were genotyped using the RT-PCR method developed by Storbeck *et al*. (2008). The primers and hybridisation probes (TibMolBio, Berlin, Germany) were as follows: LCLP, 5'-CCTGAAGGCCATACAAA-3'; LCRP, 5'-GGATACTGTCAGGGTGTG-3'; fluorescein-labelled CYP17 sensor probe, 5'-TTCTGAGCAAGGAAATTCTGTTAGA-FL; LC640-labelled CYP17 anchor probe, 640-TATTCCCTGCGCTGAAGGTGAGGA-3'. RT-PCR was carried out using a LightCycler® 1.5 instrument. Amplification reactions (20µl) contained 2 µl LightCycler® FastStart DNA Master HybProbe Master Mix (Roche Applied Science, Mannheim, Germany), 3 mM $MgCl_2$, 0.5 µM of each CYP17 primer, 0.2 µM fluorescein-labelled CYP17 sensor probe, 0.2 µM LC640-labelled CYP17 anchor probe and 10 to 100 ng genomic DNA. Following an initial denaturation at 95°C for 10 min to activate the FastStart *Taq* DNA polymerase, the 35-cycle amplification profile consisted of heating to 95°C with a 8 s hold, cooling to 52°C with a 8 s hold and heating to 72°C with a 10 s hold. The transition rate between all steps was 20°C/second. Data

were acquired in single mode during the 52°C phase using the LightCycler® software (version 3.5). Following amplification, melting-curve analysis was performed as follows: denaturation at 95°C with a 20 s hold, cooling to 40°C with a 20 s hold and heating at 0.2°C/s to 85°C with continuous data acquisition.

The sensor probe was designed to be a perfect match for the WT1 sequence and dissociated at 58°C when bound to the perfectly matched WT1 sequence. However, when bound to the mismatched sequence (WT2) dissociation occurred at 54°C. A no-template control (negative control) was also included in each assay.

Statistical analysis. For the stress test, plasma glucose and cortisol response over time for the H- and L-lines were analyzed with a regular two-way ANOVA with selection line and the period that passed since the insulin injection as factors. Differences between breed lines at specific time points were examined with Bonferonni's post-test (glucose: 95% confidence; cortisol: 94% confidence). The Chi-square test was used to analyze CYP17 genotype frequencies. GraphPad Prism (version 4) software (GraphPad Software, San Diego, California) was used for all statistical analysis.

RESULTS AND DISCUSSION

Stress test. Plasma glucose (mmol/L; which served to monitor the progress of the stress response) and plasma cortisol (log nmol/L) responses to insulin-induced stress are depicted in Figure 1. The H-line reached a hypoglycaemic state earlier than the L-line, with glucose levels of 1.9 mmol/L 30 min post insulin challenge, and recovered to baseline glucose (3.7 mmol/L) 2 hours post insulin challenge (3.3 mmol/L). Cortisol levels increased rapidly from 30 min post insulin challenge (60.3 log nmol/L), reached maximum at 60 min (120.2 log nmol/L) and returned to baseline concentrations after 2 hours. The stress response of H-line animals was completed after 2 hours with both glucose and cortisol concentrations having recovered to baseline levels.

The L-line reached maximum hypoglycaemic state at 60 min post insulin challenge with glucose levels of 2.1 mmol/L, but did not recover to baseline concentrations (3.6 mmol/L) by 2 hours post insulin challenge (2.7 mmol/L). L-line cortisol levels increased from 30 min post insulin challenge (53.3 log nmol/L), but at an apparently slower rate than the H-line. Maximum cortisol in the L-line was observed 90 min post insulin challenge (100.5 log nmol/L).

The interaction between breed line and time of measurement was highly significant ($P<0.0001$) for glucose ($F=9.22$, dfn=5, dfd=132), but not for cortisol ($P=0.3028$, $F=1.22$, dfn=5, dfd=132) responses to insulin-induced stress.

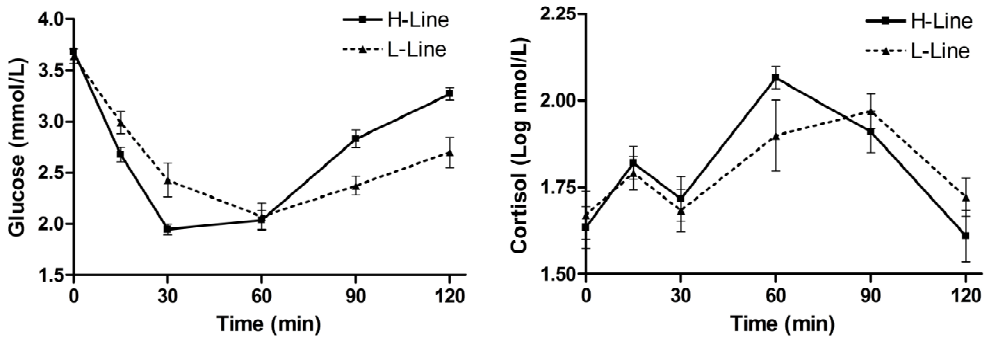

Figure 1. Merino sheep plasma glucose and cortisol response to insulin challenge.

Glucose levels were lower in the H-line at 30 minutes post insulin challenge (P<0.01), the opposite trend being observed after 90 (P<0.01) and 120 min (P<0.001). Cortisol levels were higher (P=0.0590) in the H-line 60 min post insulin challenge. The H-line animals thus had an improved ability to cope with insulin induced hypoglycaemia than the L-line, as reflected by their quicker glucose recovery to baseline and earlier peaking of cortisol at 60 min. An improved cortisol collection and detection method might limit variation in future tests.

CYP17 genotyping. Interestingly, no homozygous WT2 sheep were detected in either the H- or L-lines or among the lambs that died in 2008. Relative DNA copy number determination of sheep CYP17 has previously been done (Storbeck *et al.* 2008), indicating that the two CYP17 genetic sequences are two alleles of one gene. This finding thus warrants further investigation.

Table 1 summarizes the genotyping results obtained for the H-, L-line and lamb mortalities. There was no significant association (P=0.7617, Chi-square=0.5444, df=2) between CYP17 genotypes and designation of sample population (H-, L-line or lamb mortalities). On average 83.4 % sheep in the breeding program was heterozygous, while 16.6 % were homozygous WT1.

Table 1. Frequency distribution of CYP17 genotype in the Merino sheep breeding program

Merino flock	Homozygous WT1		Heterozygous WT1/WT2		Homozygous WT2	
	Number of sheep	Percentage	Number of sheep	Percentage	Number of sheep	Percentage
H-line	15	14.3	90	85.7	0	0
L-line	5	16.1	26	83.9	0	0
Lamb mortalities	7	19.4	29	80.6	0	0

CONCLUSIONS

The divergent breeding program was shown to result in differences in insulin-induced stress coping ability, the H-line having a higher stress tolerance than the L-line. This difference in stress tolerance could not, however, be ascribed to CYP17 genotype, since there was no association between CYP17 genotype and selection lines. One CYP17 isoform is not more advantageous for cortisol production in the adrenal gland than the other. This study rules out CYP17 as possible genotypic marker to use during selection, and suggest investigating other factors along the HPA axis or adrenal steroidogenesis that could be implicated in the stress response difference observed.

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REFERENCES

Cloete, S.W.P., Gilmour, A.R., Olivier, J.J. and Van Wyk, J.B. (2004) *Aust. J. Exp. Agric.* **44**:745.
Cloete, S.W.P., Scholtz, A.J. (1998) *Aust. J. Exp. Agric.* **38**: 801.
Engelbrecht, Y., Swart, P. (2000) *J. Anim. Sci.* **78**: 1036.
Goddard, M. (2009) "Adaptation and Fitness in Animal Populations – Evolutionary and Breeding Perspectives on Genetic Resource Management", Springer Science and Business Media, www.springer.com.
Nakajin, S., Shively, J.E., Yuan, P. and Hall, P.F. (1981) *Biochemistry.* **20:** 4037.
Storbeck, K., Swart, A.C., Snyman, M.A., Swart, P. (2008) *FEBS J.* **275**: 3934.

Addendum C

Proceedings of the World Congress on Genetics Applied to Livestock Production 9: ID628

Improving stress coping ability: Comparison between the *CYP17* genotypes of *Ovis aries* and *Capra hircus*

D. Hough[*], K. Storbeck[*], S.W.P. Cloete[†] and P. Swart[*]

Introduction

In mammals, physiological stress stimulates the release of glucocorticoids (cortisol and corticosterone) from the adrenal cortex that results in glucose production at the expense of glycolysis (Munch (1971)). Cytochrome P450 17α-hydroxylase/17,20-lyase (CYP17) plays a critical role in the production of these glucocorticoids. Abnormalities in the *CYP17* gene or protein can result in various mild to lethal disorders (Payne and Hales (2004)). In the case of the South African Angora goat, the activities of the two CYP17 isoforms was found to be the main cause for hypocortisolism that results in an increased susceptibility to cold stress (Engelbrecht et al. (2000); Storbeck et al. (2007)). In addition, Engelbrecht et al. (2000) showed that the stimulation of the hypothalmo-pituitary-adrenal (HPA) axis with insulin challenge *in vivo* resulted in less cortisol being produced in Angora goats when compared to South African Boer goats (*Capra hircus*) and Merino sheep.

Two *CYP17* genetic sequences have been identified for Angora goats, namely ACS- (GenBank accession no. EF524063) and *CYP17* ACS+ (GenBank accession no. EF524064) that is 100% homologous with Boer goat *CYP17* (GenBank accession no. AF251387) (Storbeck et al. (2007)). These sequences differ with four single nucleotide polymorphisms. In Merino sheep, two genetic sequences have been identified, namely *CYP17* WT1 (GenBank accession no. L40335) and WT2 (GenBank accession no. AF251388) (Storbeck et al. (2007)) that differ with two single nucleotide polymorphisms.

This study investigates the importance of the *CYP17* genotype in cortisol production in the Merino sheep and Angora goat adrenal gland as potential genetic markers for stress coping ability in selection programs to ultimately improve livestock fitness.

Material and methods

Animals. Merino sheep from a breeding program where divergent selection was based on maternal ranking values for number of lambs weaned per mating were used. At present, both reproduction (Cloete et al. (2004)) and stress coping ability, in terms of cortisol production in response to insulin challenge (Van der Walt et al. (2009)), is higher in the line selected in the upward deirection (H-line) than the line selected in the downward direction (L-line). The South African Angora goats used were randomly selected from the same flock.

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***CYP17* genotyping with real-time polymerase chain reaction (PCR).** Blood samples were collected from the jugular vein of adult sheep and goats, or from the heart chamber of dead lambs (2008 lambing season). The DNA isolation kit for mammalian blood (Roche, Germany) was used to isolate genomic DNA, which was then genotyped using the real-time PCR method developed by Storbeck et al. (2008).

***CYP17* copy number determination.** Relative copy number determinations were performed on the three goat genotypes (H_o, H_u and H_e) and heterozygous (WT1/WT2) sheep using quantitative PCR (Storbeck et al. (2008)). Fold change values were calculated relative to a H_o genotype calibrator using the $\Delta\Delta C_t$ method (Livak and Schmittgen (2001)).

Stress test. Merino sheep in the above mentioned breeding program (17 H-line and 21 L-line rams, 2-6 years of age) and a group of 30 Angora goats (3 groups of 10 goats for each *CYP17* genotype: 5 ewes and 5 rams, 14 months of age) were injected intravenously with human insulin at a dose rate of 0.1 IU/kg body mass after which blood samples were collected at times 0, 15, 30, 60, 90 and 120 min post insulin administration. Blood plasma glucose and free cortisol was determined with RIA. Ethics approval was obtained from the Departmental Ethics Committee for Research on Animals (DECRA ref: R08/21).

Statistical analyses. The Chi-square test was used to analyze *CYP17* genotype frequencies. For the stress test, the total HPA axis response to insulin induced hypoglycemia was calculated from the area under the curve that represented the amount of plasma cortisol produced (nmol/L) per unit plasma glucose drop (mmol/L) at times 0, 15, 30, 60, 90 and 120 min post insulin challenge. ANOVA tests were done for these responses, as well as for the relative DNA copy number determinations, followed by the Bonferonni's post-test. Graphs represent the mean and standard error of the mean. GraphPad Prism (version 4) software (GraphPad Software, US) was used for all analysis with α=0.05 for statistical significance.

Results and discussion

Merino *CYP17* genotyping. The real-time PCR method developed by Storbeck et al. (2008) to genotype Angora *CYP17* (ACS- and ACS+), was also suitable to genotype the two Merino sheep *CYP17* sequences: WT1 and WT2. The *CYP17* frequency distribution of 144 Merino sheep (112 H-line and 32 L-line, including 36 lambs that died in the peri-natal period) in the breeding program is 86.1% heterozygous WT1/WT2 and 13.9% homozygous WT1/WT1. No significant association (P=0.6722, Chi-square=0.7944, df=2) was found between *CYP17* genotypes and designation of population sample (H-line, L-line or lamb mortalities). Similar *CYP17* frequencies were observed in the case of Angora goats (Storbeck et al. (2008)). Interestingly, the homozygous WT2/WT2 genotype was not detected in Merino sheep, while in the case of Angora goats, the homozygous ACS+/ACS+ genotype was also not detected (Storbeck et al. (2008)).

Relative *CYP17* copy number determination. The absence of a homozygous genotype in both Angora and Merino suggests that either the homozygous genotype is lethal, or that there is an underlying genetic aberration for the *CYP17* gene. Storbeck et al. (2008) did a relative

CYP17 copy number determination for Angora goats with Boer goats and Merino sheep as controls. Their data revealed that three *CYP17* genotypes exist in the case of the Angora goat, namely H_o, H_u and H_e. The H_o genotype has only one *CYP17* gene, namely ACS-. In contrast, the H_e genotype has two *CYP17* genes, where the ACS- locus is always present together with the ACS+ locus. This is the reason why the homozygote for ACS+ is never detected. Crossing H_o and H_e goats would yield the proposed intermediate H_u genotype. This genotype would receive both ACS- and ACS+ loci from the H_e parent, but only the ACS- locus from the H_o parent. The ACS- : ACS+ ratio in the H_u genotype would therefore be 2:1.

Storbeck et al. (2008) also confirmed that there is only one *CYP17* gene in sheep with two alleles: WT1 and WT2. Given this data, three genotypes would be expected in the population: homozygous WT1/WT1; heterozygous WT1/WT2 and homozygous WT2/WT2. However, no homozygous WT2/WT2 genotype was detected (n=144). The possibility that this genotype is lethal is therefore considered.

The *CYP17* genotype was thus also determined for lambs that died during the peri-parturition period. However, the WT2/WT2 genotype remained undetected. It is suggested that the earlier stages in fetal or embryonic development be tested for the WT2/WT2 *CYP17* genotype. In addition, the *CYP17* genotyping test should be optimized for Merino sheep *CYP17* detection, since it was originally designed for goat *CYP17* genotyping.

Angora goat stress test. The total response of Angora goats to insulin challenge is depicted in Figure 1A. One-way ANOVA indicated that the *CYP17* genotype affected the ability of angora goats to respond to insulin-induced hypoglycemia. The Bonferonni's post-test showed a reduced response in the H_o group compared to the H_u and H_e groups. No significant difference in total response was observed between the latter groups. These results suggest that the presence of *CYP17* ACS+ in the H_e and H_u genotypes is more advantageous for cortisol production than the presence of *CYP17* ACS-.

Merino sheep stress test. When the Merino sheep responses to insulin challenge were grouped according to *CYP17* genotype, the *CYP17* WT1/WT1 group had an improved response compared to the *CYP17* WT1/WT2 group (data not shown). Interesting results were obtained where H- and L-line Merino sheep in the breeding program were subdivided into *CYP17* genotypes (Fig. 1B). Two-way ANOVA indicated that the interaction between selection line and *CYP17* genotype is significant. Bonferonni's post-test revealed that the response was the same for all groups, except for L-line heterozygous WT1/WT2 sheep where a weaker response was observed. These results indicate that the presence of *CYP17* WT1 is more advantageous for cortisol production than the presence of *CYP17* WT2 in the L-line where there is arguably an accumulation of low fitness alleles. This finding suggests that the effect of *CYP17* genotype is probably interacting with other factors along the HPA axis. *CYP17* can thus be used as a genetic marker (selection for WT1) to improve fitness in populations with an inherently low ability to cope with stress.

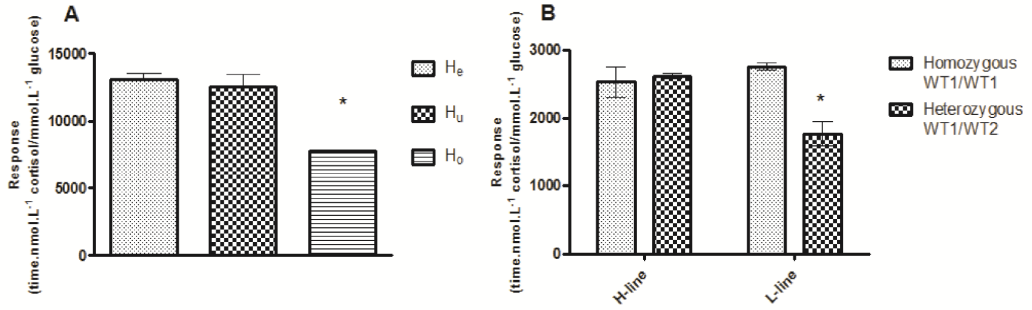

Figure 1: The total cortisol response to glucose decrease of (A) Angora goats and (B) Merino sheep (H- and L-line) grouped according CYP17 genotype.

Conclusion

The *CYP17* genotype is a factor that exerts its effect on stress coping ability via cortisol production in the adrenal cortex. This effect of the *CYP17* genotype seems to be dependent on the fitness of the sheep, or more specifically, the accumulation of other alleles that also affect cortisol production. Furthermore, genetic variation in *CYP17* exists among species which results in inherently different abilities to produce cortisol. The unique genotypes in the South African Angora goat differ not only in terms of the genetic sequences encoding for *CYP17*, but also in copy number. The genotypes in the Merino sheep, however, differ only in terms of the genetic sequences encoding the one *CYP17* gene. It is suggested that the missing WT2/WT2 genotype in sheep may be lethal or that the *CYP17* genotyping test needs to be optimised for Merino sheep. This study showed that the *CYP17* genotype is a relevant genetic marker to consider for the improvement of the stress coping ability of livestock. Further research is needed for the better understanding of the impact of *CYP17* genotypes on stress in livestock.

References

Cloete, S.W.P., Gilmour, A.R., Olivier, J.J. and Van Wyk, J.B. (2004). *Aus. J. Exp. Agr.*, 44: 745-754.

Engelbrecht, Y. and Swart, P. (2000). J. *Anim. Sci.*, 78: 1036-1046.

Livak, K.J. and Schmittgen, T.D. (2001). *Methods*, 25: 4402–4408.

Munch, A. (1971). *Perspect. Biol. Med.*, 14:265.

Payne, A.H. and Hales, D.B. (2004). *Endo. Rev.*, 25: 947-970.

Storbeck, K., Swart, A.C., Slabbert, J.T. and Swart, P. (2007). *Drug Metabol. Rev.*, 39: 467-480.

Storbeck, K., Swart, A.C., Snyman, M.A. and Swart, P. (2008). *FEBS J.*, 275: 3934-3943.

Van der Walt, D., Cloete, S.W.P., Storbeck, K. and Swart, P. (2009) In *Proc. Assoc. Advmt. Anim. Breed. Genet.*, volume 18, pages 100-103.