IDENTIFICATION OF TWO CYP17 ALLELES IN THE SOUTH AFRICAN ANGORA GOAT

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Declaration:

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.
SUMMARY

This study describes:

1. The isolation of total RNA and mRNA from Angora goat adrenals.
2. Synthesis and nucleotide sequence alignment of Angora goat CYP17 cDNA. Two DNA sequences were produced, identifying two CYP17 alleles in an Angora goat from the Swartland district.
3. The development of a CYP17 genotype test for Angora goats.
4. Genotyping of Angora goats and Boer goats with the developed genotype test.
5. Mapping of the substituted amino acids in the amino terminal of CYP17 to a specific CYP17 genotype.
6. Partial synthesis and alignment of Angora goat genomic nucleotide CYP17 sequences.
Hierdie studie beskryf:

1. Die isolering van totale RNA en mRNA van Angorabok byniere.
2. Sintese en nukleotied volgorde oplyning van Angorabok CYP17 cDNA. Twee DNA volgordes is geproduseer, en so is twee CYP17 allele in 'n Angorabok van die Swartland omgewing geïdentifiseer.
3. Die ontwikkeling van 'n CYP17 genotipe toets vir Angorabokke.
4. Genotipering van Angorabokke en Boerbokke met die ontwikkelde genotipe toets.
5. Korrelering van die omgeruilde aminosure in die aminoterminaal van CYP17 met 'n spesifieke genotipe.
6. Gedeeltelike sintese en oplyning van Angorabok genomiese CYP17 nukleotied volgordes.
Met liefde opgedra aan my ma
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ABBREVIATIONS

3β-HSD 3β-hydroxysteroid dehydrogenase
11β-HSD 11β-hydroxysteroid dehydrogenase
17OH-Preg 17-hydroxy pregnenolone
17OH-Prog 17-hydroxy progesterone
ACTH adrenocorticotrophic hormone
ADX adrenodoxin
ADXR adrenodoxin reductase
β-LPH β-lipotrophin
bp base pair
BS basic sequence
CAH congenital adrenal hyperplasia
CBG corticosteroid-binding globulin
CEH cholesterol ester hydrolase
CGRP calcitonin gene-related peptide
CHX cycloheximide
CRH corticotrophin-releasing hormone
CYP11A cytochrome P450 side chain cleavage
CYP11B1 cytochrome P450 c11
CYP17 cytochrome P450 17α-hydroxylase/17,20 lyase
CYP21 cytochrome P450 c21
cyt b5 cytochrome b5
DBD DNA binding domain
DHEA dehydroepiandrosterone
ECF extracellular fluid
FSH follicle-stimulating hormone
GIH growth hormone-inhibiting hormone
GnRH gonadotrophin-releasing hormone
GR glucocorticoid receptor
GREs glucocorticoid response elements
GRH growth hormone-releasing hormone
HPA-axis hypothalamic-pituitary-adrenal axis
HPG-axis hypothalamic-pituitary-gonadal axis
IL-1α interleukin-1α
kb kilo bases
LBD ligand binding domain
LDL low-density lipoproteins
LH luteinizing hormone
nGRE negative glucocorticoid response element
PCR polymerase chain reaction
PIH prolactin-inhibiting hormone
POMC pro-opiomelanocortin
PP-1G protein phosphatase-1G
PRD proline rich domain
RT-PCR reverse transcriptase polymerase chain reaction
SA signal-anchor sequence
StAR steroidogenic acute regulatory protein
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>TNF-α</td>
<td>tumor necrosis factor-α</td>
</tr>
<tr>
<td>TRH</td>
<td>thyrotrophin-releasing hormone</td>
</tr>
<tr>
<td>TSH</td>
<td>thyroid-stimulating hormone</td>
</tr>
<tr>
<td>VIP</td>
<td>vasoactive intestinal peptide</td>
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CHAPTER 1
INTRODUCTION

The South African mohair industry supplies close to 50% of the world’s mohair demand. Furthermore, the South African Angora goat almost unarguably produces the best quality mohair in the world. The South African Angora goat has been the topic of various research projects due to the wide spread stress intolerant phenotype among farming stocks. The stress intolerance of the Angora goat has many manifestations and places a serious burden on the mohair industry in South Africa. It seems that inbreeding practices have for many years caused the increased vulnerability to stress in these animals.

It is widely accepted that the breeding practices that produced the extraordinary quality of fleece also yielded the stress intolerant phenotype, which is causing the significant stock losses during cold spells. More than three decades ago Van Rensburg concluded that abnormally low levels of adrenal function in the Angora goat, coupled with qualitative changes in steroid hormone biosynthesis, were responsible for the stress intolerance in these animals [1]. One of the main manifestations of this intolerance is the inability of the Angora goat to withstand exposure to extreme environmental conditions. Goats are especially sensitive to the cold wet and windy weather that often prevails during winter months in the Little Karoo and Eastern Cape where most Angora goat farming takes place in South Africa.

Comparison of the increase in plasma cortisol concentrations upon intravenous injection of insulin among Angora goats and four other small ruminants showed that the Angora goat has a significantly impaired cortisol response (fig. 1.1) [2]. Engelbrecht et al., in an in-depth study of Angora goat adrenal steroidogenesis, postulated that the inability of the Angora goat to produce sufficient cortisol could be the result of a shift in the flux of steroid intermediates through the adrenal steroidogenic pathways [3]. In that study a malfunction at the level of the hypothalamic-pituitary-adrenal axis (HPA-axis) was ruled out as a major cause of the impaired cortisol response.
Adrenal steroidogenesis is a complex process, regulated by morphological zonation of steroid producing tissue together with cellular compartmentalization of the enzymes involved. The steroids produced by the adrenal cortex have target tissues throughout the body and affect sexual development and reproduction, intermediary metabolism and electrolyte balance. These physiological functions are mediated mainly by three types of steroid hormones produced by the adrenal cortex: 1) the mineralocorticoids, 2) the glucocorticoids and 3) the androgens. Production of these hormones is under strict hormonal control. Adrenocorticotrophic hormone (ACTH) from the anterior pituitary is a 39 amino acid peptide that binds to a transmembrane G-coupled protein receptor in all three zones of the adrenal cortex [4]. ACTH action promotes the biosynthesis and release of various steroid hormones. Chapter 2 gives a more detailed description of the regulation of adrenal steroidogenesis. The activities and regulation of the enzymes involved in adrenal steroidogenesis have a huge influence on the steroid hormone output of the adrenal cortex. Diseases such as congenital adrenal hyperplasia (CAH), Cushing’s syndrome and adrenogenital syndrome are a few of the well-studied conditions caused by defects in enzymes catalyzing the steroid producing reactions.

This thesis will address the role of cortisol in stress management and the biochemical processes leading to its production. Cortisol is the main glucocorticoid in goat species [5]. The mediating capacity of cortisol in intermediary metabolism, especially in carbohydrate metabolism, is well known. These properties of cortisol action are further discussed in
Chapter 2. During periods of cold stress maintenance of blood glucose levels is crucial to maintaining the mechanisms responsible for body heat production. These mechanisms are compromised in the Angora goat due to the inability of the animal to maintain a sufficient blood glucose concentration [6]. It is the aforementioned impaired cortisol response to HPA-axis stimulation that is largely responsible for the rapid drop in blood glucose concentration in the cold stressed Angora goat. In Chapter 3 adrenal steroidogenesis is discussed in more detail. It is shown that the impaired cortisol response is caused by altered fluxes through the steroid producing pathways. This alteration is the result of specificity and affinity differences of steroid producing enzymes to certain intermediates.

The work of Engelbrecht et al. gave the first insights into the biochemical mechanisms of Angora goat adrenal steroidogenesis [7]. It also indicated that one of the crucial enzymes involved in adrenal steroidogenesis, cytochrome P450 17-α hydroxylase/17,20 lyase (CYP17), was responsible for the decreased cortisol production of the Angora goat adrenal cortex. In comparative studies with Angora goat, Boer goat and Merino sheep, Angora goats were shown to produce more androgen precursors at the cost of glucocorticoid precursors when compared to the other two species. CYP17 is the only steroidogenic enzyme with dual catalytic activity. Androgen production is dependent on the lyase action and glucocorticoid production on the hydroxylation action of CYP17. The differential regulation of these activities has been the research focus in many studies investigating pubertal processes such as adrenarche, which is dependent on increased androgen production from the adrenal [8].

Despite all the research on the stress intolerance of the Angora goat, the mohair farming industry still lacks a feasible solution to their problem. According to experienced farmers in the trade there are Angora goats that are markedly less susceptible to stress than others, although they constitute the minority. Some breeders have been trying to breed hardened Angora goat stocks by employing Mendelian selection strategies, looking for characteristics they believe to confer stress tolerance. Such strategies, however, are proving unsuccessful since the properties that constitute the proper fitness are not readily physically distinguishable from those that do not.

The interesting observation by Engelbrecht et al. about the increased lyase activity of the Angora goat adrenal CYP17 prompted a further investigation of this important steroidogenic cytochrome P450-dependent enzyme. It was decided to synthesize the cDNA of CYP17 through the reverse transcriptase polymerase chain reaction (RT-PCR) for expression in non-steroidogenic tissue. This would enable reconstitution of CYP17 away from its natural
environment where the activities of other steroidogenic enzymes, and cofactors like cytochrome b5, complicates the interpretation of results. It would allow the investigation of CYP17 activity and substrate specificity to determine the exact contribution of this enzyme to the inability of the Angora goat to produce sufficient cortisol under stress conditions as shown by Engelbrecht et al [3]. The isolation of total RNA and mRNA from the adrenal cortex of a randomly selected Angora goat in the Western Cape is described in Chapter 4. The RNA isolated was used to synthesize the cDNA of CYP17 through a RT-PCR amplification. Agarose gel and sequence analysis of the RT-PCR product showed the CYP17 cDNA synthesis to be successful. Sequence analysis of the RT-PCR product, however, revealed that two copies of the Angora goat CYP17 gene exist and that different CYP17 genotypes could thus exist in Angora goat populations. In addition a unique restriction endonuclease recognition sequence was identified in one of the two CYP17 alleles. Identification of a second CYP17 allele suggested one, or even two, of the possible CYP17 genotypes to qualify as a potential marker for hypoadrenocorticism in the Angora goat.

Since hypoadrenocorticism is caused by a very definite metabolic imbalance it is believed to result from a genetic variation between goats that bear the symptoms of hypoadrenocorticism and those that do not. It should therefore be possible to find such a genetic marker and to employ it in breeding programs rather than the Mendelian breeding strategies that have been employed so far. The CYP17 genotype distribution among Angora goats of different phenotypes believed to be influenced by CYP17 activity was therefore investigated. Analysis of the Angora goat CYP17 cDNA gene structure also identified a possible methodology for identifying different CYP17 genotypes in Angora goats. The difference in the restriction profile of the two CYP17 alleles provided the basis for a genotype test that could indicate the CYP17 genotype of a goat.

The partial sequence analysis of the genomic copy of Angora goat CYP17 is described in Chapter 5. Comparisons were made with human and baboon genomic CYP17 sequences. This was done because genomic CYP17 sequences were not available for either sheep or goat. Analysis of the genomic CYP17 gene structure together with the cDNA structure aided in the development of a CYP17 genotype test. The CYP17 genotype test is fairly simple and is based on the restriction enzyme analysis of a PCR fragment spanning the polymorphic region of the genomic CYP17 gene. The PCR product of this region is submitted to endonuclease treatment with Acs I. This enzyme will cleave the PCR product to an extent determined by the CYP17 genotype due to the altered restriction enzyme profile mentioned earlier. Agarose gel
analysis of the PCR product digest with Acs I indicates the CYP17 genotype of the test subject. A blood sample of less than 1 ml from each goat, is all that is required to perform the genotype test.

Several Angora goats were subsequently genotyped in an effort to identify the different CYP17 genotypes. A further aim was to find a possible correlation between a specific genotype and a phenotype associated with the desired fitness traits. A total of 83 Angora goats and six Boer goats were genotyped. Finally, in Chapter 7, the genotype results were discussed in terms of the CYP17 allelic distribution and in terms of a correlation of the identified genotypes with described phenotypes. Some suggestions are also made for utilizing the CYP17 genotype test in further investigations of the two CYP17 allelic frequencies in different Angora goat populations. The importance of further research on Angora goat CYP17 activity in understanding the mechanism of hypoadrenocorticism is also emphasized.
CHAPTER 2
THE SOUTH AFRICAN ANGORA GOAT AND ENVIRONMENTAL STRESS: A PHYSIOLOGICAL PERSPECTIVE

2.1 Hypothalamic-pituitary-adrenal axis

Key to glucocorticoid, mineralocorticoid and androgen hormone homeostasis is the integrity of the HPA-axis. The HPA-axis consists of three endocrine organs (hypothalamus, pituitary and adrenal) and the hormones they produce. Interplay among these organs through endocrine, paracrine and autocrine action of their hormones is essential to maintaining life. Sexual maturation and reproduction [9], carbohydrate metabolism [10,11] and stress management, and also salt balance of extracellular fluid (ECF) [12] are all controlled in part by the integrated actions of adrenocortical hormones.

As mentioned earlier, in the South African Angora goat the stress management capabilities are compromised to an extent due to a malfunction at some level of the HPA-axis [2]. This manifests in the Angora goat’s inability to cope with environmental extremes such as exposure to wet and windy conditions with low temperatures. In the Little Karoo and Eastern Cape, where most Angora goat farming takes place, these conditions are often encountered in winter when surrounding mountains are covered in snow. These regions, however, offer the best grazing to Angora goats. The HPA-axis will further be discussed in terms of the hormones produced by the endocrine organs, their actions and regulation and some basic anatomical features.

2.1.1 The hypothalamus

The hypothalamus is a complex brain structure forming part of the diencephalon and consists of a variety of nuclei and nuclear areas. Many afferent and efferent neural pathways connect the hypothalamus to many different centres in the central nervous system. These include afferent pathways from the limbic lobe, midbrain, hippocampus, brain stem, medulla and thalamus. Efferent pathways project from the hypothalamus to the limbic lobe, midbrain,
The hypothalamus is central to a complex neural network that enables it to control the chemical consistency and temperature of an organism. This chapter will focus on the regulation the hypothalamus exerts through the pituitary to finally control the steroidogenic process in the adrenal cortex. A diagrammatic representation of the human hypothalamus and pituitary gland is shown in fig. 2.1.

Figure 2.1. Human hypothalamus and pituitary. Reproduced from [14].

2.1.2 The pituitary

The pituitary gland consists of an anterior and posterior lobe. Nitric oxide containing efferent nerves from the supraoptic and paraventricular hypothalamic nuclei innervate the posterior lobe via the hypothalamohypophysial tract [15]. The anterior lobe has a vascular connection with the hypothalamus and receives blood through the portal hypophyseal vessels that drain the primary plexus of capillaries on the ventral surface of the hypothalamus [16]. These portal hypophyseal vessels then split up to form a capillary network that supplies the anterior pituitary. The vascular connection between the hypothalamus and pituitary forms a
true portal system since it begins and ends with a capillary network without passing through the heart. Various neurons from the hypothalamus that secrete hypophysiotrophic hormones terminate in the median eminence and deliver their hormones to the primary plexus. These hormones are subsequently transported through the hypophyseal portal system to their target cells in the anterior pituitary.

The posterior pituitary mainly secretes vasopressin and oxytocin [17,18]. These hormones are both nonapeptides that are produced in the cell bodies of the magnocellular neurons in the supraoptic and paraventricular nuclei. They subsequently travel down the axons of these neurons and are released from the nerve endings in the posterior pituitary lobe upon depolarization of the neurons. Vasopressin (also called antidiuretic hormone – ADH) is responsible for the re-uptake of water from the collecting ducts in the kidney by increasing the permeability of the ducts to water [19,20].

Oxytocin increases contraction of smooth muscle in the breasts and uterus. Although many hormones are responsible for breast development, milk production and secretion into the ducts, ejection is dependent on oxytocin [21]. Anterior pituitary function is regulated by six hypophysiotrophic hormones released from the hypothalamus into the primary plexus of the median eminence.

These hormones are: corticotrophin-releasing hormone (CRH), thyrotrophin-releasing hormone (TRH), growth hormone-releasing hormone (GRH), growth hormone-inhibiting hormone (GIH or somatostatin), gonadotrophin-releasing hormone (GnRH) and prolactin-inhibiting hormone (PIH) [22]. Binding of these hypothalamic hormones to their membrane bound receptors in the anterior pituitary lead to the production and secretion of at least six other hormones from specific cell types.

Five secretory cell types are found in the anterior pituitary. They are: somatotrophes (growth hormone), lactotrophes (prolactin), thyrotrophes (thyroid-stimulating hormone – TSH), gonadotrophes (luteinizing hormone – LH and follicle-stimulating hormone – FSH) and corticotrophes (adrenocorticotropic hormone – ACTH and β-lipotrophin - β-LPH) [12]. These hormones modulate reproductive, energy and growth metabolism to a great extent.

The hypothalamus receives a wide array of chemical and electrical stimuli sensing the homeostatic status of an organism. In response to these stimuli it also generates chemical and electrical signals. Many efferent neural pathways from the hypothalamus innervate the central nervous system on different levels. Stimulating the pituitary also induces hormonal responses. The hypothalamus thus is a centre for integrating the nervous and endocrine systems. This
integration enables an organism to react to changes in its environment, and to maintain
homeostasis. Only ACTH forms part of the HPA-axis, since the other hormones are not
known to affect adrenal activity significantly. Therefore only the effect of ACTH on the
adrenal, and the hypothalamic response to that, will further be discussed.

2.1.3. The role of glucocorticoids in HPA-axis regulation

Although CRH and ACTH can regulate adrenocortical function independent of
hypothalamic-pituitary innervation (section 2.2), the HPA-axis is central to modulating
adrenal steroidogenesis. Glucocorticoids released from the adrenal cortex in response to
ACTH have a negative feedback effect on the hypothalamic release of CRH. This effect is
mediated by binding of glucocorticoids to their receptors.

Glucocorticoid receptors are present in many tissues of the body. Two types of receptors
exist. The type II receptor is found throughout the brain, and is believed to play an important
role in mediation of the stress response. The abundant type II receptor is far from saturated,
even at high levels of circulating glucocorticoids [23]. The mineralocorticoid receptor (type I
receptor) is confined mainly to the hippocampus. These receptors are saturated at low levels
of circulating glucocorticoids, and are believed to restrict ACTH and CRH secretion during
the nadir of the circadian rhythm [24]. When glucocorticoid levels rise, ACTH secretion is
suppressed (fig. 2.2). This is the result of glucocorticoids acting on the hypothalamus and the
pituitary (inhibiting CRH release) [25]. Lowering of ACTH and CRH levels is achieved
through a fast feedback acting on the release of ACTH and CRH, and a longer term feedback
acting on the biosynthesis thereof. The mechanism of glucocorticoid action will be discussed
later in this chapter. There are two more important factors involved in regulating HPA-axis
activity: circadian rhythms (coupled to light dark cycles) and stress factors such as the
catecholamines. The mechanisms by which these factors regulate HPA-axis activity are not
fully elucidated, and are believed to differ among species.
2.2 Intra-adrenal regulatory mechanisms

ACTH binds to typical G-coupled transmembrane protein receptors in all three zones of the adrenal. Adrenocortical steroidogenesis, however, is not solely controlled by the endocrine actions of the hypothalamus and pituitary. Diurnal variation in adrenal steroidogenesis persists in CRH knockout mice [26,27], indicating that a non-pituitary mediated mechanism for controlling adrenal steroidogenesis could exist. The adrenal gland, as a functional unit, consists of the inner medulla and the outer cortex. Although these were first thought to be two very distinct and well-separated structures, it is today widely accepted that cells of both structures are to be found in the other. Chromaffin cells from the medulla can be found as single cells or islets in all three zones of the adrenal cortex [28]. Nets of chromaffin cells have also been observed in the subcapsular region of the adrenal gland [29]. Similarly cortical cells are found in the medulla. They are also seen as islets surrounded by chromaffin tissue while some still have connections with the cortex [30]. This morphological arrangement ensures
extensive contact areas between medullary and cortical cells enhancing paracrine signalling between the two adrenal structures which forms the basis for intra-adrenal regulation of medullary and cortical function.

2.2.1 Compounds from the medullary chromaffin cells

The main secretory products of the adrenal medulla are the catecholamines epinephrine, nor-epinephrine and dopamine. There are, however, a number of other substances found in the chromaffin cells of the medulla that could have regulatory effects on adrenal steroidogenesis. Nor-epinephrine and epinephrine are secreted from the chromaffin cells in response to stimulation by the splanchnic nerve [31]. These catecholamines stimulate the secretion of cortisol, aldosterone and androstenedione if used to perfuse isolated porcine adrenal glands [32]. Furthermore, catecholamines have a stimulatory effect on the transcription of certain cytochrome P450 genes. As regulation on a transcriptional level is associated with long-term rather than acute effects, the catecholamines are able to induce prolonged secretion of corticosteroids from isolated adrenocortical cells [33].

Together with the catecholamines many different neurotransmitters, mostly peptides but also serotonin, are secreted from the chromaffin cells, where they are also produced and stored. Of these the enkephalins were discovered first and are also the most abundant medullary neuropeptides [34]. Not all the peptides have stimulatory effects on steroidogenesis, and they do not necessarily have the same action in different species. The active neuropeptides and transmitters, released from either the chromaffin cells or the nerve supply to the adrenal, form a diverse array of effectors. Amongst others, these effectors orchestrate the complex machinery driving adrenal steroidogenesis.

Co-culture studies with bovine adrenomedullary chromaffin cells and bovine adrenocortical cells established the paracrine effect chromaffin cells have on adrenocortical cells [35]. Fig. 2.3 shows the amount of cortisol released from bovine cortical cells in monolayer and in mixed culture with bovine chromaffin cells.

Different chromaffin cells exist in populations in the medulla with varying compositions of neuropeptides. Independent mechanisms are responsible for the release of the different peptides. These mechanisms are dependent on signaling molecules utilizing specific second messengers and intracellular signaling pathways [36]. Humoral and immune factors, from either intra-adrenal or extra-adrenal origin, can act as signaling molecules for the release of
the neuropeptides from the chromaffin cells. For example, interleukin-1α (IL-1α) and tumor necrosis factor-α (TNF-α) differentially regulate Met-enkephalin, vasoactive intestinal peptide (VIP), neurotensin and substance P biosynthesis in chromaffin cells [37].

The adrenocortical cells also produce the two aforementioned cytokines. Activity of the adrenocortical cells can thus alter the composition of neuropeptides within the chromaffin cells. A complete steroidogenic regulatory circuit therefore exists within the adrenal. Although this might seem a very efficient regulatory mechanism, only a small number of adrenocortical cells are in contact with medullary cells and a fast adrenocortical response would not be possible through this mechanism. Gap junctions, however, circumvent this problem. Gap junctions allow small water-soluble molecules to move quickly from the cytoplasm of one cell to the next. This allows cellular signals to be passed from a stimulated to an unstimulated cell. Gap junctions occur in all three zones of mammalian adrenal cortex [38], and have been shown to form in both human and bovine primary adrenocortical cell cultures [39].

2.2.2 Nerve supply to the adrenal cortex

It was believed many years ago that the adrenal cortex had no direct nerve supply. The nerve supply to the medulla was thought to pass straight through the cortex without branching in, or synapsing with cortical cells [40,41]. In 1971 Unsicker reported on the innervation of
the rat and pig adrenal cortex [42]. Today the adrenal cortex is known to be innervated by a rich nerve supply synapsing directly with steroid producing cells [43].

There are two distinct sources for the afferent innervation of the adrenal cortex. One is the adrenal medulla. The cortex is believed to have been the target of medullary postganglionic nerves before becoming more closely associated with the medulla during evolution. The second source of nerve supply is uncertain. These nerves, however, have their cell bodies outside the cortex and both post- and preganglionic fibres enter the cortex along blood vessels.

Many different neurotransmitters are involved in relaying signals from the neural component to the endocrine steroid producing cells. Peptidergic intrinsic nerves have been described containing VIP, neuropeptide Y, substance P and calcitonin gene-related peptide (CGRP) as neurotransmitters [44-46]. Catecholamines and acetylcholine have also been identified as neurotransmitters in nerves supplying the cortex.

Regulation of the neural component controlling adrenal steroidogenesis, i.e. the concentrations and ratios of different neurotransmitters, is complex and not well understood. It is, however, safe to say that the physiological status of an organism plays a major role in this regulation. In the same light it is difficult to say to what extent the splanchnic nerve regulates neural activity in the cortex. Studies in different animal models have, however, shown splanchnic nerve activity to increase adrenocortical sensitivity to ACTH stimulation [47]. Severing the splanchnic nerve surgically causes decreased sensitivity of adrenocortical cells to ACTH. The role of neural innervation of the adrenal cortex is probably best seen as fine tuning adrenal steroidogenesis.

2.2.3 Vascularization of the adrenal gland

The steroid hormones produced and secreted by the adrenal cortex have target cells in tissues throughout the body. These hormones have to travel along the vascular system in order to reach their target cells. Therefore, the degree of vascularization of the adrenal gland plays a vital role in the steroid delivering capacity of the gland. It is thus foreseeable that the adrenal gland is a highly vascularized gland, receiving a comparatively large amount of the cardiac output. Rat adrenals receive seven times more blood compared to the average blood to body weight distribution ratio [48]. Fig. 2.4 is a diagrammatic presentation of adrenal vascularization. In goats arterial blood is supplied from the renal and not the phrenic arteries.
Blood dissipates from these arteries into the subcapsular arteriolar plexus. From this plexus blood either enters the thin walled sinusoids supplying the cortex, or the medullary arteries that supply the medulla directly. The number of the medullary arteries varies between species. This has implications for the regulation of blood flow through the gland and also for the physiological requirements of an organism. It should be clear that the regulation of blood flow through the adrenal is important in controlling steroid hormone balance throughout the body.

As discussed earlier, both neural and endocrine mechanisms are involved in controlling adrenal activity. Blood flow through the gland is also controlled by similar mechanisms.
Splanchnic nerve activity stimulates blood flow through the gland in dogs and calves [50,51]. This could be due to the release of neuropeptides.

Many of the neuropeptides mentioned earlier have been studied to determine their influence on vasoconstriction or dilation. VIP, Met-enkephalin and CGRP cause vasodilation, while neuropeptide Y causes vasoconstriction [52,53]. Some neuropeptides were found to have no vasoactive capacity, such as substance P, neurotensin and Leu-enkephalin. The hypothalamic corticotrophe ACTH is also well known to affect blood flow through the adrenal.

It was believed for some time that ACTH constricted the medullary arteries to increase blood flow through the sinusoids in the cortex. However, in 1986, Hinson et al. showed that ACTH administration to the perfused rat adrenal resulted in an overall increase of perfusion medium flow rate [54]. Various other modulators of adrenal vasculature have been identified. They include the secretory products of the vascular endothelium, namely nitric oxide, endothelin 1 and adrenomedullin. In order for the blood flow rate to have a significant effect on steroid delivery to target tissues, it would be expected that an increase in flow rate would be met with an increase in steroid secretion.

Even if ACTH concentration were to remain constant, the rate of presentation to the target cells in the cortex would increase with any increase in blood flow. Some researchers believe this to be the main determinant for the amount of steroid secreted [55]. However, even in the absence of ACTH there is an increase in corticosterone secretion of the isolated perfused rat adrenal [54]. This effect is observed when flow rate is changed mechanically, or by the addition of vasodilators. It is also only observed in the intact adrenal. This suggests that an element that is lost when cells are dispersed, and which could therefore be a compound released from the vascular endothelium, mediates this effect. Endothelial cell products not only affect adrenal vasculature, but also modulate to some extent adrenocortical steroidogenesis.

The vascularization of the adrenal cortex is such that almost every adrenocortical cell is adjacent to a vascular endothelial cell. This arrangement is optimal for endothelial cells to exert paracrine actions on adrenocortical cells. Endothelin 1 has more than one receptor and binds to different subtypes. The distribution of these receptors through species and also the different zones of the cortex vary. Endothelin 1 has been shown to stimulate aldosterone secretion in some species. In bovine adrenal cell preparations endothelin 1 augments angiotensin II and ACTH stimulated aldosterone secretion [56]. Nitric oxide has also been
implicated as a modulator of adrenal steroidogenesis. Although nitric oxide does not influence rat adrenal responsiveness to ACTH, basal levels of steroid output are much lower in the absence of endogenous nitric oxide synthesis [57]. Adrenomedullin modulates aldosterone secretion. There has, however, been some uncertainty over whether it inhibits or stimulates aldosterone secretion. These discrepancies have been put down to the differential expression of adrenomedullin receptors. It seems only logical that there would be a connection between blood flow through the adrenal and the steroid output of the gland. This seems to be regulated to some extent in a paracrine fashion by the vascular endothelium.

2.2.4 Intra-adrenal CRH/ACTH system

CRH, as described before, is a key hypophysiotrophic hormone regulating adrenocortical activity through the activity of the HPA-axis. However, CRH is not only produced by the hypothalamus, and is found in the adrenal gland itself, where it has direct effects on adrenal activity. Although ACTH is derived from the anterior pituitary, it is also produced by the adrenal. There is thus an intra-adrenal CRH/ACTH system involved in regulating adrenocortical activity.

Bornstein et al. found evidence for a direct effect, i.e. not through pituitary activation, of CRH on adrenocortical cells. He showed that atrophy of the cortex in hypophysectomized rats could be reduced by CRH [58]. CRH also enhances adrenal responsiveness to ACTH. Ovine CRH was found to stimulate cortisol release in man independent of ACTH [59]. This effect of CRH seems not to be without mediation from other factors, either a synergistic effect with ACTH or some intermediary factor released from the medulla exist.

Intra-adrenal ACTH is also produced by the medulla [60]. This ACTH seems to be released from the medulla in response to CRH stimulation. Similarly to the central CRH/ACTH system the intra-adrenal CRH/ACTH system is also regulated by feedback mechanisms. Furthermore, the activity of the intra-adrenal CRH/ACTH system is greatly increased in hypophysectomized animals. The intra-adrenal CRH/ACTH system is believed to be of great importance in adrenocortical function.
2.3 Mechanism of ACTH action

Binding of ACTH to its receptor in the zona fasciculata leads to the activation of steroidogenesis and the release of cortisol. ACTH binds to a transmembrane G-coupled protein receptor [4]. These transmembrane hormone receptors are associated with G proteins on the inside of the cellular membrane. Most G proteins are heterotrimers consisting of α, β and γ subunits. If the receptor protein is unoccupied, the G protein α subunit is associated with GDP. Activation of the receptor by hormone results in displacement of GDP by GTP in the α subunit. This in turn leads to the dissociation of the α subunit, which then associates with adenylate cyclase (also bound to the inner cell membrane). The α subunit then stimulates adenylate cyclase to produce cAMP from ATP. The α subunit has intrinsic GTPase activity and once the GTP is hydrolyzed to GDP, the α subunit reallocates to the β and γ subunits. This activation cycle will continue while the G-coupled protein receptor is occupied by hormone. Fig. 2.5 demonstrates the cycle. cAMP produced by adenylate cyclase is an intracellular second messenger molecule that can induce changes in various metabolic pathways. This is achieved by cAMP binding to the two regulatory subunits of cAMP-dependent protein kinase, which causes the release of two catalytic subunits. The catalytic subunits can phosphorylate a number of enzymes on specific serine and threonine residues, bringing about changes in their catalytic activity. In the steroidogenic cells of the zona fasciculata cAMP induces: 1) increased rates of cholesterol ester hydrolysis to free cholesterol, 2) increased transport of free cholesterol into the mitochondria and 3) stimulation of the cholesterol side chain cleavage reaction in the mitochondria [61].

2.4 Biology of cortisol

2.4.1 Cortisol in circulation

It is evident from the above discussion that stimulation of the adrenal cortex by ACTH leads to increased release of cortisol into the bloodstream from the adrenal. There is equilibrium between free and protein-bound cortisol in circulation (fig. 2.6). Binding of cortisol to plasma proteins increases its half-life. The bound fraction is mainly bound to corticosteroid-binding globulin (CBG) and some is bound to albumin [12].
Figure 2.5. Graphic presentation of the ACTH receptor activation cycle. Box a shows ligand binding to the transmembrane G-coupled receptor and box b shows the consequent replacement of GDP with GTP. Boxes c and d show the activation of adenylate cyclase by the GTP bound $\alpha$ subunit and the subsequent hydrolysis of GTP respectively. Reproduced from [4].

The pool of free cortisol is the active concentration i.e. that which is able to cross the membrane of target cells and bind to the receptor. This equilibrium between the bound and free cortisol is influenced by various factors and has major physiological implications. CBG is produced in the liver and its production is hormonally regulated [62]. Pregnancy is also known to increase the levels of CBG. Certain illnesses, like nephrosis, cirrhosis and multiple myeloma suppress CBG levels. When CBG levels rise, more cortisol becomes bound resulting in a decrease of free cortisol. This alleviates the suppressing effect of glucocorticoids on the release of ACTH from the pituitary. An increase in ACTH will lead to a rise in cortisol and a new equilibrium is reached in which the bound cortisol is higher and
the free cortisol is normal. Changes in the opposite direction also occur. Plasma total cortisol concentrations are therefore not always indicative of glucocorticoid excess or deficiency.

**Figure 2.6.** The interrelations of free and bound cortisol. The dashed arrow indicates that cortisol inhibits ACTH secretion. The value for free cortisol is an approximation; in most studies, it is calculated by subtracting the protein-bound cortisol from the total plasma cortisol. Reproduced from [12].

2.4.2 Cortisol catabolism

The liver is the principal site for glucocorticoid catabolism [63,64]. Most cortisol is reduced to dihydrocortisol and then to tetrahydrocortisol. Glucuronyl transferase catalyzes the conjugation of the reduced cortisol to glucuronic acid. Some cortisol is converted to 17-ketosteroids and some, by 11β-hydroxysteroid dehydrogenase (11β-HSD), to cortisone, which is also conjugated to glucuronic acid. Glucuronide conjugates of cortisol are freely soluble and are excreted in the urine. The 17-ketosteroids are conjugated to sulfate and also excreted in the urine. Some 15% of the secreted cortisol is excreted in the stool via the enterohepatic circulation. Liver disease can result in a lowered capacity to catabolize cortisol. Certain stresses like surgery in humans have the same effect. This can lead to an increase of free plasma cortisol concentration to levels higher than those attained with maximal ACTH stimulation without stress. Physiological stress can thus influence cortisol levels by means other than activation of the HPA-axis.
2.4.3 Cortisol mechanism of action

Glucocorticoids, and all the other cortical steroid hormones, are derivatives of cholesterol [4]. They all contain the cyclopentanoperhydrophenanthrene steroid nucleus (fig. 3.1). Cortisol is an amphipathic compound and crosses the plasma membrane of target cells easily.

The glucocorticoid receptor (GR) is found in the cytoplasm of cortisol target cells. Human GR was cloned in 1985. It is a 777 amino acid protein, also a member of the superfamily of ligand regulated nuclear receptors [65]. The specific functions of GR are localized in so-called domains. They are the ligand binding domain (LBD), DNA binding domain (DBD) and two activation domains [66,67]. In the absence of steroid, GR is associated with two hsp90 molecules and other proteins in a multi-protein complex. Upon binding of steroid, GR undergoes a conformational change, dissociates from the complex and translocates across the nuclear membrane.

In the nucleus, GR binds to target DNA sequences termed glucocorticoid response elements (GREs) [68]. This results in activation or increased transcription of the target gene and is called transactivation. Repression of transcription is also possible for certain target genes through transrepression. In this case the DNA target sequence is called a negative GRE (nGRE). The ACTH precursor gene, pro-opiomelanocortin (POMC), has a nGRE that binds multimers of GR [69]. This transrepression of GR is responsible for the negative feedback inhibition of glucocorticoids on ACTH production in the HPA-axis.

2.5 Cortisol and carbohydrate metabolism

2.5.1 Regulation of body heat

Control of body heat in homeothermic organisms is an extremely complicated process. If the organism produces no heat, it will soon reach the temperature of the environment through simple thermal equilibration. Most non-hibernating mammals have optimum core temperatures that are maintained through reflex coordination at a very precise temperature irrespective of that of the environment. This temperature is more or less 37 °C.

There are a number of processes that produce body heat. Most involve biochemical reactions and the metabolic rate thus has a large influence on body heat. The activity of
skeletal muscle contributes greatly to body heat production. Fever is the result of heat production caused by immune responses and will not be discussed here. Heat is mostly lost through radiation and convection from the body and also through excretion of urine and stool. Ambient conditions mostly determine the rate of heat loss by radiation and convection. If an organism is exposed to extreme cold, the basal metabolic rate (BMR) is not sufficient to produce adequate heat. In such conditions shivering is an important means of body heat production. Shivering is an involuntarily action of skeletal muscle induced by central neural reflexes [70,71]. Shivering is a series of intense random contractions of skeletal muscle and requires a large amount of energy. Glucose is a key energy source for this method of body heat production.

Exposure to extremely cold weather conditions for long periods of time requires sufficient mobilization of glucose to maintain blood glucose at physiologically favorable concentrations. The main source of glucose in the post absorptive phase is stored glycogen. In a man of average build, the liver stores glycogen to a maximum of 6% of its mass (100 g), and skeletal muscle to 3% of its mass (400 g).

Depletion of the glycogen stores leads to the activation of various gluconeogenic enzymes. This activation is orchestrated by an integrated hormonal response including glucagon [72], epinephrine, norepinephrine [73] and cortisol [74,75]. Under normal conditions the effects of glucocorticoids on carbohydrate metabolism do not seem vital to sustaining life. However, when an organism is on the verge of hypothermia, which can lead to death, the contribution of glucocorticoids to maintaining adequate blood glucose concentrations is indispensable. In an organism like the Angora goat, in which the cortisol response to stress is severely impaired [3], prolonged exposure to extreme cold could be fatal.

2.5.2 Liver glycogen as blood glucose source

Regulation of glycogen stores in the liver is under control of numerous effectors. Many enzymes are involved in both glycogenesis (biosynthesis of glycogen) and glycogenolysis (breakdown of glycogen to glucose). GLUT-2 is a glucose transporter, which mediates the facilitated diffusion of glucose across the hepatocyte membrane in both directions [76]. Insulin does not strongly regulate GLUT-2 and its transport capacity is not limiting for the uptake or release of glucose. As a result the glucose concentrations in the blood and
hepatocytes are the same. This allows the liver to act as a biosensor for blood glucose concentrations.

Glycogenesis and glycogenolysis are complicated processes and the cellular machinery involved appears to be subject to cellular compartmentalization [77]. During glycogenesis glycogenin (associated with glycogen synthase) autocatalytically attaches the C-1 carbon of UDP-glucose to one of its tyrosine residues. This is the first step in the formation of a glucan chain six to seven glucose residues long, attached by α-1,4-glucosidic bonds through autocatalysis of glycogenin. Glycogen synthase then dissociates from glycogenin and further polymerizes glucose to the glucan chain using UDP-glucose as substrate. Eventually branching enzyme transfers a terminal oligo-glucan (at least six glucose units) to a neighboring glucan chain. The liberated C-1 carbon of the oligo-glucan is attached to the C-6 carbon of a glucose unit in the neighboring chain.

A mature glycogen molecule takes on the shape of a branched structure called a β-particle and consists of roughly 60,000 glucose units. Up to 40 β-particles can complex to form so-called α-rosettes.

The breakdown of glycogen (glycogenolysis) starts with the liberation of terminal glucose units through the hydrolysis of glycogen phosphorylase. This reaction requires P_i and releases glucose-1-phosphate from glycogen. When the external glucan chains are no longer than four glucose units, the transferase activity of debranching enzyme removes a maltotriose unit from the α-1,6-linked stub. The maltotriose unit is then attached to the C-4 carbon of the main chain through an α-1,4-glucosidic bond. The single remaining α-1,6-attached glucose is then removed by the α-1,6-glucosidase activity of debranching enzyme and liberated as D-glucose. Glycogen phosphorylase continues to breakdown the glycogen until only the last glucose remains attached to glycogenin. The debranching enzyme can then hydrolyse the final bond, freeing the last glucose from glycogenin. Fig. 2.7 graphically shows the biosynthesis and breakdown of glycogen.

2.5.3 The role of cortisol in maintaining sufficient blood glucose concentrations

Although many different effectors are involved, glycogenesis and glycogenolysis are mainly controlled by the phosphorylation status of glycogen synthase and glycogen phosphorylase, respectively [78,79]. Both enzymes exist in an active α, and an inactive β
form. Glycogen synthase is controlled by the reversible phosphorylation of multiple serine residues near the N and C termini [80]. Phosphorylation of the enzyme leads to inactivation through a decreased $V_{\text{max}}$. Numerous protein kinases target glycogen synthase. However, glucose and glucagon are the main effectors initiating the dephosphorylation and phosphorylation of the synthase, respectively [81].

Figure 2.7. Schematic presentation of glycogen biosynthesis (glycogenesis) and breakdown (glycogenolysis), with the main enzymes involved indicated. Reproduced from [77].

The ability of liver glycogen phosphorylase to produce glucose-1-phosphate from glycogen is dependent on intact phosphorylase kinase $\alpha$ [82]. This kinase produces the active glycogen phosphorylase $\alpha$ from the inactive $\beta$ form by phosphorylating Ser$^{14}$. Furthermore, both glycogen synthase and phosphorylase are under the control of protein phosphatase-1G (PP-1G) [83]. PP-1G dephosphorylates the enzymes and thus produces an active synthase and
an inactive phosphorylase, and in so doing promotes glycogen biosynthesis. There are many glycogenic and glycogenolytic agents. The intricacies of how they induce changes in the intra-cellular pathways controlling glycogen stores will not be discussed here. However, the main glycogenic agents are: glucose, insulin, glycogenic amino acids, fructose and glucocorticoids. Conversely the main glycogenolytic agents are: glucagon, epinephrine, splanchnic nerve activity and eicosanoids.

The focus here is on how glucocorticoids contribute to ensuring adequate blood glucose levels during periods of high glucose demand from skeletal muscle. This is achieved by glucocorticoids acting as glycogenic agents, ensuring that sufficient glycogen stores are maintained. Glucocorticoid administration in vivo results in activation of glycogen synthase and inactivation of phosphorylase [84]. However, glucocorticoids are necessary for glucagon to exert its gluconeogenic effect during periods of fasting [75]. Release of glucose (produced from gluconeogenesis) from the liver is further enhanced by glucocorticoids increasing hepatic glucose-6-phosphatase activity.

Glucocorticoids have far-reaching effects on intermediary metabolism. These include a restraint on the hypoglycemic effect of insulin [85] and also increased peripheral protein catabolism together with an increased hepatic uptake of amino acids to serve as gluconeogenic substrate [12]. Engelbrecht et al. have shown intravenous injection of insulin to cause a marked increase in blood cortisol levels in the Boer goat and Merino sheep [3]. Comparatively the Angora goat showed an impaired cortisol response to the drop in blood glucose. This impaired cortisol response is believed to be responsible for the Angora goat’s inability to maintain adequate blood glucose levels during exposure to extreme wet, windy and cold weather. The inability to produce sufficient body heat finally leads to hypothermia, and death soon follows.

The concerted effects of glucocorticoids on carbohydrate metabolism are to maintain blood glucose levels at physiologically favourable levels. These effects become even more pronounced under extremely stressful conditions when there is considerable HPA-axis activity.
2.6 Conclusion

Neuro-endocrine mediation is central to the stress response in most animals and man. The functions of the HPA-axis as a regulatory unit in this response are vital in orchestrating a proper response to noxious stimuli. In the Angora goat a malfunction in the neuro-endocrine mediated response to cold stress results in an abrupt drop in blood glucose concentration. It is the function of cortisol, together with numerous other factors, to promote a rise in blood glucose during a hypoglycemic episode. The adrenal cortex of the Angora goat fails to produce adequate amounts of cortisol due to insufficient neuro-endocrine mediation of the stress response. Finally the Angora goat suffers from critical hypoglycemia when exposed to extreme cold and this results in central collapse and death.

Adrenal steroidogenesis is central to cortisol biosynthesis. Various enzymes and co-factors are responsible to maintain a proper flux balance through the steroidogenic pathways, which ultimately produce a number of different hormones. Any defect in a steroidogenic enzyme or mediating factor could result in severe impairment or likewise an overproduction of certain steroid hormones. The next chapter focuses on the biochemistry of the adrenocortical steroidogenic processes. Special attention is given to CYP17, an enzyme that influences the balance between glucocorticoid and androgen production. Finally an overview is given of previous research implicating CYP17 in the hypoadrenocorticism of the Angora goat.
CHAPTER 3
ADRENAL STEROIDOGENESIS: KEY TO CORTISOL BIOSYNTHESIS

3.1 Anatomy and morphology of the adrenal gland

Humans and most other mammals have two bilateral encapsulated adrenal glands. These glands are situated around the superior poles of the kidneys [14]. Morphologically the adrenal gland consists of medullary and cortical tissue. The medulla occupies the centre of the gland. From an evolutionary point of view the medulla is a sympathetic ganglion in which the postganglionic nerves lost their axons and became secretory endocrine cells. Medullary cells are under the control of preganglionic nerve stimulation of the splanchnic nerve [86]. The secretory cells of the medulla are organized in interlacing cords and are densely innervated by splanchnic nerve fibres. Two main types of cells can be distinguished in the medulla, namely the epinephrine secreting type with large less dense granules and the norepinephrine secreting type with smaller, denser granules.

The adrenal cortex is made up of three distinct zones layered on top of one another. Each zone has a unique contribution to the gland’s steroidogenic output. These zones are from the outer to inner layer, the zona glomerulosa, zona fasciculata and zona reticularis. The medulla is completely surrounded by cortical tissue. Cortical tissue makes up 72% of the adrenal mass [12]. Of this the zona fasciculata constitutes 50% and the zona glomerulosa and zona reticularis 15% and 7% respectively. Adreno-medullary cells, like other cells of neural origin, do not regenerate. However, zona glomerulosa cells in addition to their aldosterone secreting function also produce new cortical cells. When the two inner zones of the cortex are removed, a new zona fasciculata and zona reticularis develop from glomerular cells attached to the capsule [87]. Fig. 3.1 shows a cross section of the adrenal gland.

Many small branches from the phrenic and renal arteries and the aorta supply the gland with arterial blood. A subcapsular arteriolar plexus diverges into the cortex and also sends blood to the sinusoids of the medulla. There are, however, a few arterioles passing directly through the cortex to the medulla from the capsule. The vascular network of the adrenal gland has evolved to accommodate a relatively large blood flow through the gland.
3.2 Hormones of the adrenal cortex

The adrenal cortex delivers steroid hormones into the general blood circulation from where it is delivered to various target cells throughout the body. Their actions have a considerable impact on sexual development and function, electrolyte balance, intermediary metabolism and stress management. The steroid hormones of the adrenal cortex are derivatives of cholesterol [4]. Similar to cholesterol, bile acids, vitamin D, ovarian steroids and testicular steroids, they also contain a cyclopentanoperhydrophenanthrene nucleus (fig. 3.2).
Gonadal and adrenocortical steroids can be classified into three categories, C$_{21}$, C$_{19}$ and C$_{18}$ according to the number of carbon atoms in the molecule [12]. The C$_{21}$ steroids have a two-carbon side chain attached to carbon 17 of the steroid nucleus. C$_{19}$ steroids have either a keto or hydroxyl group at this position. In addition to either the keto or hydroxyl group at carbon 17, the C$_{18}$ steroids have no angular methyl group attached at carbon 10 of the steroid nucleus.

Although these properties make for easy classification they are not solely responsible for the physiological actions of the hormones. The C$_{21}$ steroids are further classified as either mineralocorticoids or glucocorticoids using Selye’s terminology. However, all C$_{21}$ steroids have both mineralocorticoid and glucocorticoid activity, with one or the other activity predominating. Table 3.1 lists a number of C$_{21}$ steroids and their relative potencies as mineralocorticoids and glucocorticoids.

Although innumerable steroids have been isolated from the adrenals of many different animals, most are not secreted in physiologically significant amounts. Those that bear physiological significance are: the mineralocorticoid aldosterone, the glucocorticoids cortisol and corticosterone and the androgens dehydroepiandrosterone (DHEA) and androstenedione. Furthermore, the adrenal is known to secrete small amounts of estrogen. However, most of the circulating estrogen that is not of ovarian origin is formed in the circulation from adrenal androstenedione. The major C$_{21}$ steroids secreted by adrenocortical tissue of most animals are aldosterone, corticosterone and cortisol. The ratio of the two glucocorticoids secreted, however, differs among species. Normally one or the other would be the dominant glucocorticoid of a specific species [2]. Birds, mice and rats secrete predominantly corticosterone, whereas dogs secrete almost equal amounts of the two glucocorticoids. Humans, monkeys, cats and sheep mainly secrete cortisol.

3.2.1 Adrenal androgens and estrogens

Androgens are those steroid hormones that bring about masculinity and also promote protein anabolism and growth. However, the adrenal androgens DHEA and androstenedione have less than 20% of the potency of testosterone, the main androgen secreted by the testis.
Table 3.1. Relative potencies of C$_{21}$ steroids compared with cortisol $^{1}$

<table>
<thead>
<tr>
<th>Steroid</th>
<th>Glucocorticoid activity</th>
<th>Mineralocorticoid activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortisol</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Corticosterone</td>
<td>0.3</td>
<td>15</td>
</tr>
<tr>
<td>Aldosterone</td>
<td>0.3</td>
<td>3000</td>
</tr>
<tr>
<td>Deoxycorticosterone</td>
<td>0.2</td>
<td>100</td>
</tr>
<tr>
<td>Cortisone</td>
<td>0.7</td>
<td>1.0</td>
</tr>
<tr>
<td>Prednisolone</td>
<td>4</td>
<td>0.8</td>
</tr>
<tr>
<td>9α-Fluoro-cortisol</td>
<td>10</td>
<td>125</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>25</td>
<td>~0</td>
</tr>
</tbody>
</table>

$^{1}$ Values are approximations based on liver glycogen deposition of anti-inflammatory assays for glucocorticoid activity, and effect on urinary Na$^+$/K$^+$ or maintenance of adrenlectomized animals for mineralocorticoid activity. The last three steroids listed are synthetic compounds that do not occur naturally. Reproduced from [12].

Testicular androgen secretion is under the control of gonadotrophins from the pituitary, and is regulated through the interplay of hormones acting in the hypothalamic-pituitary-gonadal axis (HPG-axis). The HPG-axis functions in a similar fashion as the HPA-axis, but will not be discussed here. ACTH, and thus the HPA-axis, regulates adrenal androgen secretion. In both adult men and women DHEA plasma concentrations are 300 μg/dl. Adrenal androgens are responsible for the development of secondary sex characteristics since they are not as potent as testosterone. However, in certain conditions where their secretion is significantly increased, they can cause severe phenotypic alterations. In prepubertal boys it causes precocious pseudopuberty and in females pseudohermaphroditism and the adrenogenatil syndrome [8]. As mentioned earlier, the adrenal itself secretes very little estrogen. Androstenedione, however, is aromatized to form estrogen and is an important source of estrogens in males and postmenopausal woman [88].

3.2.2 Glucocorticoids

In the previous chapter the role of cortisol in carbohydrate metabolism and certain elements of its biology were discussed in some detail. Here some important properties of glucocorticoids in general will be mentioned. Basal concentrations of glucocorticoids are important in the regulation of intermediary metabolism and help the organism to cope with
various stress factors. Furthermore, immune function is also suppressed to some extent by glucocorticoids and they are therefore important in preventing the onset of autoimmune diseases [89]. Due to the anti-insulin effect of glucocorticoids in peripheral tissue (excluding heart and brain) they worsen diabetes and can lead to fatal hypoglycemia in fasting adrenalectomized individuals. The permissive effects of glucocorticoids are important for other hormone activity to be optimal. The calorigenic effects of glucagon and the catecholamines are dependent on the permissive effects of glucocorticoids [73,75] and so are the lipolytic effect [90], pressor responses and bronchodilation effect of the catecholamines [91]. The vasoconstrictive effect of norepinephrine is also dependent on glucocorticoid support. Thus adrenal insufficiency further prevents vascular compensation for the concomitant hypovolemia. Glucocorticoid insufficiency is further associated with the inability to excrete a water load, which is only rectified by the administration of glucocorticoids [92]. Circulating eosinophil [93] and basophil [94] numbers are lowered by glucocorticoids, while neutrophil [95], platelet [96] and red blood cell [97] counts are increased. Glucocorticoids help an organism deal with a wide variety of stress factors. The word stress is, however, a term used very loosely in modern times. In this context glucocorticoids promote actions that counteract the manifestations brought about by noxious stimuli that cause an increase in ACTH secretion.

3.2.3 Mineralocorticoids

Sodium concentration in the ECF is the main target of mineralocorticoid action. Mineralocorticoid action results in absorption of sodium from urine, sweat, saliva and gastric juice. Sodium diffuses from these fluids into the neighbouring cells and is actively transported from these cells into the interstitial fluid [98]. The mechanism of action of aldosterone causing sodium retention in ECF is coupled to an exchange for potassium and hydrogen ions producing potassium and hydrogen diuresis. The mechanism of action of aldosterone is similar to that of cortisol discussed in the previous chapter.

Aldosterone action results in the biosynthesis of new proteins. However, data exist which indicate that binding of aldosterone to the cellular membrane can also evoke mineralocorticoid effects [99]. Both mechanisms though seem to bring about an increased activity of the Na⁺/K⁺ ATPase transporters. It is interesting to note that in vivo the mineralocorticoid receptor has a much higher affinity for glucocorticoids than the
Aldosterone sensitive tissues, however, have the enzyme 11β-HSD [100]. This enzyme catalyzes the conversion of cortisol to cortisone and of corticosterone to its 11-oxy derivative. Aldosterone, however, is not a substrate of 11β-HSD. This prevents the glucocorticoids from having an appreciable mineralocorticoid effect. Adrenal insufficiency causing inadequate aldosterone secretion results in excessive sodium and water loss. A low sodium plasma concentration results in hypovolemia that could eventually result in circulatory insufficiency and fatal shock if untreated. Mineralocorticoid excess first causes potassium depletion due to prolonged potassium diuresis. Sodium is retained, but plasma sodium concentrations remain normal since water is also retained with the osmotically active sodium. Finally the ECF volume is significantly increased and high blood pressure results. When the ECF volume reaches a certain threshold, sodium is excreted regardless of aldosterone action on the renal tubules [101]. This is called the escape phenomenon. The precise mechanism resulting in the sodium escape is not yet known.

3.3 Biochemistry of cortisol production

The intricacies of the biosynthesis of cortisol, the main glucocorticoid secreted by the Angora goat adrenal will further be focused on in this chapter. Cortisol biosynthesis occurs primarily in the zona fasciculata and also the zona reticularis but not in the zona glomerulosa. This is because the zona glomerulosa lacks CYP17, which is crucial for cortisol production. The zona glomerulosa, however, is unique in expressing aldosterone synthase [102]. Therefore aldosterone is the only steroid with physiological significance that is secreted by the zona glomerulosa. Later in this chapter, cortisol insufficiency in the Angora goat will be discussed. As explained in Chapter 1, it is this lack of cortisol that is responsible for the Angora goat’s inability to withstand extreme environmental conditions.

Cholesterol is the precursor of all steroid hormones [4]. Acetate is the substrate for the de novo synthesis of cholesterol. However, most cholesterol used for steroidogenesis is taken up from low-density lipoproteins (LDL) in the circulation. Furthermore, ACTH upregulates LDL receptor expression in adrenocortical cells [103]. In these cells cholesterol is esterified and stored in lipid droplets. Cholesterol ester hydrolase (CEH), through the stimulation of ACTH, liberates cholesterol from its esters [104]. Section 2.3 of the previous chapter described the intracellular changes caused by ACTH. Cholesterol then is delivered to the mitochondria
where it serves as substrate for steroid production. The intermediates of cortisol production are shuttled between the mitochondria and the endoplasmic reticulum before the final reaction catalyzed by cytochrome P450c11 (CYP11B1) in the mitochondria yields cortisol. Fig. 3.3 shows the shuttling of cholesterol and other intermediates within the adrenocortical cell.

**Figure 3.3.** Graphic presentation of cholesterol delivery to the adrenocortical cell and shuttling of steroid intermediates between the mitochondria and the endoplasmic reticulum. (LDL) – low density lipoprotein, (CEH) – cholesterol ester hydrolase, (Preg) – pregnenolone, (SER) – smooth endoplasmic reticulum. Reproduced from [12].

### 3.3.1 Cholesterol delivery to the mitochondria

Free cholesterol has to be delivered to the inner mitochondrial membrane. Cytochrome P450 side chain cleavage (CYP11A) is bound to the inner membrane and is the enzyme to which cholesterol needs to be delivered. CYP11A carries out the side chain cleavage reaction on cholesterol and produces pregnenolone [105]. This reaction occurs in all steroidogenic tissues regardless of the end product produced. Delivery of cholesterol to the inner mitochondrial membrane does not occur by means of passive diffusion. It is rather the specialized function of a short-lived mitochondrial import factor called the steroidogenic acute regulatory protein (StAR). StAR is expressed in a wide variety of vertebrates ranging from birds and fish to mammals [106].

The inhibition of StAR translation by the addition of a translation inhibitor (cycloheximide (CHX), also inhibits the formation of CYP11A-cholesterol complexes. This causes a dramatic decrease in the steroidogenic flux. However, certain soluble hydroxycholesterol analogs, also substrate to CYP11A for steroid production, are able to maintain a high steroidogenic flux in
the presence of CHX [107]. This is because these analogs are not constrained by membrane barriers and therefore do not require StAR facilitated transport. StAR translation is induced by heightened cAMP and protein kinase A levels which in turn are caused by ACTH receptor activation [108]. Delivery of cholesterol to the mitochondria is thus under strict hormonal control. It would therefore appear that the delivery of cholesterol to the inner mitochondrial membrane, facilitated by StAR, has a high control coefficient in all steroidogenic pathways.

3.3.2 Enzymes involved in adrenal steroidogenesis

The family of cytochrome P450 enzymes is mainly involved in the control of adrenal steroidogenesis but 3β-hydroxysteroid dehydrogenase (3β-HSD), not a cytochrome P450, also plays a pivotal role in this process. The first reaction in steroidogenesis, as mentioned earlier, is the conversion of cholesterol to pregnenolone by CYP11A. This is a three-step reaction involving the successive hydroxylation of C22 and C20 followed by the cleavage of the vicinal diol to yield pregnenolone and isocaproaldehyde [109]. The adrenal steroidogenic enzymes are located either in the mitochondria or the endoplasmic reticulum. CYP11A1 and CYP11B1 are located in the mitochondrial inner membrane. 3β-HSD and the other two cytochrome P450 enzymes, CYP17 and cytochrome P450c21 (CYP21) are located in the endoplasmic reticulum.

Pregnenolone, produced in the mitochondria, moves to the endoplasmic reticulum where it is either substrate to CYP17 or 3β-HSD. When substrate to CYP17 it is hydroxylated at carbon 17 to yield 17-hydroxy pregnenolone (17OH-Preg), which is again substrate to CYP17. 17OH-Preg is then converted to DHEA through the lyase activity of CYP17. Pregnenolone together with 17OH-Preg and DHEA could also be substrate to 3βHSD. When substrate to 3β-HSD the intermediates (in the Δ5 isoforms) are dehydrogenated at the carbon three position and converted to the Δ4 isoforms. Pregnenolone, 17OH-Preg and DHEA yield progesterone, 17-hydroxy progesterone (17OH-Prog) and androstenedione respectively after conversion by 3β-HSD. Pregnenolone thus lies at the first junction in the steroidogenic pathway. Progesterone and 17OH-Prog (the Δ4 isoforms) are substrate to CYP17. Progesterone is hydroxylated to form 17OH-Prog, and the C17-C20 bond of 17OH-Prog is cleaved to form androstenedione. Progesterone and 17OH-Prog could, however, also be converted to deoxycorticosterone and deoxycorticisol respectively by CYP21. These two
intermediates then travel to the mitochondria where they are converted to corticosterone and cortisol, respectively, by CYP11B1. Fig. 3.4 shows a metabolic map of the steroidogenic pathways in the zona fasciculata and zona reticularis.

### 3.3.3 Cytochrome P450 mode of action and properties

Cytochrome P450 is the collective name for hundreds of enzymes which have unique spectral properties because they contain the protoporphyrin IX ring structure complexed with iron [110,111]. The reduced vs. oxidized absorption difference spectrum shows a unique maximum at 450 nm in the presence of carbon monoxide, hence the name P450. Detergent treatment of P450 containing cellular vesicles does not destroy the cytochrome, but converts it quantitatively to a solublized form with absorption maximum at 420 nm. This form of the enzyme is inactive and is referred to as P420.

Fig. 3.5 shows a typical hydroxylation catalytic cycle for cytochrome P450 [112]. The catalytic action of cytochrome P450 involves substrate binding and the subsequent uptake of electrons, which enables the binding of oxygen to the complex. During the catalytic cycle of cytochrome P450 the iron is subject to oxidation and reduction and exists in the ferric and ferrous states. Once the ternary oxygen-enzyme-substrate complex takes up a second electron, the molecular oxygen is split. The one oxygen atom is activated and the other is lost to water. The activated oxygen atom becomes incorporated into the substrate to form a hydroxylated product. The product is then released from the enzyme complex and the enzyme is regenerated to its native substrate free ferric form.

The different cytochrome P450 enzymes involved in corticosteroid biosynthesis are located in either the endoplasmic reticulum or the mitochondrial inner membrane, as mentioned earlier. Depending on their localization there are differences in the electron transfer mechanisms utilized during the hydroxylation reactions. In the mitochondria NADPH reduces adrenodoxin reductase (ADXR), which in turn reduces adrenodoxin (ADX) [109]. Reduced ADX again reduces cytochrome P450 and shuttles between ADXR and cytochrome P450. In the endoplasmic reticulum NADPH reduces NADPH-P450-reductase. The reductase then transfers the electrons directly to cytochrome P450 [113].
Figure 3.4. Schematic representation of adrenal steroidogenesis. Reproduced from [2].
3.4 Dual activity of CYP17

CYP17 is one of the most widely studied P450 cytochromes. This is mainly due to its dual catalytic activity. This enzyme is responsible for the hydroxylation at C17 of the C21 steroids, and also for the C17, C20 lyase of the C17 hydroxylated steroids [114]. CYP17 is found in the zona fasciculata and zona reticularis, but not in the zona glomerulosa. This is why the zona glomerulosa does not produce corticosteroids but only the mineralocorticoid aldosterone. However, the inner two cortical zones do produce both androgen (DHEA and
androstenedione) and glucocorticoid (17OH-Preg and 17OH-Prog) precursors. CYP17 is responsible for the production of all these precursors.

It has also been reported that human CYP17 is responsible for both the 17 and 16α-hydroxylation of progesterone [115]. This enzyme does not, however, produce any detectable amounts of androstenedione from 17OH-Prog. It is important to note that the conversion of pregnenolone to DHEA by CYP17 (fig. 3.4) occurs with the release of the intermediate 17OH-Preg from CYP17 [116]. In the steroidogenic Lydig cells of the testis pregnenolone is mainly utilized for the production of androgens, while in the zona fasciculata and also the zona reticularis, it is used for the production of glucocorticoids. CYP17 is expressed in the ovary in the theca interna cells with very low expression in the granulosa cells of humans and rats. Theca interna cells can therefore produce androgens, but the granulosa cells need androgen precursors from the theca interna cells for estrogen production [117].

Differential regulation of the two activities of CYP17 is responsible for this important shift in steroid metabolism of different tissues. It has been proposed that electron flow to the enzyme regulates the ratio of these two activities [118]. Furthermore, cytochrome b5 (cyt b5) has also been implicated in contributing to the altered 17α-hydroxylase and 17,20-lyase activities [119-121]. However, the mechanism through which cyt b5 modulates the dual activity of CYP17 is far from fully elucidated. In the testis, high 17,20-lyase activity of CYP17 is required for adequate amounts of androgen production. In human testes two different cyt b5 mRNA transcripts exist [122]. Both the cytochromes expressed were found to augment the 17,20-lyase activity of CYP17 to a similar extent. A recent paper suggests that an allosteric mechanism rather than simply electron flow plays an important role [123]. In this study Lombard et al. showed that cyt b5 exists in various multimeric configurations, depending on the physiological conditions. The differential regulation of the 17α-hydroxylation and 17,20-lyase activities of CYP17 is a complex phenomenon. It is, however, known that different tissues have different steroidogenic requirements and that various metabolic factors in these tissues influence the ratios of the two catalytic activities of CYP17.

Defects in CYP17 activity can manifest in total loss of activity, or so-called isolated 17,20-lyase deficiency. In fully developed 17α-hydroxylase and 17,20-lyase deficiency, no sex hormones are produced. This leads to the development of external female genitalia regardless of genetic sex [124]. The pathway leading to corticosterone and aldosterone production is, however, still intact. As a result elevated levels of 11-deoxycorticosterone and other
mineralocorticoids persists. This causes hypertension and hypokalemia. The loss of cortisol is compensated for by the glucocorticoid effect of the elevated corticosterone.

In isolated 17,20-lyase deficiency CYP17 still catalyzes the hydroxylation of the C21 steroids and sufficient cortisol is produced [125]. In this condition there is, however, still the loss of sex hormone production.

It is evident that alterations of any of the enzymes involved in steroidogenesis can cause a shift in the balance between the different steroid hormones produced. Such an alteration in the steroid profile of an organism could have severe clinical or phenotypic manifestations. More often than not, changes in the activities of the steroidogenic enzymes are the result of changes in the gene structure of the enzymes. The next section will discuss some genetic alterations (mutations) found in the CYP17 gene and their implications.

### 3.5 Mutations of the CYP17 gene

The nature of genetic mutations is diverse. Genes can be completely deleted from chromosomes, or large pieces of non-sense DNA can be inserted into the coding regions of genes. Conversely, large parts of the coding regions of a gene can be deleted from the gene. These insertion and deletion mutations often render the enzymes encoded by the genes inactive. Likewise point mutations that disrupt the reading frame of a gene generally result in a non-sense peptide being translated. However, point mutations that do not disrupt the reading frame can also have severe effects on enzyme activity. Such mutations usually result in one amino acid being substituted for another in an enzyme. Differences in enzyme activity from such mutations are then attributable to that specific amino acid. The identification of single amino acid substitution mutations in CYP genes has shed much light on the structure-function relation of different regions of cytochrome P450.

In a human case study a single amino acid substitution (Phe93Cys) was reported to cause CAH [126]. Hypertension is characteristic of this condition due to the overproduction of mineralocorticoids other than aldosterone. With this mutation CYP17 retained only 10% of both hydroxylation and lyase activities. Another study found that a serine to proline substitution at amino acid position 106 also resulted in the complete abolishment of CYP17 activity [127]. Both these mutations are found in a highly conserved region of CYP17. This indicates that particular region is crucial for both hydroxylase and lyase activity.
Co-expression of P450 reductase and CYP17 in COS-1 cells showed that an increase in the ratio of reductase to CYP17 augmented the 17,20-lyase activity, but not the hydroxylase activity [128]. Higher levels of the reductase are also able to increase the 17,20-lyase activity of two constructed CYP17 mutants with either threonine or alanine at amino acid 106. This indicates Ser106 to be crucial to the conformation of the active site rather than facilitating in the interaction with P450 reductase. Further site-directed mutagenesis studies have also, however, shown amino acids beyond the active site to induce changes in the two activities of the enzyme [129]. It is therefore important to understand the limitations in appointing structure function properties to certain regions of CYP17 based on kinetic differences induced by single amino acid substitutions.

In humans, only two single amino acid substitution mutations have been identified that cause isolated 17,20-lyase deficiency [130,131]. They are Arg347His and Arg358Gln. Both these mutations alter the interaction of CYP17 with P450 reductase and cyt b5. Again this points to the contribution the reducing partners have in the differential regulation of CYP17 activity.

Another region in CYP17 that seems to play a crucial role in the catalytic activity is the proline rich domain (PRD) in the amino terminal of the enzyme. This region is believed to be important in folding of the enzyme, and to prevent misfolding of the peptide prior to heme binding [132]. In addition to the PRD in the amino terminal of CYP17, there are also two other distinct sequence motifs. They are the signal-anchor sequence (SA) and the basic sequence (BS) [133]. Unlike the PRD these two sequence motifs are not essential for correct folding of the enzyme.

3.6 Previous research: CYP17 causes impaired cortisol biosynthesis in the Angora goat

From the previous discussions it is clear that adrenocortical CYP17 activity plays an extremely important role in shuttling of steroid intermediates between the androgen and glucocorticoid production pathways. Alterations in either or both the hydroxylase and lyase activities of the enzyme could severely affect the steroid output of the adrenal cortex. In previous research from our laboratory Engelbrecht et al. found Angora goat adrenal steroidogenesis to differ significantly from Boer goat and Merino sheep in terms of
glucocorticoid and androgen output [7]. The conclusions drawn from that work were that altered CYP17 activity in the Angora goat results in insufficient cortisol secretion from the cortex in response to HPA-axis stimulation. This section will further summarize the key findings of that study, which identified the primary site of the hypoadrenocorticism, and ultimately Angora goat CYP17 as the enzyme responsible for the observed hypoadrenocorticism in the Angora goat.

3.6.1 Identification of the primary site of the reduced adrenal function in the Angora goat

In a comparative study, Angora goats, Boer goats and Merino sheep were subjected to intravenous injections of insulin, CRH and ACTH. Subsequently cortisol production was measured in all three species (fig. 3.6).

![Figure 3.6](https://example.com/figure3.6.png)

**Figure 3.6.** The effect of an intravenous injection of human insulin (0.1 IU/kg), on plasma glucose and cortisol concentrations in the Angora goat, Boer goat and Merino sheep. Reproduced from [2].

CRH and ACTH function in the HPA-axis was discussed in the previous chapter. Both these compounds should elicit a cortisol response from the adrenal. Insulin, although indirectly, should also facilitate the release of cortisol from the adrenal cortex. This is because insulin induces a hypoglycemic condition that serves as a strong stress signal to the hypothalamus. Cortisol released from the adrenal cortex then aids in rectifying the hypoglycemia as discussed in Chapter 2. From fig. 3.6 it can be seen that the intravenous injection of human insulin produced significant hypoglycemia in all three species. Insulin therefore successfully induced artificial stress in the animals. Concomitantly there was an increase in plasma cortisol levels with a peak at 90 minutes after injection in the Boer goat and Merino sheep. Although
some fluctuation in cortisol levels was seen for the Angora goat, no real peak response was observed. The impaired cortisol response to hypoglycemia supported the findings of Van Rensburg (1971) [1] and Herselman (1990) [134] of the presence of hypoadrenocorticism in high fibre producing Angora goats.

Furthermore, sheep CRH produced a peak cortisol response in the Merino sheep, but not in the two goat species. This could be explained by the inability of sheep CRH to bind the goat CRH receptor efficiently in the corticotrophes of the anterior pituitary. However, ACTH did produce a definite rise in plasma cortisol levels in all three species. The response was strongest in the Merino sheep and weakest in the Angora goat. These findings suggested that the HPA-axis was functional in all three species. However, the Angora goat showed an impaired cortisol response upon HPA-axis stimulation. The adrenal gland was therefore identified as the primary site of the Angora goat’s hypoadrenocorticism. Subsequently adrenal steroidogenesis was investigated to find any anomalies that could explain the observed hypoadrenocorticism in the Angora goat.

3.6.2 Implicating CYP17 in the observed hypoadrenocorticism of the Angora goat

Angora goat adrenal steroidogenesis was again investigated in a comparative study with the Boer goat and Merino sheep. Subcellular fractions (microsomes or mitochondria) were used in the subsequent experiments determining various steroidogenic enzyme activities. The relevant steroid intermediates and cofactors were added to the cellular preparations and the steroids that were produced were analyzed. When pregnenolone was added as substrate the glucocorticoid and androgen precursors formed were compared among the species (table 3.2).

<table>
<thead>
<tr>
<th>Species</th>
<th>%Glucocorticoid steroid precursors formed (DOC &amp; DOCL)(^1)</th>
<th>%DHEA and 17OH-Preg formed</th>
<th>%A4 formed</th>
<th>%Pregnenolone remaining</th>
</tr>
</thead>
<tbody>
<tr>
<td>Angora goat</td>
<td>35.6 ± 8.9</td>
<td>34.7 ± 4.2</td>
<td>5.5 ± 2.33</td>
<td>1.06 ± 1.06</td>
</tr>
<tr>
<td>Boer goat</td>
<td>78.5 ± 13.9</td>
<td>8.8 ± 1.7</td>
<td>-</td>
<td>6.3 ± 4</td>
</tr>
<tr>
<td>Merino sheep</td>
<td>82.03 ± 6.5</td>
<td>0.0 ± 0.0</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

\(^1\)DOC: Deoxycorticosterone and DOCL: Deoxycortisol. Reproduced from [2].
The Angora goat produced only 35% glucocorticoid precursors compared to the 78% and 82% of the Boer goat and Merino sheep respectively. Angora goat microsomes, however, produced significantly more androgen precursors. Angora goat steroidogenic enzyme kinetic and specificity differences result in a higher flux of steroid intermediates through the Δ5 steroid pathway compared to the Δ4 pathway (fig. 3.4). Similar experiments were done where either only NADPH or NAD$^+$, the respective cofactors for CYP17 and 3β-HSD, were added to compare the activities of the two enzymes among the species tested. With pregnenolone and NADPH added, the production of DHEA was measured to investigate CYP17 activity. With pregnenolone and NAD$^+$ added, the production of progesterone was measured to investigate 3βHSD activity. Fig. 3.7 shows the formation of the relevant intermediates.

![Graphs showing 3β-HSD and CYP17 activity](https://example.com/fig3.7.png)

**Figure 3.7.** Comparison of 3β-HSD and CYP17 activity in Angora goats, Boer goats and Merino sheep. PROG: Progesterone, DHEA: Dehydroepiandrosterone. Reproduced from [2].

The results indicated that 3β-HSD produced progesterone at similar rates in all three species. DHEA production was, however, much faster in the Angora goat compared to the other two species. Engelbrecht et al. found in this study that Angora goat CYP17 had a higher affinity for pregnenolone than did Boer goat and Merino sheep CYP17 [7]. This resulted in an over-production of androgen precursors at the cost of glucocorticoid precursors in the Angora goat adrenal cortex. CYP17 activity was identified as an important contributing factor in the Angora goat's hypoadrenocorticism. This work led to a new hypothesis regarding the cause of the hypoadrenocorticism. The inbreeding practices over many years (Chapter 1) could have preserved a mutation in the CYP17 gene that caused the altered activity of CYP17.
3.6 Conclusion

CYP17 was implicated in hypoadrenocorticism of the Angora goat. It is clear that the dual catalytic activity of this steriodogenic enzyme plays a crucial role in shuttling of steroid intermediates through the steriodogenic pathways. Ultimately CYP17 determines to a large extent the ratio of glucocorticoid to androgen hormone production. It is the heightened affinity of CYP17 for the Δ5 steroid pathway in the Angora goat that results in an overproduction of androgens at the cost of glucocorticoid production. This impaired glucocorticoid biosynthesis forms the basis of the stress intolerant phenotype of the Angora goat. CYP17 thus remained the focus point in investigating hypoadrenocorticism in the Angora goat.

The preference of the Angora goat adrenal CYP17 for the Δ5 pathway has not been shown in other species to date. As the Angora goat CYP17 gene was never before investigated on a molecular level it was decided to synthesise the cDNA coding for the Angora goat CYP17. The cloning and expression of this enzyme in a non-steroidogenic cell line, would enable us to study the kinetics and enzymology of this hemoprotein in more detail. The following three chapters describe the experimental work investigating the Angora goat CYP17, and the development of a genotype test capable of identifying different CYP17 allelic combinations. It is finally argued that different CYP17 genotypes found in the Angora goat gene pool is at least in part responsible for the observed hypoadrenocorticism in the Angora goat.
CHAPTER 4
SYNTHESIS OF FULL LENGTH ANGORA GOAT CYP17 cDNA

4.1 Introduction

The aim of this study was to determine whether there is a genetic basis for the observed hypoadrenocorticism in the Angora goat. It has been shown that the primary cause of the Angora goat’s hypoadrenocorticism is adrenocortical insufficiency [7]. This insufficiency leads to inadequate cortisol release from the adrenal cortex upon stimulation by ACTH. The inability to secrete sufficient cortisol in response to ACTH stimulation can have severe implications. ACTH is released in response to CRH binding to its receptor on the corticotrope cells of the pituitary. CRH in turn is produced and released from the hypothalamus as a result of a wide array of neuro stimuli innervating the hypothalamus. At the level of the pituitary the neuro-endocrine connection is a complex entity, which plays a vital role in maintaining homeostasis, enabling an organism to respond to changes in the environment and to counteract them in a favourable fashion.

Various stress stimuli lead to an increase in the plasma ACTH concentration and the subsequent actions of ACTH are responsible for a concomitant rise in the plasma cortisol concentration. It is therefore clear that the inability of an organism to elevate its plasma cortisol concentration through the interplay of neuro-endocrine signals could result in a stress-intolerant phenotype.

Adrenal steroidogenesis has been studied extensively in the Angora goat, Boer goat and Merino sheep [7]. Boer goats and Merino sheep do not generally show symptoms of stress intolerance attributable to hypoadrenocorticism. Analysis of these studies revealed that adrenal steroidogenesis in the Angora goat differed markedly from steroidogenesis in the Boer goat and Merino sheep. The findings showed significant differences in CYP17 activity in terms of pregnenolone and progesterone metabolism in these species.

CYP17 became the focus of further investigations into the Angora goat’s hypoadrenocorticism. It was hypothesized that a mutation in the gene encoding CYP17 could be the cause of the differences observed in adrenal steroidogenesis between the above mentioned livestock. Such a mutation could be the result of any one of various mutations, such as a frameshift, insertion, deletion, somatic or point mutation [135], and would most
likely exist in the coding region of the gene. Mutations in the coding regions of genes are propagated to the mRNA transcripts of the gene, which are present in the cells where the gene is expressed. Furthermore, if a CYP17 mutation existed in the Angora goat gene pool, it is possible that a wild copy would also exist. Since it was reported by some breeders that their breeding stocks still have goats that are not as susceptible to environmental stress as others are, it was speculated that there were at least two alleles of the CYP17 gene in the Angora goat gene pool. The CYP17 gene structure was therefore analyzed to compare the coding region of the gene, i.e. the cDNA of the Angora goat to that of other related species.

4.2 RNA isolation and cDNA synthesis

4.2.1 Introduction

The synthesis of cDNA, encoding a specific gene, requires isolation of intact mRNA from the tissue expressing the gene of interest. CYP17 is expressed in the adrenal cortex. RNA isolation is a sensitive procedure as RNA is extremely susceptible to endonucleolytic and exonucleolytic attack from ribonucleases. In intact cells RNA is protected from endogenous ribonucleases by means of cellular compartmentalization. Homogenization of tissue destroys subcellular structures and these ribonucleases are brought into contact with the RNA. There are various methods for inactivating ribonucleases. In this study, diethylpyrocarbonate (DEPC), a strong inhibitor of ribonucleases [136], was used for this purpose. Various methods exist for the isolation of RNA from tissue, for example: phenol-chloroform extraction and centrifugation through a CsCl cushion. However, advanced technology in the field of molecular biology today provides alternative methods for the preparation of high pure RNA from tissue by employing any one of a number of commercially available kits. These kits also eliminate exposure of the researcher to harmful chemicals. A 260 nm/280 nm-absorbancy ratio of 1.9 – 2.0 for a total RNA solution is considered to be RNA of high purity [137]. Using an appropriate kit was the method of choice for RNA isolation in this study.

The cDNA of a gene bears all the information encoding the full length functional protein. Sequence comparisons of cDNAs encoding specific proteins are thus a useful method for comparing the primary structure of proteins from different species. Analysis of the cDNA sequence of a gene can provide important information regarding the integrity of a protein
expressed in a certain individual, or in a particular species. From such analysis and comparisons it can be deduced whether a mutation might have occurred in a gene, severely affecting the primary structure of a protein, or preventing the protein from being expressed. This is conceivable when large regions of DNA have been removed (deletions) or inserted (insertions) into the genomic copy of a gene, or when a premature stop codon has been introduced in the DNA as a result of mutations. Single nucleotide mutations also occur which lead to either the substitution of one amino acid for another, or disruption of the reading frame resulting in a non-sense peptide being translated.

In a preparation of total RNA mRNA, which is present together with tRNA and rRNA, is the minor constituent. Most mammalian mRNA transcripts are flanked with polyadenylic acid residues at the 3’ termini. This allows the purification of mRNA from total RNA with a kit that employs small scale affinity binding of the mRNA to an immobilized functional group of oligo(dT).

During a RT-PCR single stranded mRNA is used as template by a thermostable reverse transcriptase in the presence of various co-factors. The reverse transcriptase synthesizes a single strand DNA molecule complementary to and complexed with the mRNA molecule primed with a specific primer complementary to a target sequence in the mRNA molecule. The product is a double stranded mRNA-DNA hybrid molecule. In the subsequent stages, after dissociation of the hybrid, the primers target the complementary sequences in the single stranded DNA molecule and a thermal stable DNA polymerase amplifies the targeted DNA. A negative control reaction should be included in any RT-PCR, in which all the reagents except the reverse transcriptase are present. Amplification of DNA in this reaction is indicative of DNA contamination.

4.2.2 Experimental procedure

RNA isolation

Adrenals were obtained from a four to five year old male goat immediately after slaughtering the goat. The adrenals were subsequently flash frozen and stored in liquid nitrogen. Total RNA was isolated using the High Pure™ RNA Tissue Kit from Roche (Mannheim, Germany) according to the manufacturer’s instructions. The surrounding connective tissue was dissected from the gland. Adrenocortical tissue, 50 mg, was cut free
from the gland and put in a glass/teflon homogenizing set containing buffer from the RNA Tissue Kit. Homogenization was performed on ice and the homogenate used as starting material for the isolation. The RNA was eluted in 200 μl water and stored at −80 °C. RNA concentration and purity was determined spectrophotometrically using a Beckman DU 650 spectrophotometer from Beckman (Palo Alto, CA 94304, USA).

Total RNA, 20 μl, was used as starting material for each mRNA immobilization procedure, using the mRNA Capture Kit from Boehringer Manheim (Mannheim, Germany) according to the manufacturer’s instructions. The buffer was discarded once the mRNA immobilization was completed.

cDNA synthesis

Complementary cDNA was synthesized by reverse transcription of mRNA using the Titan™ One Tube RT-PCR system from Boehringer Manheim according to the manufacturer’s instructions. Two different RT-PCR amplifications were performed, one with total RNA and one with mRNA as template.

Genetic homology of the Angora goat to sheep was assumed to be sufficient in the design of primers used in the RT-PCR. The primers were designed complementary to target sequences upstream and downstream of the ATG translational start and TGA translational stop sites of sheep CYP17 (GENBANK accession number: L40335) respectively. All primers used in this study were purchased from Integrated DNA Technologies, Inc. (Coralville, IA 52241, USA). Specifications of the primers are listed in appendix A. The reactions were set up as follows: Three master mixes were prepared separately, I, II and III as shown in tables 4.1, 4.2 and 4.3 respectively.

(i) Template: mRNA
Master mix I was added to the tube, which contained the immobilized mRNA. Subsequently master mix III was added to the same tube.

(ii) Template: total RNA
Master mix II was prepared in a normal PCR tube and master mix III was added to this tube shortly afterwards.

The reaction mixtures were overlaid with a small drop of mineral oil purchased from Promega (Madison, WI, USA). For amplification of mRNA, RT-PCR was performed in a PCR Sprint thermocycler from Hybaid Ltd. (Ashford, Middlesex, UK) as follows: a reverse transcriptase
step at 50 °C for 30 min and an initial denaturation step at 94 °C for 2 min, followed by 10 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, and elongation at 68 °C for 3 min. A further 25 cycles were performed adding 5 s to the elongation step for each cycle, final elongation step at 68 °C for 7 min, and further storage of the reactions at −20 °C.

Table 4.1. RT-PCR master mix I

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<th>Reagents</th>
<th>Vol. (µl)</th>
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<tr>
<td>dNTPs</td>
<td>4</td>
<td>200 µM (Each)</td>
</tr>
<tr>
<td>G001 (Left primer)</td>
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<td>0.4 µM</td>
</tr>
<tr>
<td>G006 (Right primer)</td>
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<td>0.4 µM</td>
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<td>RNA template</td>
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</tr>
<tr>
<td>DTT</td>
<td>2.5</td>
<td>5 mM</td>
</tr>
<tr>
<td>RNase inhibitor</td>
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<tr>
<td><strong>TOTAL</strong></td>
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Table 4.2. RT-PCR master mix II

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</tr>
<tr>
<td>dNTPs</td>
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<td>200 µM (Each)</td>
</tr>
<tr>
<td>G001 (Left primer)</td>
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<td>0.4 µM</td>
</tr>
<tr>
<td>G006 (Right primer)</td>
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</tr>
<tr>
<td>DTT</td>
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</tr>
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<td>5 U</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td><strong>25</strong></td>
<td></td>
</tr>
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Table 4.3. RT-PCR master mix III

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<td>5 X Buffer (Supplied)</td>
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<tr>
<td>RT-AMV</td>
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<tr>
<td><strong>TOTAL</strong></td>
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</tbody>
</table>
LE agarose was purchased from Roche for agarose gel analysis of the RT-PCR products. DNA loading buffer (0.1% Orange G (w/v), 20% Ficoll (w/v), 10 mM EDTA, pH 7), 5 μl, was added to 10 μl of each RT-PCR mixture for electrophoresis. The samples were then loaded on a 1% (w/v) agarose gel prepared in TAE buffer (40 mM Tris-acetate, 2 mM Na₂EDTA·2H₂O and 20 mM acetic acid). Submerged electrophoresis was performed at 55 V for 10 min and then at 110 V until the dye reached the end of the gel. Ethidium bromide was purchased from Merck (Darmstadt) to stain gels in a 0.5 μg/ml solution for 20 min. Stained DNA fragments were then visualized on a UV transilluminator.

4.2.3 Results

RNA isolation

Spectrophotometric analysis of the purified total RNA indicated that almost 4 μg of total RNA was purified from 50 mg of adrenocortical tissue. The absorbance of a six times diluted sample of total RNA at 260 nm and 280 nm was respectively 0.8267 and 0.4164. The 260/280 absorbency ratio was 1.9856.

cDNA synthesis

Two RT-PCR amplifications were performed: (1) a one-step reaction with total RNA as template, and (2) a one-step reaction with mRNA as template. Agarose gel analysis of the RT-PCR products revealed a distinct band of approximately 1.6 kb (fig. 4.1), which is of similar size as sheep CYP17 cDNA (GENBANK accession number: L40335). Lane 5 (total RNA template) shows a light degree of DNA smearing compared to lane 4 (mRNA template). This could be as a result of non-specific DNA annealing. A negative control reaction was performed to indicate contamination of the RT-PCR with exogenous genomes, plasmids or PCR products, which amplified no fragment.
Figure 4.1. One-step RT-PCR amplifications of Angora goat CYP17 mRNA transcripts. The RT-PCR products (10 μl) were analysed by 1% agarose gel electrophoresis followed by ethidium bromide staining. Lane 1, negative control with total RNA as template; Lane 2, negative control with mRNA as template; Lane 3, DNA molecular weight marker VI (Roche); Lane 4, full length cDNA amplified in a one-step RT-PCR (mRNA template); Lane 5, full length cDNA amplified in a one-step RT-PCR (total RNA template).

4.3 Amplification and sequencing of Angora goat CYP17

4.3.1 Introduction

A RT-PCR fragment of approximately 1.6 kb was produced in an attempt to amplify Angora goat CYP17. Sequence analysis of this fragment was essential to establish whether the fragment was Angora goat CYP17 cDNA. Both strands of the cDNA had to be sequenced to be certain every base assigned to every locus of the sequence was correct. Three sets of primers were therefore used to sequence the sense and anti-sense strand of the RT-PCR fragment. Once the sequence was known, comparative analyses using other known sequences of CYP17 cDNA from different species was possible.

4.3.2 Experimental procedure

Amplification of the RT-PCR product was necessary to obtain sufficient DNA for sequencing. To further amplify the RT-PCR product it had to be purified from a preparative agarose gel. DNA was purified from agarose gel slices using a Promega Wizard® PCR preps DNA purification kit, according to the manufacturer’s instructions. The gel purified RT-PCR
product was used as template in a second round large scale (100 µl) PCR. This PCR mixture was loaded on a preparative agarose gel and the 1.6 kb DNA band was subsequently purified for sequencing.

The RT-PCR product, 12 µl, was mixed with DNA loading buffer in a 1:2.4 ratio and loaded on a preparative 1% agarose gel. The gel consisted of NuSieve® GTG low melting agarose purchased from Roche and normal LE agarose, also from Roche, mixed in a 1:3 ratio respectively. After electrophoresis the gel was stained with ethidium bromide and the DNA fragment corresponding to 1.6 kb was gel purified and eluted in water. Subsequently a second round PCR amplification was performed using 10 µl of the purified DNA as template in a total volume of 100 µl. Pwo DNA polymerase and dNTPs (mixture of dATP, dCTP, dGTP and dTTP) were purchased from Roche. The reagents were added as shown in table 4.4 and all reactions were overlaid with mineral oil (Promega) and amplified as follows: an initial denaturation step at 94 °C for 2 min, followed by 10 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, and elongation at 68 °C for 3 min. A further 25 cycles were performed adding 5 s to the elongation step for each cycle, final elongation step at 68 °C for 7 min, and further storage of the reactions at −20 °C.

Table 4.4. PCR reagents added for the second round amplification of the RT-PCR product.

<table>
<thead>
<tr>
<th>Reagents</th>
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<th>Final concentration</th>
</tr>
</thead>
<tbody>
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</tr>
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<td>10X buffer (Supplied)</td>
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</tr>
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<td>200 µM (Each)</td>
</tr>
<tr>
<td>G001 (Left primer)</td>
<td>1.5</td>
<td>0.3 µM</td>
</tr>
<tr>
<td>G006 (Right primer)</td>
<td>1.5</td>
<td>0.3 µM</td>
</tr>
<tr>
<td>DNA template</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Pwo polymerase</td>
<td>0.5</td>
<td>1.5 U</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>

The PCR amplification was electrophoresised on a 1% preparative agarose gel and the DNA fragment of approximately 1.6 kb was gel purified. The concentration of the purified DNA was determined spectrophotometrically. Subsequently the purified DNA fragment was sequenced using 13.3 ng/µl of template DNA and 1.1 ng/µl of primer DNA per sequencing reaction. Specifications of the primers used are listed in appendix A. Nucleotide sequences
were determined using the Bigdye™ Version 2 diterminator sequencing kit (model 373 A ABI, Applied Biosystems, Foster City, CA) at the central analytical facility of the University of Stellenbosch.

4.3.3 Results

The second round PCR amplification yielded approximately 3.5 μg DNA (93 ng/μl). Sequencing of this DNA produced six sequences, which were aligned with BioEdit (Hall T, 1999) to produce the complete cDNA sequence of the Angora goat CYP17 gene. The sequence generated for the RT-PCR product in this study showed Angora goat CYP17 cDNA to be 1 530 bp from the ATG translational start site to the TGA translational stop site. Furthermore, four double nucleotide base pair peaks were detected at four random loci (base pair 17, 122, 637 and 1 065) in the coding sequence on both the DNA sense and anti-sense strands. Fig. 4.2 to fig. 4.5 show the double peaks generated by the sequencing for the DNA sense and anti-sense strands. The complete sequence is shown in appendix B and is aligned with goat CYP17 cDNA sequence to determine homology. The Angora goat (Capra aegagrus) and Boer goat (Capra hircus) belong to the same genus and would therefore be expected to share a high CYP17 cDNA homology. There are only four nucleotide differences between Angora goat and Boer goat CYP17 cDNA and they are at the loci where the double peaks are found in the Angora goat cDNA sequence.
Figure 4.2. Graphic nucleotide sequence of Angora goat CYP17 cDNA showing a double nucleotide base peak at bp 17 in the cDNA. Graph A: nucleotide base at position 40 corresponds with nucleotide base 17 in the cDNA sequence. Graph B: nucleotide base at position 376 corresponds with nucleotide base 17 in the cDNA sequence.
Figure 4.3. Graphic nucleotide sequence of Angora goat CYP17 cDNA showing a double nucleotide base peak at bp 122 in the cDNA. Graph A: nucleotide base at position 145 corresponds with nucleotide base 122 in the cDNA sequence. Graph B: nucleotide base at position 271 corresponds with nucleotide base 122 in the cDNA sequence.
Figure 4.4. Graphic nucleotide sequence of Angora goat CYP17 cDNA showing a double nucleotide base peak at bp 637 in the cDNA. Graph A: nucleotide base at position 74 corresponds with nucleotide base 637 in the cDNA sequence. Graph B: nucleotide base at position 337 corresponds with nucleotide base 637 in the cDNA sequence.
Figure 4.5. Graphic nucleotide sequence of Angora goat CYP17 cDNA showing a double nucleotide base peak at bp 1 065 in the cDNA. Graph A: nucleotide base at position 502 corresponds with nucleotide base 1 065 in the cDNA sequence. Graph B: nucleotide base at position 515 corresponds with nucleotide base 1 065 in the cDNA sequence.
4.4 Discussion

Total RNA isolated from Angora goat adrenocortical tissue yielded approximately 4 μg of RNA. The one-step RT-PCR targeting Angora goat CYP17 produced a single DNA fragment of approximately 1.6 kb using either total RNA or mRNA as template (fig. 4.1). The two negative controls included in the RT-PCR produced no amplified fragments, indicating the 1.6 kb DNA fragment was derived from the RNA template. Furthermore, the size of the RT-PCR product correlated well with other CYP17 cDNA transcripts in terms of size, which strongly suggested the DNA fragment to be Angora goat CYP17 cDNA. However, only sequence analysis could truly confirm the identity of the RT-PCR product. The RT-PCR produced only one distinct amplified product indicating that the primers used did not anneal to unspecific sequences in the RNA template. Judging from the agarose gel analysis of the RT-PCR there is no considerable difference between the products of the different RNA templates used. Using total RNA as template in a RT-PCR could eliminate a costly and time consuming step in the preparation of cDNA.

The CYP17 cDNA sequence generated for the Angora goat (Capra aegagrus) (appendix B) shares 99.74% homology with Boer goat (Capra hircus) CYP17 cDNA (GENBANK accession number: AF251387). Only four nucleotide bases differed in the 1 530 bp coding region of the two sequences. This ultimately confirmed the RT-PCR product produced in this study to be Angora goat CYP17 cDNA. However, the sequencing results produced four double peaks along the 1 530 bp coding region (fig. 4.2 – fig. 4.5). This could not simply be regarded as sequencing anomalies, since these double peaks were detected in both the sense and anti-sense strand of the cDNA. Furthermore, these double peaks were detected at the loci where Angora goat and Boer goat CYP17 cDNA differed. This result therefore shows that more than one copy of CYP17 cDNA was present in the sequencing sample differing by four nucleotide bases at the indicated loci. These findings strongly indicate to the possibility that the Angora goat, from which the RNA was isolated, expressed more than one CYP17 mRNA copy in its adrenal cortex. Analysis of the cDNA reading frame, in terms of the genetic code, indicated that three of the four nucleotide substitutions translated to amino acid substitutions in the translated protein. The DNA base pair substitutions and their corresponding amino acid substitutions were as follows: position 17, C/G – Ala/Gly; position 122, C/T – Pro/Leu;
position 637, G/A - Val/Ile; position 1 065, G/A - no amino acid substitution (with positions relating to the cDNA sequence).

Furthermore, the G/A substitution at position 637 caused an alteration in the Acs I endonuclease recognition sequence. This substitution caused a change in the six base pair recognition sequence of Acs I from ‘AAATTC’ to ‘AAGTTC’. The latter sequence is not a substrate for endonuclease cleavage by Acs I.

Adrenal steroidogenesis is a complex biochemical process through which various steroid hormones are produced. These hormones are essential to maintaining homeostasis. Although cortisol is not as vital for sustaining life, since life can be sustained for longer in the absence of cortisol than aldosterone, it plays a very important role in stress management (Chapter 2). A number of adrenal enzymes are responsible for the biosynthesis of cortisol from its precursor, cholesterol. CYP17 is a microsomal P450 essential to cortisol production. This enzyme is one of the most widely studied cytochrome P450s due to its dual catalytic activity [138]. CYP17 catalyses the hydroxylation and cleavage of C21 steroids and 17-hydroxycorticoids respectively. Furthermore, this enzyme influences the ratio of androgen to corticosteroid production due to its activity in both the Δ5 and Δ4 steroid pathways. The Δ5 and Δ4 pathways lead to androgen and corticosteroid production respectively. It should be clear that any alteration in CYP17 activity could influence the balance of adrenal steroid hormone production.

Comparative studies investigating Angora goat, Boer goat and Merino sheep adrenal steroidogenesis confirmed that Angora goat adrenals favoured the production of androgens to that of the corticosteroids, compared to the two other species [7]. This seems to be the main underlying mechanism for the hypoadrenocorticism observed in the Angora goat. Furthermore, Angora goat CYP17 channels much more pregnenolone through the Δ5 steroid pathway compared to Boer goat and Merino sheep. Interestingly Angora goat CYP17 seems to channel the same amount of progesterone through the Δ4 steroid pathway as the other two species. Comparison of the other steroid producing enzymes, including CYP11A1, 3β-HSD, CYP21 and CYP11B1 showed no significant differences compared to Boer goat and Merino sheep.

Analysis of the Angora goat CYP17 cDNA sequence suggested the likelihood of an Angora goat from the Swartland farming district carrying two CYP17 copies. Since mRNA is transcribed from genomic DNA it implies that the two alleged copies of the gene would be inherently passed on to progeny in Mendelian fashion. Therefore the two genes would be
distributed throughout the gene pool. It can only be speculated when a second copy of the CYP17 gene was introduced into the gene pool, if indeed it existed. It is, however, known that inbreeding among Angora goats was common practice in the Angora goat industry [2]. This was part of ongoing attempts to produce fleece with improved quality. Concomitant with selecting for better quality fleece, was the rise in the stress intolerant phenotype among Angora goats. It remains to be seen whether these two physical traits can be separated through a breeding strategy.

In light of the possible existence of two Angora goat CYP17 alleles, three possible CYP17 genotypes could exist, namely two homozygote genotypes and one heterozygote genotype.

CYP17 activity plays a delicate role in steroidogenic homeostasis. The possibility of three different Angora goat CYP17 genotypes existing could have severe effects on the animal’s steroid hormone production. It seems likely that the Angora goat’s hypoadrenocorticism could be attributable to the existence of three CYP17 genotypes. From the preceding discussion it is conceivable that one, or possibly two, of the three proposed genotypes could be responsible for the high incidence of stress intolerance in Angora goat farming stocks. However, the Angora goat from which the cDNA was synthesized was described as hardened by the farmer. It is therefore possible that the heterozygote CYP17 genotype does not exhibit a stress intolerant phenotype. It was now vital to devise a test that could conclusively confirm the existence of a CYP17 polymorphism in Angora goats, and that could identify different CYP17 genotypes.
CHAPTER 5
DEVELOPMENT OF A PCR BASED RESTRICTION ENZYME DIGEST TEST FOR CYP17 GENOTYPING

5.1 Introduction

The generation of two different Angora goat CYP17 cDNA sequences was a noteworthy finding. Many different CYP17 mutations have been investigated in the past in other animals. These mutations often result in single amino acid substitutions, which are generally caused by point mutations. Studying the effects these individual amino acid changes have on CYP17 activity contributes to our knowledge of the structure function relationship of the enzyme [131,133]. Such studies have also contributed to our understanding of the differential regulation of the 17α-hydroxylase and 17,20-lyase activity of the enzyme. Considerable contributions were made by those specific mutations that cause so-called isolated 17,20-lyase deficiency.

The majority of these mutations were discovered because their effects on steroidogenesis presented as clinically diagnosable endocrine abnormalities. Complete abolition of CYP17 activity causes overproduction of mineralocorticoids and the inability to synthesize glucocorticoid and sex hormones [139]. Absence of CYP17 lyase activity alone will result in the failure to produce sex hormones.

Sequence analysis of the Angora goat CYP17 cDNA showed the presence of two non-identical sequences. The two 1,530 bp sequences differed only by four nucleotide bases. Whether all four of the substituted bases represent true mutations can not be told from cDNA sequence analysis alone. It is though possible that through evolution a second copy of the CYP17 gene evolved from a multi-mutation event that occurred over time. If a mutated CYP17 copy was responsible for the development of the stress intolerant phenotype among Angora goats, natural selection would most likely have expelled the mutation from the gene pool under normal circumstances. However, inbreeding practices that occurred may have preserved such a mutation.

Restriction enzyme profiles were generated for the two different CYP17 sequences, which differed only by a single Acs I recognition sequence. One of the alleles has an Acs I
recognition sequence at cDNA bp 635, which is lost in the second allele by a substitution of adenine for guanine in the Acs I recognition sequence. The altered restriction endonuclease profile between the two CYP17 cDNA sequences provided the basis for the development of a CYP17 genotype test. Development of a CYP17 genotype test would allow Angora goats to be tested, using genomic DNA, in order to determine their CYP17 genotype. Since the Angora goat described in the previous chapter produced two different CYP17 cDNA sequences the genotype test was developed using genomic DNA from that goat. This was done so that the test could be validated by producing the expected result for a CYP17 heterozygote Angora goat.

5.2 Isolation and sequencing of Angora goat genomic CYP17

5.2.1 Introduction

Genomic transcripts of many eukaryotic genes are larger than their corresponding mRNA transcripts, because they possess the intron nucleotide sequences. Each living cell of a multicellular organism has a nucleus in which genomic DNA is found. In diploid organisms each cell nucleus has two copies of each gene, one from each parent. The intron nucleotide sequences have many functions aiding in the processing of the primary transcript into mRNA upon transcription of a gene. These sequences, however, are also useful in population studies. Comparisons of the genomic nucleotide sequences of the two CYP17 alleles identified in this study in the Angora goat could therefore aid in determining the origins of the two genes. Sequence analysis of the genomic copy of Angora goat CYP17 was also necessary to aid in the development of a CYP17 genotype test, since genomic DNA would be used in the test. Genomic DNA was obtained from the same goat from which the adrenal glands were taken (Chapter 4).

5.2.2 Experimental procedure

Blood was collected from the slaughtered goat directly into EDTA-coated tubes to prevent clotting and DNA degradation and stored at 4 °C. A Wizard® Genomic DNA Purification Kit
was purchased from Promega and DNA was isolated from the blood samples according to the manufacturer’s instructions. After isolation the DNA pellet was rehydrated in water.

Three pairs of PCR primers were designed, complementary to sheep cDNA sequences, aimed at producing three overlapping PCR fragments spanning the full length Angora goat genomic CYP17 gene. Pwo DNA polymerase and dNTPs were purchased from Roche. The PCR reagents were added as shown in table 5.1. All reactions were overlaid with mineral oil (Promega) and amplified as follows: an initial denaturation step at 96 °C for 2 min, followed by 30 cycles of denaturation at 96 °C for 1 min, annealing at 55 °C for 75 s, and elongation at 72 °C for 3 min, final elongation step at 72 °C for 10 min, and further storage of the reactions at -20 °C.

Table 5.1. PCR amplification reagents with primers targeting Angora goat genomic CYP17.

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Vol. (µl)</th>
<th>Final concentration</th>
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<td>10 X Buffer (Supplied)</td>
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<tr>
<td>dNTPs</td>
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<td>200 µM (Each)</td>
</tr>
<tr>
<td>*Left primer</td>
<td>1.5</td>
<td>300 nM</td>
</tr>
<tr>
<td>*Right primer</td>
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<td>300 nM</td>
</tr>
<tr>
<td>DNA template</td>
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<td></td>
</tr>
<tr>
<td>Pwo</td>
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<td>2.5 U</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td><strong>100</strong></td>
<td></td>
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</tbody>
</table>

*Three primer pairs were used in three separate reactions. Appendix A shows the primer specifications.

The PCR amplifications, 10 µl, were loaded on a 1% agarose gel for electrophoresis (fig. 5.1) and the remaining 90 µl of each reaction was loaded on a separate 1% preparative agarose gel for electrophoresis. The DNA fragments of interest were gel purified with an Agarose Gel DNA Extraction Kit from Roche according to the manufacturer’s instructions. The purified DNA fragments were sequenced as described in section 4.3.2 using 13.3 ng/µl of template DNA and 1.1 ng/µl of primer DNA per sequencing reaction. Specifications of the primers used are listed in appendix A.
5.2.3 Results

Only two of the three PCR amplifications targeting different segments of the genomic CYP17 gene produced fragments that were suitable for sequencing. Fig. 5.1 shows the DNA fragments amplified by the three sets of primers. A very faint band is seen in lane 5 at approximately 2.5 kb. The primers used for this amplification targeted the 5' terminal segment of the gene and failed to produce a proper sequence. Lanes 3 and 4 show the fragments produced by the primer pairs aimed at the 3' terminal segment and middle segment of the gene respectively.

Figure 5.1. PCR amplifications of different segments of Angora goat genomic CYP17. Amplification mixtures (10 µl) were analysed by 1% agarose gel electrophoresis followed by ethidium bromide staining. Lane 1, negative control reaction; Lanes 2 and 6, 1 kb DNA Ladder (Promega); Lane 3, primers targeting 3' terminal segment of gene; Lane 4, primers targeting middle segment of gene; Lane 5, primers targeting 5' terminal segment of gene.

Sequencing of these two fragments showed them to overlap and include the TGA translational stop site. The two segments were not completely sequenced on both sense and anti-sense strands and therefore some bases assigned could be incorrect. The sequences generated for Angora goat genomic CYP17 were aligned with BioEdit (Hall T, 1999). This partial genomic sequence was aligned with the Angora goat CYP17 cDNA sequence (appendix C), which allowed the determination of Angora goat CYP17 exon and intron sizes as shown in table 5.2. Since genomic CYP17 sequences were not available for sheep or Boer goat, comparisons were made with human and baboon CYP17 genomic sequences to draw an estimated genomic
CYP17 gene map (fig. 5.2). Table 5.2 shows all the intron and exon sizes for human and baboon CYP17 and those available for the Angora goat.

**Table 5.2.** Genomic CYP17 intron and exon sizes (indicated by nucleotide base pair length) compared for human, baboon and Angora goat. Human and baboon sizes obtained from [141].

<table>
<thead>
<tr>
<th></th>
<th>Human</th>
<th>Baboon</th>
<th>Angora</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exon 1</td>
<td>297</td>
<td>297</td>
<td>/</td>
</tr>
<tr>
<td>Intron A</td>
<td>1668</td>
<td>1757</td>
<td>/</td>
</tr>
<tr>
<td>Exon 2</td>
<td>143</td>
<td>143</td>
<td>/</td>
</tr>
<tr>
<td>Intron B</td>
<td>229</td>
<td>235</td>
<td>/</td>
</tr>
<tr>
<td>Exon 3</td>
<td>230</td>
<td>230</td>
<td>/</td>
</tr>
<tr>
<td>Intron C</td>
<td>662</td>
<td>641</td>
<td>/</td>
</tr>
<tr>
<td>Exon 4</td>
<td>87</td>
<td>87</td>
<td>/</td>
</tr>
<tr>
<td>Intron D</td>
<td>694</td>
<td>1012</td>
<td>/</td>
</tr>
<tr>
<td>Exon 5</td>
<td>216</td>
<td>216</td>
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<td>Intron E</td>
<td>232</td>
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<tr>
<td>Exon 6</td>
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<tr>
<td>Intron F</td>
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<td>533</td>
</tr>
<tr>
<td>Exon 7</td>
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<td>104</td>
<td>104</td>
</tr>
<tr>
<td>Intron G</td>
<td>518</td>
<td>520</td>
<td>524</td>
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<tr>
<td>Exon 8</td>
<td>284</td>
<td>284</td>
<td>287</td>
</tr>
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</table>

/ Not available

**Figure 5.2.** A graphic presentation of the Angora goat genomic CYP17 gene showing eight exons (yellow) and seven introns (A to G). This gene map is an estimate, since the Angora goat genomic CYP17 has not been sequenced completely. Similarity to Human and Baboon genomic CYP17 was assumed. The four nucleotide substitutions and their respective amino acid substitutions are shown (numbers according to the 1530 bp cDNA sequence). The Acs I recognition sequence is indicated, with the substituted base in red.
5.3 Development of a CYP17 genotype test

5.3.1 Introduction

The complete cDNA and partial genomic sequences for Angora goat CYP17 obtained in this study provided sufficient information to devise a strategy for developing a CYP17 genotype test. The nucleotide base substitution in exon 3 (fig. 5.2) of Angora goat CYP17 resulted in the alteration of the recognition sequence of the endonuclease Acs I. The altered restriction profile of the two CYP17 genes formed the basis of the genotype test developed in this study. A strategy was selected whereby genomic DNA of an experimental animal is subjected to a PCR using primers spanning the nucleotide substitution in exon 3. The primers were designed complementary to sequences predicted to be in exon 2 and exon 4 according to the gene map (fig. 5.2). A PCR product produced with these primers was estimated to be approximately 1.2 kb. The CYP17 genotype of the animal would determine whether the PCR fragment possesses or lack the Acs I recognition sequence. Subsequent endonuclease treatment of the PCR fragment with Acs I should produce DNA fragments that have a distinct agarose gel banding pattern indicating the CYP17 genotype of the test subject upon electrophoresis. A CYP17 heterozygote should produce three DNA fragments, one at the size of the undigested PCR fragment, and two smaller fragments.

5.3.2 Experimental procedure

A PCR amplification was prepared with genomic DNA template from the CYP17 heterozygote Angora goat described in Chapter 4. Three similar amplification reactions were performed in a total volume of 50 µl. The primer specifications are listed in appendix A. Pwo DNA polymerase and dNTPs were purchased from Roche. The PCR reagents were added as shown in table 5.3. The reaction mixtures were overlaid with mineral oil (Promega) and amplified as follows: an initial denaturation step at 96 °C for 2 min, followed by 30 cycles of denaturation at 96 °C for 1 min, annealing at 55 °C for 75 s, and elongation at 72 °C for 3 min, final elongation step at 72 °C for 10 min, and further storage of the reactions at –20 °C.
Table 5.3. PCR amplification reagents with primers targeting the nucleotide substitution in exon 3 of Angora goat CYP17.

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<th>Reagents</th>
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<td>200 µM (Each)</td>
</tr>
<tr>
<td>Acs LP (Left-primer)</td>
<td>1.5</td>
<td>600 nM</td>
</tr>
<tr>
<td>Acs RP (Right-primer)</td>
<td>1.5</td>
<td>600 nM</td>
</tr>
<tr>
<td>DNA template</td>
<td>2.0</td>
<td></td>
</tr>
<tr>
<td>Pwo</td>
<td>0.25</td>
<td>1.25 U</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td><strong>50</strong></td>
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</tr>
</tbody>
</table>

Once the amplification reactions were completed the three mixtures were pooled and 30 µl DNA loading dye added for electrophoresis on a 0.8% preparative agarose gel. DNA fragments of the predicted size at approximately 1.2 kb were cut from the gel and purified with an Agarose Gel DNA Extraction Kit (Roche), according to the manufacturer’s instructions. The purified DNA was eluted in a total volume of 50 µl water and subsequently placed in a vacuum oven at 40 °C until the DNA was completely dry. Water, 22 µl, was used to reconstitute the dried DNA, which was then digested (90 min) at 50 °C with Acs I (4 U) purchased from Roche in the digest buffer supplied by the manufacturer in a total volume of 25 µl. A second digest was performed with Acs I on the same PCR fragment of the same goat with 8 units of Acs I and was incubated for three hours at 50 °C. The reaction mixtures were overlaid with a small drop of mineral oil (Promega). DNA loading dye, 5 µl, was added to the reaction mixtures once the digest was completed and then analysed on a 0.8% agarose gel.

5.3.3 Results

Two prominent DNA fragments were produced (fig. 5.3) in the PCR with primers targeting the polymorphic region in exon 3 of Angora goat CYP17. One of the fragments was approximately 1.3 kb, close to the predicted size of 1.2 kb. The second fragment had a higher electrophoretic mobility than any of the fragments of the DNA marker and could be no more than a few hundred base pairs.
Figure 5.3. PCR amplification of the polymorphic region in exon 3 of Angora goat CYP17. The amplification reactions (3 X 50 µl) were loaded on a 0.8% preparative agarose gel for electrophoresis followed by ethidium bromide staining. Lane 1, DNA marker DNAλ/Eco RI and DNAλ/Hind III (Promega); Lanes 2, 3, 4 and 5 received equal amounts of the PCR amplification mixture.

The smaller DNA fragment was likely the product of the Acs LP and Acs RP primers (20 base pairs each) binding to unspecific sequences in the genomic DNA template.

The Acs I digest of the 1.3 kb PCR fragment obtained with the Acs LP and Acs RP primers produced an agarose gel banding pattern as it was predicted for a CYP17 heterozygote Angora goat (fig. 5.4). Half of the PCR fragments of a CYP17 heterozygote should not have the Acs I recognition sequence, while the other half should have the recognition sequence resulting in the three bands seen in lanes 3 and 4 in fig. 5.4.
Figure 5.4. Acs I restriction digest of the PCR fragment spanning exon 3 of a heterozygote CYP17 Angora goat. The restriction digest reactions (20 μl) were analysed by 0.8% agarose gel electrophoresis followed by ethidium bromide staining. Lane 1, DNA marker DNAλ/Hind III (Promega); Lane 2, the undigested PCR fragment obtained from amplification of exon 3 with the Acs LP an Acs RP primers; Lane 3, Acs LP an Acs RP PCR fragment digested with Acs I (4 U) for 90 min; Lane 4, Acs LP an Acs RP PCR fragment digested with Acs I (8 U) for 3 hrs.

5.4 Discussion

The CYP17 genotype test described in this chapter produced results as it was predicted for a CYP17 heterozygote Angora goat. Two PCR fragments were produced with the primers aimed at the G/A nucleotide substitution in exon 3 of CYP17. Only one, however, was near the predicted size of 1.2 kb (fig. 5.3). It is not surprising that another fragment was amplified since genomic DNA was used as template and therefore non-specific binding of the primers was likely to occur.

The first test was done on the genomic DNA of the goat from which the adrenals were used to synthesize CYP17 cDNA. This goat was believed to might have expressed two different CYP17 copies because of the two different CYP17 cDNA sequences produced from its adrenocortical RNA. Therefore any genotype test should produce the same result if indeed two CYP17 alleles were present. The agarose gel banding pattern of the Acs I digested PCR fragment was as predicted (fig. 5.4). One fragment was the same size as the undigested PCR
fragment and two smaller fragments resulting from Acs I cleavage of half of the PCR fragments. Further validation for the test was the same result produced by a second Acs I digest of the same fragment, which received 8 U of Acs I and was incubated for three hours. This result concluded that the banding pattern was not the result of incomplete digestion by Acs I.

The genomic copy of the Angora goat CYP17 gene has not yet been fully sequenced. The primers designed for amplifying Angora goat CYP17 mRNA and genomic sequences were complementary to sheep CYP17 cDNA sequences since the Boer goat (Capra hircus) cDNA sequence was not available at the time. These primers worked for all applications except for amplifying the 5' terminal segment of Angora goat genomic CYP17. Failure of this PCR, however, could also have resulted from the right primer being designed across an intron-exon border. This would prevent the primer from binding properly and thus no fragment will be amplified. Failure to amplify this segment of the gene could not be due to inadequate binding of the left primer, since this primer was successfully used to amplify Angora goat CYPI7 cDNA. Another possibility is that the primers were designed to far apart and the polymerase did not have sufficient time to copy the full length of DNA in the elongation period. A new PCR strategy for amplifying this segment should, however, solve the problem. Completing the genomic sequence of the two Angora goat CYPI7 alleles would aid in population analysis, investigating the origins of the two genes.

The developed CYP17 genotype test showed that a G/A nucleotide substitution mutation did indeed occur in exon 3 of the tested Angora goat. Further testing of more goats was now possible to determine if a true CYP17 polymorphism existed in Angora goats. Finding a correlation between a stress susceptible phenotype and any one of the CYP17 genotypes is now a possibility by genotyping Angora goats with different stress susceptible phenotypes.
CHAPTER 6
CYP17 GENOTYPING OF ANGORA GOATS FROM DIFFERENT POPULATIONS

6.1 Introduction

Previous research indicated that Angora goat adrenals produced abnormally high levels of androgens at the cost of glucocorticoids [7]. This shift in steroidogenesis is responsible for the inadequate amounts of cortisol produced by the adrenal cortex. Hypoadrenocorticism is the condition that develops from such a steroidogenic imbalance. Since three CYP17 genotypes exist theoretically, they might each induce a unique balance between the metabolic flux through the Δ5 and Δ4 steroid pathways. These pathways lead to androgen and glucocorticoid production, respectively. This condition is ultimately responsible for the high mortality among Angora goats suffering from exposure compared to the more hardened Boer goat. Hypoadrenocorticism in the Angora goat also leads to a high mortality rate among Angora goat kids, low conception rates and spontaneous abortions in breeding stocks [2]. These hardships have become synonymous with Angora goat farming. It would therefore be of great value to the farming community if a genetic marker was found for hypoadrenocorticism in the Angora goat. If a positive correlation could be found between the stress intolerant phenotype and a CYP17 genotype, it would strongly suggest that genotype to be a reliable marker.

Using the CYP17 genotype as a marker for stress susceptibility with reasonable certainty would necessitate that a specific genotype first be correlated with a stress related phenotype. A test was therefore developed to genotype Angora goats without using adrenal tissue. Genomic DNA isolated from a blood sample is subjected to a PCR based test to determine the combination of the two identified CYP17 copies. Since a goat receives one copy of each gene from each parent, three combinations are possible. A number of goats were subsequently genotyped. Genotyping was done using Angora goats, which had been categorized into stress intolerant and stress resistant groups. Goats were obtained from two areas, a designated farm in the Little Karoo and the Grootfontein experimental farm in the Eastern Cape. The Little Karoo goats were divided into oily skin and dry skin groups as phenotypic marker for stress
resistance and stress intolerance respectively. Four of the Grootfontein goats were classified as either strong or weak reproducers.

The amino terminal of CYP17 contains a proline rich domain (PRD) of 13 amino acids that is highly conserved among various species (appendix D). This domain is believed to be of significant importance in correct folding of the enzyme [132,133]. Proline residues may play an especially important role, as this amino acid normally induces a bend in the peptide backbone of a protein. Replacement of a proline in the conserved region by site directed mutagenesis has been shown to cause severe impairment of CYP17 activity [132]. Sequence analysis of Angora goat CYP17 cDNA identified a possible substitution mutation (Pro41Leu), in the PRD of the amino terminal (fig. 5.2). A Pro/Leu substitution in this region could have adverse effects on Angora goat adrenal steroidogenesis. Mapping the Pro41Leu substitution to a CYP17 genotype could therefore aid in understanding a genotype-phenotype correlation.

### 6.2 CYP17 genotype testing of Angora goats with different stress susceptible phenotypes

#### 6.2.1 Introduction

The focus of this study was to find a marker for stress susceptibility in the Angora goat. Therefore the CYP17 genotype test, which was developed in this study, was used to genotype several goats from different areas categorized as either stress resistant or stress intolerant. If a positive correlation was found between a certain CYP17 genotype and a stress related phenotype, it would strongly suggest that CYP17 genotype as a marker for stress susceptibility in the Angora goat.

Angora goats from the Little Karoo were phenotypically divided into two groups of 10 each for CYP17 genotyping. One group had oily skins and one group had dry skins. According to an experienced breeder goats with oily skins were much better insulated against cold compared to dry skin goats and therefore resisted cold stress better. Thus the oily skin goats are classified as stress resistant and the dry skin goats as stress intolerant. Four Angora goat ewes from Grootfontein were also selected for genotyping. These goats were classified as either strong or weak reproducers. This was done by determining the number of foetuses each ewe carried after impregnation for a number of reproduction cycles. Only one of the four
ewes was classified as a weak reproducer. Fertility could also be a potential phenotypic marker for susceptibility to stress, since Angora goat breeding stocks are known to have low conception rates attributable to the discussed stress intolerance [2]. A further 61 goats from Grootfontein were genotyped to investigate the distribution of the different CYP17 alleles.

The identification of two CYP17 alleles in the Angora goat led to speculation that one of the genes was passed on to the Angora goat from the Boer goat through cross-breeding that occurred. Therefore six Boer goats were randomly selected from a Little Karoo farm to be genotyped with the CYP17 genotype test developed for the Angora goat.

6.2.2 Experimental procedure

Peripheral blood was drawn from all goats to be genotyped directly into EDTA-coated vacutainer tubes to prevent clotting and DNA damage. Samples were stored at 4 °C. Genomic DNA was isolated from the blood samples with a Wizard® Genomic DNA Purification Kit (Promega) according to the manufacturer’s instructions. CYP17 genotyping was subsequently performed on all samples as described in section 5.4.2.

6.2.3 Results

Photos of all agarose gels showing the CYP17 genotyping results for all the goats tested (89 goats) are shown in appendix E. Some gels do not show DNA markers, since the DNA fragments indicating the CYP17 genotype were known to have a lower electrophoretic mobility than the dye in the loading buffer, which was used to indicate when sufficient electrophoresis took place. From the 89 goats successfully genotyped in this study the CYP17 homozygote with the Acs I recognition sequence in exon 3 was never identified. The two identified CYP17 genotypes are designated A^-A^- and A^+A^- for the homozygote (without the Acs I recognition sequence in exon 3) and heterozygote respectively. Genotype results were obtained for all goats except for two Little Karoo Angora goats. Table 6.1 shows the CYP17 genotype results for the 18 Little Karoo Angora goats, six Boer goats and the four Grootfontein ewes. CYP17 genotype results for the Angora goats from Grootfontein that were not phenotypically categorised are not listed in table 6.1. The CYP17 genotype distribution for these goats was 14 homozygotes and 47 heterozygotes. Figure 6.1 shows a typical agarose gel banding pattern resulting from the CYP17 genotype test for the A^-A^- and A^+A^- genotypes.
Table 6.1. Results for the CYP17 genotype test performed on goats from different populations as indicated.

<table>
<thead>
<tr>
<th>Origin of Goats</th>
<th>Goat no. in Appendix E</th>
<th>Phenotype</th>
<th>CYP17 Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Little Karoo</td>
<td>1</td>
<td>Dry skin</td>
<td>A'A'</td>
</tr>
<tr>
<td>Angora goats</td>
<td>2</td>
<td>Oily skin</td>
<td>A'A</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>/</td>
<td>A'A</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>/</td>
<td>A'A</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>/</td>
<td>A'A</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>/</td>
<td>A'A</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>/</td>
<td>A'A'</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>/</td>
<td>A'A</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>Oily skin</td>
<td>A'A'</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>Dry skin</td>
<td>A'A</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>/</td>
<td>A'A</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>/</td>
<td>A'A</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>/</td>
<td>A'A</td>
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<td></td>
<td>14</td>
<td>/</td>
<td>A'A</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>Oily skin</td>
<td>A'A</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>Dry skin</td>
<td>A'A</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>/</td>
<td>A'A</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>/</td>
<td>A'A</td>
</tr>
<tr>
<td>Grootfontein</td>
<td>S1</td>
<td>Strong reproducer</td>
<td>A'A</td>
</tr>
<tr>
<td>Angora goat ewes</td>
<td>S2</td>
<td>Strong reproducer</td>
<td>A'A</td>
</tr>
<tr>
<td></td>
<td>S3</td>
<td>Strong reproducer</td>
<td>A'A</td>
</tr>
<tr>
<td></td>
<td>W1</td>
<td>Weak reproducer</td>
<td>A'A</td>
</tr>
<tr>
<td>Little Karoo</td>
<td>B1</td>
<td>/</td>
<td>A'A</td>
</tr>
<tr>
<td>Boer goats</td>
<td>B2</td>
<td>/</td>
<td>A'A</td>
</tr>
<tr>
<td></td>
<td>B3</td>
<td>/</td>
<td>A'A</td>
</tr>
<tr>
<td></td>
<td>B4</td>
<td>/</td>
<td>A'A</td>
</tr>
<tr>
<td></td>
<td>B5</td>
<td>/</td>
<td>A'A</td>
</tr>
<tr>
<td></td>
<td>B6</td>
<td>/</td>
<td>A'A</td>
</tr>
</tbody>
</table>

/ Not available or not relevant – See discussion

Figure 6.1. A 0.8% agarose gel stained with ethidium bromide showing the typical banding pattern of an $A^+A^-$ and an $A^-A^-$ Angora goat as produced by the CYP17 genotype test described in Chapter 5. Lane 1, an $A^-A^-$ Angora goat; Lane 2, DNA marker $\text{DNA}_{\lambda}/\text{Eco RI}$ and $\text{DNA}_{\lambda}/\text{Hind III}$ (Promega); Lane 3, an $A^+A^-\text{Angora goat.}$
6.3 Mapping Pro41 to the A\(^\text{A}^\text{A}\) CYP17 genotype

6.3.1 Introduction

According to the Angora goat CYP17 cDNA sequence a Pro41Leu substitution is found in the PRD of the amino terminal. It is conceivable that such an amino acid substitution might have a significant effect on CYP17 activity since it is found in the PRD, a highly conserved region of CYP17 \[132\]. It was therefore decided to map either one of the two amino acids found at position 41 to the A\(^\text{A}^\text{A}\) CYP17 genotype. In order to do this, the PRD of genomic CYP17 of an A\(^\text{A}^\text{A}\) CYP17 goat had to be sequenced.

6.3.2 Experimental procedure

A PCR primer was designed downstream of the C/T nucleotide substitution in exon 1. This primer (Pro/Leu RP) was used together with primer G001 to amplify the segment spanning the PRD of CYP17. Primer specifications are listed in appendix A. Two PCR amplifications were performed, one with genomic DNA template from an A\(^\text{A}^\text{A}\) CYP17 Angora goat and one with genomic DNA template from an A\(^\text{A}^\text{A}\) CYP17 Angora goat. Pwo DNA polymerase and dNTPs were purchased from Roche. The PCR reagents were added as shown in table 6.2. All reactions were overlaid with mineral oil (Promega) and amplified as follows: an initial denaturation step at 96 °C for 2 min, followed by 30 cycles of denaturation at 96 °C for 1 min, annealing at 55 °C for 75 s, and elongation at 72 °C for 3 min, final elongation step at 72 °C for 10 min, and further storage of the reactions at −20 °C.

Table 6.2. PCR amplification reagents with primers targeting exon 1 of Angora goat CYP17.

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Vol. (µl)</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>dH(_2)O</td>
<td>74.5</td>
<td></td>
</tr>
<tr>
<td>10 X Buffer (Supplied)</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>dNTPs</td>
<td>10</td>
<td>200 µM (Each)</td>
</tr>
<tr>
<td>G001 (Left primer)</td>
<td>1.5</td>
<td>300 nM</td>
</tr>
<tr>
<td>Pro/Leu RP (Right primer)</td>
<td>1.5</td>
<td>300 nM</td>
</tr>
<tr>
<td>DNA template</td>
<td>2.0</td>
<td></td>
</tr>
<tr>
<td>Pwo</td>
<td>0.5</td>
<td>2.5 U</td>
</tr>
<tr>
<td>TOTAL</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>
DNA loading dye, 20 µl, was added to the amplification mixture once the reaction was completed and then loaded on a 0.8% preparative agarose gel for electrophoresis. A DNA fragment produced by the PCR amplification was cut from the gel and purified with an Agarose Gel DNA Extraction Kit from Roche according to the manufacturer's instructions. The purified DNA fragments were sequenced as described in section 4.3.2 using 13.3 ng/µl of template DNA and 1.1 ng/µl of primer DNA per sequencing reaction. The right primer used in the PCR amplification was used for sequencing the fragment.

6.3.3 Results

DNA sequences of CYP17 exon 1 were produced for an A'A' and an A'A Angora goat (fig. 6.2). The graphic nucleotide sequences clearly show a double peak at position 122 of exon 1 for the A'A' Angora goat and a single peak at position 122 for the A'A Angora goat. A cytosine peak was found at position 122 for the A'A' Angora goat, which coded for Proline at position 41 in the CYP17 peptide.

**Figure 6.2.** Graphic nucleotide sequences of Angora goat genomic CYP17 (exon 1). Graph A shows the sequence of an A'A'Angora goat and graph B of an A'A Angora goat. Position 125 in both sequences corresponds to position 122 of exon 1.
6.4 Discussion

Development of a CYP17 genotype test for the Angora goat enabled the screening of 83 Angora goats and six Boer goats. One aim of this experiment was to find a possible correlation between the different CYP17 genotypes, and a physical characteristic of the Angora goat associated with stress intolerance. Finding such a correlation could help in breeding programmes aimed at producing more hardened Angora goats. Six Boer goats were also genotyped to see whether they also carried a second CYP17 allele. This was done because Boer goats have been used in the past in breeding programs with Angora goats. The Boer goat, although not a good fleece producer, has many properties aiding its adaptation to extreme environmental conditions. Cross-breeding was aimed at introducing these qualities into Angora goat populations. Although a hardened cross-breed was produced, these goats never produced the same quality of fleece as purebred Angora goats. Furthermore the six Boer goats genotyped produced the same size PCR product as the Angora goats, which upon digestion with Acs I showed the same agarose gel banding pattern after electrophoresis as a CYP17 heterozygote Angora goat. These results certainly lay the foundation for further investigation into the presence of two CYP17 genes in the Boer goat.

Results were obtained for a total of 83 Angora goats and six Boer goats after genotyping 84 Angora goats and six Boer goats. One Angora goat blood sample was lost and another failed to produce a PCR product. All the Grootfontein Angora goats were heterozygotes. Only one, however, was identified as a weak reproducer. Unfortunately this goat had the same CYP17 genotype as the three strong reproducers. No substantiated conclusion could therefore be made from this result alone. It could, however, be speculated that CYP17 heterozygotes are strong reproducers. After the genotype tests for the Little Karoo Angora goats were completed, the breeder was only able to conclusively categorize six of the 20 goats he provided in terms of oily and dry skin. This was unfortunate, since the statistical analyses of the results were compromised. However, a positive correlation was seen among six of the goats (table 6.1). Three had oily skins and three dry skins. The three oily skin goats were heterozygotes and the three dry skin goats were homozygotes. According to the breeder the oily skin goats are hardened compared to the dry skin goats.

The goat described in Chapter 4 from which the CYP17 cDNA was prepared had also been described as hardened. It therefore seems that there is a correlation between the A^A^-CYP17 genotype and the properties generally observed in hardened Angora goats. Whether the A^-A^-
CYP17 genotype (without the Acs I recognition sequence in exon 3) could serve as a marker for hypoadrenocorticism could not conclusively be determined from these results. The current evidence would, however, suggest it to be a good candidate marker. Biochemical analysis of the two different CYP17 enzymes encoded by the different CYP17 genes could clarify the role of CYP17 in hypoadrenocorticism in the Angora goat.

If it is assumed that only the two alleles identified for the Angora goat and Boer goat CYP17 gene exist, the gene could be termed polymorphic. This is because in the population of 83 Angora goats successfully genotyped, both alleles have a significant frequency. If the two CYP17 alleles are designated A⁻ and A⁺, and the homozygote identified is A⁻A⁻, then the allelic frequencies for A⁻ and A⁺ can be calculated with the formula \( P(A^-) = 0.5 \cdot P(A^-A^-) + P(A^-A^+) \) where \( P(A^-A^-) \) and \( P(A^-A^+) \) are the respective genotypic frequencies and \( P(A^-) \) the A⁻ allelic frequency. For A⁺ the equation would be, \( P(A^+) = 0.5 \cdot P(A^+A^-) + P(A^+A^+) \) where \( P(A^+) \) is the allelic frequency for A⁺ and \( P(A^+A^+) \) the genotypic frequency for the unidentified A⁺A⁺ genotype. From the genotype results \( P(A^-A^-) = \frac{24}{83} = 0.29 \) and \( P(A^-A^+) = \frac{59}{83} = 0.71 \) respectively. The genotypic frequencies, for any polymorphic gene in a population, must add up to 1 [135]. The allelic frequencies can now be calculated as: \( P(A^-) = 0.5 \cdot (0.71) + 0.29 = 0.645 \) and \( P(A^+) = 0.5 \cdot (0.71) + 0.0 = 0.355 \) (the A⁺A⁺ genotype was not identified in the tested population, therefore the 0.0 value in the latter equation).

If a Hardy-Weinberg relation existed between the allelic and genotypic frequencies, the A⁺A⁺ genotype would have a frequency of 0.355² = 0.126. However, factors such as mutation, genetic drift, gene flow and selection all alter the Hardy-Weinberg relation between genotypic and allelic frequency. The number of Angora goats tested in this study is relatively small and the interpretations made in terms of population genetics are therefore limited. The A⁺A⁺ genotype that was not identified could possibly be lethal. Should that be the case, the A⁺A⁻ CYP17 genotype must have a selective advantage above the A⁻A⁻ genotype for it to have such a high genotypic frequency. It could be argued that if the CYP17 heterozygote does not cause hypoadrenocorticism, or at least not a severe form thereof, it could produce the selective advantage that would explain its high genotypic frequency.
CHAPTER 7
GENERAL DISCUSSION

The aim of this study was to further investigate the underlying mechanism of hypoadrenocorticism in the South African Angora goat. Engelbrecht et al. previously identified hypoadrenocorticism as the primary cause of the Angora goat’s inability to withstand exposure to extreme environmental conditions [2]. It has been a long time since reduced adrenal function was diagnosed in habitually aborting Angora goats [141]. The reduced adrenal function is responsible for the observed hypoadrenocorticism in the Angora goat, which ultimately results in inadequate cortisol release from the adrenal cortex in response to HPA-axis stimulation.

Previous research on Angora goats showed an abrupt drop in blood glucose concentrations to be largely responsible for insufficient body heat production in the cold stressed Angora goat [6]. Cold stress activates the HPA-axis. This is followed by the release of CRH from the hypothalamus, which stimulates the corticotrophs of the anterior pituitary to secrete ACTH into the general circulation. ACTH then binds to its membrane bound receptor in the adrenal cortex and activates various intracellular signaling pathways. This activation promotes the biosynthesis and release of the glucocorticoids. Glucocorticoid release from the adrenal cortex is a central event in the mammalian response to stress [142]. The ability of glucocorticoids to counteract the manifestations of noxious stimuli helps both man and animal to cope with a wide variety of stresses encountered in everyday life. Any physiological or pathological condition that impairs the glucocorticoid response to stress could have severe implications for an organism if the stressful stimulus is intense and present for long periods of time.

To many farmers Angora goat farming in South Africa is synonymous with large numbers of goats that easily succumb to exposure during cold wet winter nights. The reason for this is the inability of the goats to produce sufficient body heat. This problem has received a great deal of attention from various research groups. It is generally accepted that the hypoglycemia, induced in the Angora goat by cold stress, precipitates hypothermia. Finally hypothermia causes central collapse and death follows. Wentzel developed an alkali-ionophore-treated whole grain that improved the animal’s energy status, which aided in body heat production during cold stress [143]. This nutrient supplement strategy towards combating the Angora goat’s susceptibility to cold stress was, however, not financially viable. A better understanding
of the molecular mechanisms of hypoadrenocorticism could therefore aid in farming with and breeding of Angora goats. It was strongly believed that a genetic predisposition to stress susceptibility might exist in the Angora goat. This prompted an investigation into the CYP17 gene of the Angora goat.

CYP17 of the Angora goat was synthesized by isolating total RNA and mRNA from a randomly selected Angora goat. Sequence analysis of the cDNA produced two unique sequences differing only by four isolated DNA nucleotides. These results indicated the possibility that two copies of the CYP17 mRNA were expressed in the adrenal cortex of a single Angora goat. A possible CYP17 heterozygote was thus identified in a small Angora goat breeding stock in the Western Cape. Both Angora goat CYP17 sequences consisted of a 1530 bp coding region from the ATG translational start to the TGA translational stop site. However, these sequencing anomalies could have been caused by the reverse transcriptase during synthesis of the cDNA since this enzyme is not subject to a high degree of stringency. Therefore the CYP17 genotype test was developed, which successfully genotyped several goats and also proved the nucleotide substitution at position 637 (cDNA sequence) to be a true mutation. Furthermore the C/T nucleotide substitution at position 122 is also believed to be a true mutation. This is because exon 1 of a CYP17 homozygote and heterozygote (as identified by the genotype test described in Chapter 5) Angora goat was sequenced using genomic DNA as template. The sequencing templates for these reactions were produced by conventional PCR, which was preformed using a proofreading thermostable polymerase. This enzyme, unlike the reverse transcriptase, is subject to a high degree of stringency. The homozygote produced only a C peak at position 122 and the heterozygote produced a double C/T peak, as was originally shown from the cDNA sequence. Whether this mutation is linked with or segregated from the G/A substitution mutation at position 637 is undetermined. To determine this exon 1 of many more CYP17 homozygote Angora goats (Acs I tested) need to be sequenced. The other two possible base mutations have not been investigated and can thus not be confirmed as true mutations. Sequencing of the cloned Angora goat CYP17 cDNA constructs could, however, confirm those nucleotide substitution mutations and assure the correct assignment of each base to the right CYP17 allele.

Cloning of the two Angora goat CYP17 cDNA constructs has, to date, been unsuccessful. However, a CYP17 cDNA sequence is available on GENBANK for Capra hircus, the Boer goat. This sequence is 100% homologous to the Angora goat (Capra aegagrus) sequences generated in this study, except for the four loci with the substituted bases. Furthermore, the
bases at the four loci differing in the two Angora goat sequences are in the Boer goat always one of the two nucleotides identified in the heterozygote Angora goat. It therefore strongly suggests that the one Angora goat CYP17 cDNA sequence would be identical to the Boer goat sequence. This likelihood is further supported by the cross-breeding practices that occurred between Angora goat and Boer goat breeding stocks.

Identification of a CYP17 heterozygote Angora goat theoretically implied the existence of two homozygote CYP17 genotypes. To identify such possible homozygote genotypes, several goats had to be screened for the different CYP17 combinations. This was done by the genotype test as described in Chapter 5. A further objective was to find a possible correlation between the CYP17 genotypes and a phenotype associated with either stress tolerance or stress intolerance. For this purpose some Angora goats were categorized according to certain stress related phenotypes and then genotyped. From the 89 goats genotyped in this study, the CYP17 homozygote without the AcI recognition sequence in exon 3 was never identified.

Of all the Angora goats genotyped, 20 were from the Little Karoo. An attempt was made to categorize them phenotypically according to whether they had oily or dry skins, but only six were confidently categorized, three with oily skins, associated with stress tolerant characteristics, and three with dry skins, associated with stress intolerant characteristics. Of the 20 goats only 18 were successfully genotyped. Although statistically inconclusive, there was a 100% genotype to phenotype correlation for the six goats that were successfully identified according to their phenotypes. The three oily skin goats were CYP17 heterozygotes, and the three dry skin goats were homozygotes. The four ewes from the Grootfontein agricultural research farm were all CYP17 heterozygotes. Of these goats one was a weak reproducer and the other strong reproducers. Although thus far only a small number of goats have been genotyped, it seems likely that the identified CYP17 heterozygote genotype is associated with a more hardened phenotype among Angora goats.

It should further be noted that all the goats tested were adults. The allelic and genotypic frequencies therefore only extend to the adult Angora goat population. Hypoadrenocorticism, however, is responsible for high kid mortality and high numbers of spontaneous abortions among pregnant ewes [2]. If a CYP17 genotype is responsible for the fatalities among young goats and the abortions, CYP17 genotype frequencies will very likely differ in a population of Angora goat kids and aborted embryos. Further investigations should therefore determine the CYP17 genotype of aborted fetuses and young goat corpses.
Sequence results from the Angora goat CYP17 cDNA revealed four possible DNA nucleotide substitutions between the two copies. Of these only three translated into amino acid substitutions. One of these amino acid substitutions at amino acid position 41 from the amino terminal of the protein is in a highly conserved region of CYP17 [133]. Furthermore, this amino acid substitution is caused by the nucleotide substitution in exon 1 of Angora goat CYP17, which is believed to be a true mutation. This 13 amino acid conserved stretch (amino acid 32 to 44) is called the proline rich domain (PRD). The PRD is believed to play an important role in the correct folding of the enzyme, especially by stabilizing the conformation before heme binding [132,133]. Substitution of the prolines in this region for other amino acids through site directed mutagenesis greatly impairs CYP17 activity. Of the two CYP17 copies identified in the Angora goat, one has a proline at position 41 and the other a leucine.

Sequencing data from the genomic copy of CYP17 from a CYP17 homozygote Angora goat mapped the proline at position 41 to the CYP17 homozygote identified in this study. Furthermore, this CYP17 homozygote seems to produce a more stress intolerant phenotype associated with hypoadrenocorticism. If the leucine at position 41 were to abolish or significantly impair CYP17 activity, the A⁺A⁺ genotype (leucine at position 41) would hardly be associated with hypoadrenocorticism that is caused by a heightened flux through CYP17 activity. The same premise would explain why the CYP17 heterozygote produces a lower flux through the Δ5 steroid pathways compared to the identified A⁺A⁻ homozygote. This would explain why the identified CYP17 homozygotes are those bearing characteristics associated with hypoadrenocorticism.

Any predictions made regarding the influence a specific Angora goat CYP17 mutation could have on adrenal steroidogenesis are purely speculative. For such conclusions to be made, the enzyme kinetics and substrate specificity of the two different CYP17 enzymes encoded, will have to be done through in vitro expression and analysis.

The six genotyped Boer goats were all CYP17 heterozygotes. Although no A⁻A⁻ homozygotes were identified among Boer goats they are believed to exist, since only a very small number of Boer goats were tested. The Boer goat population in the Eastern Cape is very hardened. It should be noted that one genetic variant that causes a stress intolerant phenotype in one species would by no means necessarily have the same effect in another. Furthermore, the GENBANK sequence for Capra hircus (Boer goat) CYP17 cDNA made no mention of a polymorphism and showed a leucine at position 41.
In the final analysis it would seem that the different CYP17 genotypes present in the South African Angora goat population could possibly contribute to the stress intolerant phenotype. The only way in which a CYP17 genotype could precipitate such an effect is to alter the balance of adrenocortical steroid output. This is well in accordance with the previously identified hypoadrenocorticism in the Angora goat. This condition does indeed cause a shift in the flux through the steroidogenic pathways, and ultimately results in inadequate cortisol release from the adrenal cortex [7]. The two cDNA constructs encoding Angora goat CYP17 are still not cloned. This needs to be done before the two enzymes could be expressed separately in non-steroidogenic tissue culture. *In vitro* studies of the activities of the different CYP17 enzymes encoded by the two cDNA constructs, would conclusively confirm whether a CYP17 genotype is responsible for the altered steroidogenic output in the Angora goat adrenal. It could further predict to what extent any of the three CYP17 genotypes could confer hypoadrenocorticism to an Angora goat.

The CYP17 genotype test developed in this study now enables those investigating the stress susceptibility of the Angora goat to further interpret their findings in terms of a genetic variation strongly believed to influence the Angora goat’s stress response. Engelbrecht et al. made the first contributions towards understanding the molecular mechanism of the Angora goat’s impaired stress response [7]. With a better understanding today of hypoadrenocorticism, as seen in the Angora goat, and also with the knowledge of the genetic polymorphism found in a gene involved with adrenal steroidogenesis more focused research strategies can be developed. This study has identified at least two Angora goat CYP17 alleles. However, more might exist since four nucleotide substitutions were detected in the cDNA sequence of which only two were shown to be true mutations. The foundation is thus laid by this work for further in depth investigations into Angora goat CYP17 allelic distributions.

To obtain more reliable numbers for the allelic frequencies in the Angora goat population of the two identified CYP17 alleles, many more goats need to be genotyped. Such testing could provide useful information about the distribution of stress intolerance compared to that of the different CYP17 genotypes. It is also important that consensus be reached as to what physical properties indeed produce a hardened goat for such testing to have any significance. Whether selecting for a specific CYP17 genotype produces a more hardened goat that still produces fleece of sufficient quality remains to be seen.
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APPENDIX A

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LP - Left primer
RP - Right primer

All primers used were dissolved in water.
APPENDIX B

Angora 1 and Angora 2 represent the two CYP17 cDNA sequences and Goat represents the *Capra hircus* CYP17 cDNA sequence. The Angora goat nucleotide bases that differ from the goat sequence are indicated with red arrows. The Acs I recognition sequence is indicated in blue (bp 635).

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APPENDIX C

Angora 1 and Angora 2 represent the two CYP17 cDNA sequences and genomic represents the partial Angora goat genomic CYP17 sequence.
ANGORA 1
GENOMIC
ANGORA 2

1860 1870 1880 1890 1900

.................................| 490
ANGORA 1
GENOMIC
ANGORA 2

1910 1920 1930 1940 1950

.................................| 490
ANGORA 1
GENOMIC
ANGORA 2


.................................| 516
ANGORA 1
GENOMIC
ANGORA 2

2010 2020 2030 2040 2050

.................................| 516
ANGORA 1
GENOMIC
ANGORA 2

2060 2070 2080 2090 2100

.................................| 566
ANGORA 1
GENOMIC
ANGORA 2

2110 2120 2130 2140 2150

.................................| 566
ANGORA 1
GENOMIC
ANGORA 2

2160 2170 2180 2190 2200

.................................| 616
ANGORA 1
GENOMIC
ANGORA 2

2210 2220 2230 2240 2250

.................................| 616
ANGORA 1
GENOMIC
ANGORA 2

2260 2270 2280 2290 2300

.................................| 716
ANGORA 1
GENOMIC
ANGORA 2

2310 2320 2330 2340 2350

.................................| 716
ANGORA 1
GENOMIC
ANGORA 2
ANGORA 1
GGTGAAGATCGAGGTGCGCCAGGCCTGGAAGGAAGCCCAGGCTGAGGGTA 766
ANGORA 2
GGTGAAGATCGAGGTGCGCCAGGCCTGGAAGGAAGCCCAGGCTGAGGGTA 766

2360 2370 2380 2390 2400
ANGORA 1
GTCACCTGATGA 777
ANGORA 2
GTCACCTGATGA 777

2410
ANGORA 1
AGCTCCCCCGCSTC 2415
ANGORA 2

Appendix D shows the conserved proline rich domain (PRD) of CYP17. Amino acids 32 to 44 are indicated using one letter amino acid abbreviations. Angora 1 and Angora 2 represent the PRD sequences for the A^-A^- and A^+A^- CYP17 genotypes respectively.

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32 33 34 35 36 37 38 39 40 41 42 43 44
APPENDIX E

The numbers for each lane correspond to the goat numbers in table 6.1 except for numbers 19 – 79, which represent the goats from Grootfontein that were not phenotypically categorized. Of these the following numbers were homozygotes: 20, 23, 25, 39, 55, 56, 60, 66, 67, 69, 72, 73, 74 and 75. M1 represents DNA marker DNAλ/Eco RI and DNAλ/ Hind III (Promega) and M2 represents the 1 kb Plus DNA ladder (Life Technologies).