Regulation of Wool and Body Growth: Nutritional and Molecular Approaches

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Abstract

The partitioning of nutrients between wool and muscle growth involves complex physiological and metabolic events. The first experiment in this study aimed to develop an understanding of the processes controlling growth and the partitioning of protein between the skin and the whole body. Novel selection of phenotypically-distinct Merino sheep, that differed widely in wool production and body weight enabled investigation of both traits concurrently. The manipulation of feed intake and measurement of wool growth rate (WGR), body weight (BW), nitrogen (N) balance and plasma insulin-like growth factors (IGFs) addressed the hypotheses that the partitioning of protein between wool and body growth is regulated by the IGFs (IGF-I and IGF-II), and that these differences in partitioning are expressed to a greater extent at a high level of intake (1.8 x maintenance versus 0.9 x maintenance).

The quantity of protein (nitrogen) retained and partitioned between wool growth and body growth was related to plasma IGF-I concentration but not plasma IGF-II concentration. The proportion of retained N that was partitioned to wool or body growth did not differ between phenotypic groups, at either intake level. Despite significant variation in WGR between phenotypic groups when selected in the field prior to conducting the pen study, similar WGRs were measured for all phenotypic groups during the experiment. This variability and sensitivity of wool growth to the environment and nutrition was the focus of the second experiment, which investigated the partitioning of sulphur-containing amino acids (SAA) to wool production.

South Australian Merino sheep selected using estimated breeding values (EBVs) for wool and body traits, formed two genetic selection groups of sheep that were divergent in fleece weight (W) yet similar in body weight, follicle density and fibre diameter. Investigation of wool growth variability in divergent groups (+W, -W), without the confounding of information due to variable follicle numbers and body size, enabled physiological and

metabolic comparisons with studies of other fleece-weight (FW) selection lines and the examination of molecular determinants of genetic differences in fibre growth and composition, in industry-relevant sheep. The general hypothesis tested was that differences in wool production between selection groups arise from variability in the partitioning of sulphur to the skin and follicles, and/or variability in the expression of different keratin genes in response to sulphur supply.

Wool growth response to nutrition (low-sulphur diet versus high-sulphur diet) was similar for both groups, but some differences in wool characteristics between selection groups were identified. Although fibre diameter was also similar, the +W selected sheep produced longer fibres contributing to divergent wool production. Wool sulphur (S) concentration was lower in +W sheep, as for other FW selection flocks, but the total output of S in the wool of these sheep was greater, indicating higher uptake and utilisation of S by the wool follicles of +W sheep. This occurred despite similar apparent S digestion, S balance and plasma SAA concentration. To maintain the high levels of wool growth measured in this study, both selection groups lost weight and required SAA from mobilised tissue protein to support this wool growth.

Greater fibre production despite equivalent supply of S to follicles in +W sheep compared to –W sheep, may be the result of greater efficiency of S uptake into the follicles or differential expression of keratin genes in the skin of +W sheep. Expression of keratin genes including keratin-associated protein (KAP) 2 (a high-S gene), KAP 4 (an ultra-high S gene), KAP 6 (a high glycine/ tyrosine gene) and the intermediate filament gene K 2.10 did not differ significantly between divergent +W and –W groups. KAP 2 and K 2.10 independently accounted for approximately 5% of the variation in WGR but no genes examined were significantly related to the S content of the fibre produced. Expression of the oASCT2 neutral amino acid transporter was detected in skin from these sheep however quantification of differential expression levels between genotypes was not possible suggesting a more sensitive

technique may be required to detect low levels of expression. Thus it appears that greater WGR in the +W selection group was related to greater follicle rate processes and possibly transporter activity or number, but not differential expression keratin genes.

Declaration

This thesis contains no material which has been accepted for the award of any degree or diploma in any University or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference is given.

I give consent to this copy of my thesis, when deposited in the University library, being available for loan and photocopying.

Megan Bray

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Abbreviations

µg:	Microgram
µl:	Microlitre
µm:	Micron
+FW:	Sheep selected for plus-fleece weight
-FW:	Sheep selected for minus-fleece weight
+W:	Sheep selected for plus CFW EBV
-W:	Sheep selected for minus CFW EBV
+W+BW:	Sheep selected for high clean fleece weight and high body weight phenotype
+W-BW:	Sheep selected for high clean fleece weight and low body weight phenotype
-W+BW:	Sheep selected for low clean fleece weight and high body weight phenotype
-W-BW:	Sheep selected for low clean fleece weight and low body weight phenotype
0.9xM:	0.9 times the feed requirement to maintain body weight
1.8xM:	1.8 times the feed requirement to maintain body weight
ad libitum:	Without restraint
ALB	Agarose load buffer
oASCT2	Neutral amino acid transporter
bp:	Base pair
BW:	Body weight
C:	Control sheep (unselected)
cDNA:	Complementary DNA
CFW:	Clean fleece weight
DEPC:	Diethyl pyrocarbonate
DTT:	Dithiothreitol
DMD:	Dry matter digestibility
DM:	Dry matter
DNA:	Deoxyribose nucleic acid
EBV:	Estimated breeding value
et al:	and others
FD:	Fibre diameter
FL:	Fibre length
FL: FD:	Fibre length to fibre diameter ratio
FGLB:	Formaldehyde gel load buffer
FRB:	Formaldehyde running buffer
g:	Gram
GAPDH:	Glyceraldehyde-6-phosphate dehydrogenase
GH:	Growth hormone
HGT:	High glycine-tyrosine wool proteins/ genes
HPLC:	High performance liquid chromatography
HS:	High sulphur-containing diet *or* high sulphur wool proteins/ genes
IF:	Intermediate filaments
IGF-I:	Insulin-like growth factor-I
IGF-II:	Insulin-like growth factor-II
IRS:	Inner root sheath
KAP:	Keratin associated protein
kb:	Kilobase
kg:	Kilograms
LS:	Low sulphur-containing diet *or* low sulphur wool proteins/ genes
mg:	Milligram
mol%:	Molar percent
MQ water:	Milli-Q- filtered water

mRNA:	Messenger RNA
N:	Nitrogen
ng:	Nanogram
NIRS:	Near-infrared spectrometry
ORS:	Outer root sheath
PCR:	Polymerase chain reaction
pmol:	Picomol
RNA:	Ribose nucleic acid
rRNA:	Ribosomal RNA
Real-time RT-PCR:	Real-time reverse-transcriptase polymerase chain reaction
S:	Sulphur
SAA:	Sulphur-containing amino acids
SARDI:	South Australian Research and Development Institute
SDF:	Selection demonstration flock
SDS:	Sodium dodecyl sulphate
SEM:	Standard error of the mean
SSC:	Sodium citrate/ Sodium chloride buffer
TAE:	Tris-acetate EDTA buffer
TRN:	Trichohyalin
UHS:	Ultra-high sulphur wool proteins/ genes
v/v:	Volume : volume
WGR:	Wool growth rate
WW:	Weaning weight selection lines

Chapter 1

Introduction

1. Introduction

The complexity associated with nutrient utilisation for peripheral tissues such as skin and wool, and growth and maintenance of the whole body, has particular implications to production from Merino sheep, where economic returns from both wool and meat are important. Of particular relevance are the insulin-like growth factors (IGFs), which produce morphogenic and mitogenic effects, and are necessary in cell differentiation and cell cycling (Jones and Clemmons, 1995). For example, administration of IGF-I increases net protein retention across the hindlimb (Oddy and Owens, 1996) and increases amino acid use for protein synthesis and blood flow in the skin of sheep (Harris et al, 1993a; Hocking-Edwards et al, 1995). A direct role of the IGFs in the coordination of nutrient use, in the skin and the whole body, is yet to be elucidated.

The production of fibre is limited by the supply of sulphur-containing amino acids (SAAs) cysteine and methionine, however our knowledge of the mechanisms involved in SAA utilisation by the skin and wool follicle remains incomplete. A large proportion of the information pertaining to this has been gained from investigations of SAA use in fleece-weight selection lines of sheep that produce divergent quantities of wool. Any variability in the digestion and absorption of SAAs, their supply to the skin, SAA uptake by the follicle and expression of any number of genes by the follicle to produce keratin proteins, may contribute to differences in wool growth. The digestibility of SAA is similar between high (+FW) and low (-FW) fleece weight lines (Piper and Dolling, 1969), and plasma cysteine concentration and wool S concentration are consistently higher in +FW Merinos, but not in +FW Romneys (Piper and Dolling, 1966; Williams et al, 1972a; Sun et al, 1991; Sun et al, 1994). Nutritional and genetic variability may be responsible for some of these inconsistencies, but further investigation is warranted. No investigation of mechanisms controlling cysteine uptake into the follicle, nor the expression of different keratin genes or proteins, in FW selection lines

have been undertaken, but these remain as probable sources of wool growth variation between high- and low- wool producing sheep.

Nattrass (2000) isolated and sequenced the oASCT2 neutral amino acid transporter from sheep follicles and reported higher levels of expression in the follicle compared with other tissues, although comparative work in skin from sheep producing divergent quantities of wool was not investigated. A greater proportion of high S wool proteins have been extracted from the wool of a low- than high- producing sheep (Gillespie and Reis, 1966), suggesting that SAA uptake into the wool is associated with the rate of wool growth. Current techniques in molecular biology enable investigation of wool (keratin) proteins at the gene expression level in sheep producing divergent quantities of wool.

The general aim of this thesis was to understand the utilisation of nutrients for growth of wool and the whole body, by comparing nutritional, hormonal and molecular variability in sheep well-characterised for wool and body growth. More specifically, aims were to:

a) investigate the association of insulin-like growth factors (IGFs) and nutrition with the partitioning of protein (nitrogen) between wool and body growth,
b) investigate the partitioning and utilisation of sulphur-containing amino acids (SAA) for wool growth in Merinos genetically divergent in wool production and
c) examine the influence of nutrition on the expression of keratin genes in Merinos genetically divergent in wool production.

Chapter 2

Literature Review

2.1 Wool growth

2.1.1 Cellular and molecular events in fibre formation.

The wool follicle forms as a downward growth of the epidermis that begins *in utero* at approximately day 50 of gestation (primary follicles) in the sheep, followed by the development of the sweat glands and arrector pili muscle 20 days later (Figure 2.1). Secondary follicles are usually smaller, do not have the same associated structures as primary follicles and form at approximately day 85 of gestation. Fibre formation begins soon after the follicles have formed, as cells of the lower bulb region proliferate, differentiate and express numerous proteins, in particular keratin intermediate filaments (IFs) and keratin associated proteins (KAPs). In this review the physical structure of the follicle, the cellular events involved in fibre formation and the expression of keratin genes in fibre cell types are described.

2.1.1.1 Follicle structure and fibre formation

2.1.1.1.1 The skin

The follicle is comprised of a number of cell types organised concentrically within the skin (Figure 2.1). The epidermal cell layer forms a continuous, protective outer layer under which the supportive framework of the dermis is integrated, providing elasticity and support. The dermis also supplies the epidermis with nutrients via a vast capillary network. Skin, in sheep, contributes to approximately 14% of the total protein mass of the animal (Lobley, 1994) and an additional 15-25% of total body protein is produced in wool (MacRae et al, 1993). The proportion of whole body protein in wool is influenced by genotype, the amount and composition of the diet consumed. Liu et al (1998) estimated that approximately 18% of protein synthesis in the skin of Merinos contributes to wool growth, intermediate to the values reported in Romney ewes (Harris et al, 1994) and Suffolk cross lambs (Nash et al, 1994). The skin itself contains a large proportion of sulphur-containing amino acids (SAA), as does the fibre, and high proline and hydroxyproline concentrations contribute to the large quantity of

collagen protein in the skin. The fractional synthetic rate of protein in skin is high compared to the muscle (between 5% and 18%/d in skin and 2% to 3%/d in muscle, depending on nutrition) (Harris and Lobley, 1991; Liu et al, 1998) thus it can provide a highly-labile source of proteins for use elsewhere in the body if required.

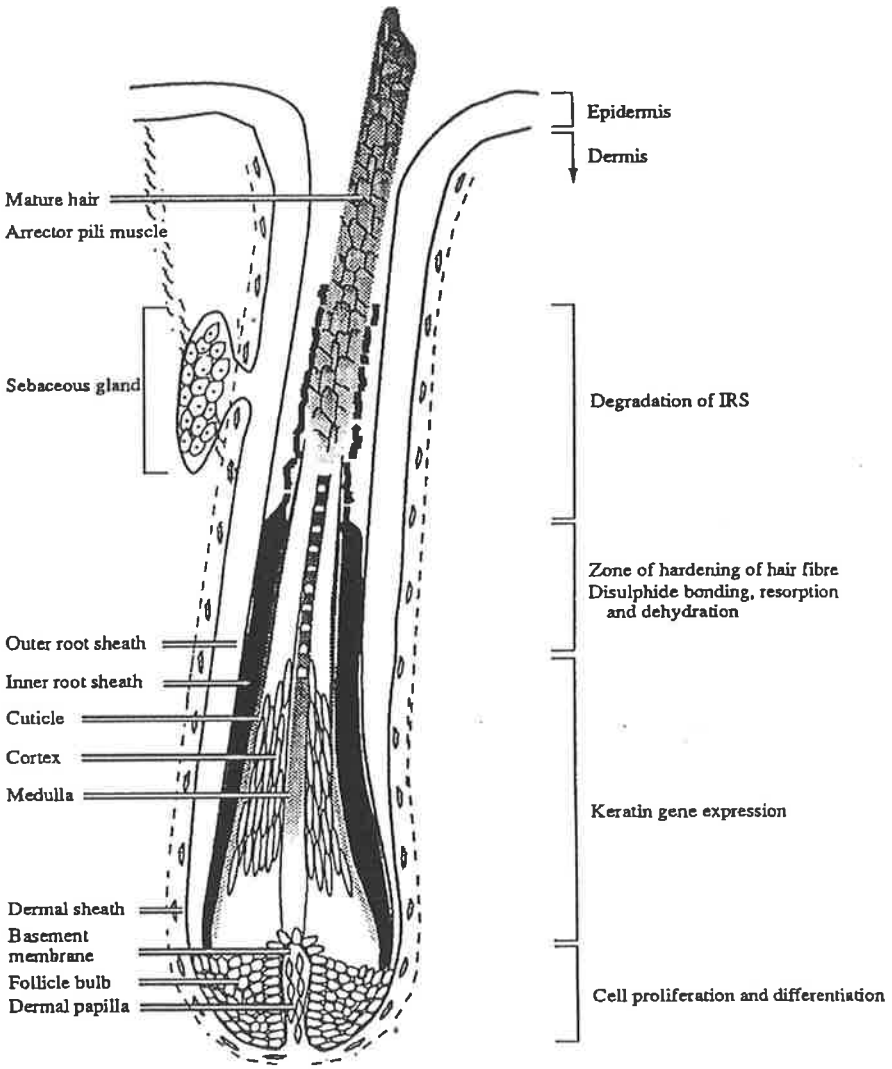

Figure 2.1: Diagrammatic representation of the hair follicle (Powell and Rogers, 1997).

2.1.1.1.2 The follicle bulb

Follicle bulb cells proliferate rapidly and are located at the base of the follicle, providing a source of cells for production of fibre and supportive layers of the follicle including the inner root sheath (IRS) (Figure 2.1). The rate of proliferation is influenced to a large extent by nutrition; a turnover rate of 20 hours may be expected when a Merino is fed a high level of nutrition (30g/kg body weight), and approximately 35 to 40 hours when fed a low level (10g/kg body weight) (Wilson and Short, 1979). In the wool follicle bulb, cells that

are destined to form the fibre or inner root sheath (IRS) layer surround a small group of mesenchymal cells the dermal papilla cells. These cells play an important regulatory role in follicle initiation (Reynolds and Jahoda, 1992), maintenance of the hair cycle (Holbrook et al, 1993) and possibly in signalling to follicle stem cells, thus stimulating fibre growth (Cotsarelis et al, 1990). The supply of follicle bulb cells and their distribution as they differentiate into the various layers of the fibre and IRS (Section 2.1.3.3.4) will influence the rate of wool growth, which averages between 300-400μm in length per day in the Merino (Hynd, 1989b).

2.1.1.1.3 The outer root sheath (ORS)

The outer root sheath (ORS) layer of cells is continuous with the epidermis and its main function is to support the fibre and the follicle (Powell and Rogers, 1994). The ORS maintains its own cell population, unlike the fibre and IRS, with cellular proliferation occurring along the length of the ORS. It has been suggested that follicular stem cells reside throughout this layer in the Merino wool follicle (Bray et al, 1998), supplying the follicle with a pluripotent cell population. ORS cells express epidermal type keratin genes, as opposed to follicular keratin genes that are expressed in other fibre and follicular cells (Section 2.1.1.2) (Coulombe et al, 1989).

2.1.1.1.4 The inner root sheath (IRS)

The inner root sheath (IRS) consists of three concentric cell layers : Henle's layer (the outermost layer, adjacent to the ORS); Huxley's layer; and the IRS cuticle. These cell layers all differentiate from the cells of the bulb matrix. Cells of the IRS migrate up the follicle, supporting the growing fibre until it reaches the level above the sebaceous gland, where the fibre emerges from the skin and IRS cells are lost from the skin surface. Aside from this supportive role, the IRS cuticle cells are involved in the development of a scale pattern associated with the fibre (Orwin, 1989). Differentiation of the IRS cells is marked by the formation of trichohyalin protein granules, which form a matrix between the keratin filament proteins (Section 2.1.1.2). Variation in the rate of IRS cell differentiation and changes in the

trichohyalin molecule distinguish the three IRS cell types from one another (reviewed by Powell and Rogers, 1997).

2.1.1.1.5 The fibre cortex and cuticle

Hair and wool fibre is comprised of cortical, cuticular and medullary cells that are derived from the bulb cell population. However, in fine fibres including Merino wool, the medulla is absent and therefore, is not discussed further in this review. Cortical cells are approximately 100μm long and 5-10μm wide, and the spindle shaped cells align in an overlapping fashion along the length of the fibre (Rogers et al, 1998). Two main cortical cell types, orthocortical and paracortical cells, differ in their distribution and pattern of expression of the two major keratin gene groups, the intermediate filament proteins (IF) and keratin associated proteins (KAP). These cell types position bilaterally within the fibre, contributing to wool characteristics including crimp definition. Orthocortical cells follow the outside of the fibre crimp and have a lower sulphur (S) concentration than paracortical cells, with IF and KAP matrix proteins grouped closely together in an organised pattern. Paracortical cells align with the inside of the crimp and have a higher S concentration, with IF and KAP matrix proteins forming a continuous mass. In addition, orthocortical cells have more IF proteins, and paracortical cells more KAP matrix proteins. The rate of supply of differentiated cortical cells from the bulb, their final elongated size and their arrangement within the fibre influences the length and diameter of the wool fibre produced.

The cuticle consists of a single layer of overlapping, flattened cells surrounding the cortical cells. Each cuticle cell is comprised of three layers, the A, exocuticle and endocuticle layers, which together act as a barrier to the fibre, but one which allows dyes and water to penetrate (Montagna and Parakkal, 1974 ; Orwin, 1989).

2.1.1.2 Keratin gene expression in the wool fibre

As cells of the follicle differentiate and migrate from the follicle bulb region, keratin intermediate filament (IF) and keratin associated proteins (KAP) genes are sequentially

expressed in cortical and cuticle cells, whilst trichohyalin is the predominant protein produced in IRS cells. The initial stages of keratinisation and fibre formation involve expression of IF genes, then KAP genes are expressed and a protein matrix forms between the IFs. This sequence of gene expression is depicted in Figure 2.2. The nomenclature associated with the keratin genes was recently reviewed by Powell and Rogers (1997) and briefly, IFs are referred to as K 1 (type I) or K 2 (type II) proteins, and KAPs are numbered KAP 1 through to KAP 11, depending on the gene sequence and where in the follicle it is expressed.

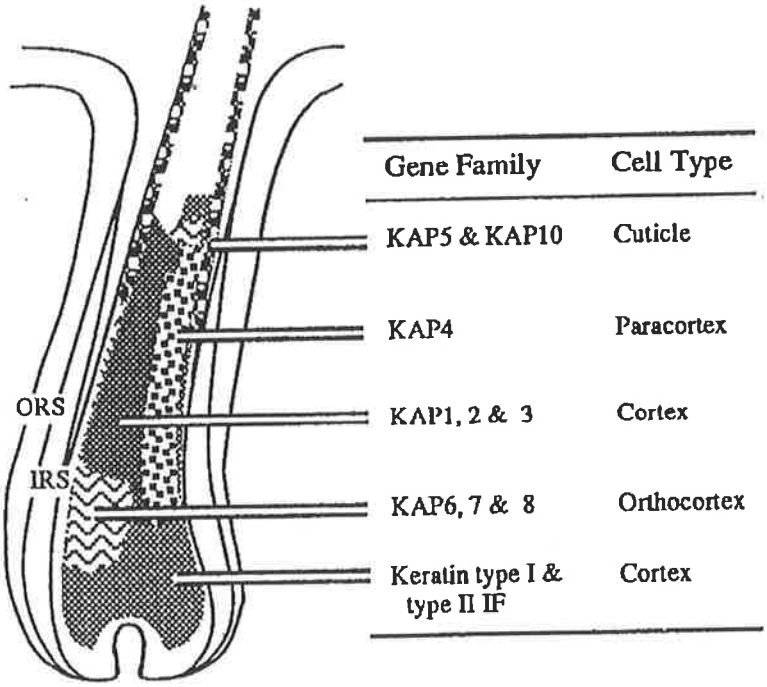

Figure 2.2: Localisation of gene expression in cortical and cuticle cell types in the fibre (Powell and Rogers, 1997).

2.1.1.2.1 Keratin intermediate filaments

Intermediate filament (IF) genes are the first keratin genes expressed in fibre cells, with low levels of expression initially localised to central bulb cells and with increased expression as the cells migrate to the lower shaft region. IF proteins have a low cysteine content (5 mol % cyst) and form 8-10 nm long filaments in the cell cytoplasm. Cysteine residues at the end domains of these filaments allow extensive disulphide bond formation and cross linking with the KAP proteins that form the fibre matrix (Section 2.1.1.2.2). There are two main sub groups of IFs, type I and type II, which are expressed concurrently and form a heteropolymeric structure, the fundamental unit of keratin IFs. Sequence data and gene expression patterns of IFs in various species have been reviewed extensively (Powell and Rogers, 1997). Of relevance here, the expression of K 2.12 (type II IF) in the Merino wool follicle is localised in cells from the mid-bulb region, followed by concurrent expression of K 2.10 and K 2.9 in cells further up the wool follicle shaft, and corresponding simultaneous expression of a number of type I IF genes (Powell et al, 1992).

2.1.1.2.2 Keratin associated proteins (KAP)

Expression of the KAPs in the fibre matrix occurs sequentially in the lower to mid follicle region, following the commencement of IF protein synthesis (Figure 2.2). KAPs are grouped depending on their amino acid composition, including glycine-tyrosine rich (HGT) KAPs and cysteine-rich KAPs, the latter of which has both high sulphur (HS) and ultra high sulphur (UHS) subgroups. Differential expression and formation of these proteins inevitably influences the properties of the fibre produced, including crimp definition and fibre strength (Campbell et al, 1975; Reis, 1979).

The glycine-tyrosine rich KAPs

High glycine-tyrosine (HGT) genes are the first KAPs expressed in fibre cells, initially in the lower to mid bulb region. In general, HGT KAPs contain high proportions of glycine and tyrosine residues, between 34 and 65% of total residues, and are synthesised primarily in orthocortical cells. KAP 6 is the major family of HGT proteins, containing approximately 80

amino acids, 11 mol% of which are cysteine. In addition KAP 7 and KAP 8 are unique HGT proteins containing approximately 84 and 61 amino acid residues respectively, of which approximately 6 mol% are cysteine (Powell and Rogers, 1994).

The cysteine rich KAPs

Genes encoding the high sulphur (HS) KAPs (KAP 1, 2, 3) are co-expressed initially in the orthocortical cells following HGT KAP synthesis, and then in all cortical cells soon after. These proteins are comprised of between 94 and 181 amino acid residues, and between 16 and 24 mol% of these residues are cysteine (Powell and Rogers, 1994). Later, ultra high sulphur (UHS) KAPs are expressed, including the predominantly paracortical KAP 4 gene family (Powell, unpublished) which contains approximately 200 amino acid residues, more than 30 mol% of these being cysteine. The KAP 5 gene family (UHS), containing 33 mol% cystine and 27 mol% glycine, is expressed in differentiated cuticle cells well above the follicle bulb (MacKinnon et al, 1990), as is the KAP 10 family. Furthermore, a number of KAP gene families including the UHS KAP 9 family (McNab et al, 1989), the LS KAP 11 family (Huh et al, 1994), and possibly up to 100 other KAP genes yet to be identified have an unknown effect on the expression pattern and fibre structure.

2.1.2 The importance of sulphur-containing amino acids (SAA) for wool growth

The supply and composition of absorbed protein is the most important nutritional limitation to wool growth. Availability of the sulphur-containing amino acids (SAA; methionine and cysteine) is the major nutritional influence affecting the rate of wool production. Reis and Schinkel (1963) demonstrated the effect of SAA supply on this relationship, by infusing SAAs abomasally, which led to a 40% - 130% increase in the rate of wool growth and a 30% increase in the S concentration of the wool. Marston (1928) assumed that wool contained a constant concentration of sulphur (S) and that the majority of this S was derived from cystine residues. An inverse relationship between the rate of wool growth and its concentration of S was initially hypothesised by Ross (1961, 1964) and subsequently

confirmed in Merino lines divergently selected for fleece weight (Piper and Dolling, 1966) and in phenotypically distinct sheep (Reis et al, 1967).

Initial trials designed to stimulate wool production by increasing dietary cystine intake were unsuccessful (Du Toit et al, 1932 and Marston, 1932, cited by Reis and Schinkel, 1963), in contrast to the positive growth response when supplied subcutaneously (Marston, 1935). SAAs are present in a large proportion of pasture species, however once ingested, the microflora and fauna of the rumen rapidly degrade them rendering them unavailable post-ruminally, unless incorporated into microbial protein. Tissue and plasma protein turnover (Downes et al, 1965), and the transulphuration of SAAs via the transulphuration pathway (Figure 2.3), operating primarily in the liver and to a lesser extent in the skin (Egan et al, 1984), can improve the supply of cysteine to the follicle. Intermediates involved in the metabolism of methionine to cysteine, including homocysteine, that have been infused intravenously or abomasally, have stimulated wool production (Doyle, 1981; Reis et al, 1990). Therefore, supply and metabolism of SAAs can affect their utilisation for wool growth.

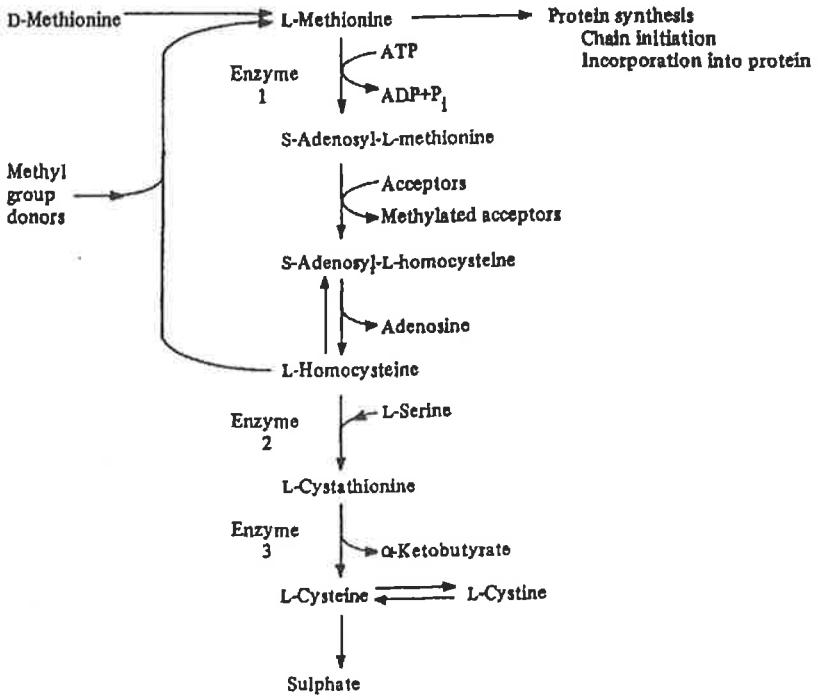

Figure 2.3: The transulphuration pathway: production of cysteine via the transulphuration of methionine (adapted from Finkelstein, 1970).

2.1.3 Selection lines for wool growth in sheep

The generation of single trait, fleece weight (FW) selection flocks in the 1950s contributed to our improved understanding of physiological parameters associated with wool production. This review focuses on three separate selection flocks that were developed in Australia and New Zealand; the Merino clean fleece plus (+FW) and fleece minus (-FW) flocks at Trangie (Turner et al, 1970), Merino +FW and –FW flocks at Cunnamulla (Pattie and Barlow, 1974), and Romney greasy fleece weight selected (+FW) and control (C) lines at Massey University in New Zealand (Blair, 1981). Other fleece weight selection flocks have been developed but the majority of published research refers to the three aforementioned flocks. In this review, information from these flocks regarding physiological and metabolic variability, skin, follicle and fibre characteristics, and some endocrinological comparisons, between genetically high and low wool producers, are summarised.

2.1.3.1 Nutrition and wool growth

In general +FW selected Merino sheep are more responsive to improved nutrient supply, such that the increase in wool growth associated with improved nutrition is proportionally greater than in –FW selected sheep (Williams, 1966; Williams et al, 1972b; Williams, 1995). The potential rate of wool growth of a sheep is genetically independent of its capability to increase body weight with improved nutrition (Williams, 1987). The degree of skin wrinkling is highly variable across high and low wool-producing sheep, thus it can be concluded that variation in wool production across selection lines largely stems from differences in the output of wool per unit area of skin, and not from consistent differences in the surface area, body size or wrinkling of the skin.

2.1.3.1.1 Efficiency of converting feed to wool

Indoor trials have demonstrated the superiority of genetically-high wool producers in the efficiency of converting crude protein, dry matter intake and SAA into wool fibre production (Dolling and Moore, 1960; Ahmed et al, 1963; Williams, 1966). This superior

efficiency has been replicated in field trials, in which the difference in efficiency between +FW and -FW sheep was at least 40%, depending on the availability of feed in the pasture (Hamilton and Langlands, 1969). The difference in efficiency between genetically divergent groups was greatest at high levels of nutrition, but absolute efficiency of wool production was highest at low levels of nutrition (Williams, 1966).

2.1.3.1.2 Feed intake and digestive functions

Some trials have shown a higher level of intake in +FW sheep when feed was offered in unlimited quantities (Ahmed et al, 1963; Hamilton and Langlands, 1969), but others have not found this to be the case (McClelland et al, 1986; Clark et al, 1989; Faichney et al, 1998) and in some instances, differences in intake were only apparent when the diet was of a high quality (Thomson et al, 1989).

The +FW sheep have maintained their superior efficiency of wool production in a range of situations despite variability in *ad libitum* feed intake suggesting that differences in the digestive function between high- and low- producing sheep may exist. However, no studies have found this to be the case. Piper and Dolling (1969) and McClelland et al (1986) found no consistent difference in the apparent dry matter digestibility of feed between +FW and –FW Merinos or +FW and C Romneys respectively. Similarly Faichney et al (1998) and Thomson et al (1989) found no difference in the digestibility of nitrogen (N), nor was there a difference in the retention of N (McCutcheon et al, 1987; Thomson et al, 1989; Sun et al, 1994; Cronje and Smuts, 1994) or the digestion and retention of S (Sun et al, 1994).

Differences in N utilisation and formation of nitrogenous by-products, such as urea and creatinine, have been inconsistent among FW selection lines. +FW sheep from Romney selection lines generally have lower plasma urea and creatinine concentrations compared to C sheep (McCutcheon et al, 1987; Thomson et al, 1989; Clark et al, 1989), possibly indicating altered kidney function and preferential conservation of amino acids for productive functions, such as wool growth. The difference between plasma concentrations in +FW and C sheep, and the clearance rates of these compounds, varied depending of the level of nutrition (Thomson

et al, 1989) and between different trials (Sun et al, 1994). In contrast, sheep from both Merino FW selection lines had similar plasma urea (Hough et al, 1988), plasma creatinine concentration (Williams et al, 1991), and clearance of both compounds (Williams et al, 1991).

The retention of N was similar in +FW and –FW South African Merinos but -FW sheep partitioned a smaller proportion of consumed N to wool production and gained body weight at a faster rate (Cronje and Smuts, 1994). No other comparisons of the partitioning of consumed or retained nitrogen or sulphur have been made in Australian or New Zealand FW selection lines, but differences in partitioning in these lines may account for differences in wool or body growth.

2.1.3.1.3 Metabolism of sulphur-containing amino acids (SAA)

The provision of adequate SAA such as cysteine, is vital for the production of fibre (Section 2.12). Variation in wool growth between +FW and –FW or C selection lines indicates that these groups differ in metabolism and utilisation of SAA. Williams et al (1972a) observed low concentrations of cystine in the plasma of +FW sheep, from as early as 7-10 days of age (Williams, 1984). This was generally the case for Merino selection lines (Hough et al, 1988; Williams et al, 1986; Williams and Thornberry, 1996) but was not found in the +FW and C Romney lines (Sun et al, 1994; Harris et al, 1993b; Miller et al, 2000). The concentration of glutathionine (GSH), a cysteine-containing tripeptide in the red blood cells, was lower in +FW Merinos compared to –FW sheep (Williams and Thornberry, 1996) but again, this was not evident in Romney selection lines (Sun et al, 1994).

Evidence suggests that +FW selected sheep clear a bolus dose of ^{35}S-cysteine more readily from the plasma (Williams and Thornberry, 1991). Sulphate, a product of cystine oxidation, is detected in similar concentrations in the plasma of +FW and –FW sheep, but a bolus dose of ^{35}S-sulphate is cleared more readily from +FW sheep, indicating a lower rate of irreversible loss of sulphate (Williams, 1995). This suggests that the availability of SAAs is similar for both groups but that +FW sheep utilise more for the production of wool (Williams, 1995).

2.1.3.2 Supply and uptake of nutrients by the skin and follicle

2.1.3.2.1 Skin vascularity and blood flow rate

The organised, rectangular arrangement of blood vessels surrounding the generally straight follicles of the +FW Merinos, contrasts with the distorted, irregular blood vessels surrounding the generally tangled follicles of the –FW sheep (Nay, 1966; Nay and Johnson, 1967). Difficulties associated with accurate measurement of blood flow rates and vasculature in the temperature-responsive, mobile skin mean few studies have been conducted in any FW selection lines. Harris et al (1993b) found no difference in skin blood flow rate between FW and C Romney sheep, and that there was no relationship between WGR and blood flow. In FW selected Merinos, Hales and Fawcett (1993) found a greater rate of blood flow per unit area of skin in +FW Merinos and a low to moderate correlation between skin blood flow and WGR, as did Hocking-Edwards and Hynd (1994) in strong-wool versus fine-wool sheep. The relationship between blood flow and WGR remains unclear because of the technical difficulties and the poor repeatability associated with measuring blood flow in skin.

2.1.3.2.2 Uptake of SAA into cells of the follicle and fibre

The mechanisms involved in uptake of cysteine into the cells of the follicle have not been investigated in any of the FW selection lines but, FW selected Romneys accrued 28% of ^{35}S-cysteine into skin compared with 11 % in C sheep (Harris et al, 1990) suggesting the capacity of the skin and follicles to transport SAA may be a limiting step. Uptake was localised in the cells of the keratogenous zone in unselected sheep (Downes et al, 1962; Chapman and Gemmel, 1973) and, in cultured wool follicles, cysteine uptake was mediated by a sodium-dependent transport mechanism, suggested to be an ASC type amino acid transporter (Wilson, 1995). The ASCT2 wool follicle amino acid transporter was isolated and sequenced from Corridale follicles, and was expressed discretely in the cortical cells of the keratogenous zone which is the primary site of cystine uptake by wool follicles (Nattrass, 2000). Therefore, differences in the affinity of this transporter for the substrate, cysteine, or

variability in the number of transporters themselves, could result in divergent fibre production.

2.1.3.3 Skin and follicle characteristics in FW selection lines

2.1.3.3.1 Follicle density

The density of follicles per unit area will influence the total amount of fibre synthesised per unit area of skin. Selection for FW in Merino lines increased the average follicle density of +FW sheep compared to –FW sheep (Williams, 1973; Williams, 1976; Williams and Winston, 1987). The total amount of follicular material and the rate of cellular activity of the follicles may contribute to increased wool production in +FW sheep and, in addition, the higher ratio of secondary to primary follicles in +FW sheep indicated that the secondary follicles contributed to this higher density (Williams and Winston, 1987). However, in the Romney breed there was no difference in the density of follicles between FW and C selected sheep, despite divergent wool growth, indicating the follicles themselves were more active and thus produced more wool (Kelly et al, 1993). Therefore, both an increase in follicle density and / or an increase in follicle activity influence wool growth variability between selection lines.

2.1.3.3.2 Follicle bulb and dermal papilla cells

Follicle bulb dimensions relate to the number and size of bulb cells destined for the formation of fibre or IRS, and possibly indicate the quantity of fibre produced. Bulb diameter was identical in +FW and –FW Merino lines (83μm diameter) (Williams and Winston, 1987) but +FW selected Romney sheep had significantly larger bulb diameters (~126 μm) than C sheep (~119 μm) (Kelly et al, 1993). This is further complicated by the supply of nutrition, as some researchers have reported an increase in bulb size with improved nutrition (Wilson and Short, 1979) and others showed no change (Williams and Winston, 1987). +FW Merinos had deeper follicle bulbs (1.52 mm) than –FW Merinos (1.38mm) (Williams and Winston, 1987) but +FW and C Romneys follicles were of a similar depth (~1.4 – 1.5mm deep) (Kelly et al,

1993). The significance of follicle depth is unclear but Nay (1966) described deeper follicles with more organised vasculature in +FW sheep.

Bulb diameter is an indicator of the number of follicle bulb cells, but a measure of bulb volume combined with the turnover and migration rate of bulb cells gives a more informative indication of fibre output. +FW Merinos and Romneys had greater bulb cell volume and a higher proportion of these cells was mitotically active, but there has been no demonstration of a significant difference in the number of bulb cells per volume of bulb tissue between +FW and –FW or C sheep (Kelly et al, 1993; Williams and Winston, 1987) or between fine and strong wools (Hocking- Edwards and Hynd, 1992). Therefore, selected Merinos had deeper follicles of a similar diameter whereas selected Romneys had follicles with a greater diameter but the same depth in the skin. There has been no definitive demonstration of a difference in the turnover rate of bulb cells.

There has been limited comparative investigation of the dermal papilla and its dimensions in +FW and –FW or C lines. Holle (1992) showed a strong relationship between the volume of dermal papilla cells and the area of bulb cells with +FW Romneys (Holle et al, 1994), and suggested that +FW Merinos (Williams and Winston, 1987) had a greater volume of dermal papilla cells compared to C and –FW sheep respectively. The comparative dimensions and activity of the dermal papilla requires further investigation in divergent fleece-producing sheep.

2.1.3.3.3 Cortical cell characteristics

The dimensions of follicle cortical cells and the rate of their production ultimately determines the length and diameter of the wool fibre produced. Williams and Winston (1987) found no difference in the dimensions of cortical cells (approximately 100 μm long x 6 μm wide) between +FW and –FW sheep, and therefore no difference in the cortical cell volume. However, the estimated number of cortical cells produced each day was higher in +FW sheep and the average volume of cortical cells was more responsive to nutrition in +FW lines compared to –FW lines (Williams and Winston, 1987). In contrast, +FW Romney sheep had a

greater cortex diameter thus a higher estimated cortex cell volume, than C Romney sheep (Holle et al, 1994). This variability may be attributable to the different techniques used by Williams and Winston (1987) and Holle et al (1994) but the volume of cortical cells, the extent of elongation and the rate of supply of cells to the cortex remain possible sources of wool growth differences.

2.1.3.3.4 Distribution of bulb cells to fibre or IRS

Wool growth variation between FW selection groups may arise from differences in distribution of bulb cells between the IRS and the fibre cortex, as well as some possible cell death and resorption after division, but technical difficulties associated with measuring and quantifying this means definitive answers are not available. A number of different techniques have been employed in an attempt to understand the proportion of bulb cells to the fibre but none have sufficiently accounted for the wool growth rate variation described in the FW selection lines. In separate experiments utilising +FW and C Romney sheep, Kelly et al (1993) suggested that 30-40% of cells labelled in the bulb region migrated into the fibre, whereas Holle et al (1994), measuring histological skin sections, estimated approximately 70-80% of bulb cells formed the fibre. Hocking-Edwards and Hynd (1992) measured the size of cortical cells and calculated that between 20-60% of bulb cells entered the fibre in fine and strong wool Merinos. The large variation in the estimated proportion of bulb cell migration to the fibre may indicate inaccuracies with the techniques utilised. It is however, generally accepted that this proportioning of bulb cells between the IRS and fibre is genetically determined, with minimal influence of nutrition (Wilson and Short, 1979; Hynd, 1989a) and this remains as a possible source of wool growth variation between +FW and –FW or C sheep.

2.1.3.3.6 Skin characteristics

Variable rates of protein synthesis and degradation in the skin can lead to differences in skin composition and in the amount of wool produced, but this has not been addressed in FW selection lines. The skin of +FW Merinos was 19% heavier per unit area than –FW sheep

(Hales and Fawcett, 1993) but was in fact lighter (not significantly so) in +FW compared to C Romney sheep (Harris et al, 1993b). Skin from +FW Merino sheep was thicker, more fragile (Williams and Morley, 1994), and more wrinkly (Williams, 1987), with a smaller network of collagen fibres than in the skin of –FW sheep and lower skin collagen content. Therefore, the skin of +FW sheep had relatively more non-collagenous proteins including cystine and non-peptide bound α-amino nitrogen (Williams and Morley, 1994). The concentration of enzymes involved in the regulation of active transport of metabolites into cells (sodium potassium adenosine triphosphatase and γ-glutamyl transpeptidase) and the control of cell division (ornithine decarboxylase) in the skin did not differ significantly between Merino selection lines (Williams et al, 1996).

2.1.3.4 Fibre characteristics in FW selection lines

2.1.3.4.1 Wool sulphur (S) concentration

A negative association between the S concentration of wool and the quantity of wool produced has been identified in studies of phenotypically diverse wool producing sheep (Reis et al, 1967). The -FW sheep from Merino selection lines generally have higher wool S concentrations than +FW sheep (Piper and Dolling, 1966) but because of the lower rates of wool production, –FW sheep accrue less total S in wool. The association has been consistently reported in Merino selection lines (Williams et al, 1972b; Hough et al, 1988; Williams, 1995) but inconsistently in Romney FW lines (Antram et al, 1991), and varies in the latter flocks depending on seasonal variation in wool growth, the age of the animal and position of sampling on the body (Sun et al, 1991). The similar wool S concentrations and total wool S outputs in Romney selection lines (McCutcheon et al, 1987; Sun et al, 1994) was because the rate of wool growth between the lines actually did not differ at the time of the experiments and suggests further investigation of S output in wool in the Romney selection lines, when the rate of wool growth differs.

There is limited information regarding the expression of different S-containing keratin proteins in wool from FW selection lines. When radiolabelled cysteine was infused intravenously into sheep from Merino FW selection lines, there was no difference in the level of radioactivity measured in high-sulphur (HS) and low-sulphur (LS) protein fractions between the lines (Williams, 1973), suggesting similar proportions of proteins. Gillespie and Reis (1966) found the proportion of HS proteins in the wool from a low-producing, high wool-S Merino to be greater than in the wool from a high-producing, low-wool S Merino (25% compared to 20% of total wool protein). +FW sheep generally have a low crimp frequency, related to the straighter follicles, and less well defined bilateral segmentation of orthocortical and paracortical cells in the fibre (Kaplin and Whitely, 1978). In crimp frequency selection lines, high crimping wool contained 10% more HS proteins than low crimping wool (Campbell et al, 1972). Up to 9% of all wool proteins from high crimping wools were ultra-high S proteins (UHS), a proportion which varies with nutrition, but these proteins were not present in low crimping wool at any nutritional level (Campbell et al, 1975).

Nutrition has a large influence on the types of wool proteins produced. The provision of SAA via abomasal or intravenous infusions, increased the S content of wool and increased the proportion of HS proteins (Gillespie et al, 1969). Furthermore, a reduced supply of nutrients caused a proportional decrease in HS proteins but minimal changes in LS proteins in wool (Campbell et al, 1975), leading to decreases in wool S concentration of between 20 to 30% (Gillespie and Reis, 1966). More recently the expression of genes encoding these proteins have been investigated with an increase in the expression of KAP 4.2, a gene encoding an ultra high S protein, in response to the intravenous infusion of cysteine in Corridale sheep (Fratini et al, 1994). Given the different wool proteins in sheep growing different wool types and quantities, and the influence of nutrition on protein production, variation in wool S protein types (ie. LS, HS and UHS) in the FW selection lines is probable and the investigation of genes encoding these proteins would be warranted.

2.1.3.4.2 Fibre length and fibre diameter

Variability in volumetric fibre growth, determined by fibre diameter (FD) and fibre length (FL), will affect the total quantity and quality of wool produced. Differences in the proportional response of FD and FL to nutrition or to genotype may cause this change in production. Data from Ahmed et al (1963) indicate higher FL:FD ratio in +FW selected sheep compared to –FW sheep, however some conjecture surrounds the differential response of FD and FL to nutrition, in the FW lines. Some +FW selected sheep increased FD in response to improved nutrition to a greater extent than –FW sheep (Williams, 1966) and in response to additional casein in the diet (Williams, 1995), thus the FL:FD ratio decreased. Williams and Winston (1987) found increased FD and FL in +FW and –FW sheep with improved nutrition, but the FL:FD ratio remained constant. A consistent response in FD and FL to nutrition in sheep from the FW selection lines has not been demonstrated.

2.1.3.5 Endocrine relationships in FW selection lines

2.1.3.5.1 General endocrine relationships with fibre growth in FW selection lines

Plasma insulin (Hough et al, 1988) and cortisol (Williams et al, 1986) concentrations were similar in +FW and –FW selection lines. Growth hormone (GH) and its association with wool growth is somewhat inconsistent, exerting both positive (Johnsson et al, 1985) and negative (Wynn et al, 1988) effects on wool growth; in Merino selection lines, the plasma concentration of GH was similar for both groups (Hough et al, 1988). Plasma thyroxine concentration however, was significantly lower in +FW Merino sheep (Hough et al, 1988) but Sun et al (1994) found no consistent difference in thyroxine concentration between FW and C Romney sheep. There has been no investigation of the insulin-like growth factors (IGF) or their associated receptors and binding proteins in sheep from FW selection lines despite the suggestion that the IGF system could play a major role in the regulation of fibre growth and quality (Nixon and Moore, 1998).

2.1.3.5.2 The insulin-like growth factor (IGF) system

Insulin-like growth factors (IGFs) are single-chained polypeptides that circulate in blood in two major forms, IGF-I and IGF-II, and have similar structural homology and shared biological activity with insulin. The anabolic effects of IGF-I in mediating GH action (GH/IGF-I axis) is in contrast to the less well understood activities of IGF-II.

IGFs bind to plasma membrane glycoprotein receptors, including the type 1 (IGF-1R) and type 2 (IGF-2R) IGF receptors, and the insulin receptor, and elicit mitogenic and morphogenic effects on cells (DeMeyts et al, 1994). The IGF-1R, which binds IGF-I and insulin with greater affinity than IGF-II, consists of two alpha subunits containing the ligand binding sites and two beta subunits which traverse the plasma membrane and contain cytoplasmic tyrosine kinase activity (Breier and Gluckman, 1991). The IGF-2R has greater affinity for IGF-II, less for IGF-I and no affinity for insulin, and is identical to the mannose-6-phosphate receptor involved in lysozome targeting (Breier and Gluckman, 1991).

A family of six IGF-binding proteins (IGFBP) bind with both IGF-I and IGF-II with very high affinity, primarily to control the distribution of IGFs in interstitial fluids and thus mediate circulating IGF concentrations, and control the binding of IGFs to cell surface receptors. Expression of IGFBP-1 is restricted primarily to the liver (Wood et al, 1990) with highest plasma concentrations detected in the fetus, and production regulated by blood insulin levels (Clemmons, 1997). Similarly for IGFBP-2, highest levels are detected in fetal blood and it is the second most abundant BP in circulation, binding with greatest affinity with IGF-II (Baxter, 1994). IGFBP-3 binds approximately 90-95% of all IGFs in circulation and is regulated by age, nutritional status and growth hormone concentration of the animal (Clemmons, 1997). IGFBP-4 consistently inhibits the action of IGF-I despite minimal concentrations in serum. IGFBP-5 and –6 circulate in very low levels in serum with stimulatory and inhibitory roles that are less well defined than for other BPs.

2.1.3.5.3 Do the IGFs stimulate fibre growth?

The anabolic effects of the IGFs on body growth is discussed in Section 2.2.1.5, but a role for IGFs in the skin and follicle, and in mediating fibre production directly, is less well understood. Expression of the type I and type II IGF receptors and the insulin receptor have been described in wool follicles (Nixon and Moore, 1998). Variable expression of these receptors in outer and inner root sheath cells, matrix, dermal papilla and sebaceous gland cells has been reported (Dicks et al, 1996; Nixon et al, 1997). A number of the IGFBPs have been identified in the dermal papilla, the border of the dermal papilla and the matrix cells, and in the dermal sheath in human skin (Batch et al, 1996) and rat skin (Hynd and Penno, 2001). Furthermore mRNA encoding both IGF-I and IGF-II has been localised to the skin and follicle (Adams et al, 2000). Together the expression of the IGFs, and their binding proteins and receptors suggests an important paracrine and autocrine role in fibre growth.

Infusion of IGF-I into the skin of sheep has increased the rate of blood flow, the net uptake of oxygen and amino acids, and the synthesis of protein by the skin (Harris et al, 1993a; Hocking-Edwards et al, 1995; Lobley et al, 1997). However, this effect is transient and no increase in follicular activity, including the number of cells replicating in the bulb, or fibre output was measured (Harris et al, 1993a). *In vitro,* both IGF-I and IGF-II stimulated fibre growth from human hair follicles in a dose-dependent manner (Philpott et al, 1994). Transgenic mice (Su et al, 1999) and sheep (Damak et al, 1996) showing increased expression of the IGF-I transgene in the hair and wool follicle respectively have been successfully produced. A significant increase in the elongation of vibrissae (whisker fibre) during the first neonatal hair cycle was observed in IGF-I transgenic mice (Su et al, 1999). In transgenic sheep, over-expressing IGF-I in the wool follicle, a 6% increase in clean fleece production at yearling shearing compared to non-transgenic sheep was found (Damak et al, 1996) but this was not maintained in the second year (Su et al, 1998). The fibre produced by transgenic sheep was larger in diameter compared to non-transgenic wools (Su et al, 1998) and was one

of the first studies to improve a production trait in domestic animals without apparent adverse effects on health or reproduction.

2.2 Body growth

Increased fibre and body growth in sheep as feeding increases above maintenance contrasts with decreasing body weight but continual wool production when nutrient supply is below maintenance requirements (Marston, 1948; Ferguson, 1962). Body weight loss at low feeding levels provides a source of amino acids available to the skin for wool growth. In fact, skin requirements for energy and amino acids at sub-maintenance nutrition rival the importance of visceral organ demands (Harris et al, 1990). Growth depends on the quantity and composition of available nutrients, and is regulated by endocrinological and biochemical mechanisms. The competition or cooperation of nutrient use between peripheral tissues, such as the skin, versus growth of the whole body, and the control of growth by physiological, environmental and endocrine factors will determine differential rates of production in different tissues (Black and Reis, 1979). Modelling of the partitioning of nutrient use in the late 1970s (Black and Reis, 1979) coincided with studies of nutrient use in fleece weight (FW) selection lines (Section 2.1) and lines developed using body weight criteria, as will now be discussed.

2.2.1 Selection lines for body growth in sheep

Body weight selection lines developed in sheep have used yearling or weaning live weight selection criteria to provide a research tool to assist in understanding growth mechanisms in sheep. In Australia the Merino weaning weight (WW) selection lines were established at the Trangie Research Centre in the 1950's (Pattie, 1965a, b) alongside fleece weight selection lines (FW) (Turner et al, 1970). In New Zealand, a number of flocks were developed using weaning weight or yearling weight selection criteria in Romney sheep (McEwan et a, 1989; Clark et al, 1989; Johnson et al, 1995; Morris et al, 1996). Physiological and endocrinological information from these sheep selection lines are reviewed briefly here,

with respect to competition between the skin and the body as a whole for nutrients and the role of endocrine factors, specifically insulin-like growth factors (IGFs).

2.2.1.1 Liveweight and body composition

Liveweight differed significantly between high weaning weight (+WW) and low weaning weight (-WW) Merino selection lines at birth, to approximately 100 days of age (Pattie and Williams, 1967), with little further divergence in growth after 12 months of age (Speck, 1991). Despite this difference in liveweight, the proportional composition of the carcass at 4 months of age (Pattie and Williams, 1966) and proportions of chemical and dissected fat, protein and muscle (Thompson et al, 1985b) in mature animals, was similar between +WW and –WW sheep. Perry et al (1992) reported similar growth patterns for the skeletal system in Merino selection lines and in Romney WW and control (C) selection lines; fat and muscle measurements were comparable at the same liveweight (McEwan et al, 1989).

2.2.1.2 Feed intake and conversion efficiency

Feed intake was higher in +WW sheep compared to –WW sheep prior to weaning (Pattie and Williams, 1966; Oddy et al, 1989b) and after weaning (Pattie and Williams, 1967; Herd et al, 1993) but when intake was adjusted for liveweight, it did not differ between groups. Variability in pre-weaning intake may be a major determinant of live weight potential. The *ad libitum* supply of reconstituted dried milk in WW selection lines led to a greater intake and rate of body weight gain in +WW selected Merinos (Oddy et al, 1989b). There was no difference in the efficiency of growth (gross food conversion efficiency or growth efficiency) in either mature sheep (Herd et al, 1991, 1993) or in growing lambs (Pattie and Williams, 1966). Therefore, +WW sheep consumed more feed and grew more than –WW sheep but feed intake per unit liveweight and growth efficiency were similar.

2.2.1.3 Protein turnover and nitrogen (N) balance

The retention of N was higher in +WW Merino lambs, reflecting both a greater intake of N and a greater use of consumed N for synthesis of body protein (Oddy et al, 1989b)

however, N retention did not differ in adult Merinos between WW selection lines (Speck, 1991). As feed intake increased, 14 month old +WW sheep gained body protein at a faster rate than –WW sheep, due to decreased protein degradation in the hindlimb of +WW sheep and despite similar rates of protein synthesis for both selection lines (Oddy et al, 1989a). The increase in protein gain in +WW sheep was also supported by lower rates of amino acid oxidation (assessed using labelled phenylalanine) in the whole body, lower rates of blood flow to the hindlimb and lower oxygen use by the hindlimb (Oddy et al, 1995).

2.2.1.4 Relationships with wool growth

Wool growth rate did not differ between +WW and –WW Merinos but the average liveweight and feed intake did differ significantly, therefore the efficiency of wool production (wool growth per unit feed intake) in –WW sheep was 33% greater than for +WW sheep (Herd et al, 1993). Oddy et al (1989b) related this reduced efficiency of wool growth in +WW sheep to an increase in deposition of body protein and consequential decrease in N use for wool protein synthesis. Similarly in South African Merinos selected for divergent fleece weight, low producers partitioned less N (as a proportion of N intake) to wool growth and gained more weight than high wool producers (Cronjé and Smuts, 1994).

2.2.1.5 General association of endocrine factors and body growth in WW selection lines

Body growth requires the coordination of many biological signals that facilitate the partitioning of nutrients for growth. Growth hormone (GH) does not appear to play a major role in generating any liveweight differences between WW selection lines, as the concentration of plasma growth hormone (GH), the GH response to infusions of arginine or growth hormone-releasing factor, GH binding to hepatic receptors and the plasma IGF-I response to GH infusion, was similar for all sheep (Speck, 1991). Little variability between WW lines in plasma cortisol (Oddy et al, 1995), prolactin (Speck, 1991) and thyroid hormone concentration (Speck, 1991) indicates these hormones are also unlikely to play a major role in creating liveweight differences. A relationship between protein gain in the hindlimb and

plasma insulin concentration in +WW sheep but not –WW sheep, suggests that greater protein gain in +WW sheep may in part be related to greater insulin responsiveness, however mean line concentrations did not differ (Oddy et al, 1995).

Plasma insulin-like growth factor –I (IGF-I) is the only endocrine substance to differ significantly between WW selection lines. Furthermore, despite no investigation of IGF-II concentrations in any weaning or yearling weight selection lines in sheep, a possible role for IGF-II in the regulation of feed intake (Lauterio et al, 1987) suggests both IGF-I and IGF-II could be important in regulating the utilisation of nutrients for body growth.

2.3 Project aims and hypotheses

In the first experiment (Chapter 4) reported in the following chapters the association of insulin-like growth factors (IGFs) and nutrition with the partitioning of protein (nitrogen) between wool and body growth was investigated, in Merino sheep, phenotypically divergent in both wool and body weight. The hypotheses tested were that the partitioning of nitrogen between wool and body growth is associated with plasma concentrations of IGFs, and that these differences in partitioning are expressed to a greater extent when a high intake is supplied.

In the second experiment (Chapter 5) the partitioning and utilsation of sulphur-containing amino acids (SAA) for wool growth was studied in Merinos genetically divergent in wool production and selected from a commercial-orientated research flock. The general hypotheses tested were that differences in wool growth between genetically divergent sheep arise from variability in efficiency of converting sulphur into wool, in digestion and retention of sulphur, in partitioning of sulphur to wool and in wool growth response to nutrition.

The final experimental chapter (Chapter 6) addressed the effect of nutrient use on the expression of keratin genes in these genetically diverse groups of Merino sheep. It was hypothesised that the high-wool producers (+W) expressed higher levels of low-sulphur genes, lower levels of high-sulphur and ultra high-sulphur genes, and were more responsive in wool growth due to additional nutrient supply because of the production of a higher proportion of low-sulphur genes compared to low-wool producers (-W).

Chapter 3

Materials and Methodology

3.1 Sheep selection data

3.1.1 The SARDI Selection Demonstration Flocks

Animals used in experiments described in this thesis were selected from the South Australian Research and Development Institute (SARDI) Selection Demonstration Flocks (SDF), based at the Turretfield Research Centre at Rosedale. These flocks were established in 1995, with the primary objective of "genetic improvement of the profitability of South Australian Merino sheep", by reducing fibre diameter whilst improving clean fleece weight (Ponzoni et al, 1999). To address the general criterion, three selection flocks and one control flock participate, with selection based on measured performance, professional sheep classer assessment or a soft rolling skin approach to selection. Rams used for breeding in the SDF were initially sourced from top studs throughout Australia, the sires used thereafter produced within each selection group flock, as were all replacement breeding ewes. The rams used for Experiment 1 (Chapter 4) and Experiment 2 (Chapters 5 and 6) in this thesis were indiscriminately selected from all four flocks within the SDF project; selection criteria were solely to meet the experimental objectives and are outlined in Sections 4.2.1.1 and 5.2.1.1.

3.1.2 Follicle density measurement and calculation

The number of follicles per unit area of skin was calculated for SDF rams in 1997 (n=374) and 1998 (n=408). Skin samples were taken using local anaesthesia (Lignocaine with adrenaline; Troy Laboratories, NSW) from each ram at 16 months of age with a trephine instrument, and the skin sample was removed using surgical scissors. Biopsies were immediately fixed in 10% buffered formalin then stored in 70% ethanol. Samples were prepared by removing excess wool with a scalpel, then processed, embedded, sectioned and stained as outlined in Appendix 1.1.1 and 1.1.2.

Follicle density was determined using Chromatic Colour Image Analysis System Version 3.0 (Leading Edge Pty.Ltd, Adelaide). The number of follicles ('n') was counted over five random sites of known area ('a') for each skin section, the average value representing the total number of follicles per unit area of skin. This value was adjusted to account for

shrinkage of skin samples during fixation and processing by measuring the actual size of sectioned trephine sample ('s'), using BioQuant Intro Program (BioQuant Systems, Nashville), and comparing it to the size of the skin biopsy when it was first taken (original area = 78.5mm^2).

$$\text{Follicle density (number of follicles / mm}^2) = \frac{\text{'s'}}{78.5} \times \frac{\text{'n'}}{\text{'a'}}$$

The total number of follicles for each individual sheep was calculated by determining the estimated skin surface area (based on body weight; Bennett, 1973) and multiplying this by the follicle density (the number of follicles per unit area).

3.1.3 Other selection data

Accurate records of birth type and birth weight for example, were kept for all animals from the SDF's, at birth, weaning, 10 months and 16 months of age. Fleece and body weight data used in the selection procedure for the experiments discussed here were measured at 16 months of age and fixed variables, such as type of birth or rearing, were used in subsequent analysis where appropriate.

3.2 Wool measurements

3.2.1 Wool growth rate

Midside wool growth was measured by tattooing an area of skin on the midside of the sheep and clipping within this boundary (Langlands and Wheeler, 1968). Sheep were restrained in a swivel crush then positioned on their side, and an area covering approximately 12-14 cm square was clipped on the midside, a hand space down from backbone, over the last rib. A wire template, dipped in Indian Ink, imprinted the desired area on the clipped region of skin. Local anaesthetic (Lignocaine with adrenaline; Troy Laboratories, NSW) was administered sub cutaneously along the templated outline with a 25 gauge needle, then the epidermal layer of skin was cut with a scalpel along the template line to the depth of the muscle fascia. Indian ink was applied into the cut surface using a syringe and blunt needle attachment, taking care not to overfill the area to avoid smudging. Animals were kept indoors

following the procedure and healing was complete within 3 weeks, at which time the area was carefully clipped to remove any remaining dried surface ink and blood, revealing a clearly defined tattooed outline in the skin.

Wool grown within the tattooed boundary was clipped at designated times using Andis clippers and surgical sized blades (Andis, no.40). The wool was removed as close to the skin surface as possible and stored in a plastic sealable bag until washing. The outline of the tattoo was traced onto a clear plastic sheet and the area of the clipped patch determined by measuring the area of the traced patch on an image analysis system (Bioquant Intro Program, BioQuant Systems, Nashville).

Wool growth measurements taken in Experiment 1 (Chapter 4) were conducted over 28-day periods. In Experiment 2 (Chapters 5 and 6), both mid side areas were tattooed (left and right side) and wool samples were taken every ten days, alternating from right side to left. Thus, each wool sample taken represented approximately 20-days growth on each side and permitted four samples to be taken throughout the experiment.

Each wool sample was conditioned for 24 hours prior to weighing to establish greasy fleece weight (20 +/-2°C ; 65+/- 2% relative humidity). Samples were washed using gentle agitation to avoid felting in pre- weighed, tight weaved, calico bags to avoid loss of short fibres (Appendix 1.2). Wool was dried in an oven at 60 °C for 72 hours, then placed in a desiccator for 20 minutes prior to weighing for clean fleece weight.

Patch wool growth (WGR^A), expressed in mg /cm^2 / d, was calculated as follows,

$$WGR^A = (CFW / n) / \text{`x'}\ cm^2$$

where CFW = amount of clean, dry wool collected from the patch area (mg)

n = number of days wool growth at collection

'x' = wool patch area measured from tracing

Whole body wool growth rate (WGR^B), expressed as g / d, was calculated using the equation of Bennet (1973), that is

$$WGR^B = WGR^A \times (0.094 \times BW^{0.67})$$

3.2.2 Wool fibre diameter and length

Wool fibre diameter was measured using the OFDA100 (Optical-based Fibre Diameter Analysis) system (BSC Electronics, Pty. Ltd), for more than 3000 fibres per wool sample. Fibre length was calculated by incorporating wool growth rate, fibre diameter and follicle density measurements into the following equation,

$$WGR^B = \pi \times (FD/2)^2 \times FL \times \delta \times N$$

WGR^B = Whole body, clean wool growth rate (g / d)

FD = Fibre diameter (μm)

FL = Fibre length (μm)

δ = Specific gravity of wool, assumed to be 1.3041 (Connell and Andrew, 1974)

N = Total number of follicles producing fibre per unit area

3.3 Nitrogen (N) and sulphur (S) balance

3.3.1 Collection protocol

Sheep were introduced to metabolism cages prior to each collection period to acclimatise them to the confined environment. During the collection period, faeces and urine were collected from segregated collection vessels under the cage every 24 hours just prior to morning feeding, at which time residual feed was collected. The weights of daily urine and faecal outputs were recorded, and 5% and 1% sub samples respectively were bulked over the collection period and stored at –20°C until analysis. Residual feed was weighed and dried in an oven at 90°C for 48 hours to determine dry matter content, allowing accurate intake records to be made during the collection time. To maintain urine pH below 4 (to avoid N losses through volatilisation), a known amount of 6N hydrochloric acid was added to collection vessels each day and pH monitored daily.

3.3.2 Nitrogen analysis

Nitrogen (N) balance was calculated for all sheep in Experiment 1 (Chapter 4), from samples collected over a seven-day balance period. N content was determined in duplicate,

using the Leco system to determine faecal and feed N contents and a standard Kjeldahl digestion for urine N concentration. Feed and faecal N contents were determined using the Leco method, conducted in the Department of Agronomy and Farming Systems, at the Roseworthy Campus of Adelaide University. Urine samples were filtered, digested at 400°C in an AIM 500 Block Digestion System and distilled using a Kjeltec System 1026 Distilling Unit once cool. Distilled samples were titrated with 0.1M hydrochloric acid and N content determined.

3.3.3 Sulphur analysis

Sulphur (S) balance was determined for all sheep in Experiment 2 (Chapter 5) from samples collected over a six-day collection period. S analysis was carried out by digestion with nitric and perchloric acid, and analysed using Inductively Coupled Plasma Atomic Emission Spectrometry (ICPAES), by Waite Analytical Services.

3.4 Blood sample collection and analysis

3.4.1 Experiment 1 (Chapter 4)

Serial blood samples were taken from a jugular catheter inserted one day prior to sampling, for the analysis of plasma insulin-like growth factor (IGF) concentrations in sheep from Experiment 1. A small area on the neck was clipped and a 14 gauge catheter placement unit (Optiva, Johnson & Johnson Medical) was used to insert 1mm(id) by 1.5mm(od) polyethylene medical grade catheter tubing (Dural Plastics and Engineering, NSW) to a depth of approximately 10 centimeters into the jugular vein. The tubing was held in place by securing with leukoplast tape (Beiresdorf, Hamburg, Germany) around the neck. A blunt 18 gauge needle and cap were inserted into the sampling end of the tubing. Catheter patency was maintained by flushing the catheter twice a day with 5-10ml of sterile heparinised, 0.9% sodium chloride (Heparin sodium-porcine mucosa at 50U/ml saline, David Bull Labs via Fauldings; Baxter Healthcare Pty Ltd., NSW).

Blood samples (5ml) were collected in sterile heparinised tubes on ice (Greiner Bio-one, Austria) at 10 minute intervals from 9am – 1pm and 9pm - 1am (n=50) for all 48 sheep.

Blood samples were centrifuged at 2200rpm for 10 minutes at 4 -8°C, then plasma was separated from each sample, aliquoted into 1ml samples and stored at –20°C until analysis.

Radio-immuno assay of plasma samples was carried out in the Department of Obstetrics and Gynecology at Adelaide University, using the methodology of Owens et al (1990), to determine IGF-I and IGF-II concentrations. Between- and within- assay coefficients of variation were 11% and 10% respectively for the IGF-I assay, and 9% and 19% respectively for the IGF-II assay.

3.4.2 Experiment 2 (Chapter 5)

Blood samples were taken on three occasions throughout Experiment 2 between 9am and 11am, prior to morning feeding, for the analysis of plasma sulphur-containing amino acid (SAA) concentrations. Sampling periods were as follows; field sample (taken prior to entering the animal house), mid-experiment sample (3 weeks after feeding treatments commenced) and final sample (six weeks after feeding treatments commenced). Pressurized, heparinised vaccutainers (Greiner Bio-one, Austria) with 18 gauge needles were used to take single blood samples directly by venipuncture of the jugular vein. Samples were centrifuged at 2000g for 10 minutes; plasma was removed and mixed with an equal volume of 20% TCA solution in a fresh tube. The sample was vortexed and re-spun in the centrifuge, to precipitate protein. Protein free plasma (supernate) was aliquoted and stored at –20° until analysis for amino acid concentration.

The concentration of the plasma sulphur amino acids (SAA) cystine and methionine, were determined using pre-column derivitisation and HPLC analysis of final plasma samples from Experiment 2. The methodology was adapted from that used for pig plasma samples (Reverter et al, 1997) and was developed in conjunction with the Pig and Poultry Production Institute (PPPI) at Roseworthy. Briefly, an external standard norleucine (2.5mM), was combined with 360µl of TCA treated plasma and 200µl sodium hydroxide (NaOH) to optimise the pH of the plasma solution between 7 and 9. Large contaminants were removed by filtering using a 0.45µm syringe filter and the plasma solution left at room temperature for

4-6 hours to permit conversion of cysteine molecules to cystine (manufacturer's correspondence). The plasma solution was derivitised using an AccQfluor reagent (Waters AccQTag kit) prior to separation of amino acid fractions using HPLC (Waters Alliance 2690XE Separation module, 474 Scanning Fluorescence detector and 150mm AccQTag Column) and determination of SAA concentrations (Waters Millennium 32 Chromatography Manager).

3.5 Development of a method for determination of wool sulphur concentration using Near Infrared Reflectance Spectrometry (NIRS)

The measurement of S concentration in Merino wool using NIRS was developed as part of this thesis, as an extension of work carried out using Romney wool (Corsan et al, 1998) and preliminary calibrations on Merino wool conducted by staff in our laboratory, in conjunction with the Pig and Poultry Production Institute (PPPI) at Roseworthy. Clean wool samples were evenly loaded into a quartz window ring cup designed for the Foss NIRSystem Model 6500 Spectrophotometer used for work in this thesis (Foss NIRSystem Inc., Silver Spring, MD, USA). Samples were scanned at 2nm intervals at a wavelength between 1100 and 2500nm and the predicted wool S content was calculated using Intrasoft International (ISI) NIRS 3 version software and calibration data documented previously (Hynd et al, 2000).

A sub-group of wool samples taken during Experiment 2 was digested with nitric and perchloric acid for analysis using Inductively Coupled Plasma Atomic Emission Spectrometry (ICPAES; Waite Analytical Services, Adelaide) to indicate the precision of the NIRS technique. The same sub-group of wool samples was measured using NIRS and the calibration established by Hynd et al (2000). Regression analysis of wool S concentration measured by NIRS and ICPAES is shown in Figure 3.1. The R^2 value was high (0.72) and thus, NIRS was used to measure wool S in this thesis.

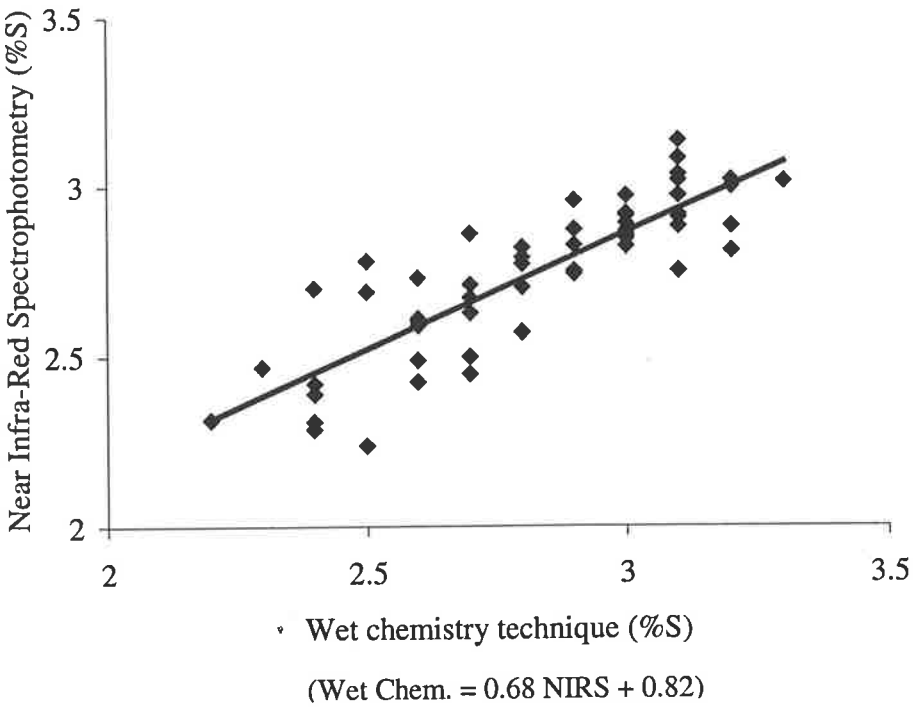

Figure 3.1: Comparison of the S concentration of wool samples measured with NIRS and a wet chemistry technique.

3.6 Detection of keratin gene mRNA expression in skin

Molecular biology protocols to facilitate northern blot analysis, used to detect messenger RNA (mRNA) encoding keratin genes in sheep skin samples from Experiment 2 (Chapter 6), were carried out essentially as described by Sambrook et al (1989). The protocols include RNA extraction, northern transfer, polymerase chain reaction (PCR) to produce DNA probe fragments, ethanol precipitation, phenol extractions, and oligolabelling and kinasing of DNA to radiolabel the probes. The constituents of each solution are detailed in Appendix 1.3.

3.6.1 Skin tissue collection

Skin biopsies (protocol from Section 3.1.2) taken from all 32 sheep during Experiment 2 were snap frozen in liquid nitrogen and stored at –80°C. RNA was extracted from skin samples from eight +W sheep (4 HS diet and 4 LS diet) that produced the greatest quantity of wool at the end of the experiment, and eight –W sheep (4 HS diet and 4 LS diet) that produced the lowest quantity of wool at the end of the experiment (day 65).

3.6.2 RNA extraction from skin tissue

3.6.2.1 RNA extraction and preparation

Total RNA was extracted from whole skin biopsies using the acid-guanidinium isothiocyanate methodology of Chomczsynski and Sacchi (1987). Briefly, whole skin biopsies were homogenised in Solution D (Appendix 1.3.2), sequentially mixed with 2M sodium acetate, water-saturated phenol and a chloroform/ isoamyl alcohol solution, then chilled for 15 minutes. The RNA-containing aqueous phase was separated by centrifugation, combined with an equal volume of isopropanol, then RNA was precipitated out of solution during centrifugation. Total RNA was resuspended in 500µl DEPC treated milli Q ultra pure water and stored at –80°C.

3.6.2.2 Determination of RNA integrity and concentration

The integrity of RNA extracted from whole skin was assessed using small analytical 1% agarose gels, prepared using double size microscope slides and a horizontal mini gel apparatus (home-made). RNA solutions, prepared by combining 2µl RNA sample and 2µl agarose load buffer (ALB; Appendix 1.3.2), were electrophoresed in 1 x TAE buffer (Appendix 1.3.2) at 100V. A SSP-1 DNA marker (Appendix 1.3.8) was run for each gel to compare molecular size. Following electrophoresis each gel was stained with ethidium bromide (1µg/ml), viewed under UV light and photographed using the GelDoc 1000 System (BioRad, USA). The integrity of 18S and 28S rRNA bands indicated the quality of each RNA sample. RNA concentration was determined by measuring the absorbance at A_{260}, using a Pharmacia GeneQuant spectrophotometer, assuming $A_{260} \approx 1.0$ for 40µg/ml solution of RNA.

3.6.3 Northern blot procedure

3.6.3.1 RNA sample preparation and electrophoresis

The northern blot procedure was carried out essentially as described in Sambrook et al (1989), with each RNA sample run in triplicate, that is, electrophoresed on three different northern blots. Briefly, 10µg of each aqueous RNA sample was precipitated with 0.1 v/v 4M sodium chloride and 2.5 v/v absolute ethanol at –20°C for 20 to 30 minutes. Each sample was

centrifuged at 15000rpm for 15 minutes and the precipitated RNA was resuspended in 10µl formamide, 2 µl 5 x formaldehyde running buffer (FRB; Appendix 1.3.2), 3.5 µl formaldehyde and 4.5 µl MQ water. Thoroughly vortexed samples were incubated at 65°C for 15 minutes, chilled on ice and 2µl 10x formaldehyde gel loading buffer (FGLB; Appendix 1.3.2) added to each. Samples were electrophoresed on a large 1% formaldehyde-agarose gel, containing 10µg/ µl ethidium bromide.

3.6.3.2 Gel preparation and sample loading

Large (20cm x 20cm x 0.5cm; 200ml), 1% formaldehyde-agarose gels enabled the fractionation of 16 RNA samples at one time. The gel contained 125ml agarose (2g agarose in 125ml), 35.7 ml formaldehyde, 39.3 ml 5 x FRB (Appendix 1.3.2) and 10ul (10ug/ul) ethidium bromide. Each gel was pre-run at 50V in 1 x FRB (Appendix 1.3.2) for 30 minutes prior to loading the RNA samples, including RNA markers (Appendix 1.3.8; Life Technologies, RNA Ladder). Samples were then electrophoresed at 40V for 20 minutes and fractionated overnight for approximately 16-18 hours, at 25V with re-circulating buffer (1 x FRB).

3.6.3.3 Northern Transfer

Gels were de-stained and equilibrated twice in 10 x SSC (Appendix 1), for 20 to 30 minutes each time, at room temperature and with gentle agitation, prior to photographing under UV light using the GelDoc1000 System. Gels were transferred to a Zeta probe GT membrane in 10 x SSC using a vacuum blotting apparatus (2016 Vacugene, LKB), at 40cm water pressure for 90 minutes. RNA samples were UV-crosslinked to the Zetaprobe membrane using a Stratagene 'strata-linker' set at 'Auto crosslink' (1200MJ) and the membrane was then dried at 65°C for 30 minutes.

3.6.4. Hybridisation probe fragments

The DNA probes used for this work are outlined below.

Trichohyalin (TRN)

Trichohyalin is an abundant protein, expressed in the inner root sheath cells of the wool and hair follicle. A gene-specific 331bp *DraI / EcoRI* 3'non-coding fragment (Fietz et al, 1993) was prepared via PCR by amplifying the ovine cDNA sequence from a pGEM-2 plasmid, using SP6 and T7 oligonucleotide primers.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)

A DNA fragment encoding GAPDH, an intermediate enzyme vital to the functioning of the glycolytic pathway for energy production, is commonly used as a 'house-keeping' gene probe in northern blots. The ovine GAPDH cDNA was isolated and cloned into a pBluescript I-KS plasmid and partially sequenced (Nattrass, 2000). The 200bp fragment was excised from the 1kb cDNA insert by *HindIII / SfiI* digestion and subcloned into pBluescript. This fragment was amplified using PCR, utilizing T7 and RSP primers.

Keratin associated protein 4.2(KAP 4.2)

The KAP 4 family of genes encode ultra high sulphur (UHS) proteins expressed predominantly in paracortical cells of the wool fibre. The KAP 4.2 fragment contains the full coding region consisting of 770bp (B.C. Powell, unpublished) and was amplified by PCR from pGEM7-Zf(+) plasmid clone, using SP6 and T7 primers.

Keratin intermediate filament type II protein (K2.10)

The 220bp, K2.10 *PstI* probe fragment was derived from the wool follicle K2.10 type II intermediate filament cDNA, and included 18bp encoding 6 amino acids from the carboxy-terminal end of the protein and 202bp from the 3' non-coding region (Powell et al, 1992). The *PstI* fragment was amplified from a pGEM2 plasmid clone using PCR, with the SP6 and T7 primers.

Keratin associated protein 2.12 (KAP 2.12)

A 300bp *PvuII* restriction fragment, including 75% of the coding region of the high sulphur BIIIA gene family, encoding 99 amino acid residues (14 to 112) (Powell, unpublished) was a gift from S.Bawden (SARDI Livestock Systems Alliance, South Australia).

Keratin associated protein 6.1 (KAP 6.1)

The glycine/tyrosine–rich type II KAP 6.1 fragment, was amplified from a pGEM3-Zf(+) plasmid using PCR and SP6/T7 primers. The KAP 6.1 gene sequence between *AvaI / HinfI* sites contained approximately 250bp of predominately coding sequence, with 14bp of 3'non-coding region (Fratini et al, 1993).

Human 28S ribosomal RNA (28S rRNA)

A 29-mer anti-sense oligonucleotide sequence from a region highly conserved among higher eukaryotes was used. The base sequence is as follows;

5'- AAA ATC AGA GTA GTG GTA TTT CAC CG -3'

oASCT2 transporter

A 360bp *HindIII/ BamHI* Bluescript plasmid subclone fragment contained the 5'coding region of the ovine ASCT type 2 neutral amino acid transporter (Nattrass, 2000). The fragment was obtained using a DNA digest of the pBluescript plasmid with BamHI, followed by gel fractionation and purification using the UltraClean™ GelSpin DNA Purification kit (MoBio Laboratories Inc.).

3.6.5 Preparation of DNA sequences (probes)

3.6.5.1 Polymerase chain reaction (PCR)

The PCR methodology was described by Saiki et al (1988) as in Sambrook et al (1989) and the conditions varied depending on the fragment that was being amplified. All reaction volumes were 25µl, the contents of which are outlined in Appendix 1.3.2, and were overlayed with mineral oil. The DNA amplification was performed in a Robocycler (Stratagene, USA) and Taq DNA polymerase enzyme (Amplitaq, Applied Biosystems) was added during the

extension step of the first cycle of forty, to activate DNA synthesis. Promoters for the SP6 and T7 primers, for example, were as follows; 1 cycle = 94°C/ 5 minutes, 40 cycles = 94°C / 30 seconds, 48°C/ 1 minute, 72°C/ 2 minutes.

PCR products were treated with Proteinase K (100µg/ml) to remove Taq polymerase and were phenol extracted to further purify the DNA sequence (Appendix 1). The DNA was ethanol precipitated with 0.1 v/v 4M sodium chloride, 2.5 v/v absolute ethanol and 10µg glycogen at –20°C for an hour, pelleted, dried under vacuum then resuspended in 100µl of sterile water. The DNA was electrophoresed in a 2% agarose gel in 1xTAE solution alongside SPP1/ EcoR1 and pUC19/ HpaII DNA size markers (Appendix 1.3.8; Geneworks, Adelaide) to demonstrate the integrity of the DNA, then photographed using a GelDoc 1000 System for image analysis (BioRad, USA). The concentration of DNA was estimated by comparing PCR fragment amounts, with the DNA quantities in the size marker bands (SPP1 marker; Appendix 1.3.8).

3.6.5.2 Plasmid digest

Restriction endonuclease digestion of the oASCT2 DNA plasmid stock (100ng/µL, approx. 5µg in 50µL) was performed at 37°C for 2-3 hours. The integrity and concentration of the DNA was determined as described in Section 3.6.2.2. The DNA digest was precipitated with 0.1 v/v 4M sodium chloride and 2.5 v/v absolute ethanol and electrophoresed in a 1% agarose gel in 1 x TAE, at 110mA (140V) for 15 minutes. After ethidium bromide staining, agarose containing the DNA fragment was cut from the gel under UV light, using a sterile scalpel blade and the DNA was purified from the gel slice using an UltraClean™ GelSpin DNA Purification kit (MoBio Laboratories Inc.).

3.6.6 Pre-hybridisation of northern membranes

Northern blot filters were pre-hybridised in 10ml of (pre) hybridisation solution (Appendix 1.3.2). Incubations were carried out in glass bottles (four filters per bottle), at 42°C in a hybridisation incubator (Hybaid, UK) for approximately 1 hour.

3.6.7 Radiolabeling and hybridisation of northern membranes

Probe DNA (Section 3.6.4), for all fragments except 28S rRNA (see next paragraph), was labelled with random primers and α-^{32}P-dCTP using a MEGAprime labelling system kit (Amersham, UK). Unincorporated radiolabel was removed from the radiolabelled probe DNA by eluting probe solution through a size-separation column (Sepharose CL-6B). To probe northern membranes effectively, approximately 100-150ng of labelled DNA was required per probing (bottle), with a specific radioactivity of approximately 10^8 cpm/µg. Radiolabelled DNA probe was denatured at 100°C for five minutes then added to 10ml of hybridisation solution (Appendix 1.3.2). The pre-hybridisation solution was removed, the denatured DNA probe/ hybridisation mixture added, and hybridisation of northern blots carried out at 42°C, for approximately 16-20 hours.

One µg of the 29-mer 28S rRNA oligonucleotide was labelled by kinasing in a 100µl reaction. The reaction was performed in 70mM Tris-HCl (pH7.6), 10mM $MgCl_2$ and 5mM DTT, and contained 100µCi of γ-^{32}P-rATP and 40 units of T4 Polynucleotide Kinase enzyme (New England Biolabs, Genesearch, Australia).

3.6.8 Washing, exposure, quantification and stripping of probed membranes

3.6.8.1 Washing probed membranes

Non-specifically bound probe DNA was removed from the northern membranes by washing with 2xSSC/0.5%SDS, initially at room temperature, then at 65°C if required. The level of hybridisation was estimated using a hand held geiger counter, to determine if further washing was required to remove non-specific binding.

3.6.8.2 Exposure and quantification of probed membranes

Membranes were sealed in plastic and exposed to phosphorimage screens (Fuji, BAS-MP) in autoradiograph cassettes (Fujix BAS-2040 cassette). Hybridisation was detected using phosphorimage analysis (STORM 860, Molecular Dynamics, USA) and quantification made using ImageQuant 1.0 software (Molecular Dynamics, USA). All expression (quantification) levels were standardised relative to the 28S rRNA measured for each individual sample.

3.6.8.3 Washing (stripping) of probed northern membranes

Hybridised, radiolabelled DNA was stripped from the northern membranes by washing membranes twice, using gentle agitation for 20 minutes, in 0.1xSSC / 0.1%SDS heated to 100°C. After each probe was stripped, examination of northern filters with a hand-held geiger counter verified that the majority of radioactivity had been removed from the filters. To eliminate the possibility that an initial probe could interfere with the signal from a subsequent probe fragment, probes were applied sequentially to detect transcripts of largely differing sizes.

Chapter 4

Quantification of nitrogen partitioning in sheep selected for wool and body growth phenotypes

4.1 Introduction

The partitioning of nutrients between body tissues, including peripheral production systems such as wool and muscle growth, involves complex physiological and metabolic events. Sheep breeds such as the Merino are often considered dual purpose animals for the production of meat and wool, and are therefore a very useful animal model when considering nutrient use between two tissues that are predominately composed of protein but have very different functionality and amino acid compositions. This experiment involved identification of Merinos from the same flock, using detailed genetic and rearing information, with considerably different wool and body growth, to develop an understanding of the processes controlling growth and the partitioning of protein between the skin and the whole body.

Different experimental genetic lines of Merino sheep have been developed in Australia and elsewhere, dating back to the early 1950s. Of interest here, lines selected for high or low weaning weights (WW) (Oddy et al, 1995), or selected for high or low fleece weights (FW) (Turner et al, 1970; Williams, 1987) have permitted the investigation of numerous aspects of metabolism and physiology. For example, sheep from the high FW selection lines have a greater wool growth response to abomasal methionine and cystine infusion (Williams et al, 1972b), have 20% lower plasma cysteine levels (Williams et al, 1972a) and a lower wool sulphur concentration (Piper and Dolling, 1966) than low FW sheep. Sheep from high WW selection lines show no differences in growth efficiency (Thompson et al, 1985a), little difference in the rate of energy expenditure (Thompson and Parks, 1985) and do not differ significantly in the rate of whole body wool growth (Herd et al, 1993). Selection for one production trait of interest, however, is not an ideal approach to investigate the regulation of partitioning between two traits of economical importance, namely meat and wool production.

In the following experiment, sheep selected for divergence in both wool and body growth were used to investigate the partitioning of nutrients between wool and body components. Sheep were selected from the Turretfield Selection Demonstration Flock, which is representative of the diversity in wool and body weight traits seen in typical breeding flocks

throughout South Australia (Ponzoni et al, 1999). The phenotypic differences between selected groups were 20-30 kg in bodyweight and 3-4 kg in annual fleece weight, despite being the same age (16 months) and being raised in the same flock and under the same environmental and management conditions. An understanding of the processes responsible for regulating wool and body growth may allow future manipulation of nutrient partitioning to these tissues.

Growth or 'production' is the result of the interaction between genetic drive, supply of nutrients or substrates, and various environmental factors. These processes are coordinated and modulated by the endocrine system (Breier and Gluckman, 1991). Hormones including those of the growth hormone/ insulin-like growth factor (GH/IGF-I) axis, and their associated binding proteins and receptors have received considerable attention in recent years (Hossner et al, 1997; Nixon and Moore, 1998; Su et al, 1999) and are important in the regulation of nutrient use by different tissues within the body, including muscle and skin.

Higher plasma IGF-I concentrations have been reported in sheep selected for weaning weight (Oddy et al, 1995) whilst infusion of the growth factor into the hindlimb of sheep (Oddy and Owens, 1996) has reduced protein degradation. *In vitro* promotion of hair growth with IGF-I (Philpott et al, 1994) and a transient increase in skin protein synthesis when IGF-I was infused into the skin of sheep (Lobley et al, 1997) lend further support to the involvement of IGF-I in the control of nutrient use. The roles of IGF-II are less well defined but are thought to be predominant mainly in the fetus. Despite the apparent roles of the IGFs in nutrient use in either muscle or skin, there is limited evidence of a relationship between the growth factors and the partitioning of nutrients between these two tissues. The release of the IGFs and the responsiveness of tissues to them depends on the level of intake and the composition of the diet (Bass et al, 1991; Clarke et al, 1993; Davenport et al, 1995; Oldham et al, 1999), and hence nutrition may also play a role in the partitioning between muscle and wool.

In the experiment discussed in this chapter, the novel approach to selection enabled investigation of wool and body growth concurrently using phenotypically-distinct Merino sheep, highly variable in wool growth and body weight. Feed intake was manipulated to elucidate differential responses in the production of IGFs and their action, and in the partitioning of protein (nitrogen) to skin and the whole body. The hypotheses tested were that the partitioning of nitrogen between wool and body growth is associated with plasma concentrations of the IGFs, and that these differences in partitioning between wool and body growth are expressed to a greater extent when a high intake is supplied.

4.2 Methodology

4.2.1 Experimental methodology

4.2.1.1 Selection of experimental sheep

Experiment 1 was conducted at the Waite Campus of the Adelaide University, over a period of 140 days and used forty-eight, 1997-born South Australian Merino rams from the SARDI Selection Demonstration Flocks (SDF) (Section 3.1.1). Data (at 16 months of age) were used to identify four distinct phenotypic groups (n=12) differing in wool growth rate (WGR) and body weight (BW), and follicle density was measured to ensure similar total follicle numbers (Section 3.1.2) for all four groups. The selection groups are referred to as follows; high WGR, high BW (+W +BW), high WGR, low BW (+W –BW), low WGR, low BW (-W –BW) and low WGR, high BW (-W +BW). The distribution of BW and WGR for the entire flock and for selected individuals used in the analysis at 16 months of age, is shown in Figure 4.1. The selection group means for WGR, BW, total follicle number and fibre diameter are shown in (Table 4.1).

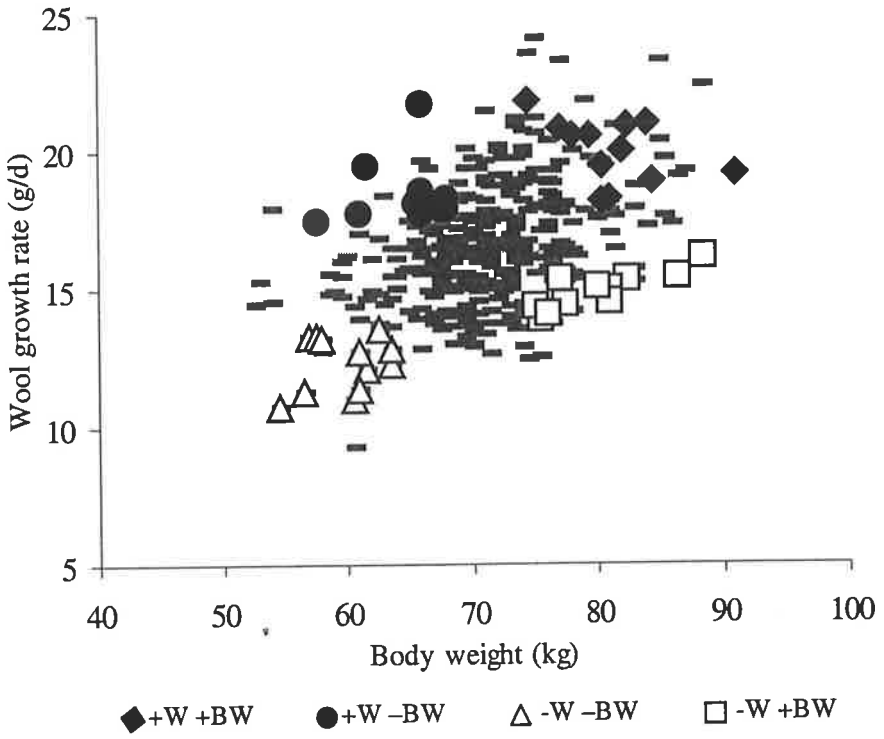

Figure 4.1: Distribution of body weight (BW) and average wool growth rate (WGR) phenotypes for all 1997-born Selection Demonstration Flock rams, including those selected for Experiment 1.

Table 4.1: Average BW, WGR, fibre diameter and total number of follicles at 16 months of age, for each phenotypic selection group.

	+W +BW	+W -BW	-W -BW	-W +BW	Pooled SEM
Body weight (BW) (kg)	81.2 ^{c}	65.0 ^{b}	60.0 ^{a}	79.8 ^{c}	1.23
Wool growth rate (WGR) (g/ d)	20.0 ^{d}	18.4 ^{c}	12.4 ^{a}	14.9 ^{b}	0.32
Fibre diameter (μm)	21.4	21.0	20.0	21.6	0.42
Total follicle number (x10^{6} follicles)	93	88	86	82	5.13

Different superscripts within a row differ at $p<0.05$

4.2.1.2 Housing and experimental measurements

The rams were 18 months of age and were shorn 3 months prior to the commencement of the experiment, which was conducted indoors, with sheep housed individually in pens and

monitored daily. The large number of sheep were randomly segregated into 3 experimental sub-groups (A, B, C) of 16 sheep per sub-groups. Each sub-group contained individuals representing all treatments equally and thus, facilitated efficient wool sampling, urine and faeces collection and blood sampling. Body weight was measured once a week using electronic scales, the required feed intake level for maintenance of BW indoors calculated (MAFF Bulletin, 1975) and the feed offered adjusted accordingly on a weekly basis. Water was available *ad libitum* and feed refusal and wastage was recorded daily. All sheep were drenched (Ivomec®, Merial) approximately one month before the experiment commenced.

The schedule of sampling is outlined in Figure 4.2. A number of problems were encountered during the study that altered the initial experimental design, details of which are provided in Appendix 2.

Measurement of wool growth rate (WGR) is described in Section 3.2.1. Nitrogen (N) balance was determined over a seven-day period, involving the collection of feed, faeces and urine samples (Section 3.3.1) and samples were analysed using the Kjeldahl and Leco techniques (Section 3.3.2). Serial blood samples were taken from a jugular catheter inserted one day prior to sampling (Section 3.4.1), for the analysis of plasma insulin-like growth factor concentrations (Section 3.4.1).

4.2.1.3 Experimental nutrition

The sheep were fed a pelleted diet for the first two months of the experiment, to maintain body weight (Sheep Nut, Ridley Agriproducts, Murray Bridge). Requirements were calculated using the equation for sheep maintained indoors (MAFF Bulletin, 1975).

The nutritional requirements were reassessed after two months and a modified diet was fed during experimentation, consisting of 65% sheep nut pellets and 35% lucerne chaff, the energy and protein contents of which were determined (Feedtest, Hamilton) and are displayed in Table 4.2. The pellet/chaff ration was fed for four weeks to maintain body weight and adapt animals to the new nutritional regime, prior to applying experimental levels of intake. Individuals were then randomly assigned to a low or high intake level of the pellet/chaff diet

(0.9 maintenance requirements (0.9xM) or 1.8 maintenance requirements (1.8xM); n=6). The two levels of intake were supplied for the remainder of the trial.

Table 4.2: Nutritional composition of the pellet and lucerne chaff used in Experiment 1.

	Pellet A	Lucerne chaff
DMD (%DM)	82	73
Crude Protein (%DM)	15.2	22.2
ME (MJ/ kg DM)	12.4	10.6

4.2.2 Statistical methodology

REML Variance Components Analysis (GENSTAT, 5th Edition) was used to analyse data on wool growth, body weight and N balance (Section 4.3.1 and 4.3.2) using a model containing the fixed factors "selection group" and "intake level", and "sub group" as a random effect.

A general linear regression approach (GENSTAT, 5th Edition) was used in Section 4.3.1.4. The fitted maximal model related the rate of wool growth to that of bodyweight gain, dietary treatment and selection group, and the two and three way interactions associated with these factors. The reduced model for which any fixed effects, explanatory variates and interactions that were not significant at the 5% level were systematically removed was,

WGR = BW gain + Intake.

A general linear regression model (GENSTAT, 5th Edition) was used for Sections 4.3.3 and 4.3.4 to relate body weight, wool growth and N partitioning ('x' variables) to IGF-I and IGF-II concentrations. The fitted maximal model consisted of the variable of interest (IGF-I or IGF-II), selection group and dietary treatment, and the two and three way interactions associated with these factors. The final reduced model was derived in the same manner as above and was,

'x' = IGF-I (or –II) + Intake (unless otherwise stated in the text).

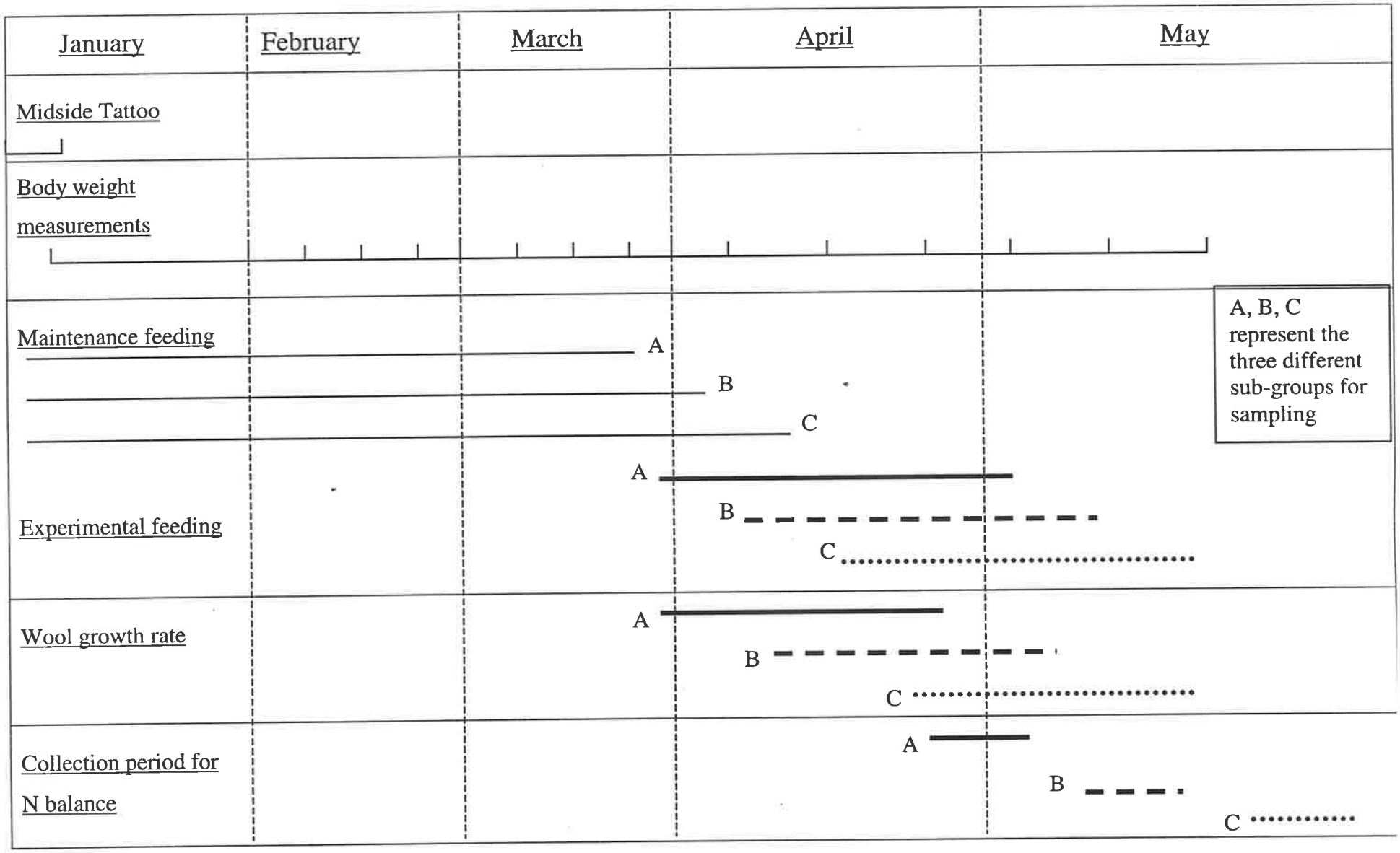

Figure 4.2: Experimental schedule for sampling for Experiment 1

4.3 Results

4.3.1 Wool growth (WGR) and body weight (BW)

4.3.1.1 Experimental phenotypes

The average BW and WGR during the experiment and previously when selected in the field for each selection group and dietary treatment are compared in Figure 4.3. Open symbols represent animals on the high (1.8xM) intake and closed symbols represent animals on the low (0.9xM) intake; this legend is used in all Figures throughout this experimental chapter.

Low intake

When the sheep were fed 0.9xM and housed indoors there was no difference in the average BW gain between the selection groups, therefore the selected BW groups remained significantly different for this trait (ie. bodyweight). However, the magnitude of differences in WGR between groups differed when compared during the experiment, than when compared to field data. That is, the WGR of +W sheep was approximately 15% less during the experiment than in the field. In contrast, the WGR of -W sheep was approximately 15% greater during the experiment than in the field. As a consequence, the wool phenotypes selected in the field were not expressed indoors at the low level of nutrition, and thus WGR did not differ significantly between the four selection groups (Figure 4.3)

High intake

Sheep fed the high level of nutrition responded differently to sheep fed the low intake when housed indoors. All selection groups increased BW by 10 to 15 % during the experiment and as such, +BW sheep remained heavier than –BW selected sheep. However, +W-BW sheep produced similar amounts of wool to both the –W+BW and –W-BW groups (approximately 22.0g/d) yet the +W+BW sheep produced significantly more wool (26.2g/d) than the other three selected groups, therefore the phenotypic differences in WGR selected for in the field were again not maintained during this experiment (Figure 4.3).

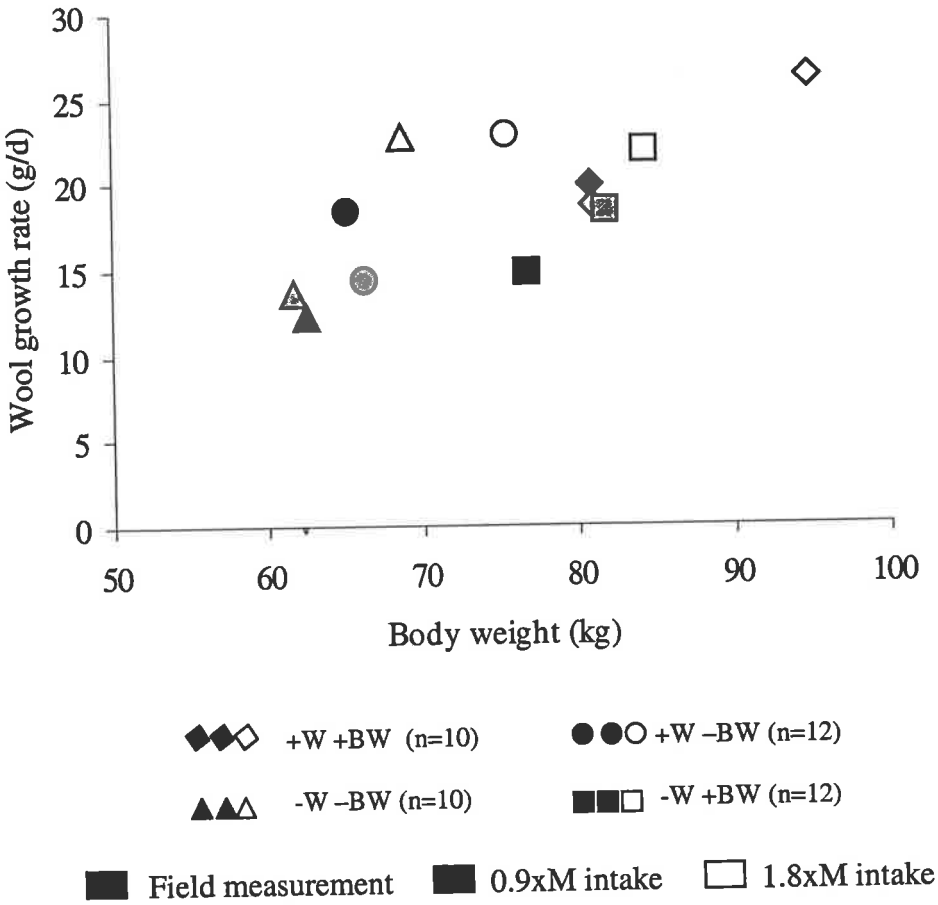

Figure 4.3: Wool growth (W) and body growth (BW) phenotypes in the field, and when sheep consumed low (0.9 x maintenance) or a high (1.8 x maintenance) intake during the experiment.

4.3.1.2 Wool growth rate (WGR)

There was no significant group x intake interaction for WGR thus the data presented in Table 4.3 represents the WGR for each selection group averaged across the two levels of intake. There was no significant difference between selection groups in WGR, as measured from the midside patch (WGR ^{A} ; mg/cm^2/d) during the experiment (Table 4.3). However, sheep fed 1.8xM grew significantly more wool than sheep fed 0.9xM (1.33 vs 0.98 mg/cm^2/d; $p<0.001$). For whole body WGR (WGR ^{B}) all sheep selected for +BW grew similar amounts of wool (22.5 g/d vs. 20.2 g/d for +W and –W groups respectively) but the +W+BW group grew significantly ($p<0.05$) more wool than the two groups selected for –BW characteristics (18.6 g/d vs. 18.2 g/d for +W and –W groups respectively).

Table 4.3: Average midside (A) and whole body (B) wool growth rate (WGR) for each selection group and level of intake.

		WGR ^{A} (mg/cm^2/d)	WGR ^{B} (g/d)
Selection group	+W +BW	1.19^{a}	22.5^{b}
	+W -BW	1.13^{a}	18.6^{a}
	-W -BW	1.17^{a}	18.2^{a}
	-W +BW	1.11^{a}	20.2^{ab}
	Pooled SEM	0.08	1.37
Intake supply	Low	0.98^{a}	16.3^{a}
	High	1.33^{b}	23.4^{b}
	Pooled SEM	0.06	0.94

Different superscripts within a column differ at $p<0.001$.

No interactions were significant so comparisons are made *within* each main effect (that is *within* selection group and intake level).

^{A} WGR (mg/cm^2/d) calculated from the amount of clean wool produced from a tattooed midside patch of known area.

^{B} WGR (g/d) The total body WGR calculated from patch WGR^{A} and a surface area estimation (0.094 x BW $^{0.67}$; Bennett, 1973).

4.3.1.3 Body weight (BW) and BW gain

There was no significant difference in the average rate of BW gain between the four selection groups (Table 4.4). At the end of the experiment the average BW of all groups differed significantly from each other ($p<0.001$). That is, +W+BW had the highest BW (87.8kg) followed by -W+BW (83.1kg) , then +W–BW (70.8kg), and the lowest average BW was for the -W–BW group (65.3kg). Sheep consuming the high level of nutrition (1.8xM) had the greatest BW gain during the experimental period and consequently the greater final BW (Table 4.4).

Table 4.4: Average body weight gain (BW gain) and body weight (BW), for each selection group and level of intake.

		BW gain (g/d)	BW (kg)
Selection group	+W +BW	261	87.8^{d}
	+W -BW	193	70.8^{b}
	-W -BW	120	65.3^{a}
	-W +BW	212	83.1^{c}
	Pooled SEM	76.8	1.66
Intake supply	Low	23^{a}	72.7^{a}
	High	370^{b}	80.8^{b}
	Pooled SEM	62.8	1.14

Different superscripts differ within columns at $p< 0.001$

4.3.1.4 The relationship between wool and body growth

Approximately 33% of the total variation in WGR was accounted for by BW gain (6%; $p=0.05$) but the majority was accounted for by the level of intake (27%; $p<0.001$). Figure 4.4 depicts the relationship between WGR and BW gain for sheep fed 0.9xM (dark symbols) and 1.8xM (open symbols) intake levels, and shows that there was a greater rate of wool growth and BW gain generally measured in sheep fed the high level of intake.

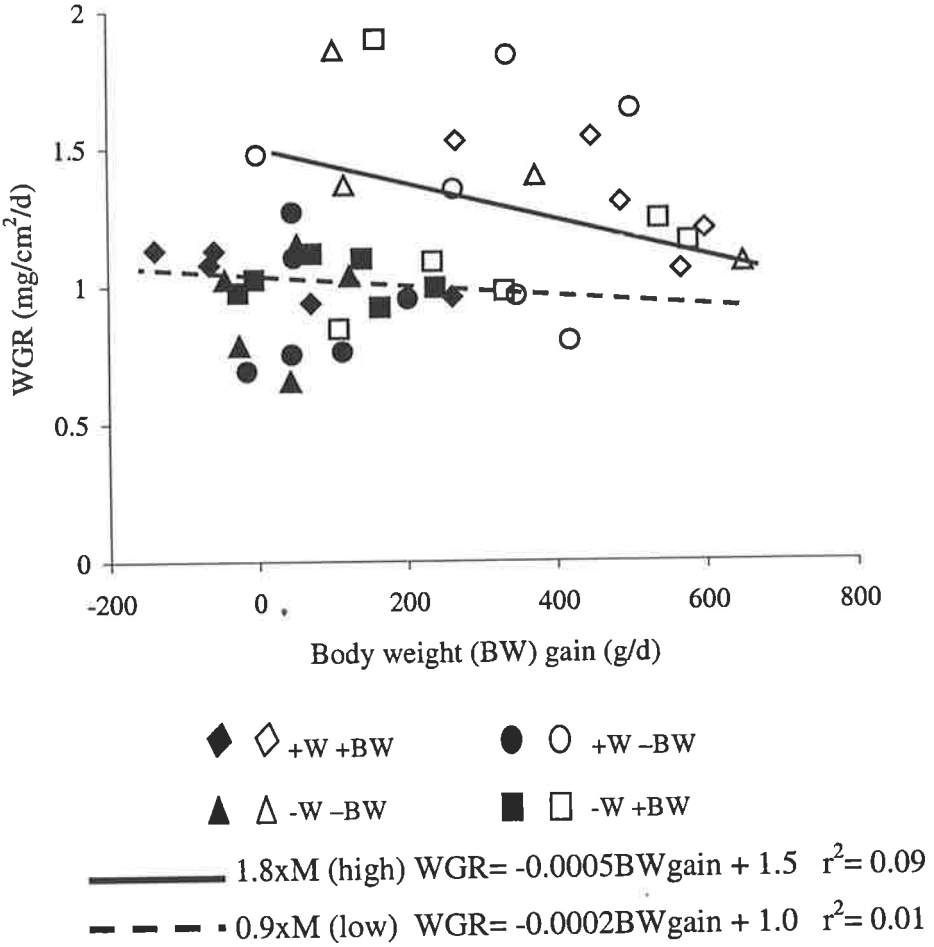

Figure 4.4: The relationship between the rate of wool (WGR) and body (BW) gain in sheep selected for BW and WGR phenotypes, and fed high (open symbols) and low (closed symbols) levels of intake.

4.3.2 The retention, utilisation and partitioning of nitrogen for wool production.

4.3.2.1 N retention and utilisation

The data in Table 4.5 describe the components of N balance. Sheep selected for +BW consumed significantly more N than sheep selected for –BW ($p<0.001$) due to their higher levels of intake that were calculated from their bodyweight (see Section 4.2.1.3). When expressed per kg of BW, N intake did not differ between groups (Appendix 2).

Sheep fed 1.8xM consumed approximately double the N of sheep fed the 0.9xM intake ($p<0.001$). Despite this, there was no significant difference in the apparent digestibility of N between sheep fed the high or the low level of intake, or between the selection groups. There was also no difference in the whole body retention of N between selection groups, but sheep

that consumed the high level of intake retained more N than sheep fed the low level of intake ($p<0.001$). Sheep fed 1.8xM utilised proportionally more of the apparently digested N than sheep fed the low intake (approximately 50% vs 32%; $p<0.001$). The utilisation of apparently digested N did not differ between selection groups.

Table 4.5: Nitrogen consumption, digestion, retention and utilisation.

	Intake level	+W +BW	+W -BW	-W -BW	-W +BW	Average pooled across selection groups	Pooled SEM
Intake N	Low	27.5^{b}	23.3^{a}	22.1^{a}	26.9^{b}	25.0	1.56
(gN/d)	High	59.0^{e}	47.2^{d}	42.9^{c}	48.2^{d}	49.3	
Digestibility N[1]	Low	72.5	72.3	71.3	72.1	72.1	3.08
(%)	High	72.0	70.9	72.1	72.3	71.8	
Retained N[2]	Low	6.1	5.2	5.6	6.5	5.8^{a}	1.49
(gN/d)	High	21.3	16.0	19.1	17.6	18.5^{b}	
Utilised N[3]	Low	30.0	30.1	34.0	34.2	32.0^{a}	5.71
(%)	High	50.4	47.4	62.7	51.3	52.9^{b}	

Different superscripts differ at $p<0.05$ for ;
N intake, comparisons between selection group x intake level (interaction significant, $p<0.05$)
All other variables, comparisons between intake levels (pooled across selection groups; group x intake interaction and selection group differences were not significant at $p<0.05$).

[1] The apparent digestibility of N calculated as (N excreted in faeces /d) / (N consumed /d), expressed as a percentage.

[2] The amount of N retained, g/d, calculated as the difference between the amount of N consumed /d and that excreted in faeces *and* urine, expressed as N g/d.

[3] The proportion of N utilised by the body, '2' (N retention) divided by the amount of N digested /d (N consumed – N excreted in faeces), expressed as a percentage.

NB: Pooled SEM refers to pooled standard error of the mean

4.3.2.2 N partitioning

The proportions of retained N that was partitioned to wool or to other body components are shown in Table 4.6 (the total amount of retained N partitioned to wool and other body components is described in Appendix 2). There were no significant differences in the proportion of retained N that was partitioned to wool or body growth between selection groups. However, the partitioning of N differed when sheep were consuming different intakes

($p<0.001$). That is, equal proportions of N were partitioned between wool and other body components when the low level of intake was consumed but only 20% of retained N was used for wool growth and 80% for other body components when sheep were consuming the high level of intake (Table 4.6).

Table 4.6: The proportion of nitrogen partitioned between wool and other body components.

	Intake level	+W +BW	+W -BW	-W -BW	-W +BW	Average pooled across selection groups
N wool [1] (%)	Low	59	51	50	52	53
	High	19	23	18	19	20
N body [2] (%)	Low	41	49	50	48	47
	High	81	77	82	81	80

Note : standard error associated with intake main effect 4.33 and group x intake interaction 9.80.
[1] The proportion of retained N (Table 4.5) that is partitioned to wool (assumed the protein content of wool to be 100%, therefore N content 16%), multiplied by the wool production (Table 4.3) and expressed as a percentage of retained N.
[2] Retained N (Table 4.5) minus the quantity of N produced in wool (above), expressed as a percentage of retained N.

4.3.3 The association of insulin-like growth factor-I (IGF-I) with WGR and BW, and the partitioning of nitrogen.

Table 4.7 summarises the relationships between IGF-I and various body and wool growth measurements. The mean plasma IGF-I concentrations for selection groups and intake levels are tabulated in Appendix 2.

Table 4.7 : Summary of the regression analysis of various body and wool growth measurements with the main effects of level of intake and plasma IGF-I concentration.

	Percentage of variation accounted for by main effects			
Variable	Final model	IGF-I concentration	Intake	Group
BW gain (g/d)	48	21***	27***	ns
BW (kg)	85	41***	6***	38***
N retained in body [1](gN/d)	79	10***	69***	ns
Proportion of retained N in body components (%)	45	ns	45***	ns
WGR [2] (mg/cm^2/d)	33	8*	25***	ns
WGR [3] (g/d)	48	24***	24***	ns
Proportion of retained N in wool (%)	47	2 ns	44	1 ns

*** p<0.001, ** p<0.01, * p<0.05, ns not significant (removed from final model; p>0.05), the number indicates the proportion of each main effect that contributes to the variation.
[1] The amount of N partitioned to body components excluding wool, calculated as retained N (Table 4.5) minus N excreted in wool ([3]), expressed in g/d.
[2] WGR per unit area (mg/ cm^2/ d), measured by wool patch
[3] WGR, total body (g/ d), calculated from the wool patch measurement and surface area estimation (derived from body weight)

4.3.3.1 IGF-I and body growth

Approximately 85% of the variation in BW could be accounted for by feed intake (6%, $p<0.001$), plasma IGF-I concentration (41%, $p<0.001$) and selection group (38%, $p<0.001$) (Table 4.7). A significant, positive association between final BW and plasma IGF-I concentration was identified for both the high and the low intakes (Figure 4.5). 48% of the variation in the rate of BW gain was accounted for by intake level (27%; $p<0.001$) and IGF-I concentration (21%; $p<0.001$).

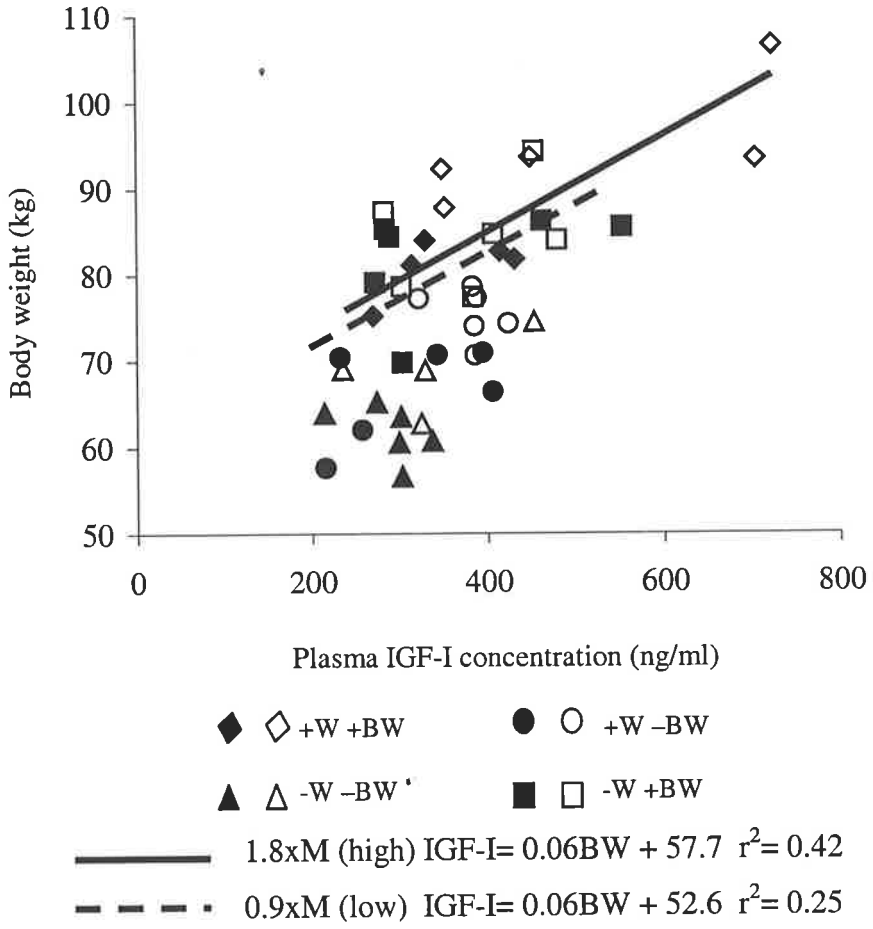

Figure 4.5: The association between plasma IGF-I concentration and body weight (BW) for sheep selected for high and low clean fleece weight (CFW) and BW, and fed the same diet, at a high (open symbols) or low (closed symbols) intake.

4.3.3.2 IGF-I and wool growth

33% of the variation in WGR (mg/cm^2/d) and 48% of the variation in whole body WGR (g/d) was accounted for by the feed intake and the plasma concentration of IGF-I (Table 4.7). Only 8% of the WGR variation could be accounted for by variation in plasma IGF-I concentration, and when expressed as whole body WGR (g/d), this increased to 24% (Figure 4.6) but most of the variation in WGR was related to feed intake level (approximately 25%).

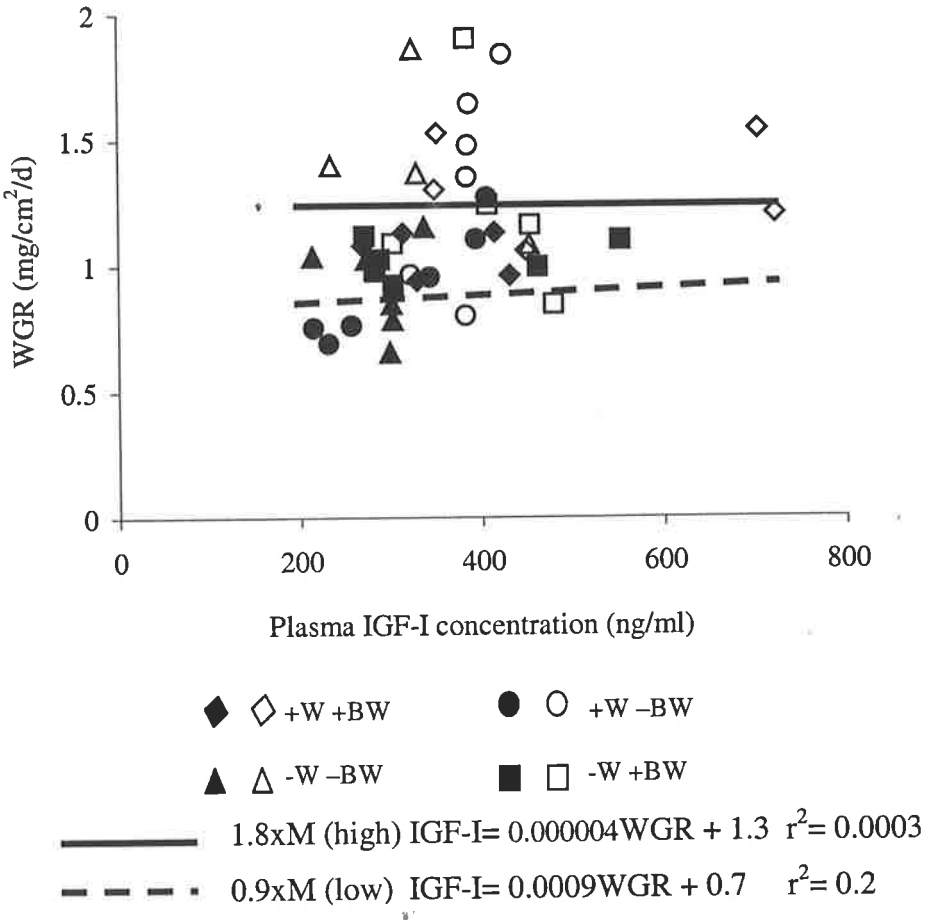

Figure 4.6: The association between plasma IGF-I concentration and wool growth rate (WGR), for sheep selected for high and low clean fleece weight (CFW) and body weight (BW), and fed the same diet, at a high (open symbols) or low (closed symbols) intake.

4.3.3.3 IGF-I and the partitioning of protein (N) to body growth

The partitioning of retained N to body components excluding wool was significantly associated with the level of intake and the plasma IGF-I concentration (Table 4.7). Both main effects accounted for a significant ($p<0.001$) proportion of the total variation (79%) in the amount of N partitioned to body components other than wool. However, most of this was explained by the level of intake (69% variation) with a much smaller component explained by plasma IGF-I concentration (10% variation) (Figure 4.7). There was no significant association between plasma IGF-I concentration and the proportion of total retained N that was partitioned to body growth (Table 4.7).

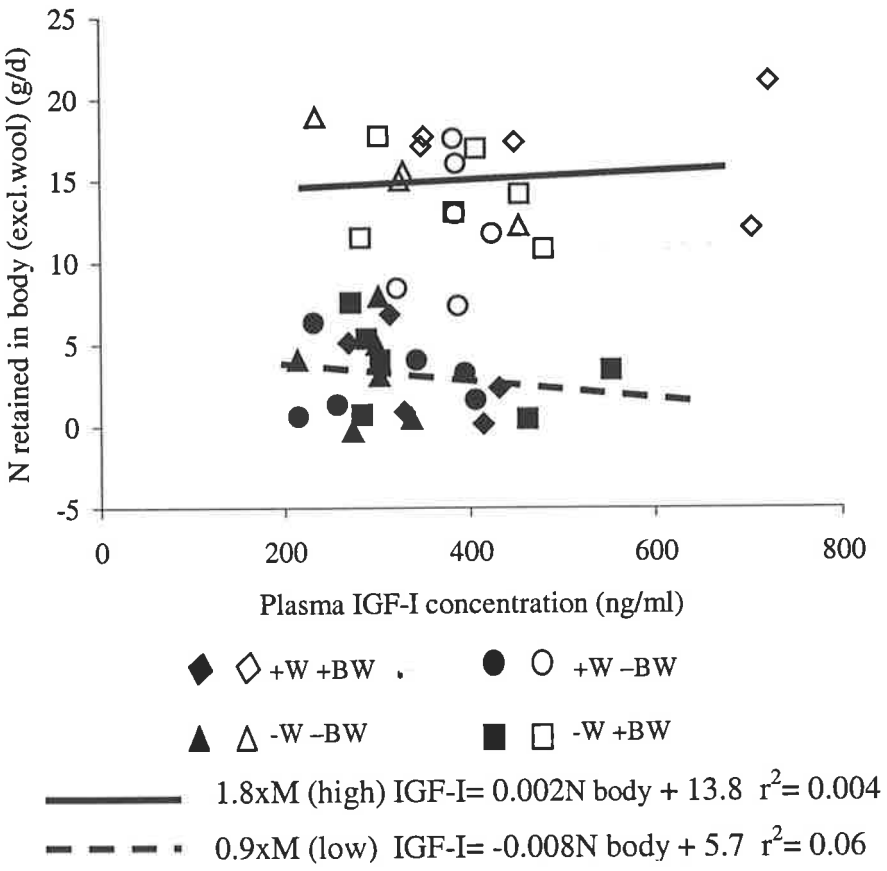

Figure 4.7: The association between plasma IGF-I concentration and the amount of nitrogen partitioned to body growth (excluding wool), for sheep selected for high and low clean fleece weight (CFW) and body weight (BW), and fed the same diet, at a high (open symbols) or low (closed symbols) intake.

4.3.4 Regression analysis of insulin-like growth factor-II (IGF-II) with WGR and BW, and the partitioning of nitrogen.

Table 4.8 summarises the relationships between IGF-II and various body and wool growth measurements. The mean plasma IGF-II concentrations for selection groups and intake levels are tabulated in Appendix 2.

Table 4.8: Summary of the regression analysis of various body and wool growth measurements with the main effects of level of intake and plasma IGF-II concentration.

Variable	Percentage variation accounted for by main effects			
	Final model	IGF-II concentration	Intake	Group
BW gain (g/d)	41	ns	41***	ns
BW (kg)	79	7***	11***	61***
N retained in body [1] (gN/d)	78	10***	68***	ns
Proportion of retained N in body components (%)	44	ns	44***	ns
WGR [2] ($mg/cm^2/d$)	32	4 ns	28***	ns
WGR [3] (g/d)	41	7*	34***	ns
Proportion of retained N in wool (%)	45	4 ns	41***	ns

*** $p<0.001$, ** $p<0.01$, * $p<0.05$, ns not significant (removed from final model; $p>0.05$), the number indicates the proportion of each main effect that contributes to the variation.

[1] The amount of N partitioned to body components excluding wool, calculated as retained N (Table 4.5 and 4.6) minus that excreted in wool ([3]), expressed in g/d.

[2] WGR per unit area ($mg/ cm^2/ d$), measured by wool patch.

[3] WGR, total body (g/ d), calculated from the wool patch measurement and surface area estimation (derived from body weight)

4.3.4.1 IGF-II and body growth

There was a significant ($p<0.001$) positive relationship between BW gain and the level of intake consumed, with 41% of the variation in gain accounted for by feed intake. None of the variation in BW was significantly accounted for by plasma IGF-II concentration (Table 4.8). The selection group accounted for the majority of the variation (61%, $p<0.001$) in BW at the end of the experiment. The low negative association between BW and plasma IGF-II (7%; $p<0.001$) is shown in Figure 4.8, for the high and low intake levels.

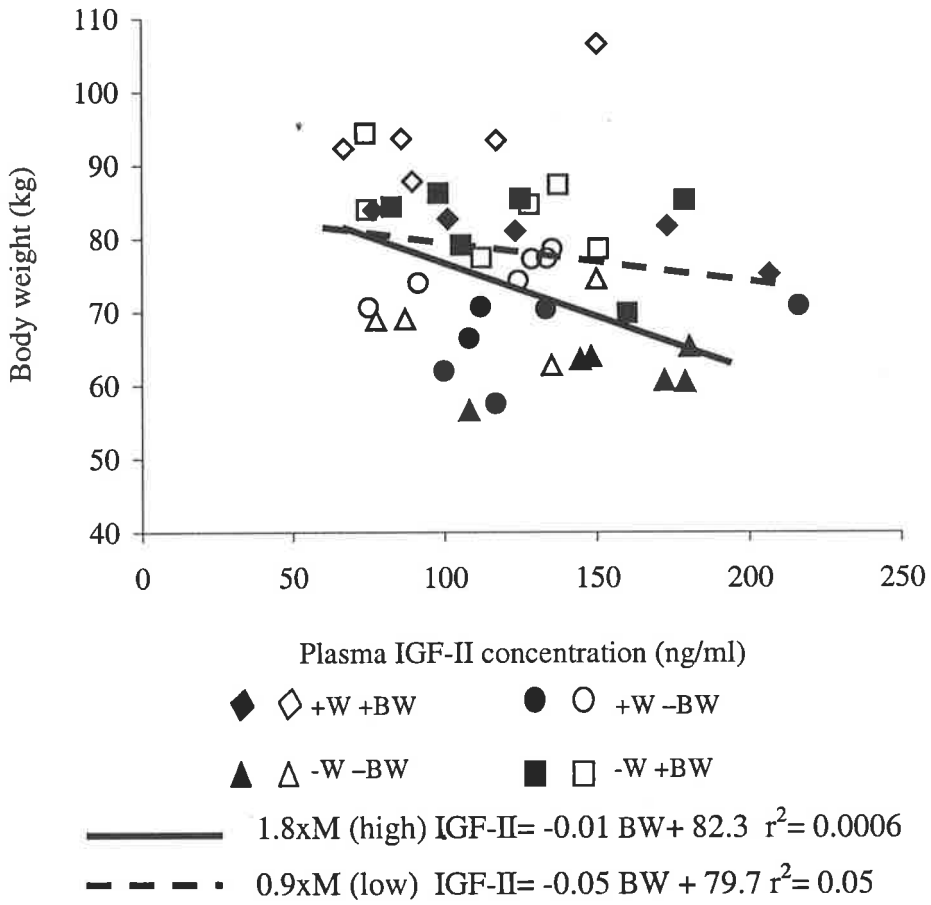

Figure 4.8: The association between plasma IGF-II concentration and BW at the end of the experiment, for sheep selected for high and low clean fleece weight (CFW) and body weight (BW), and fed the same diet, at a high (open symbols) or low (closed symbols) intake.

4.3.4.2 IGF-II and wool growth

There was no significant relationship between WGR per unit area of skin (mg/cm2/d) and plasma IGF-II concentration, however 28% of the variation in WGR was accounted for by feed intake ($p<0.001$; Table 4.8 and Figure 4.9). When WGR was expressed for the whole body (g/d), 34% of the variation was accounted for by intake level ($p<0.001$) and 7% by plasma IGF-II concentration ($p<0.05$).

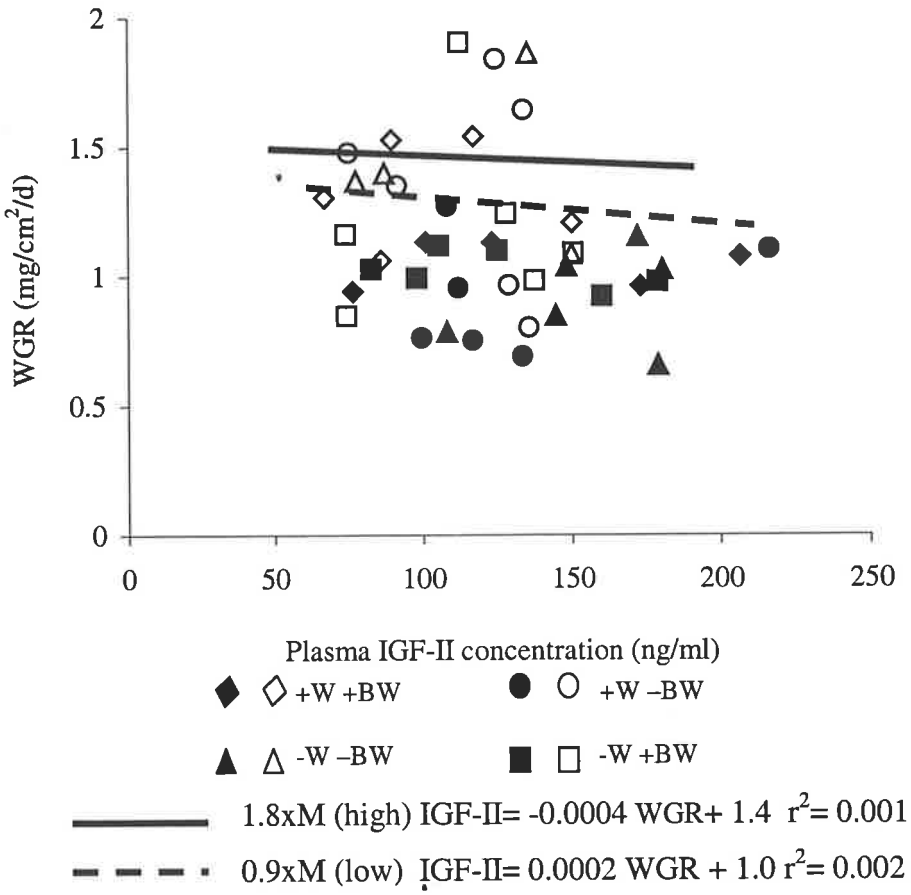

Figure 4.9: The association between plasma IGF-II concentration and WGR, for sheep selected for high and low clean fleece weight (CFW) and body weight (BW), and fed the same diet, at a high (open symbols) or low (closed symbols) intake.

4.3.4.3 Plasma IGF-II concentration and the partitioning of protein (N) to body growth

The level of intake (68%, $p<0.001$) and the plasma IGF-II concentration (10%, $p<0.001$) together accounted for 78% of the variation in the amount of retained N partitioned to body components excluding wool, such that as intake increased and plasma IGF-II concentration decreased, the amount of N partitioned to body components excluding wool increased (Table 4.8; Figure 4.10). The proportion of retained N that was partitioned to body components excluding wool was positively associated with the level of intake (44%, $p<0.001$) but not related to plasma IGF-II concentration (Table 4.8).

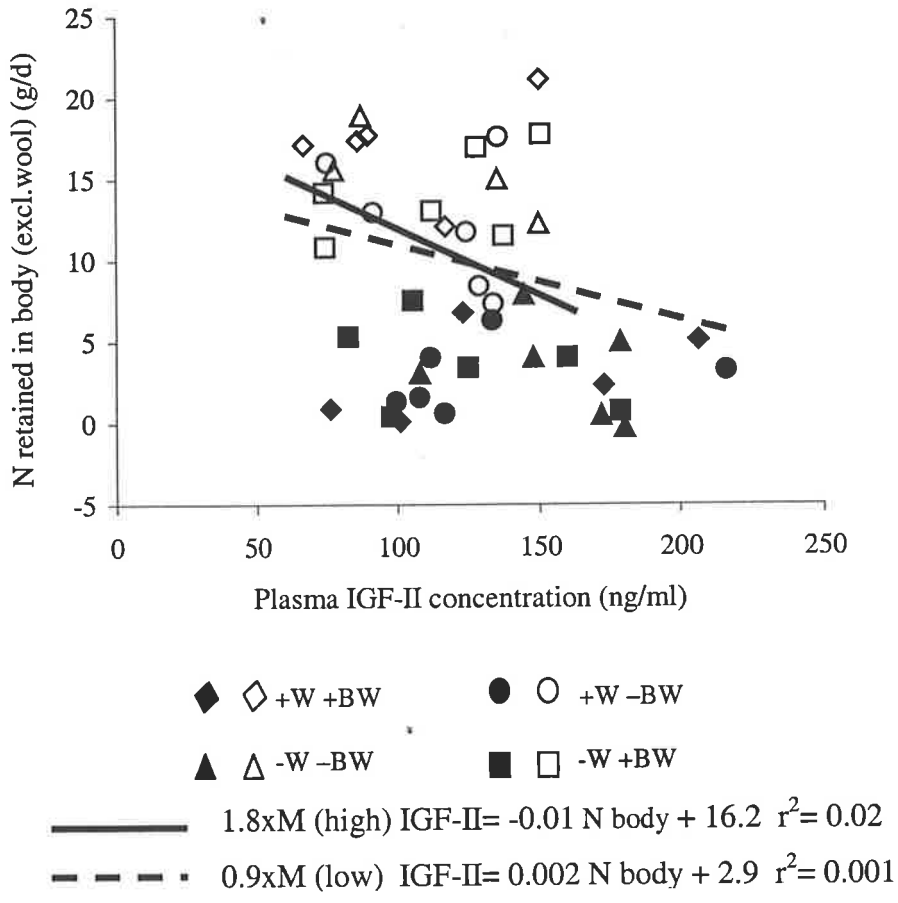

Figure 4.10: The association between plasma IGF-II concentration and the amount of nitrogen partitioned to body growth, for sheep selected for high and low clean fleece weight (CFW) and body weight (BW), and fed the same diet, at a high (open symbols) or low (closed symbols) intake.

4.4 Discussion

The objective of the study was to examine the relationship between the insulin-like growth factors (IGFs), wool and body growth, and the partitioning of retained N between these functions, when sheep were fed two different levels of nutrition. The quantity of retained protein (N) that was partitioned to wool growth and to a lesser extent body growth, was related to the plasma concentration of IGF-I but not IGF-II. Therefore, the first hypothesis was partially supported, that is that partitioning of nitrogen between wool and body growth is associated with plasma concentrations of IGFs. However, because the selected groups did not significantly differ in the proportion of retained N partitioned between wool and body growth at low or high intakes, the second hypothesis was not supported. Large unexpected shifts in WGR during the experiment compared to the initial selected phenotypes meant there were no differences in the rate of wool growth or body weight gain between the selected groups (phenotypes), during the experimental period. Possible reasons for this will now be considered.

4.4.1 Why did the selected phenotypes for wool change during the experimental period?

Sheep that were initially selected for +W grew 40% more wool than –W producers (19.2 vs. 13.6 g/d), and +BW sheep weighed 30% more than the –BW group (80.5 vs. 62.4 kg) when grazing prior to the commencement of the experiment. The differences in these phenotypes were similar to those described for other divergent, single trait selection flocks. For example, ewes from the Trangie weaning plus and weaning minus selection lines (+WW, -WW) differed in live weight by 35% when housed indoors (Herd et al, 1991). Romney fleece weight selected (+FW) and control lines differed in greasy wool production by 20% (Blair, 1986). The difference in the rate of wool production between Merino fleece weight selection lines varied between 16% (Williams et al, 1972b) and 80- 90% (Williams, 1995) when trialed indoors. Given the differences between + and – sheep for body and fleece weight in other

flocks that have involved indoor studies, the lack of phenotypic difference in WGR expressed in this study was not anticipated.

There have been reports describing the phenotypic variation in wool traits when grazing compared to when sheep were studied indoors. In Merino FW selection lines, for example, the difference between the two lines in fleece production was greatest when sheep were grazing outdoors and the variation decreased when the sheep were studied indoors (Graham, 1968). Similarly, in Merino staple strength (SS) selection lines no variation in SS was measured between high and low selected lines when sheep were studied inside (Schlink et al, 1998). These data indicate that significant genotype x environment interactions influence these wool traits, suggesting that the results from one study may not necessarily apply to other circumstances. This is relevant to the current study, highlighting the difficulty in predicting wool production traits and the partitioning of nutrients to wool, and has ramifications for industries such as shedded sheep enterprises, where sheep are generally selected on the basis of field performance, but then moved indoors.

Following the current study, estimated breeding values (EBVs) for clean fleece production were calculated using bloodline records and wool data collected from the flock at their first shearing. Using the calculated figures, the sheep were re-classified into the four phenotypic groups, but the newly-selected groups were identical to those generated using the initial raw data. This suggests that the effect of genotype on the expressed wool phenotypes during the experiment was far less influential in comparison with environmental effects.

Environmental effects such as the extremes in weather conditions, including temperature and wind exposure, which would be experienced in the field are removed when sheep are housed indoors. Altered feeding behaviour, including elimination of grazing activity, and the supply of a non-pasture diet once a day when indoors may have influenced wool growth. Pritchard et al (1986) showed some superiority of grazing selection in high wool producing sheep. The housing of sheep in pens alters, if not removes, the usual social interaction amongst the flock that may, for example, be beneficial for individuals that are

normally low in the heirachial structure of a flock. Furthermore, van Adrichem et al (1993) found isolation of sheep from their usual flock environment to individual housing increased the metabolic rate and decreased the apparent digestibility of feed.

The increased interaction with humans on a daily basis may be more stressful for some individuals and may have led to suppressed rates of wool or body growth. Numerous studies have investigated the endocrinological changes in sheep in response to interactions with humans (Hargreaves and Hutson, 1990; Hall et al, 1998; Goddard et al, 2000), with Weisinger et al (2000) describing a decrease in feed intake associated with the administration of such stress hormones. The parasitic status of the sheep when selected in the field was not known, but if some sheep had a greater parasite burden than others, a reduction in the time spent grazing and herbage intake (Hutchings et al, 2000) may have led to lower initial wool production in the field. Thus the differential responses of individuals or selection groups as a whole to these types of conditions may be possible reasons for the variation in the wool production measured in the field versus penned conditions.

The deviation from the initially-defined wool phenotypes meant all selection groups produced wool at similar rates during the experiment (ranging from 0.8 to 1.9 mg/cm^2/d). Similarly the average rate of BW gain did not differ between selection groups but values for individuals within groups ranged significantly. Therefore, despite the overall similarities in group means during the experiment, the variation in BW and WGR for individuals enabled the relationship between the IGFs and wool and body growth and partitioning to be investigated.

4.4.2 Wool growth and the IGFs

There are no reports of the relationship between IGF-I or IGF-II concentration and wool growth in FW selection flocks to date, although there is no difference between selection lines in the concentration of related hormones such as insulin and growth hormone (Hough et al, 1988). The regression analysis reported here showed that the level of intake was the major

source of variation in WGR, such that WGR increased when the intake of N increased. There was no difference between any of the four selection groups in the relationship between IGF-I or IGF-II and WGR or the partitioning of N to wool, suggesting all groups responded to the level of feed intake in a similar fashion. Although IGF-I and to a lesser extent IGF-II concentration was weakly related to WGR and the partitioning of retained N to wool, the direct association between IGF-I or IGF-II with wool growth is unclear. Furthermore, less than 45% of the variation in WGR and partitioning was accounted for by the models described in this thesis that suggests sources in addition to those outlined in the models, such as other environmental factors, were responsible for most of the variation.

It is of interest to note that there were two individuals from the +W+BW selection group that had extremely high plasma IGF-I concentrations (approximately 700ng/ml) and consequently were points of high leverage in the analysis. Despite this, the individuals remained in the analysis because of their correspondingly high BW (93 kg and 107 kg; Figure 4.5) and high whole body WGR (30.2 g/day and 25.9 g/day; data not shown). Such high circulating IGF-I levels may indicate excessive endocrine production of IGF-I by the liver, perhaps due to poor negative feedback systems operating within the growth hormone (GH) / IGF-I axis, or higher circulating levels of IGFBP-3 that mediates the circulating levels of IGF-I. Nevertheless, the relationship between plasma IGF-I concentration and body or wool growth, described in this work was greatly diminished if these sheep were removed from the analysis (data not shown).

Despite the relatively weak association, IGF-I has been shown to promote fibre growth *in vitro* (Philpott et al, 1994), increase oxygen and cysteine uptake as well as skin blood flow during skin infusions (Harris et al, 1993a; Hocking- Edwards et al, 1995), and shown some positive relationships with fibre growth in transgenic sheep, although generally inconsistently (Damak et al, 1996). In comparison, there is limited evidence to indicate a role in fibre production for IGF-II, and this is further supported by the non-significant relationships described in this experiment. Most work to date has focused on a somewhat interventionist

approach whereby, for example, the infusion of IGFs and production of IGF transgenic sheep have been studied. To elucidate the role of the IGFs, if any, in the regulation of fibre production, an alternative approach should be applied in which the paracrine and autocrine synthesis and activity of the IGFs are investigated. For example, investigation of the expression of IGFs and their receptors and binding proteins, in the skin of sheep producing wool at different rates may indicate a possible role in partitioning of nutrients or wool growth. This issue was not explored in the current study because of the lack of significant differences in wool production between groups. Nevertheless, the small role for circulating IGF-I concentration, but not IGF-II, in regulating wool growth and the partitioning of nutrients (N) to wool growth that was observed, suggests that further studies of the growth factors at the tissue level in divergent wool producing sheep is warranted.

4.4.3 Body growth and the IGFs

The strong positive association in this experiment between plasma IGF-I concentration and bodyweight (41%), and between plasma IGF-I concentration and bodyweight gain (21%) (Table 4.7) supports the anabolic relationship described for meat-type sheep (Wylie et al, 1997), Merino crosses (Gatford et al, 1996) and Merinos from the weaning weight selection lines (Speck et al, 1989). There are reports in sheep of positive relationships between IGF-I concentration and live weight (Mears, 1995) and IGF-I concentration and the predicted carcass lean weight (Cameron and Cienfeogos-Rivas, 1994). Comparatively less work has focused on IGF-II as it is generally considered to be a fetal growth factor. There was no significant relationship between bodyweight or bodyweight gain and plasma IGF-II concentration derived from this experiment.

4.4.4 Nutrition and the partitioning of nutrients, WGR and BW.

Intake accounted for the majority of the variability in wool and body growth that was attributable to the models. The lack of any difference in the rate of wool or body growth between selection groups was matched by the lack of variability in the proportion of retained

N that was partitioned to wool or body growth. Notwithstanding this, the partitioning of retained N and the apparent digestion, retention and utilisation of N were significantly affected by nutrition. When sheep consumed the high level of intake, more N was apparently digested, retained and utilised (Table 4.5) and a greater proportion of retained N was partitioned to body growth rather than wool growth (Table 4.6). Plasma IGF-I concentrations were higher and plasma IGF-II concentrations lower in all selection groups fed the high level of intake compared to the same groups fed the low level of intake supporting previous reports (Bass et al, 1991; Hua et al, 1995; Oldham et al, 1999).

The comparatively greater proportion of retained N that was partitioned to wool in sheep fed the low intake (50%), compared to when sheep were fed the high intake (20%), becomes important when the rate of body growth is compromised at low feeding rates. The apparent paradox of continual wool growth when body growth may be limited at low feeding was documented as early as the late 1940's (Marston, 1948; Ferguson et al, 1949). Liu et al (1998) discussed the differential changes in protein synthesis in the skin versus the muscle in response to nutrition and stated that partitioning favoured WGR at sub maintenance feeding in a study using Merinos from Western Australia, but at supra maintenance feeding, partitioning favoured BW. Therefore, sheep consuming the low intake partitioned proportionally more retained N to fibre production despite producing wool at an overall slower rate. Cronje and Smuts (1994) showed that sheep fed a maintenance diet partitioned 8% of the N consumed to wool compared with 5% when fed *ad libitum*, similar to the 10% and 7% of N intake partitioned to wool in this experiment when fed the low and high levels of intake respectively.

The low level of intake supplied during this study was designed to be slightly below maintenance requirements. However, the BW gain was positive for all groups suggesting that the calculated maintenance requirements were in excess of actual requirements. This may have been because the rams were approaching two years of age and the N requirement for body growth would likely be lower than for growing sheep.

4.5 Summary

The quantity of retained protein (N) that was partitioned to wool growth and to a lesser extent body growth, was related to the plasma concentration of IGF-I but not IGF-II. At both the low and high level of intake, the selected groups did not significantly differ in wool and body growth, or in the proportion of retained N partitioned to these functions. This work confirms the significant association of IGF-I but not IGF-II with increasing BW and to a lesser extent with WGR. The study emphasised the significant genotype x environment interactions responsible for the wool phenotype expressed in the field and under experimental conditions.

Chapter 5

Sulphur utilisation in sheep with different estimated breeding values (EBVs) for wool growth.

5.1 Introduction

The production of wool within a flock of sheep is typically highly variable, both in fibre quality and the quantity produced. Since the amino acids containing sulphur (S), namely cystine and methionine, are usually the first limiting nutrients for wool growth (Reis, 1979) it is important to understand how an animal digests, utilises and partitions S-containing amino acids (SAA) in order to explain sources of variation in wool growth.

The previous chapter demonstrated the high variability and sensitivity of wool growth to the environment and nutrition, in sheep selected based on phenotype in the field and subsequently studied indoors. In the following experiment, the sources of variability in wool growth between individuals were further investigated, focusing on the partitioning of S to wool growth as this may limit the phenotypic expression of genetic differences in the rate of wool growth.

The improvement of fibre production when additional SAA are administered has been shown consistently over many years. Researchers have used a number of techniques to increase the supply of SAA and subsequently improve wool growth, including the use of abomasal infusions (Reis and Schinkel, 1961, 1963), sub-cutaneous injections (Stephenson et al, 1991), rumen protected supplements (Mata et al, 1995), intravenous injections (Reis et al, 1989), arterial infusions directly into a patch of skin (Harris et al, 1997), feeding diets high in SAA (Reis and Schinkel, 1963), and the use of solid licks or water supplements (Fenn and Leng, 1989). Despite this extensive evidence supporting the requirement of SAA for maximal wool growth, our understanding of the mechanisms involved in S utilisation by the skin and follicle remains incomplete.

A number of important researchers have investigated SAA metabolism and the production of wool using fleece weight selection lines, including the Merino fleece weight (FW) selection lines developed at Trangie in NSW (Pattie and Barlow, 1974) and Cunnamulla in QLD (Brown and Turner, 1968), and the Massey University Romney selection flocks in New Zealand (Blair et al, 1984, 1985). These studies have shown small, non-significant

differences in feed intake (Williams, 1979) and no difference in digestive function (Piper and Dolling, 1969) between selected flocks. No variation in the plasma sulphate concentration (Williams, 1995) or S and sulphate balances (Sun et al, 1994) existed between the lines, however in general low wool-producing sheep (-FW) have a higher plasma concentration of cystine (Williams et al, 1972a) from an early age (Williams, 1984). The association between the supply of blood to the skin and wool growth in FW selection lines is not clear (Hales and Fawcett, 1993; Harris et al, 1993b) and no mechanistic studies of nutrient uptake into the follicle itself have been conducted in sheep differing in wool-growing capacity. The S content of wool in +FW sheep is lower than that produced by -FW selected sheep (Piper and Dolling, 1966), but the total S output in the wool is higher for the +FW sheep. The selection for wool growth has also seen a divergence in the follicle population, with a greater total number of follicles in +FW sheep compared to –FW sheep (Brown and Turner, 1968; Barlow, 1974).

In the following experiment sheep were selected using estimated breeding values (EBVs). This enabled selection of two 'genotypic' groups of sheep that were divergent in fleece weight (W) yet similar in body weight and fibre diameter, thus providing an improved model for more detailed investigation of wool growth variability, than has been examined previously. The sheep were sourced from the SARDI Selection Demonstration Flock (SDF), to generate a large range of wool growth rates (WGRs) between selected individuals. The selection strategy allowed the investigation of differences in wool growth between divergent groups of sheep, without confounding due to variable follicle numbers and body size. The experiment was designed to re-confirm previous information from FW selection flocks in industry-relevant Merino sheep and to precede a detailed molecular investigation of the basis of genetic differences in fibre growth and composition.

The general hypothesis tested was that differences in wool production between selection groups arise from variability in the partitioning of nutrients to the skin and follicles, and/or differences in the ability of the follicles to utilise the nutrients supplied under different nutritional and environmental conditions. Specific hypotheses tested were:

a. that +W producers are more efficient at converting feed (and S) into wool (and wool S proteins)

b. that +W producers digest and retain more S than -W producers

c. that +W producers partition a greater proportion of retained S to wool production and less to other body components

d. that +W sheep fed a high S diet partition a greater proportion of SAA to wool production than –W sheep also fed the high S diet

5.2 Methods

5.2.1 Experimental methodology

5.2.1.1 Selection of experimental animals

Experiment 2 was conducted in the Livestock Research Centre at the Roseworthy Campus with thirty-two, 1998-born, South Australian Merino rams from the SARDI Selection Demonstration Flocks (SDF) (Section 3.2.1). Estimated breeding values (EBV) were calculated using ASReml (Gilmour et al, 1998) and follicle density measured (Section 3.2.2) providing the data for selection of two groups of sheep distinctly divergent in wool production: high producers (+W) and low producers (-W). The two groups had similar total numbers of wool follicles and similar body size, and were 20 months of age at the commencement of the experiment, with 4 months of wool growth. The average EBVs, and wool and body growth measurements at selection are tabulated in Table 5.1.

Given the variation in wool growth measured in Experiment 1 when sheep were studied indoors, a number of adjustments were made to the experimental animal model in Experiment 2. The ratio of calcium to phosphorus in the diet was appropriate (refer to Appendix 2.1.1 for description), the experimental period was limited to 65 days in duration and EBVs were used as selection criteria, thus the possibility of phenotypic changes in wool production between field and indoor measurements was reduced.

Table 5.1: The average measured and EBV (estimated breeding value) of clean fleece weight (CFW), body weight (BW) and fibre diameter (FD), for +W and –W groups when selected at 16 months of age.

		+W	-W	SEM
Measurement	CFW (kg)	6.9 [b]	4.4 [a]	0.08
	BW (kg)	75.9	74.5	0.74
	FD (μm)	21.4	22.0	0.38
	Foll.dens (no./ mm^2)	52	47	2.50
EBVs	W	10.2 [b]	-7.2 [a]	0.84
(%derivations)	BW	2.9	3.7	0.73
	FD	0.16	0.31	0.18

Note : superscripts differ at $p<0.001$ within the main effect of selection group.

5.2.1.2 Measurements

Figure 5.1 outlines the experimental design for Experiment 2. The trial was conducted with sheep housed individually in pens and monitored daily. Body weight was measured weekly using electronic scales. Water was available *ad libitum* and feed refusal and wastage was recorded daily. All sheep were drenched for gastrointestinal parasites (Ivomec®, Merial) approximately one month before the experiment commenced.

Measurement of midside wool growth rate (WGR) and wool sulphur (S) concentration are described in Sections 3.2 and 3.5 respectively. An optical fibre diameter analysis machine (OFDA 100, BSC Electronics Pty. Ltd) was used to measure fibre diameter. Clean fibre yield was determined as the percentage of clean wool obtained from the greasy wool sample (Section 3.2) and fibre length was calculated using the equation outlined in Section 3.2.2 Blood samples were taken between 9am and 11am prior to morning feeding on three occasions and plasma extracted (Section 3.4.2) for the analysis of S-amino acids (SAA) using pre-column derivatisation, high performance liquid chromatography (HPLC) (Section 3.4.2). S balance was determined over a six-day period (Section 3.3.1). Feed, faeces and urine

samples were digested with nitric/ perchloric acid and the S concentration was measured using inductively coupled plasma atomic emission spectrometry (ICPAES) at the Waite Analytical Services laboratory in Adelaide (Section 3.3.3).

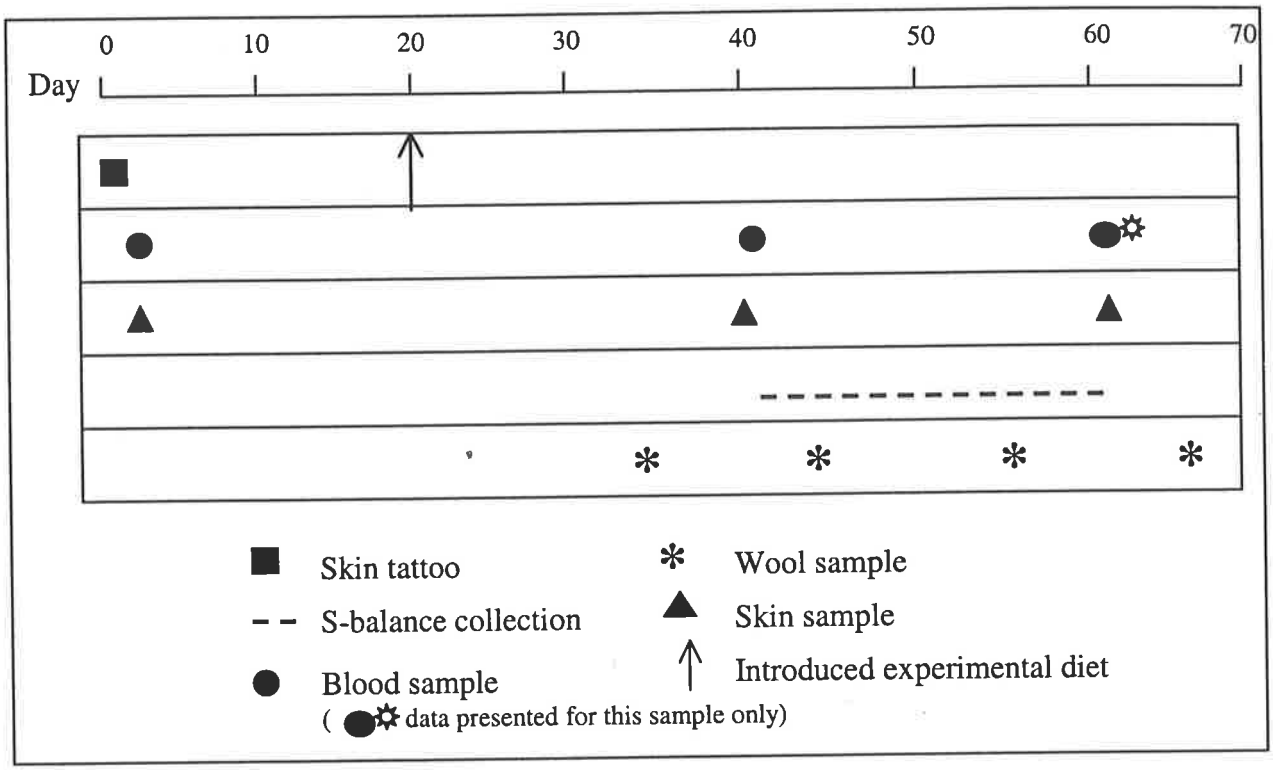

Figure 5.1: Experimental design for Experiment 2.

5.2.1.3 Experimental nutrition

Sheep from each selection group (n=16) were randomly and equally allocated to a high S (Pellet B) and low S (Pellet A) diet, with both diets containing comparable energy concentrations. The diet consisted of 65% pellet (A or B) and 35% lucerne chaff, Pellet A was a commercial sheep pellet (Ridley's Agriproducts, Murray Bridge) and Pellet B was produced at the Roseworthy feed mill. It contained the following proportions of constituents (by weight, air dry); 45% lupins, 25% soybean meal (HiPro solvent extracted Soybean meal, North Melbourne Stockfeeds), 7.5% linseed meal, 7.5% Copra meal (Natural coconut meal, imported by Mataranka Grains Pty Ltd, Qld) and 15% fishmeal (Seapep).

Table 5.2 indicates the dry matter digestibility (DMD), crude protein (CP) and metabolisable energy (ME) of pellets A and B, and lucerne chaff (Feedtest, Hamilton, Victoria). The S concentration of all feeds was determined using inductively coupled plasma

atomic emission spectrometry (ICPAES). Diet A (Pellet A and lucerne chaff) was fed to maintain body weight (MAFF Bulletin, 1975) and Diet B (Pellet B and lucerne chaff) was fed at 1.5 times maintenance requirements for energy, such that Diet B supplied approximately twice the quantity of S that Diet A provided. The quantity of feed offered was adjusted weekly if required, based on changes in bodyweight.

Table 5.2: Diet constituents for Experiment 2.

	Pellet A	Pellet B	Lucerne chaff
DMD (%DM)	82%	82%	73%
Crude Protein (%DM)	15.2	41.1	22.2
ME (MJ/ kg DM)	12.4	12.9	10.6
S (mg/kg)	1845	3650	3700

5.2.2 Statistical methodology

REML Variance Components Analysis (GENSTAT, 5th Edition) was used to analyse data for wool growth rate (WGR), body weight (BW), wool S concentration, fibre diameter (FD) and fibre length (FL) throughout the experimental period. A model containing the fixed factors selection group, dietary treatment and experimental time period, and the two-way and three-way interactions between these factors, was employed for the analysis of each response variate. A similar fixed model was used in Section 5.3.2 for data collated from days 51-65 of the experiment, where the fixed model included selection group and dietary treatment, and the two-way interaction between these factors.

The variation in WGR and its association with variables such as wool S concentration and S retention (Section 5.3.3) were investigated using a General Linear Regression approach (GENSTAT, 5th Edition). The fitted model consisted of the variable (eg. S retention), selection group and dietary treatment, and the two-way and three-way interactions associated with these factors. The final reduced model, for which any fixed effects, explanatory variates and interactions that were not significant at the 5% level were systematically removed, is indicated for each analysis in Section 5.3.3.

5.3 Results

5.3.1 Response to nutrition over time

5.3.1.1 Wool growth rate (WGR)

Figure 5.2 shows the change in WGR (mg/ cm^2/ d) for high-wool producing (+W) and low-wool producing (–W) selection groups, and sheep consuming diets high (HS) or low (LS) in sulphur concentration. Wool samples were taken four times throughout the experiment (day 35, 45, 55 and 65) from a midside tattooed patch. There was a two-way interaction (p=0.005) between selection group and diet. Averaged over all sample times, the +W sheep grew the most wool, when fed the high sulphur (HS) diet (+WHS=1.91mg/cm^2/d). The –W sheep on the LS diet grew the least amount of wool (-WLS= 0.96mg/cm^2/d LS diet), whilst wool growth was intermediate for the –W sheep on the HS diet and the +W sheep on the low sulphur (LS) diet (-WHS=1.25mg/cm^2/d and +WLS=1.35mg/cm^2/d respectively). As the experiment progressed, the difference in WGR between sheep fed the HS diet versus sheep fed the LS diet increased (diet x time; p<0.001). After 35 days of the experiment, sheep on the HS diet grew 15% more wool than those on the LS diet, and by the end of the experiment (day 65) this difference had increased to approximately 70%. Sheep selected for +W and fed the HS diet (+WHS) produced significantly more wool at all sampling times and sheep selected for –W and fed the LS diet (-WLS) consistently grew less wool than all other groups. There was no significant group x time or group x diet x time interaction (p>0.05) indicating both +W and –W selection groups responded similarly over time.

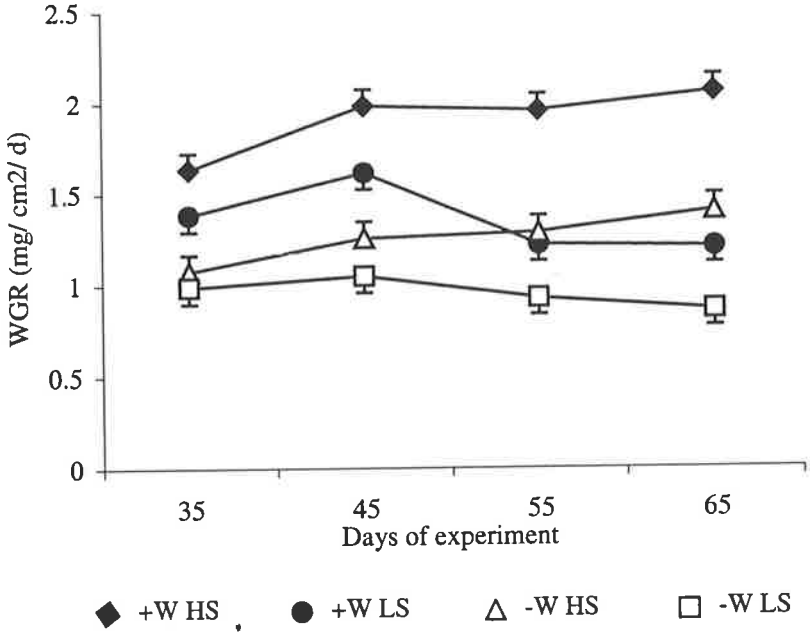

Figure 5.2: Change in mean WGR (± S.E.M) for each selection group and experimental diet, over time.

The trend in whole body WGR (g/d), was similar to that described above. A significant two-way group x diet interaction ($p<0.001$) indicated +W sheep grew more wool when consuming either diet (34.1g/d HS diet or 23.0g/d LS diet), compared to –W selected sheep (22.0g/d HS diet and 16.3g/d LS diet). Sheep consuming the HS diet increased WGR by approximately 30% from day 35 of the experiment to day 65 (23.6 g/d to 30.9 g/d respectively) compared to a 15% decrease in WGR for sheep consuming the LS diet (20.3 g/d at day 35 to 17.4 g/d at day 65) (diet x time; $p<0.001$). Again, there was no significant group x time, or group x diet x time interaction ($p>0.05$) indicating both groups responded similarly in the rate of WG.

5.3.1.2 Body weight (BW) and gain

Figure 5.3 shows the average body weight (BW) for each selection group and experimental diet throughout the experiment. The group x diet interaction ($p<0.05$) indicated no difference in BW between groups fed the LS diet (approximately 75.5kg), but when the HS diet was fed, +W sheep were heavier than –W sheep (81.4kg for +WHS and 78.9 kg for

–WHS sheep). There was a two-way diet x time interaction ($p<0.05$) whereby sheep fed the HS diet increased BW over the experimental period (approximately 3.5kg increase) but the BW of sheep fed the LS diet remained steady.

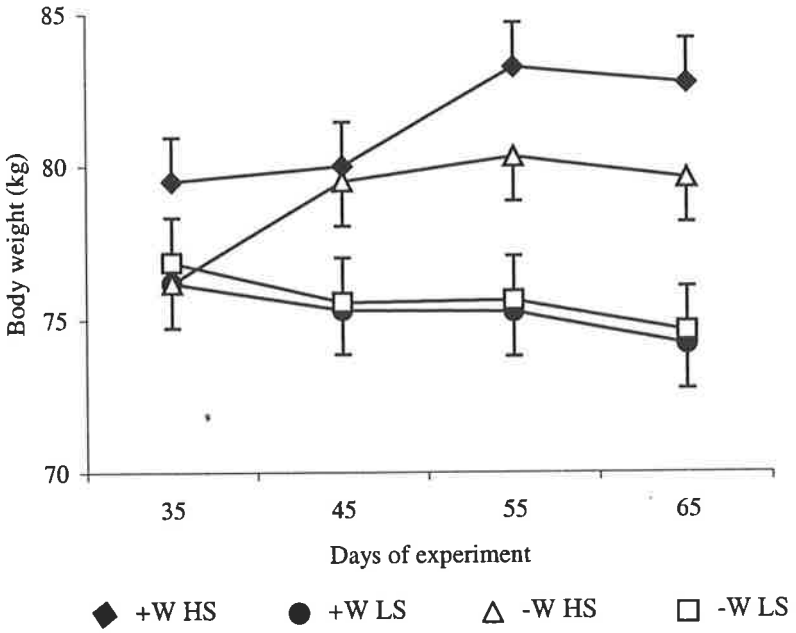

Figure 5.3: Change in mean body weight (± S.E.M) measured throughout the experiment for each selection group and experimental diet.

5.3.1.3 Wool S concentration

The average S concentration of wool (Figure 5.4) was greatest in sheep selected for -W compared to +W sheep (2.87% S vs. 2.77% S; $p<0.001$; data not shown). Sheep fed the HS diet produced wool with a higher S concentration than sheep fed the LS diet (2.86% S vs. 2.79% S respectively; $p<0.01$). The lack of a time x diet interaction ($p=0.09$) indicated no variation in the S content of wool due to diet throughout the experiment, although on day 65, sheep fed the HS diet produced wool with a higher S concentration than sheep fed the LS diet. -WHS sheep had the highest wool S concentration (2.90%) throughout the experiment and +WLS sheep had the lowest (2.74%), with -WLS and +WHS intermediate to these values. There was no significant group x diet, group x time, or group x diet x time interactions.

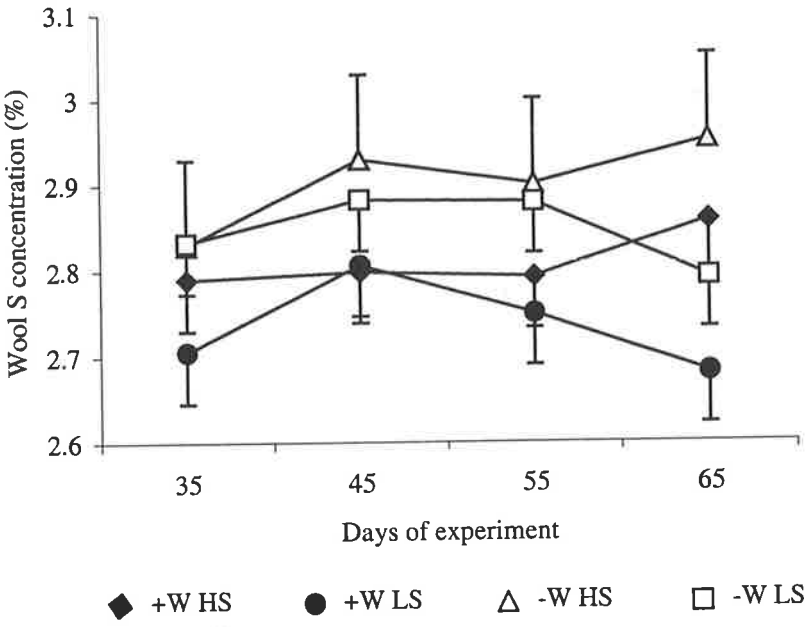

Figure 5.4: Change in the average S concentration (± S.E.M) of wool for each selection group and dietary treatment, over time.

The output of S in wool per day was calculated from the total whole body WGR per day and the S concentration of the wool produced (Figure 5.4). A significant group x diet interaction ($p<0.001$) showed +WHS sheep exported over 950mg of S per day, compared to approximately 630 mg of S for both +WLS and –WHS sheep and 464 mg of S for –WLS sheep. Sheep fed the HS diet increased the output of S in wool by 35%, from day 35 (660mg/d) to day 65 (894mg/d), and sheep on the LS diet decreased wool S output by 15% from day 35 (561mg/d) to day 65 (475mg/d) (diet x time; $p<0.001$).

5.3.1.4 Fibre length

Averaged over time and diet, sheep selected for +W produced longer fibres (471µm/d) than –W sheep (274µm/d) ($p=0.003$) (Figure 5.5). A significant diet x time interaction ($p=0.006$) meant fibre length increased by approximately 40% during the experiment for sheep consuming the HS diet (366 µm/d to 516 µm/d, at day 35 to day 65 of the experiment respectively) and decreased by 20% for those consuming the LS diet (312 µm/d to 249 µm/d, at day 35 to day 65 of the experiment respectively).

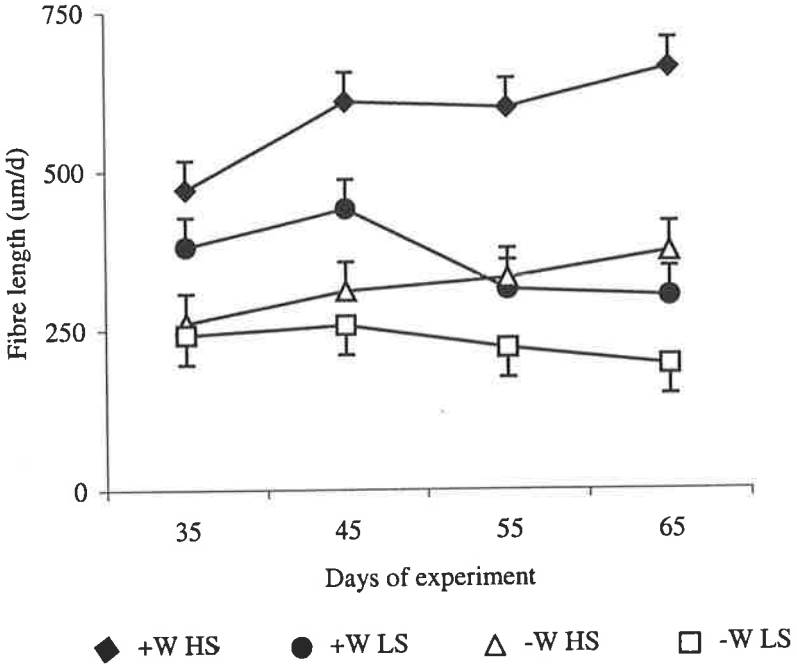

Figure 5.5: Change in the average fibre length (± S.E.M) produced per day, for each selection group and dietary treatment over time.

5.3.1.5 Fibre diameter

There was no significant difference in the average diameter of fibre produced for each selection group ($p=0.5$) but there was a significant group x diet interaction ($p<0.05$) such that +W sheep were more responsive to the diet in FD than –W sheep (Figure 5.6). +W sheep produced wool with a higher FD than –W sheep when fed the HS diet (25.4μm vs. 24.6μm respectively), however on the LS diet, +W sheep had a lower FD than –W sheep (22.8μm vs. 23.2μm respectively). Dietary effects on FD were apparent at day 45 of the experiment onwards, as sheep on the HS diet produced wool with a higher FD compared to sheep on the LS diet (diet x time interaction; $p<0.001$).

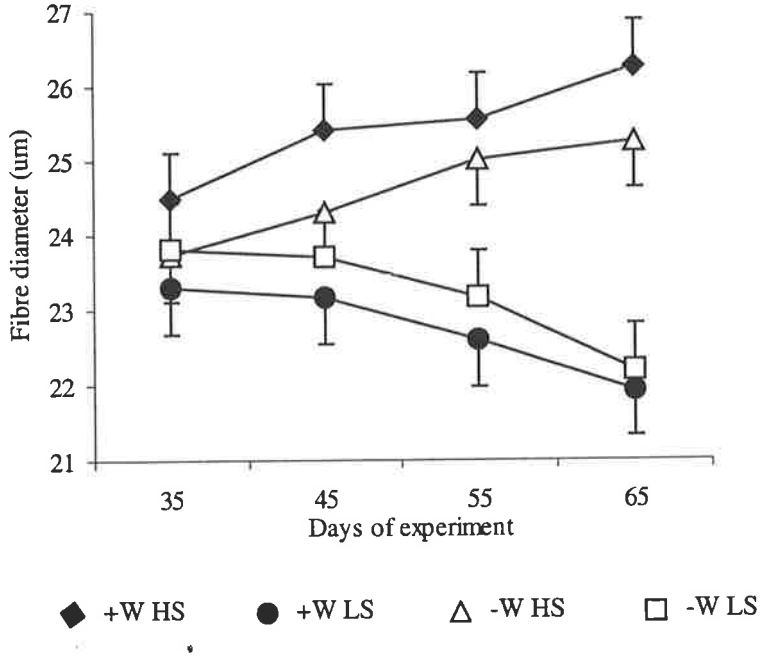

Figure 5.6: Change in the average diameter of fibre (± S.E.M) produced, for each selection group and dietary treatment over time.

5.3.2 S utilisation and related WGR parameters (measured during days 51-65 of trial)

5.3.2.1 Response in WGR to nutrition

Sheep in this experiment were not fed the LS diet followed by the HS diet but sheep from the same selection group were fed either the LS *or* the HS diet, thus a true response to nutrition is not described. Figure 5.7 shows the mean WGR at day 65, for each selection group fed the LS diet and each selection group fed the HS diet. When fed the LS diet, +W sheep produced 40% more wool than –W sheep, and when fed the HS diet +W sheep grew 51% more wool than –W sheep.

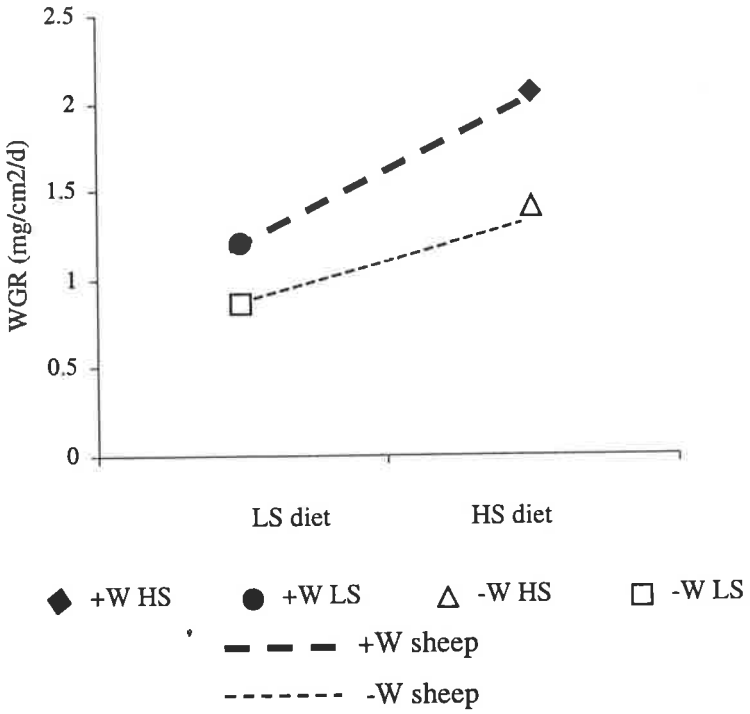

Figure 5.7: The average WGR for sheep in the +W and –W selection groups, fed the LS or the HS diet, and measured at completion of the study (day 65).

5.3.2.2 Sulphur (S) balance

Table 5.3 summarises the apparent digestion, retention and utilisation of S, and the use of S for wool production for each selection group (+W and –W) and for each diet (HS and LS); there was no group x diet interaction for any of the variables measured unless stated. More detail relating to the S balance components are tabled in Appendix 2. There was no significant difference between the +W and –W sheep in the amount of dry matter (DM) and S consumed, the excretion of S in faeces and urine, or the amount of S retained in the body. Although not significant, +W sheep retained more than two and a half times the S that –W sheep retained (219mg/d vs 81mg/d). +W sheep exported 40% more S in wool compared to –W sheep ($p<0.001$), that is 803 mg/d compared to 563 mg/d. However more S was exported in wool than was retained and hence an additional 480 and 590 mg of S per day was required for both groups from other sources, presumably from mobilised body tissue.

Sheep consuming the HS diet ingested more feed DM per day ($p<0.001$) and S per day ($p<0.001$) than sheep on the LS diet, and excreted 45% more S in faeces ($p<0.001$) but still

apparently digested more S ($p<0.001$). Sheep fed the HS diet excreted more than double the S in urine ($p<0.001$) compared with sheep fed the LS diet, yet they retained significantly more S than sheep fed the LS diet ($p<0.05$). The amount of S exported in the fibre was greatest in sheep that consumed the HS diet ($p<0.001$), thus the amount of S apparently required from mobilised body tissue pools was higher in sheep on the HS diet (606mg/d) compared to the LS diet (464mg/d), but not significantly so ($p=0.25$).

Table 5.3: Mean sulphur (S) consumption, digestion, retention and output in wool per day (per head) for each diet and selection group.

	HS diet	LS diet	+W group	-W group	Pooled SEM
Intake DM (g/d)	1367[b]	1065[a]	1225	1207	15.5
S consumed (g/d)	5012[b]	2628[a]	3847	3793	127.3
S excreted in faeces (g/d)	1598[b]	1111[a]	1378	1331	137.6
Apparent digestibility (%)[1]	68.3[b]	57.6[a]	62.8	63.1	2.6
S excreted in urine (g/d)	3141[b]	1511[a]	2270	2381	74.6
S balance (retention) (g/d)[2]	273[b]	27[a]	219	81	87.7
Total S output in wool (mg/d)[3]	879[b]	487[a]	803[b]	563[a]	33.1
S apparently mobilised from body tissue (mg/d)[4]	606	464	588	482	87.9

Note: different superscripts within a row and within each main effect differ at $p<0.05$.
1. The proportion of consumed S that was not excreted in faeces.
2. S consumed minus S excreted in faeces or in urine.
3. The concentration of S in wool multiplied by the WGR (g/d) (Table 5.4)
4. Total S output ('3') minus the S retained ('2')

5.3.2.3 WGR and associated measurements

The mean values for various wool parameters measured at day 65 are shown in Table 5.4. There was a significant group x diet interaction ($p<0.05$) for WGR (g/ d) and fibre length, such that +WHS sheep grew a significantly greater quantity of longer wool than –WHS sheep. The –WHS sheep grew more wool with a greater fibre length than +WLS sheep, which in turn grew more wool than the –WLS sheep. When fed the LS diet +W sheep grew 40% more wool

than –W sheep, but when fed the HS diet this difference was 51%.

There was no group x diet interaction for WGR (mg/ cm^2/ d) and wool S concentration, but WGR was significantly higher and wool S concentration lower for +W sheep compared with –W sheep ($p<0.001$). There was no significant difference in the fibre diameter or clean wool yield between +W and –W sheep ($p>0.05$). Sheep fed the HS diet produced significantly ($p<0.001$) more wool (per unit area and whole body growth) with a higher clean wool yield and wool S concentration, as well as fibre diameter, than the sheep consuming the LS diet.

Table 5.4: A comparison of WGR, wool S and fibre length, diameter and yield for each selection group and dietary treatment at day 65 of the experiment (day 65).

	+WHS	+WLS	-WHS	-WLS	Pooled SEM
WGR (mg/ cm^2/ d)	2.05	1.20	1.40	0.86	0.09
WGR (g/ d)	37.2^d	20.3^b	24.7^c	14.5^a	1.63
Wool S concentration (%)	2.86	2.68	2.95	2.79	0.06
Fibre length (µm/day)	706^d	296^b	390^c	195^a	49.9
Fibre diameter (µm)	26.2	21.9	25.2	22.2	0.64
FL : FD ratio	26.7	13.3	15.6	8.6	1.83
Clean wool yield (%)	71.4	68.7	69.5	63.2	2.08

Note: values with different superscripts differ within a row at $p<0.05$.

5.3.2.4 The efficiency and utilisation of feed (and S) for wool growth.

The efficiency of feed and S utilisation for wool growth can be expressed a number of different ways. Table 5.5 indicates that +W selected sheep were more efficient than –W sheep at converting ; feed into wool (Efficiency A; $p<0.001$), S consumed into S in wool (Efficiency B; $p<0.001$) and S digested into S in wool (Efficiency C; $p<0.001$). The +W sheep utilised 9.5% of digested S (in the whole body) compared to 1.6% in –W sheep, although this difference was not significant ($p>0.05$). Wool S production per unit of S retained (Efficiency

D) was negative and not significantly different between +W and –W sheep, indicating that the output of S in wool was greater than the amount of S retained in the whole body (Table 5.3), and therefore a portion of S used for wool growth appeared to come from the mobilisation of tissue S.

Sheep fed the HS diet were more efficient at converting feed into wool (Efficiency A; $p<0.001$), however when this was expressed per g of S fed (Efficiency B), the difference was lost ($p=0.7$). Sheep fed the LS diet were more efficient than those on the HS diet at converting digested S into S in wool (Efficiency C; $p<0.05$), however the whole body utilisation of digested S was greater in sheep fed the HS diet (7.7%) compared to the LS diet (3.3%) although this difference was not statistically different ($p>0.05$). The amount of S in wool was greater than the retention of S in the whole body for sheep fed on both diets and there was no difference between diets in Efficiency D.

Table 5.5: The efficiency of wool growth, partitioning of S to wool and utilisation of digested S, for each diet and selection group.

	HS diet	LS diet	+W group	-W group	Pooled SEM
Efficiency A[1]	22.5^{b}	16.6^{a}	23.2^{b}	16.0^{a}	0.969
Efficiency B[2]	175.6	185.0	209.5^{b}	151.1^{a}	8.95
Efficiency C[3]	259^{a}	324^{b}	338^{b}	245^{a}	16.1
Utilisation[4]	7.7	0.8	7.6	1.0	3.41
Efficiency D[5]	-977	-1024	-932	-1069	1396

1. mg of wool produced per g of feed DMI
2. mg wool S produced per g of S consumed
3. mg wool S produced per g of S apparently digested
4. Percentage of S apparently digested that is retained in the whole body
5. mg wool S produced per mg of S retained

Note: values with different superscripts within each main effect differ at $p<0.001$ (except $p<0.05$ for Efficiency C for the main effect of diet).

5.3.2.5 Plasma concentration of S-containing amino acids (SAA)

There was no effect of diet or selection group on the plasma concentration of the SAA (Table 5.6), in blood samples taken at the end of the trial (day 65). Given this, and that the largest differences were expected at this time, following feeding on the HS and LS diets for six weeks, further analysis of the remaining blood samples (Figure 5.1) was not warranted.

Table 5.6: Mean concentration of sulphur containing amino acids in plasma, for each selection group and experimental diet, at the completion of the experiment (day 65).

	HS diet	LS diet	+W group	-W group	Pooled SEM
Plasma cyst(e)ine (pmol/µl)	90.0	90.7	89.9	90.9	4.03
Plasma methionine (pmol/µl)	22.1	19.8	20.8	21.1	1.22

5.3.3 Regression relationships with WGR

Table 5.7 indicates the percentage of variation in WGR that could be accounted for by a range of measured variables and the main effects of selection group and dietary treatment.

Table 5.7: Summary of the percentage variance in WGR that was accounted for by the final reduced model, at the completion of the experiment.

Variable	Percentage of variation accounted for by…				
	Final model	Variable	Selection Group	Dietary treatment	Interactions (see text)
S retention	70***	15**	19***	36***	ns
Wool S concentration	79***	ns	31***	42***	6*
Plasma cyst(e)ine	75***	ns	24***	48***	ns
Plasma methionine	75***	7**	24***	44***	ns
Clean wool yield	82***	36***	11***	31***	4*

*** $p<0.001$, ** $p<0.01$, * $p<0.05$, ns not significant ($p>0.05$), the number indicates the proportion of each main effect that contributes to the variation.

5.3.3.1 *WGR and S retention*

(final model; WGR = retained S + G + D)

The main effects of S retention (15%), diet (36%) and selection group (19%) contributed in total to 70% of the variation in WGR ($p<0.001$) and there were no significant two-way or three-way interactions. The weak, positive relationship ($p=0.001$) between retained S and WGR was highly variable between treatment groups but significant (Figure 5.8).

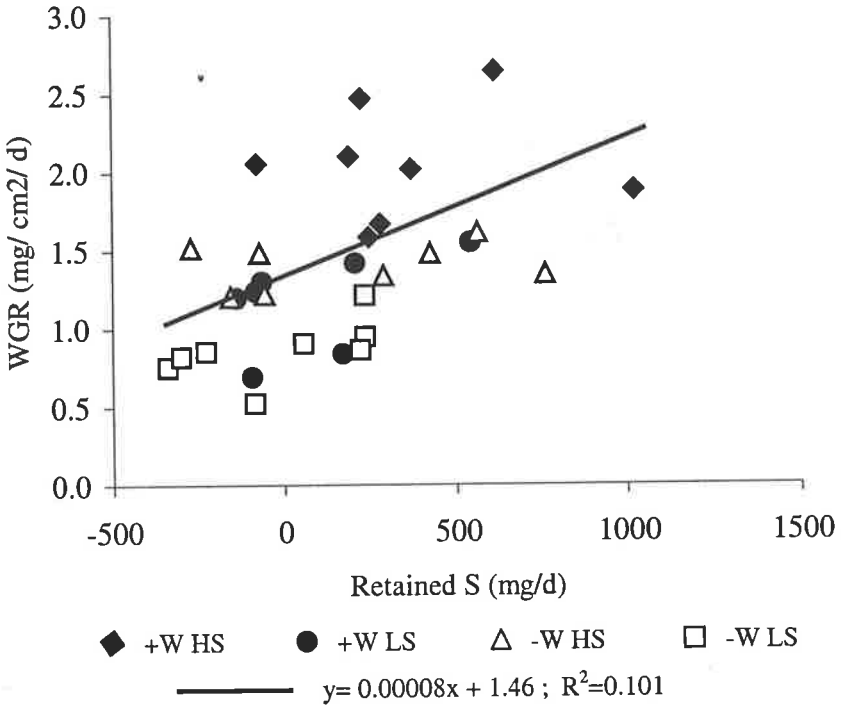

Figure 5.8: The relationship between WGR (mg/cm^2/d) and retained S (mg/d), for individuals from each selection group and dietary treatment at day 65 of the experiment.

5.3.3.2 WGR and wool S concentration

(final model; WGR= Wool S + G + D + Wool S*D)

Approximately 79% of the variation in WGR was explained by the final model ($p<0.001$). The main effects of diet (42%) and selection group (31%) contributed to the majority of the variation ($p<0.001$) (Table 5.7) but there was no significant relationship with the S concentration of wool. Figure 5.9 shows the association between WGR and wool S for the two different diets ($p<0.05$), such that wool S concentration decreased as WGR increased for sheep fed the HS diet only.

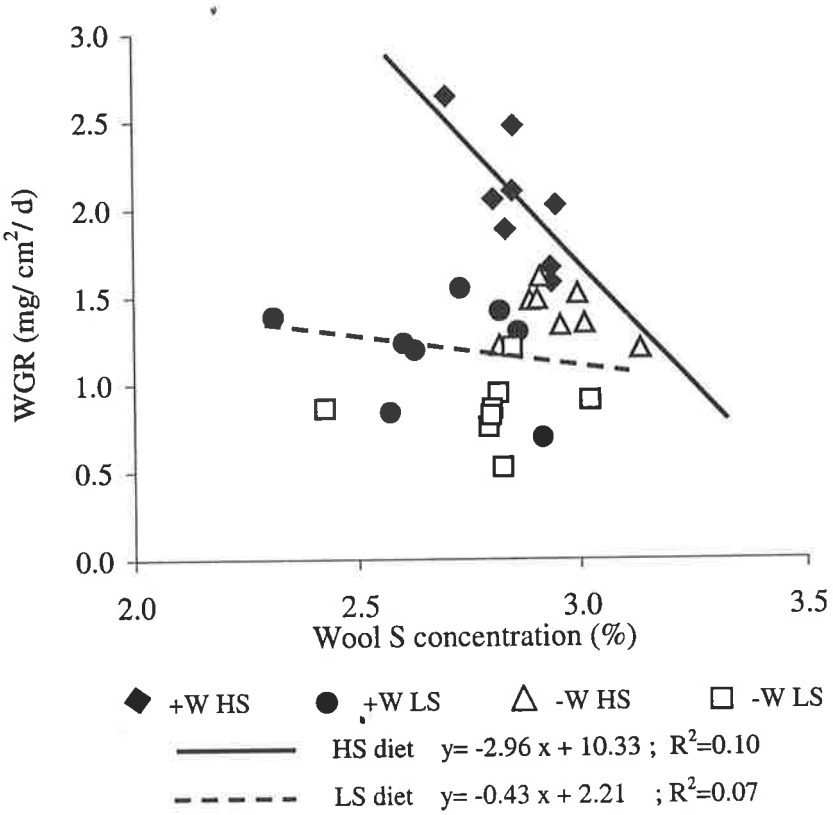

Figure 5.9: The relationship between WGR (mg/cm^2/d) and wool S concentration (%S), for sheep on the HS diet and sheep on the LS diet at day 65 of the experiment.

5.3.3.3 WGR and plasma SAA concentration

(final model; WGR= cys + G + D)

(final model; WGR= meth + G + D)

Although the final model incorporating plasma cyst(e)ine, group (24%) and diet (48%) accounted for 75% of the variation in WGR there was no significant effect of plasma cyst(e)ine concentration (Table 5.7; figure not shown). Plasma methionine (7%), selection group (24%) and dietary treatment (44%) accounted for a significant proportion of the 75% total variation in WGR. Figure 5.10 shows the significant ($p<0.01$) but weak linear relationship between plasma methionine and WGR/d.

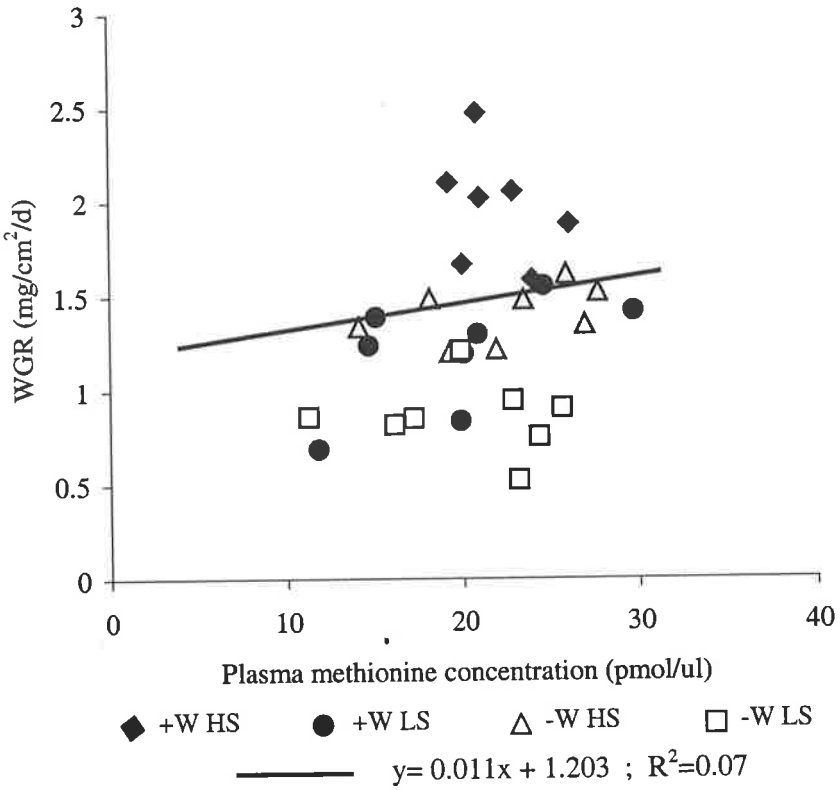

Figure 5.10: The relationship between WGR ($mg/cm^2/d$) and plasma methionine concentration (pmol/µl), for individuals from each selection group and dietary treatment at day 65 of the experiment

5.3.3.5 WGR and yield (of clean fleece from greasy fleece).

(final model : WGR = yield + G + D + yield*G)

The reduced model accounted for 82% of the variation in WGR ($p<0.001$). Figure 5.11 demonstrates the positive association between WGR and fleece yield, which contributed to 36% of WGR variation ($p<0.001$). In addition, the main effects of selection group and diet significantly ($p<0.001$) contributed to 11% and 31% of the variation respectively. Although significant ($p<0.01$), the two-way interaction between yield and selection group was responsible for only 4% of WGR variation, as sheep selected for +W yielded more clean wool in comparison with –W selected sheep (see Table 5.6).

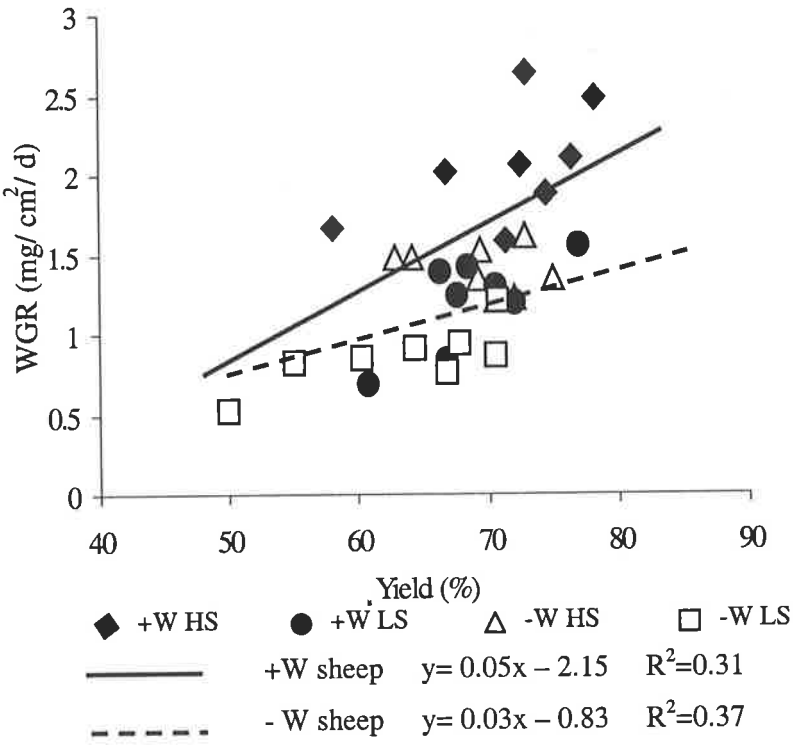

Figure 5.11: The relationship between WGR (mg/cm^2/d) and yield (% clean wool), for individuals from each selection group and dietary treatment at day 65 of the experiment

5.4 Discussion

Selection of sheep for this experiment using estimated breeding values (EBVs) and supply of judicious nutrition generated some of the largest differences in WGR documented to date, with +W sheep producing in excess of 28 g/d on average and approximately 50% more wool than –W selected sheep. These sheep, derived from the industry-relevant SARDI Selection Demonstration Flock, were of a similar body weight and had similar follicle numbers, in contrast to other Merino FW selection lines (Brown and Turner, 1968; Barlow, 1974). The genetically diverse groups of sheep produced wool of variable quantity and composition, thus enabling the hypotheses to be tested without confounding due to differences in total follicle material. The experimental model is therefore suitable to investigate potentially small differences at the follicle level in sheep that are relevant to the Merino wool industry of today.

5.4.1 Components of WGR variability

The difference in WGR between +FW and –FW selected sheep generally becomes more marked when SAA supply is high (Williams et al, 1972b), that is, +FW sheep are more responsive in WGR to an increased nutritional supply. Whilst sheep fed the HS diet received more energy per day than sheep fed the LS diet, the extra energy is unlikely to have had a major effect on wool growth relative to the significant effects of the additional SAA (Reis, 1969; Black et al, 1973; Reis et al, 1992). Williams et al (1972b) reported a 16% difference in WGR between Merino FW lines fed a basal diet, and an approximate 50% difference in WGR when cystine or methionine was infused abomasally. Because the sheep in the experiment described in this thesis were not fed the LS diet then the HS diet, a true response to nutrition is not described, but dietary effects within each selection group can be discussed. The 40% difference in wool growth between +W and –W sheep on the LS diet compared to the 51% difference on the HS diet (Figure 5.7) suggests a similar scenario, although not as extreme, to that described by Williams et al (1972b).

Fibre production per follicle is a function of fibre length (FL) and diameter (FD), and variation in WGR can be attributed to one or both of these components. At the completion of the current study there was no difference in the FD of +W and –W sheep (24.1μm and 23.7μm respectively) but wool FL and the FL:FD ratio was greater in +W sheep than –W sheep (500μm/d versus 290μm/d and 20:1 versus 12:1 respectively). These results indicate that wool FL was the major source of variation in WGR between selection groups.

Williams (1966) showed the differences in WGR between +FW and –FW Merinos when restriction-fed (69% difference) and *ad libitum*-fed (103% difference) was the result of different genotypic responses in the average fibre diameter and not fibre length. Furthermore, FL:FD remained similar in +FW and –FW sheep on two different levels of feeding (Williams and Winston, 1987), which is in agreement with the work of Downes and Sharry (1971) and Reis and Sahlu (1994), suggesting the ratio is independent of nutrition. However, a change in the ratio of FL:FD in response to improved protein nutrition was shown by Hynd (1989b) and Sherlock et al (2001), and was seen in the present study, whereby the HS diet increased FL:FD ratio due to disproportionate increase in FL compared to FD.

Sherlock et al (2001) showed an increase in wool FL growth in response to both intake level and increased cysteine supply in Romney ewes fed a high and low intake level, and infused intravenously with cysteine or saline. It was suggested that the increase in FL response was the result of increased protein filling of cortical cells, thus increased cortical cell length, and/or an increase in the migration of bulb cells to the cortex. Therefore, it appears that the supply of the HS diet, at a higher level of intake than the LS diet, affected FL and follicle rate processes similarly in the current experiment.

5.4.2 The efficiency of wool production

5.4.2.1. Are +W sheep more efficient at converting feed, including SAA, to wool?

In this experiment, +W selected sheep required significantly less feed DM per unit of wool production. This is consistent with other fleece weight (FW) selection lines, as

established in the early 1960's (Dolling and Moore, 1960; Ahmed et al, 1963; Williams, 1966; Hamilton and Langlands, 1969). They also grew more wool per unit of S consumed. This greater efficiency in +W sheep supports hypothesis 'a' in this chapter and suggests that +W sheep are able to absorb or retain S more efficiently, deliver more S to the skin, or that the follicles of +W sheep utilise S for fibre growth more efficiently. The relative contributions of each step to improve the efficiency of converting consumed S into wool S can be gauged by analysing the proportions of digested and retained S being converted to wool.

5.4.2.2 Do +W sheep have a greater digestive capacity than –W sheep?

There was no difference in the apparent digestibility of S between the +W and –W groups with approximately 60% of S consumed being digested, although, +W sheep were more efficient than –W sheep at converting this digested S into wool S. There was little difference in the digestive capacity of +FW and –FW or control (C) sheep from Romney FW lines (Sun et al, 1994; McCutcheon et al, 1987) or Merino FW lines (Piper and Dolling, 1969; Williams et al, 1988; Lee and Williams, 1994). In the present experiment, an average of 30-40% of the total S consumed was excreted in faeces, a proportion similar to reports elsewhere (Langlands et al, 1973; Revell et al, 1994; Sun et al, 1994). Similarities in the digestion of S between +W and –W sheep suggest +W sheep retain or utilise more S, or partition more S to wool production, to enable the greater WGR and wool S output measured here.

5.4.2.3 Do +W sheep have lower plasma SAA concentrations?

In the early 1970s researchers first reported a significant difference in the plasma concentration of cyst(e)ine between Merino sheep selectively bred for or against wool growth (+FW or –FW) (Williams et al, 1972a) whereby –FW sheep had a higher concentration than +FW sheep, a difference measured as early as 7-10 days of age (Williams, 1984). It was hypothesised that the difference in plasma cyst(e)ine concentration is a consequence of greater cyst(e)ine incorporation into wool fibres (Williams and Thornberry, 1991). This

difference was not described in Romney +FW and C lines (Sun et al, 1994; Miller et al, 2000).

In the current study there was no difference in plasma cyst(e)ine or plasma methionine concentrations between selection groups and there was no relationship between plasma cyst(e)ine and WGR. This was despite significant WGR variability between selection groups that was comparable to that described in FW selection lines. Sheep from both +W and –W groups were losing body weight and mobilising tissue SAA stores, a factor that may have contributed to the lack of difference between groups in plasma SAA concentration. Blood samples were taken prior to morning feeding, effectively after a 25 to 30 hour fast, and Williams et al (1986) have described a linear rise in plasma SAA concentrations following fasting in Merino sheep, possibly masking any relationships between plasma SAA and WGR.

5.4.2.4 *Do +W sheep utilise and/or retain more S for wool growth?*

The +W sheep retained approximately two and a half times the quantity of S that the –W sheep retained, as a result of less S excreted in urine, although these differences were not significant. Both groups excreted approximately 60% of the S they consumed in their urine, which is approximately twice that found in other studies; for example, proportions of consumed S excreted in urine in sheep range between 20-40% S (Langlands et al, 1973; Revell et al, 1994; Sun et al, 1994). Despite this, the values discussed in this thesis are considered valid, as the supply of S in the diet in this experiment exceeded the quantity supplied in the aforementioned publications by between 1400 and 1800mg S per day; that is 40-50% more S was supplied.

Given that only 15% of the variation in WGR between +W and –W sheep was attributed to the amount of S retained and that the utilisation of digested S was similar for both groups, it appears unlikely that digestion (and absorption) or retention were the major sources of WGR differences. No relationship between WGR and retained S was found by Sun et al (1994), in which the average WGR was similar to that measured in the current study but S

retention was far greater. Williams (1976) on the other hand showed a positive relationship between WGR and retained S, as did Revell et al (1994). The significant yet small relationship in this experiment, and the retention of approximately two and a half times the S in +W than –W sheep, would contribute, at least in part, to the greater rate of wool growth measured in the +W sheep, partially supporting the second hypothesis, that +W sheep digest and retain more S than –W sheep.

5.4.2.5 Do +W sheep partition more retained S to wool production?

The output of S in wool fibres ranged from between 400 to 900 mg S/ day, indicating the large demand for SAA exerted by wool follicles. At the end of the experiment (Day 65) +W sheep required more than 20% of the S they consumed to grow 70% more wool than –W sheep, which utilised just 14% of the S consumed for wool growth. The large difference in wool S output suggests that –W sheep follicles were either not supplied with the same amount of S as +W sheep, or that they do not have the same potential to convert the SAA they are supplied with into wool growth.

In his work with the Trangie Merino FW selection lines, Williams (1995) also found +FW sheep had a higher output of S in wool compared to –FW sheep. In the current study, both +W and –W sheep increased wool S output when fed the HS diet compared to the LS diet but in contrast with this, Williams reported that only +FW sheep increased wool S output in response to improved nutrition. The wool S output was lower (0.029 mg/cm^2/d; Williams, 1995) than that found presently (average 0.038 mg/cm^2/d), most likely due to the significantly lower S content of the diet in Williams' trial. Outputs of S in wool comparable to those documented in this study have been reported previously (Sun et al, 1991; Sun et al, 1994; Mata et al, 1995).

In the study reported here the amount of S retained in the whole body was less than the amount of S exported in wool. Thus the third hypothesis, that suggested +W sheep partition more S to wool and less S to other body compartments, and the fourth hypothesis, that

suggested that +W sheep partition more S to wool than –W sheep fed the same diet, could not be addressed. That is, sheep in this experiment retained up to approximately 270 mg S /d but the output of S in wool was far in excess to this, ranging from 400 to 900 mg S /d (Table 5.3). This suggests that S for wool growth was, at least in part, obtained from a source in addition to the diet, presumably through body protein mobilisation. Alternatively, the calculation of S balance, WGR or wool S concentration may be inaccurate, thus the validity of these measurements and calculations are addressed in the following section.

5.4.3 Calculations of S partitioning to wool production

5.4.3.1 Determination of S intake

Inaccurate measurement of S intake in the current study appears unlikely. Sheep were fed once a day at 0900 hours and the majority of the feed was consumed by all animals within the first one hour, thus residual feed measurement and spillage was minimal. The S content of the feed was analysed by a certified feed testing service (Waite Analytical Services, Adelaide) and compared favourably with the predicted S content of each diet, estimated from published data.

The provision of additional S via the water supply was possible given the estimations of Bray (1965) who suggested that up to 1500 mg of S per day could be supplied from highly sulphated water. After the experiment had been completed, data were obtained from the South Australian water authority (SA Water) that indicated that sulphate concentrations of between 60 to 70 mg/litre were typical for water in the Roseworthy area. During this experiment it was not possible to measure water intake, however, if it was assumed that sheep were consuming similar quantities of water to that being excreted in urine, then water intake may have supplied up to 190 mg S per day, less than 4% of the total dietary intake of S. The addition of this proportionally small quantity to S intake did not alter the significance of the S balance results.

5.4.3.2 Excretion of S in faeces and urine

The overestimation of S excreted in faeces and urine is a possible source of error when calculating S balance. In a comparison of the use of S and nitrogen (N) balances in sheep, Bray and Mucha (1984) describe S balance as a superior estimate of body change and wool growth, in comparison with calculations using N balance. Despite possible losses of urinary sulphate as a precipitate (Prien and Frondell, 1947 cited by Bray and Mucha, 1984), negligible losses of sulphide in faeces (Warth and Krishnan, 1935 cited by Bray and Mucha, 1984), and some losses of volatile S in faeces and urine (Kandylis and Bray, 1982), Bray and Mucha (1984) conclude the possibility of mismeasurement of S excretion to be minor. In any case an underestimation of the loss of S in excreta due to unaccounted for losses in volatiles or precipitates would also lead to underestimation of apparent losses of S from mobilised body tissue, and therefore appears unlikely.

5.4.3.3Calculation of WGR

A miscalculation of whole body wool production, extrapolated from mid side patch data, would have a direct effect on the prediction of the output of S in wool produced. Whole body wool production was calculated in the current experiment by combining the estimated skin surface area, calculated using body weight, and the wool growth measured per unit area (Bennet, 1973). In longer term studies, the whole body WGR has been determined by measuring patch WG over a defined period of time as a proportion of total WG in the patch over 12 months, and multiplied by whole body fleece growth over the 12 months (see Thompson et al, 1994). The majority of other studies in sheep have used lambs, hoggets or adult ewes that have a markedly smaller body size (less than 50kg bodyweight), thus smaller surface area (less than 1.1metres^2) than the rams used in the current experiment (approximately 80kg body weight and 1.77 metres^2).

There is large variation between breeds and within strains of the same breed in the rate of wool production per unit area. In the current experiment, average wool production ranged

from 0.9 to 1.9 mg/cm^2/d across groups and diets, a rate which was comparable with other studies with Romney sheep (Sun et al, 1994; Harris et al, 1997), Merinos (Williams, 1984; Thompson et al, 1994) and English Leicester-Merino crosses (Reis and Schinkel, 1964). Thompson et al (1994) recorded whole body WGR of approximately 25g/d in South Australian Merino wethers, that were grazing pasture and were 20-50% lighter in body weight than sheep from this experiment. Also, Romney sheep that grew comparable amounts of wool per unit area as sheep in my experiment (1.65mg/cm^2/d), produced 17.5g wool/d and released 570mg S /d in the wool, with an average body weight that was 50% lighter (40kg) than sheep in my experiment (Harris et al, 1997). Thus, the average output of wool at the end of this experiment of 29g/ d and output of S of up to 800mg S /d in wool (Table 5.3 and 5.4) is reasonable given the comparison with other studies.

5.4.3.4 Measurement of the S content of wool

The measurement of wool S concentration has traditionally been determined using a wet chemistry technique (Reis and Schinkel, 1963; Antram et al, 1991), a time-consuming process involving relatively expensive and toxic chemicals. The use of Near Infrared Reflectance Spectrometry (NIRS) eliminates the need for harsh chemicals and is a quick and cost-effective method, with the retrieval of the wool sample following analysis. Corson et al (1998) developed the approach with Romney sheep wool samples and the extensive validation of the NIRS technique used in this thesis for Merino wool is described in Section 3.5.

The range of S concentrations in wool samples measured here were between 2.29%S and 3.15%S, and supported the accepted view that –W sheep have a higher wool S concentration than +W sheep (2.87% vs. 2.77% respectively in the present study). The NIRS measured concentrations were within the normal documented range for Romney and Merino wool fibres (Piper and Dolling, 1966; Sun et al, 1991; Sun et al, 1994; Williams, 1995) and were validated with the results from the standard wet chemistry technique, indicating that the measurement of wool S concentration in the current experiment was accurate.

The valid estimation of S consumption and retention, WGR and wool S concentration, indicates an additional source of S was available to supply the demands of the wool follicle. The mobilisation and supply of amino acids from protein turnover in body tissues may provide this source of SAA. The rates of protein turnover in tissues required to supply an adequate amount of S for the measured WGR are calculated and discussed below.

5.4.4 Mobilisation of S for wool growth

At the completion of the current experiment (day 65) sheep from both selection groups were losing BW (approximately 140g/d) whilst maintaining wool growth at considerable rates, particularly when fed the HS diet. Although it could be reasoned that insulation from wool growth may enhance animal survival, it appears somewhat counterintuitive that sheep fed at or above maintenance should lose body weight, but this may be explained in part by the relative time span over which measurements were taken. For example, WGR /d was calculated over a 20 day period, S balance /d was averaged from a six-day collection period, and BW was taken at one point in time, thus direct comparisons between these variables may require some caution.

The degradation of protein from body tissues, as part of protein turnover, mobilises amino acids from tissue protein that may be used by other tissues in the body. In fact, the flow of amino acids from the breakdown of tissue protein has been estimated as being up to two to three times that supplied by nutrition (Reeds and Lobley, 1980). As the output of S in the wool of all sheep in this experiment was greater than the amount of S accounted for by the S balance (retention), the likelihood that between 350 and 600 mg S /d was mobilised from tissues including skin and muscle is explored.

Together, skin (14%) and muscle (32%) contribute 45-50% of the whole body's protein mass and approximately 25% and 18% of the whole body protein synthesis occurs in these tissues respectively (Lobley, 1994). The fractional synthetic rate of protein (FSR) in the skin is approximately 3 to 4 times higher than that in muscle (13-20%/d vs 1.5-3%/d; Liu et al,

1998). When the relative proportions of protein synthesis and mass for each tissue are considered, the absolute contributions of each tissue to whole body protein kinetics are similar (Harris and Lobley, 1991). The liver and gastro-intestinal tract may also supply SAA from protein breakdown but these sources are not considered further in this study as the rates of synthesis and degradation, and overall contribution to total body protein mass is small in comparison with the skin and muscle combined. The rate of turnover (synthesis and degradation) is rapid in the skin and, despite being less so in the muscle, both tissues are considered highly labile (Harris and Lobley, 1991). The measurement of protein degradation rate is difficult, relative to measuring synthesis, thus limited data are available for protein turnover rates, especially for skin. The animals in this study were mature, so tissue (protein) growth would be minimal and it would appear that rates of protein degradation were higher than the rates of protein synthesis, in order to supply amino acids required for wool growth.

To determine the feasibility that up to 600mg S/d could be supplied by mobilisation of tissue proteins, calculations using data from previous studies and measurements conducted here are made to estimate the rates of protein breakdown that would be required. The S-amino acid (SAA) concentration in skin and muscle was reviewed by Harris and Lobley (1991). The skin cysteine content was 2.7% and methionine content 1.1%, compared to 1.0% and 1.8% respectively in muscle. Based on the S concentration of each SAA, the proportion of S (from cysteine and methionine) was equivalent to 1.0% in the skin and 0.7% in muscle. The total body, skin and muscle content of N, protein and S was estimated for an 80kg sheep (Table 5.8). It was estimated that a sheep of this weight would have approximately 7kg of skin (70 g S) and 16kg of muscle (107 g S) (MacRae et al, 1993; Lobley, 1994), containing a total pool of S in excess of 175g.

Table 5.8: The calculated nitrogen (N), protein and sulphur (S) content of skin and muscle, for an 80 kg Merino ram.

	(grams)
Total body nitrogen[1] (g)	2000
Total body protein[2] (g)	12 500
Skin protein content[3] (g)	1750
Total skin weight[4] (g)	7000
Total skin sulphur[5] (g)	70
Muscle protein content[6] (g)	4000
Total muscle weight[7] (g)	16 000
Total muscle sulphur[8] (g)	107

1. Estimated for an 80kg sheep based on MacRae et al (1993)
2. Calculated from [1] multiplied by 6.25
3. Calculated from Lobley (1994), skin protein 14% total body protein mass.
4. [3] multiplied by 4; as skin comprised 75% water, 25% protein
5. Harris and Lobley (1991)
6. Muscle protein 32% total body protein (Lobley, 1994).
7. [6] multiplied by 4, assuming muscle 75% water, 25% protein.
8. Harris and Lobley (1991)

The estimates in Table 5.9 indicate the total weight of muscle or the total weight of skin that would be mobilised (/d), in order to meet the calculated wool S requirements of the sheep in this study. In addition, the required proportion of total muscle or skin weight to be mobilised is shown in Table 5.9. On average, +W and –W sheep would require mobilisation of 72 to 88g of muscle /d or 49 to 60 g of skin /d, to supply adequate S. There was no significant difference in the mobilisation requirements for sheep from either selection group and the weight of muscle loss or skin loss or a combination of both seems conceivable, as the BW loss during this time averaged 140 g /d (Table 5.9).

Table 5.9: Prediction of the required loss of muscle or skin weight, and loss as a proportion of total tissue protein, necessary to supply adequate S for wool growth, averaged for diet and selection group.

	HS diet	LS diet	+W selected	-W selected	SEM
S to be mobilised (mg/d)	606	464	588	482	87.87
Muscle[1] (g/d)	91 (23)	70 (17)	88 (17)	72 (18)	13.17 (3.29)
Skin[2] (g/d)	61 (15)	47 (12)	60 (15)	49 (12)	8.91 (2.23)
BW loss [3] (g/d)	-116	-165	-140	-140	59.9
Proportion of total muscle mass to be mobilised [4] (%)	0.6	0.5	0.6	0.5	0.08
Proportion of total skin mass to be mobilised [5] (%)	0.9	0.7	0.8	0.7	0.13

1. Required muscle mass to be mobilised /d (S to be mobilised / total muscle S pool) * muscle wt
2. Required skin mass to be mobilised /d (S to be mobilised / total skin S pool) * skin wt
NOTE: bracketed values are the g protein /d required from 1 (muscle) and 2 (skin).
3. BW gain (loss) excluding wool, expressed in g/d.
4. Percentage of total muscle mass required to be mobilised; ('1'/ total muscle weight)*100.
5 Percentage of total skin mass required to be mobilised; ('2' /total skin weight)*100.

The fractional synthetic rate (FSR) of skin and muscle in sheep is approximately 17% and 2.5% respectively (Lui et al, 1998), indicating that an 80kg sheep would synthesise approximately 300 g protein (11.5g S) in the skin and 100 g protein (2.6g S) in the muscle per day. If the difference in the fractional degradation rate (FDR) and FSR was 0.5% in muscle (therefore 3% FDR), this would be sufficient to supply adequate S to meet the requirements described here. Similarly a 1% difference between FDR and FSR in skin (therefore 18% FDR) would supply sufficient S. Therefore, the S exported in wool fibres that was not accounted for by the S balance could have been mobilised from either muscle or skin or a combination of both to meet the requirements. This has relevance to future studies, particularly long-term trials, whereby bodyweight may be compromised over an extended period of time resulting in continued loss of production effecting project outcomes and animal health.

5.5 Summary

The +W sheep in the current experiment produced approximately 50% more wool than –W sheep, primarily due to increased FL and not FD. These sheep produced wool more efficiently and retained more S (although not significantly), but the digestive and absorptive capacity of both selection groups did not differ. All sheep mobilised a similar quantity of S from tissue proteins to support the S requirement for fibre growth. Regardless of the source of the mobilised S, +W sheep utilised approximately 45% more S to produce wool. Thus more efficient uptake of SAA into the follicle or differential expression of wool keratin genes in the follicle, for example, may be responsible for the greater level of fibre production in +W sheep.

Chapter 6

Wool follicle gene expression in sheep with different estimated breeding values (EBVs) for wool growth.

6.1 Introduction

Greater fibre production is related to lower wool sulphur (S) concentration (Piper and Dolling, 1966; Chapter 5 of this thesis) and increased responsiveness of wool growth to improved nutrition in genetically superior Merinos, compared to genetically lower wool producers (Williams, 1966). This relationship in Merino fleece weight (FW) selection lines raises a number of questions in relation to the production of different wool keratin proteins and the response in production of these proteins to an improved supply of sulphur-containing amino acids (SAA). In the experiment reported in Chapter 5, Merino sheep were selected using estimated breeding values (EBVs), to form two genetically distinct selection groups; high wool (+W) producers and low wool (-W) producers. It was proposed that +W and –W sheep have variable expression of the different wool keratin proteins and that the increased supply of SAA via a high S-containing diet (HS diet) would differentially influence the expression of these genes in +W and –W sheep, leading to differences in wool growth rate (WGR) and wool S concentration.

The wool fibre cortex comprises keratin-associated proteins (KAPs) and intermediate filaments (IFs). As the genes encoding these proteins are sequentially expressed along the length of the follicle, extending from the bulb region, proteins are formed and harden, in a process of keratinisation. The KAPs are characterised by their proportion of specific amino acids; for example, genes encoding proteins high in cysteine residues are high sulphur (HS) genes. Other KAP groups include the ultra-high sulphur (UHS) and high glycine-tyrosine (HGT) genes. Early work conducted by Gillespie and Reis (1966) found varying concentrations of HS and LS proteins in wool from two sheep producing different quantities of wool and more recently, the infusion of cysteine increased the expression of an UHS gene in the skin of a Corridale sheep (Fratini et al, 1994). Thus it would appear that the expression of the various keratin proteins may be under genetic and nutritional control.

The experiment reported in this chapter examined the expression of HS genes (KAP2.12), UHS genes (KAP 4.2), HGT genes (KAP 6.1) and a low sulphur (LS) IF gene

(K2.10) using the northern blot molecular technique, with RNA samples extracted from the skin of +W and –W sheep fed LS and HS diets (see Chapter 5). Expression of trichohyalin (TRN), a protein synthesised predominantly in the inner root sheath (IRS) cells of the follicle and glyceraldehyde-6-phosphate dehydrogenase (GAPDH), a 'house-keeping' enzyme involved in a crucial step of the glycolytic pathway, was also examined. The apparent digestion and net retention of SAA were similar for both +W and –W sheep (Chapter 5) yet the rates of wool production differed by 50%, thus the following hypotheses were addressed here;

a. that +W sheep produce more wool of a lower S concentration as a result of greater expression of LS keratin genes,
b. that –W sheep have higher levels of expression of HS and UHS genes in the skin compared to +W sheep, and thus produce less wool with a higher S concentration,
c. that +W sheep are more responsive in WGR to improved nutrition because they utilise additional SAA for the production of more LS proteins, whereas –W sheep convert additional SAA into HS or UHS proteins.

6.2 Methodology

6.2.1 Experimental methodology

Sixteen of the thirty-two Merino sheep (eight +W sheep and eight –W sheep; equally distributed between nutritional treatments) were selected from the experiment described in Chapter 5, based on divergent WGR at the end of the trial (day 65). These sheep were representative of the selection groups, with an average WGR of 32.5 g/d and 17.7 g/d and wool S concentration of 2.77%S and 2.87%S respectively for the +W and –W sheep (compared with 28.7 g/d and 19.6 g/d WGR, and 2.77% and 2.87% wool S concentrations, for the entire 32 sheep used in Chapter 5). Skin samples were taken under local anaesthetic (Lignocaine with adrenaline; Troy Laboratories, NSW) at the beginning (day 0) and the end (day 65) of the two-month trial, using a trephine (Section 3.1.2). Samples were snap frozen

and stored at –80°C until RNA was extracted using a method adapted from Chomczsynski and Sacchi (1987) (Section 3.6.2). RNA northern blots were prepared (Section 3.6.3) and probed using ^{32}P-dCTP labelled gene sequences to quantify differential keratin gene expression (Section 3.6.4 to 3.6.8). All expression levels were standardised for the concentration of 28S rRNA in the sample (Section 3.6.8).

Each northern was probed with 28S rRNA, the inner root sheath protein sequence TRN and the house-keeping sequence GAPDH, sequentially, and relative levels of expression of the three sequences for each sample, on each northern, was compared by densitometry (Section 6.3.4), to evaluate their use for standardisation of total RNA levels in each sample.

6.2.2 Statistical methodology

REML Variance Components Analysis (GENSTAT, 5th Edition) was used to examine the differential expression of the wool keratin genes between selection groups and dietary treatment, and the two-way interaction between these fixed effects, at the beginning (day 0) and the end of the study (day 65). The phenotypic correlation between the keratin gene expression, and wool growth and composition was determined using GENSTAT (5th Edition). The variation in WGR, wool S concentration and wool S output was partitioned to the expression of the keratin genes, selection group and dietary treatments, using a General Linear Regression approach (SAS/STAT, Version 8.2). The fitted model consisted of the variable (eg. KAP 2) and selection group for day 0 samples, and for data from day 65, the variable (eg. KAP 2), selection group, dietary treatment and the two-way interactions associated with these factors. The final reduced models, for which any fixed effects, explanatory variates and interactions that were not significant at the 5% level were systematically removed were,

WGR (or wool S, or S output) at day 0 = gene expression + group

*WGR (or wool S, or S output) at day 65 = gene expression + group + diet + group*diet,*

unless stated otherwise in the text.

6.3 Results

6.3.1 Northern gel blots

Figures 6.1 to 6.7 show an example of one northern gel, containing samples from selected individual sheep representative of the –W group fed the LS diet (-WLS), the -W group fed the HS diet (-WHS), the +W group fed the HS diet (+WHS) and the +W group fed the LS diet (+WLS), and probed with sequences encoding keratin genes or standardising sequences as indicated. Each Figure shows samples taken on day 0 (left track) and samples taken on day 65 (right track), for each representative sheep.

Although 10μg of RNA was loaded for each sample, the densitometry reading for each probed sample was standardised to the densitometry reading for the same sample when probed with 28S rRNA (Figure 6.1). This corrects for discrepancies in RNA concentration between samples when quantifying gene expression, thus direct visual interpretation of the probed northern blots is misleading. The probed RNA samples in Figures 6.1 to 6.7 show discrete and strong probing of the mRNA to the correct size transcript in each case, and indicate that the RNA extracted and probed was of a high quality.

6.3.2 Gene expression in the skin

Expression levels for all keratin genes, glyceraldehyde-6-phosphate dehydrogenase (GAPDH), and trichohyalin were similar for sheep from +W and -W selection groups at day 0. There was no difference in the expression levels of any of the genes, between different selection groups and dietary treatments, in samples from day 65. Figures 6.8 to 6.13 show the level of expression at day 65, in arbitrary units, of GAPDH (Figure 6.8), Trichohyalin (Figure 6.9), KAP 2.12 (HS; Figure 6.10), KAP 4.2 (UHS; Figure 6.11), KAP 6.1 (HGT/LS; Figure 6.12) and K 2.10 (IF; Figure 6.13), for each selection group and dietary treatment interaction. No significant variability in gene expression between the two selection groups was detected at day 0 and hence these data are not presented.

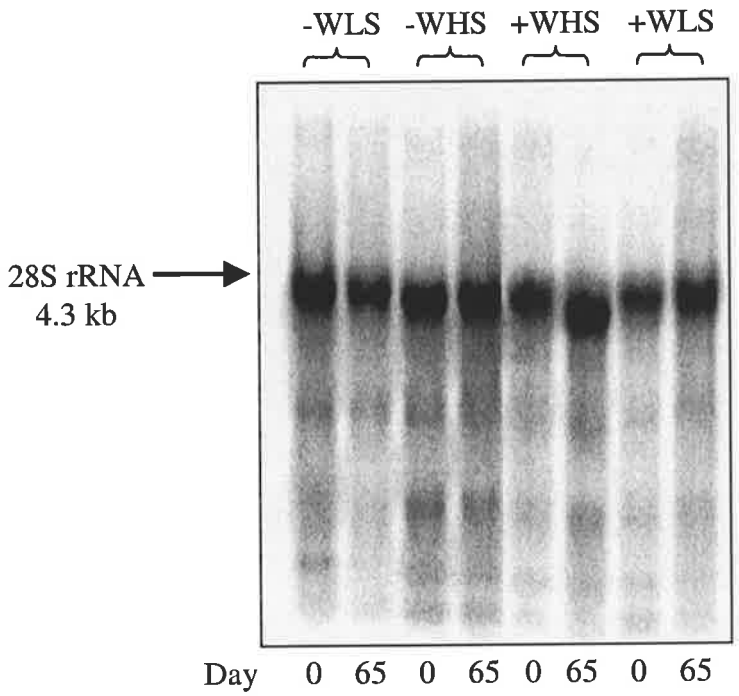

Figure 6.1 : Northern blot probed with a 29mer oligonucleotide 28S rRNA kinased sequence, radiolabelled with γ^{32}P- rATP. The hybridised and washed filter was exposed to a phosphor-image screen for 3 hours.

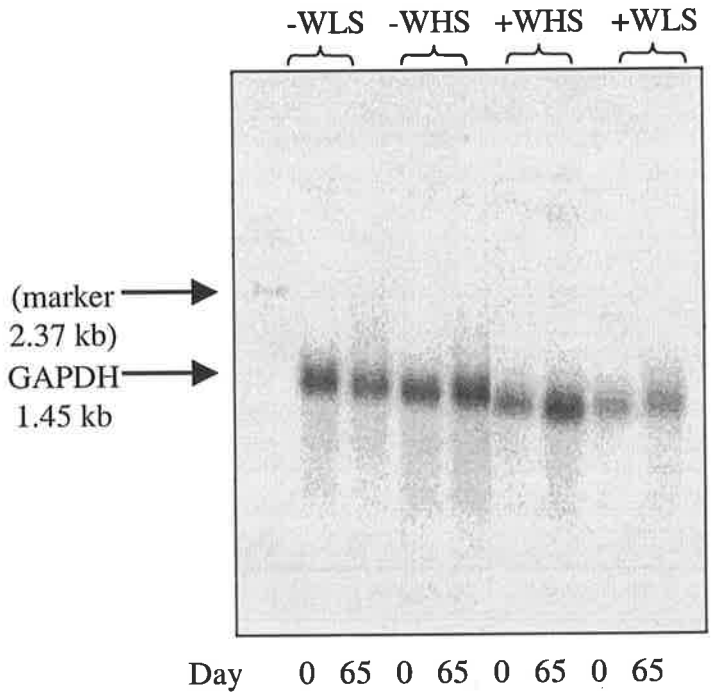

Figure 6.2 : Northern blot probed with 200 bp *HindIII / SfI* glyceraldehyde-6-phosphate dehydrogenase (GAPDH) fragment, radiolabelled with α^{32}P- dCTP. The hybridised and washed filter was exposed to a phosphorimage screen for 45 hours.

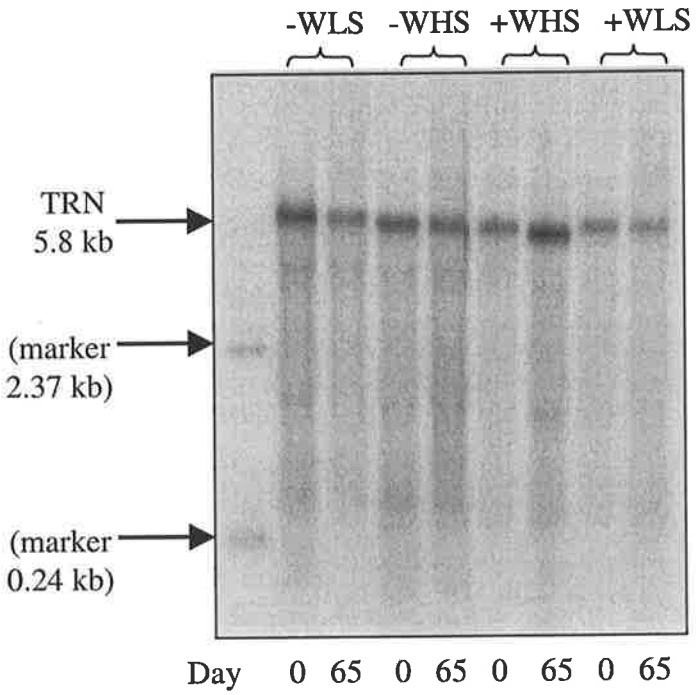

Figure 6.3 : Northern blot probed with 331 bp *DraI / EcoRI* 3'-non-coding Trichohyalin fragment radiolabelled with α^{32}P- dCTP (Fietz et al, 1993). The hybridised and washed filter was exposed to a phosphorimage screen for 65 hours.

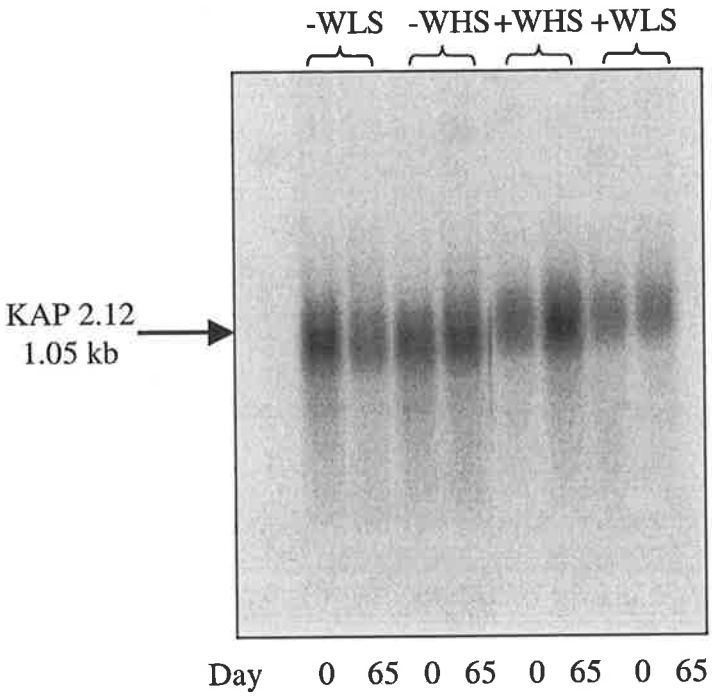

Figure 6.4 : Northern blot probed with 330 bp *PvuII* 75%-coding region of KAP 2.12 fragment (Powell, unpublished) radiolabelled with α^{32}P- dCTP. The hybridised and washed filter was exposed to a phosphorimage screen for 18.5 hours.

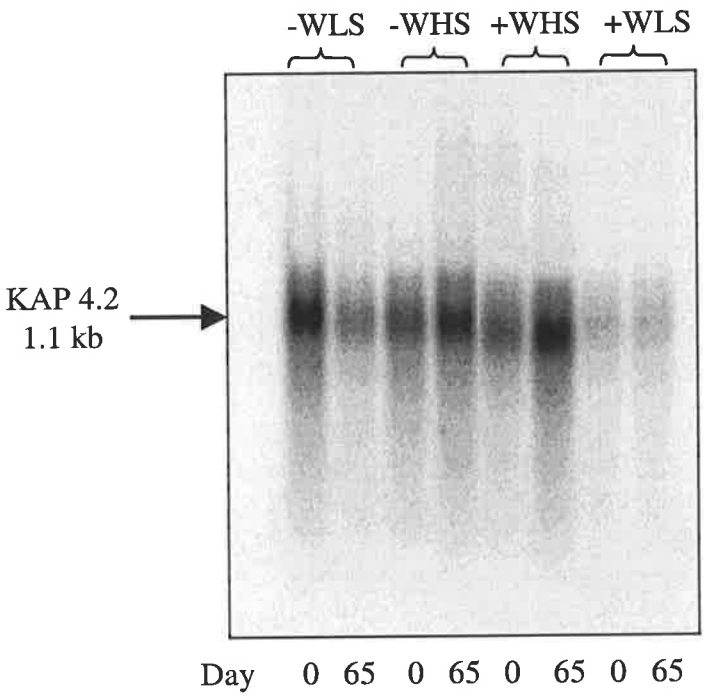

Figure 6.5 : Northern blot probed with 770 bp *EcoRI* coding KAP 4.2 sequence (Powell, B.C. unpublished), radiolabelled with α^{32}P- dCTP. The hybridised and washed filter was exposed to a phosphorimage screen for 21 hours.

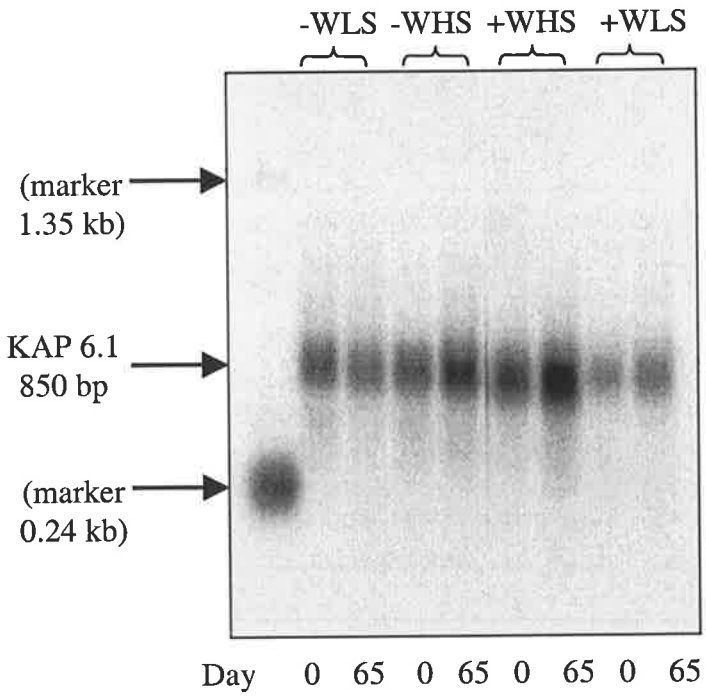

Figure 6.6 : Northern blot probed with 250 bp *AvaI/ HinfI*, predominantly coding KAP 6.1 sequence (Fratini et al, 1993), radiolabelled with α^{32}P- dCTP. The hybridised and washed filter was exposed to a phosphorimage screen for 65 hours.

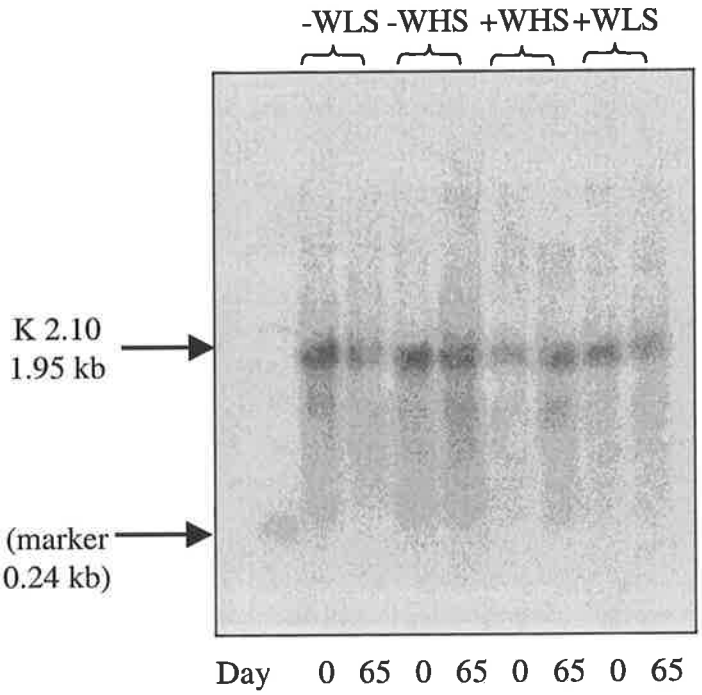

Figure 6.7 : Northern blot probed with 220 bp *PstI* predominantly 3'-non-coding K 2.10 sequence (Powell et al, 1992), radiolabelled with α^{32}P- dCTP. The hybridised and washed filter was exposed to a phosphorimage screen for 46 hours.

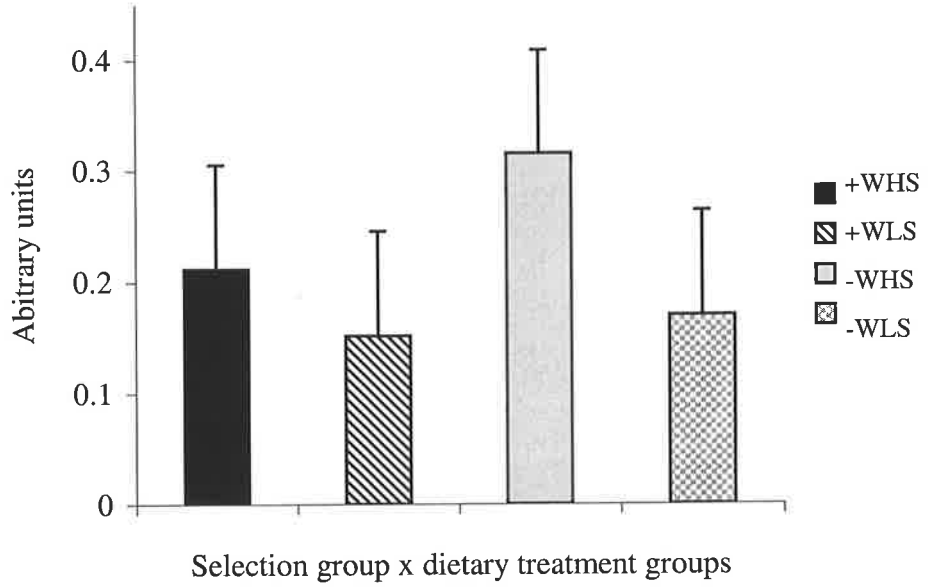

Figure 6.8: Comparative expression of GAPDH in the skin of sheep from different selection groups and dietary treatments (n=4), on day 65 of the experiment (mean ± SEM).

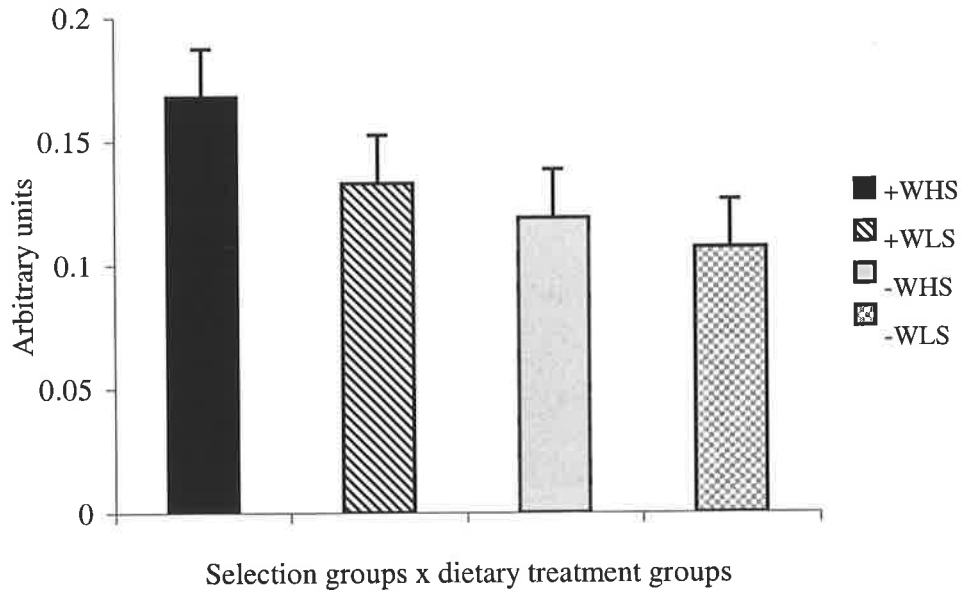

Figure 6.9: Comparative expression of trichohyalin (TRN) in the skin of sheep from different selection and dietary groups (n=4), on day 65 of the experiment (mean ± SEM).

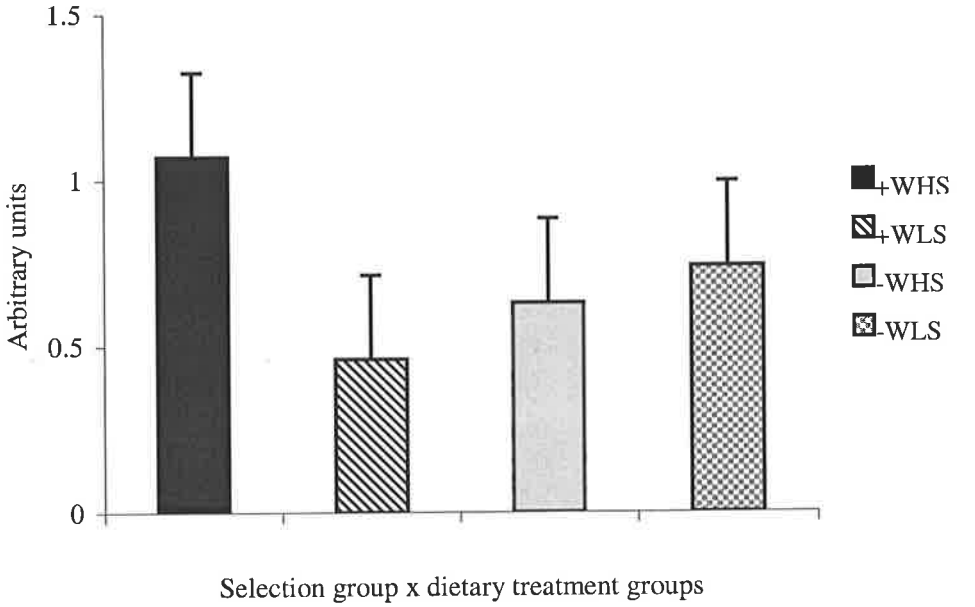

Figure 6.10: Comparative expression of KAP 2.12 in the skin of sheep from different selection and dietary groups (n=4), on day 65 of the experiment (mean ± SEM).

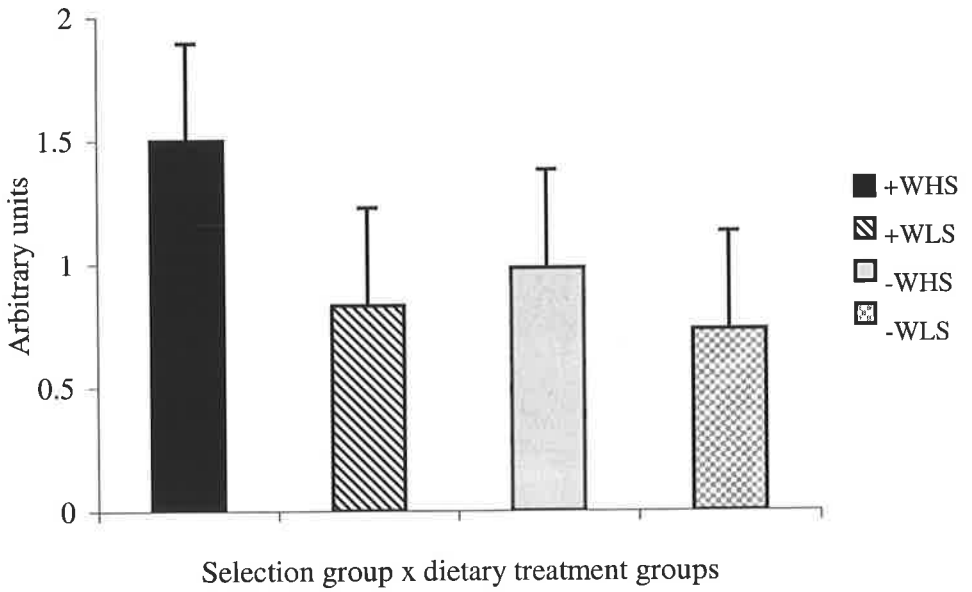

Figure 6.11: Comparative expression of KAP 4.2 in the skin of sheep from different selection and dietary groups (n=4), on day 65 of the experiment (mean ± SEM).

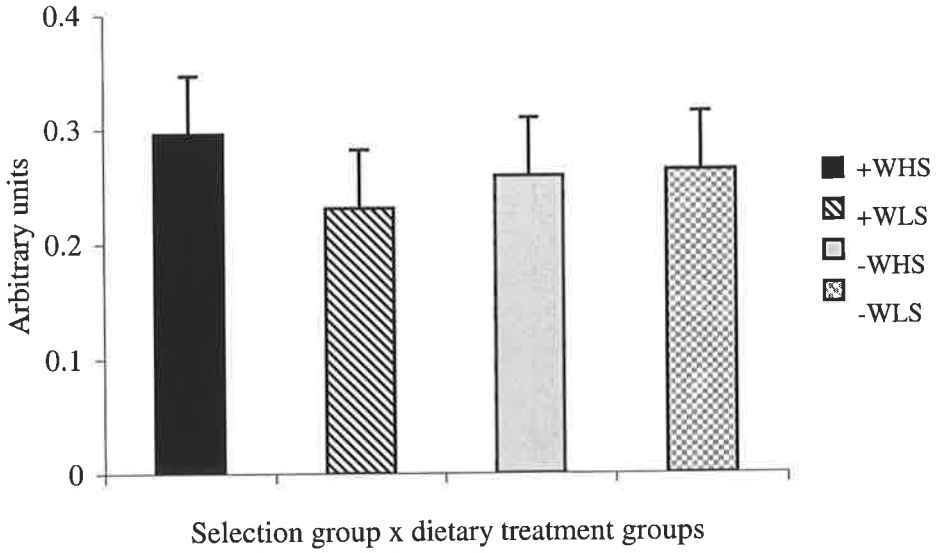

Figure 6.12: Comparative expression of KAP 6.1 in the skin of sheep from different selection and dietary groups (n=4), on day 65 of the experiment (mean ± SEM).

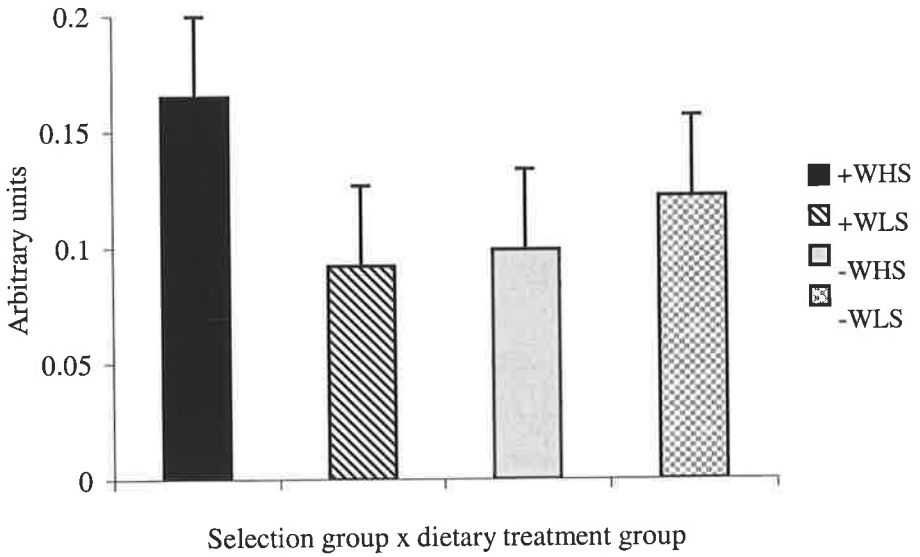

Figure 6.13: Comparative expression of K 2.10 in the skin of sheep from different selection and dietary groups (n=4), on day 65 of the experiment (mean ± SEM).

6.3.3 Wool growth and composition variability, and the association with keratin gene expression

6.3.3.1 Keratin gene expression at the commencement of the experiment (day 0)

At the start of the study period (day 0), prior to the assignment of experimental diets, expression of the HS keratin gene KAP 2 accounted for 16% of the variation in WGR ($p<0.05$), but no relationship between WGR and KAP 4, KAP 6 or K2.10 expression levels was found (Table 6.1). In comparison, selection group accounted for more than 28% of WGR variation in all models ($p<0.01$).

There was no association between selection group, or expression of any of the keratin genes examined, and wool S concentration or total S output, at the commencement of the experiment (day 0), with the exception of the model containing KAP 4 + group, in which approximately 44% ($p<0.05$) and 20% (ns) of the variation in wool S concentration was attributed to selection group and KAP 4 respectively (Table 6.1).

Table 6.1: The percentage of variation in WGR, wool S concentration or wool S output accounted for by keratin gene expression and selection group at the beginning of the study (day 0)

	Percentage of variation accounted for by:					
	WGR		*Wool S concentration*		*Wool S output*	
Variable	Gene	Group	Gene	Group	Gene	Group
KAP 2	16 *	28 **	2 ns	32 ns	<1 ns	7 ns
KAP 4	6 ns	47 **	20 ns	44 *	<1 ns	11 ns
KAP 6	<1 ns	57 **	<1 ns	31ns	8 ns	8 ns
K 2.10	1 ns	61 **	11 ns	27 ns	<1 ns	11 ns

ns not significant; * P<0.05; ** P<0.01

6.3.3.2 Keratin gene expression at the completion of the experiment (day 65)

WGR variability and wool keratin gene expression

The expression of KAP 2 and K2.10 genes accounted for only 5% and 4% of the WGR variation respectively ($p<0.05$), and KAP 6 and KAP 4 were not significantly related to WGR (Table 6.2). The majority of the variation in WGR was related to selection group and dietary treatment, such that together, group and diet significantly accounted for more than 75% of the variation. The group x diet interaction in the models containing KAP 4 and KAP 6 had a significant but small effect on WGR (Table 6.2).

Table 6.2: The relationship between WGR variability and selection groups, dietary treatment and the expression of wool keratin genes, at the completion of the experiment (day 65).

	Percentage of WGR variation accounted for by:			
Variable	Gene expression	Group	Diet	Group x Diet
KAP 2	5 ***	46 ***	32 ***	na
KAP 4	2 ns	43 ***	33 ***	3 *
KAP 6	0 ns	49 ***	40 ***	3 *
K 2.10	4 *	44 ***	36 ***	na

ns not significant; * P<0.05; ** P<0.01; *** P<0.001; na not applicable (interaction ns thus removed from model)

Wool S and wool keratin gene expression

There was no significant association between any keratin genes, selection groups or dietary treatments, and the concentration of S in the wool at the completion of the trial (Table 6.3).

Table 6.3: The relationship between wool S variability and selection groups, dietary treatment and the expression of wool keratin genes, at the completion of the experiment (day 65).

	Percentage of wool S variation accounted for by:		
Variable	Gene expression	Group	Diet
KAP 2	3 ns	8 ns	22 ns
KAP 4	11 ns	13 ns	10 ns
KAP 6	9 ns	9 ns	15 ns
K 2.10	1 ns	8 ns	21 ns

ns= not significant

Wool S output and wool keratin gene expression

A small but significant percentage of the variation in S output in wool was accounted for by KAP 2 (4%), KAP 4 (3%) and K2.10 (3%), but there was no association with KAP 6 expression (Table 6.4). More than 70% of the variation in S output was significantly accounted for by selection group and dietary treatment combined.

Table 6.4: The relationship between wool S output variability and selection groups, dietary treatment and the expression of wool keratin genes, at completion of the experiment (day 65).

	Percentage of S output variation accounted for by:			
	Gene expression	Group	Diet	Group x Diet
KAP 2	4 *	39 ***	39 ***	na
KAP 4	3 *	35 ***	37 ***	2 *
KAP 6	1 ns	41 ***	45 ***	na
K 2.10	3 *	37 ***	43 ***	na

ns not significant; * P<0.05; ** P<0.01; *** P<0.001; na not applicable (interaction ns, thus removed from model)

6.3.3.3 Correlations between keratin gene expression, wool composition and wool growth.

Table 6.5 shows the phenotypic correlations between keratin genes, WGR, wool S concentration and wool S output at the completion of the trial (day 65). The correlation coefficients (r-values) between expression levels of all four keratin genes investigated in this work were moderate to high, ranging from 0.37 to 0.76 (Table 6.5; bold text). WGR was moderately correlated with KAP 2, KAP 4 and K 2.10 expression (r= 0.40 to 0.49) and poorly with KAP 6 expression (r= 0.19) (Table 6.5). There was a moderate correlation between wool S concentration and KAP 4 (r= 0.39) and with KAP 6 (r= 0.37), but KAP 2 and K2.10 expression were not correlated with wool S concentration. The expression of trichohyalin was positively and moderately correlated with all keratin genes examined (r= 0.28 to 0.37), moderately correlated with WGR (r= 0.53) and poorly correlated with wool S concentration (r= 0.11) (Table 6.5).

Table 6.5: Correlation coefficients (r-value) between keratin gene expression and wool growth and composition (Values in bold indicate the correlations between all keratin genes)

	KAP 2	KAP 4	KAP 6	K 2.10	TRN	WGR	Wool S	S output
KAP 2	1.00							
KAP 4	**0.62**	1.00						
KAP 6	**0.45**	**0.37**	1.00					
K 2.10	**0.76**	**0.68**	**0.55**	1.00				
TRN	0.29	0.37	0.35	0.28	1.00			
WGR	0.44	0.49	0.19	0.40	0.53	1.00		
Wool S	-0.06	0.39	0.37	-0.05	0.11	0.06	1.00	
S output	0.42	0.52	0.24	0.39	0.54	0.99	0.20	1.00

Significant at $p<0.05$ when $r>0.47$ (Snedecor and Cochran, 1980)

6.3.4 A comparison of northern blots probed with 28S rRNA, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and trichohyalin (TRN) sequences, for use as standards to assess RNA quantity

Figure 6.14 shows an example of one northern blot, probed consecutively with 28S ribosomal RNA, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and trichohyalin (TRN) transcripts. It demonstrates the variability in probing intensity for each sample, thus the assumed RNA concentration for each sample, when probed with 28S rRNA versus GAPDH versus TRN.

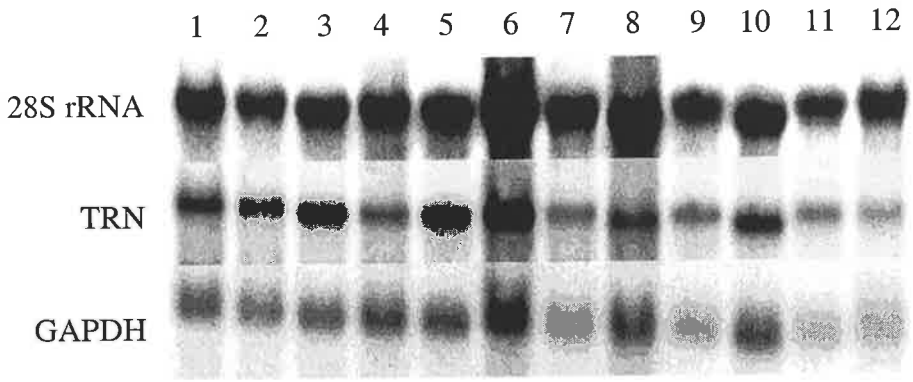

Figure 6.14: An example of a northern blot probed with 28S rRNA, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and trichohyalin (TRN) sequences consecutively, to assess their use as standardising sequences for total RNA levels in each sample. Samples 1-12 are representative of typical RNA samples from skin.

Quantification of keratin gene expression in this chapter was standardised to levels of 28S rRNA expression, however GAPDH and TRN have been used for this purpose in other experiments to examine gene expression in sheep skin. Table 6.6 summarises the mean expression levels of all genes examined for samples from day 65 (pooled for selection groups and dietary treatments) and standardised using 28S rRNA, GAPDH or TRN expression levels. The values reported are relative to values derived when standardising with 28S rRNA, hence values for 28S rRNA are equal to one. Expression levels when standardising with GAPDH or TRN for each of the genes were variable when compared to 28S rRNA standardising. Values

for GAPDH standardising were similar to that for 28S rRNA standardising for some keratin genes (eg. KAP 2) but were highly variable, with some gene expression values (eg. 28S rRNA) up to 66% different to values obtained using 28S rRNA to standardise (Table 6.6). Values obtained using TRN were more consistent when compared to 28S rRNA standardising, with gene expression values less than 10% different to the levels when standardising with 28S rRNA (Table 6.6).

Table 6.6: A comparison of the expression levels of keratin genes standardised to 28S rRNA, GAPDH or TRN, for samples taken at the completion of the study (day 65)

Standardising sequence	Relative gene expression (relative to expression when standardised with 28S rRNA)						
	KAP 4	KAP 2	KAP 6	K2.10	28S rRNA	GAPDH	TRN
28S rRNA	1.0	1.0	1.0	1.0	1.0	1.0	1.0
GAPDH	1.12	1.08	1.34	1.28	1.66	1.0	1.38
TRN	0.97	0.97	1.04	1.05	1.10	1.04	1.0

6.3.5 Expression of the oASCT2 amino acid transporter in the skin

The probing of northern blots with a 360bp HindIII/ BamHI oASCT2 transcript (Figure 6.15) was poor, thus the determination of differential expression levels of oASCT2 between different selection groups and dietary treatments was not accurate. Figure 6.15 demonstrates this faint probing of a northern gel exposed for more than 48 hours.

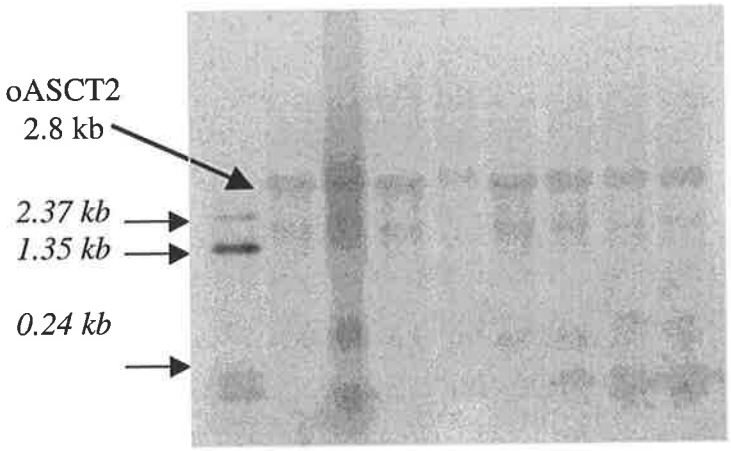

Figure 6.15: Northern blot probed with 360 bp *HindIII/ BamHI* Bluescript plasmid subclone oASCT2 fragment, radiolabelled with α32P- dCTP. The hybridised and washed filter was exposed to a phosphorimage screen for more than 48 hours. The 0.24 - 9.5 Kb RNA Ladder molecular weight markers are indicated on the left.

6.4 Discussion

The experiment described in this chapter is the most comprehensive investigation to date of wool follicle keratin gene expression in the skin. Previous work has examined production of different wool proteins, in sheep producing different quantities of wool but not in fleece weight (FW) selection lines. This experiment showed the relationship between wool growth, wool composition, and the expression of different keratin genes in Merino sheep that had well-defined wool characteristics. The experimental design permitted the use of large numbers of animals to generate statistically powerful data, not generally seen in work investigating gene expression in this way. This is the first comparative investigation of keratin gene expression in Merino sheep selected for divergent fleece production and supplied with diets of varying concentrations of sulphur-containing amino acids (SAA).

6.4.1 Expression of wool keratin genes did not differ between +W and –W sheep

The +W sheep selected for the study presented in this chapter produced more wool, with a lower S concentration than –W sheep, as expected, but the level of expression of keratin genes in the skin of all sheep was similar at the beginning and the end of the experiment. Sheep were selected using EBVs to generate two selection groups, and although these were not true 'selection lines', the variation in average wool growth was similar to that described for Merino FW selection lines (Williams, 1995). Although wool S concentration varied between +W and –W selection groups, the variation in the current experiment was much less than that described in Merino FW selection lines (Piper and Dolling, 1966), and may be the reason for similarities in gene expression levels.

The initial hypotheses proposed that –W sheep produced less wool with a higher wool S concentration as a result of greater expression of the UHS and HS genes, and that +W sheep produced more wool with a lower wool S concentration, due to greater expression levels of the LS genes. However from the current study it is apparent that expression levels of keratin genes in the skin were similar for +W and –W sheep, despite variability in wool production

and wool S concentration.

6.4.2 Limited association of keratin gene expression with WGR and wool S concentration

A general linear regression approach was used to relate keratin gene expression with WGR and wool S concentration. The remaining discussion will focus on comparisons at the end of the experiment (day 65), as keratin gene expression did not differ between selection groups at the commencement of the trial and dietary treatments were not imposed at this early time. All keratin genes examined in samples from the end of the study were moderately to highly correlated with one another, suggesting that expression of the genes under investigation are controlled by similar factors.

Principal Components analysis was undertaken, because of the high correlations between genes and because it condensed the keratin gene information into one or two components to associate with WGR and wool S concentration. The first principal component (PC1) accounted for 71% of the standardised variance of the keratin gene data, and was a measure of all keratin genes, as shown by equal loadings of all genes (Appendix 2.3) but there was no association between PC1 and WGR or wool S concentration. None of the remaining principal components accounted for a large proportion of the standardised variance (less than 15%) and were not significantly related to WGR or wool S concentration. Combined, the principal components analysis was not easily interpreted biologically so other methods of analysis were explored.

The HS, UHS and IF gene expression levels were moderately to highly, and positively, correlated with WGR, thus as wool growth increased, the expression of the keratin genes also increased. Examination of each of the genes individually (with group and diet in the regression model) accounted for a very small proportion of the WGR variation (KAP 2 and K2.10) and none of the variation in wool S concentration, suggesting factors other than differential expression of the genes examined were responsible for the variation described between groups and diets. The poor relationship between WGR and keratin gene expression,

and the high correlation of keratin genes with each other, meant that when all genes were combined in an additive regression model together (data not shown), it was difficult to ascertain the relative role each gene may have played in determining wool growth and composition. Although the specific genes examined here were considered representative of the different KAP and intermediate filament gene families that comprise the proteins of the wool fibre, it is possible but unlikely that expression of genes other than those investigated may have been associated with WGR.

In conclusion, similarities in keratin gene expression in the skin between +W and –W sheep suggest that wool produced from sheep in both selection groups was similar in composition, but that +W sheep produced significantly more of it. This differs from reports in other FW selection lines (Williams et al, 1972b), that showed larger differences in wool S concentration between selection groups, and raises the following question; how do +W selected sheep produce more wool when expression of keratin genes in the skin is similar?

6.4.3 Do +W and –W sheep differ in their uptake of SAA into the follicle?

The +W sheep had a higher total S output in fibre than –W sheep (approximately 800 mg S/d vs 550 mg S/d), indicating that the follicles of the +W Merinos utilised more SAAs for wool production. As there was no significant difference in the retention of S or the concentration of SAA in the plasma of +W and –W sheep (Chapter 5), and if the relationship between blood supply to the skin and WGR is considered poor (Harris et al, 1993b) it suggests the follicles of the +W and –W sheep were supplied with similar quantities of SAAs. Therefore, the efficiency of the follicles to take up SAA into follicle cells may have been greater in +W sheep or rate of expression of wool keratin proteins in the cortical cells may have differed in the +W and –W sheep examined in this experiment.

Downes et al (1962) identified that SAA uptake was localised to the cortical cells of the keratogenous zone, which was later identified as being mediated by a sodium-dependent transport system (Wilson, 1995) that has recently been isolated, sequenced and named an

oASCT2 neutral amino acid transporter (Nattrass, 2000). Nattrass found the greatest levels of expression of this transporter in wool follicle RNA, with less expression in the kidney, adipose tissue and lung, and little or no expression in the brain or skeletal muscle using northern blot analysis. oASCT2 expression was localised using in situ hybridisation to the cortical cells of the keratogenous zone (Nattrass, 2000), identical to where L-cysteine is taken up (Wilson, 1995), with no expression in the inner or outer root sheath cells. Transfer of SAA into the follicle via the oASCT2 transporter follows Michaelis-Menten kinetics, however it was suggested that nutrient supply to the IRS or ORS cells may occur via diffusion or an unknown transporter, or that the capillary network surrounding the dermal papilla delivers the required SAA (Nattrass, 2000). It was proposed in the current experiment that variation in wool growth may arise from differences in the number or type of transporters associated with the follicles or that the affinity of the transporters for the SAAs may differ between the +W and –W sheep, the former of which was addressed in this work.

The northern blot technique (Section 6.6) was employed to investigate expression of oASCT2 in +W and –W sheep skin, an approach used by Nattrass (2000) in various other sheep tissues, such as the liver and muscle. However the approach was not sensitive enough to detect differential quantities of the low levels of mRNA encoding oASCT2 thus selection group variability was not readily detectable. Although exposure of the hybridised filters exceeded 48 hours (as for Nattrass, 2000) expression signals were very low and therefore quantification was considered to be inaccurate. A technique such as poly-A+ (mRNA) northern analysis or Real-Time Reverse Transcription Polymerase Chain Reaction (RT-PCR) analysis could be used in the future, to permit detection and quantification of the low levels of oASCT2 mRNA, and is worth pursuing.

6.4.4 Do +W and –W sheep differ in follicle rate processes or cellular differentiation?

The efficiency of follicle rate processes, such as bulb cell turnover time, and cellular differentiation, including the fate of bulb cells, may vary between +W and –W sheep,

irrespective of SAA uptake into the follicle, and lead to differences in fibre production.

The follicle bulb

The total output of cells from the follicle bulb is determined by the size of the germinative region of the follicle bulb and the proliferative activity of this cell population (Hynd, 1989a). For example, the volume of bulb region and the rate of cell turnover in this region may be greater in +W sheep, thus potentially supplying more cells to the developing fibre. The volume of germinative tissue (bulb volume minus dermal papilla volume) was greater in +FW-selected sheep in both the Romney breed (Holle et al, 1994) and in Merinos (Williams and Winston, 1987), however in both +FW and –FW or control (C) sheep, a similar proportion of these cells were labelled as 'dividing cells'. Thus it appears that more cells in total are supplied from the follicle bulb for the formation of the fibre and IRS, in FW-selected sheep.

The activity and size of the bulb cell population is dependent on nutrient supply (Short et al, 1965; Wilson and Short, 1979), in particular SAAs (Hynd, 1989b) and the presence or absence of various growth factors (Chapman et al, 1982; Hynd, 1989b). No definitive studies of bulb cell production in FW selection lines have yet shown a significant relationship with hormones or growth factors, but this remains as a possible source of WGR variation in +W and –W sheep. For example, thyroxine has been shown to increase the rate of bulb cell division (Hynd, 1994), and an increase in the number or sensitivity of hormone receptors such as the thyroxine receptor in follicle cells, may lead to higher rates of wool growth in +W sheep.

Proportion of bulb cells entering the fibre cortex versus the inner root sheath cell layer

Attempts have been made to accurately quantify the proportioning of differentiating bulb cells into IRS and fibre cortical cells (Wilson and Short, 1979; Hynd, 1989b; Holle et al, 1994), assuming limited cell loss through cell death and resorption after division, although this assumption has been questioned (Orwin and Woods, 1982). Nevertheless, the complexities involved in measuring this proportioning of cells means the available data are

somewhat variable and possibly inaccurate. Values in the range of 10% to 20% (Wilson and Short, 1979) and 70% to 80% (Holle et al, 1994) of bulb cells entering the fibre have been described. Despite these variances, genetic control is thought to be the major influence on the proportioning of bulb cells, with little sustained effect of nutrition (Wilson and Short, 1979; Hynd, 1989b). However, the only significant difference in the proportion of bulb cells forming the IRS or fibre between FW selection lines is described by Hynd (1989a) using data from Williams and Winston (1987), in which +FW sheep produced comparatively more cortical cells. This remains as a likely source of WGR variation for further investigation, once the technical difficulties and inaccuracies of measurement can be overcome.

Fibre cortical cell production

The rate of production and keratinisation of cortical cells, as well as the dimensions of the cells themselves, directly influences the quantity of fibre produced. Williams and Winston (1987) found little variation in cortical cell volume in the fibre, but in +FW sheep, the cell volume is more sensitive to nutrition, increasing by up to 25% with improved nutrition yet remaining relatively constant in the –FW sheep. The daily volumetric output of cortical cells is also greater in +FW Merinos and similarly the output is significantly greater in strong wool Merinos, than that in fine wool Merinos (Hocking-Edwards and Hynd, 1992). Given the large variation in wool growth and much smaller variation in wool composition (wool S concentration) between selection groups in the current study, it seems likely that +W sheep produced a greater number of cortical cells per day compared to –W sheep. Although in contrast to Williams and Winston (1987), the +W sheep here were equally responsive to nutrition in WGR as –W sheep. Hynd (1989b) proposed that the increased responsiveness of the +FW sheep to nutrition resulted from an increase in cortical cell size, and the lack of nutritional responsiveness in –FW sheep was due to a change in cortical cell composition rather than size (as their cortical cell size was already approaching a maximum).

6.4.5 The northern blot technique

Northern blot analysis was used to examine mRNA expression levels in the skin, an approach that has been used to quantify mRNA for keratin proteins in sheep skin previously (Fratini et al, 1994; Jenkins and Powell, 1994; Bawden et al, 1998). Northern blots indicate both the quality and quantity of RNA and, although use of the approach is widespread, there is limited documentation about the sensitivity of the technique.

Data generated for this Chapter were derived from RNA samples from 16 different sheep skin samples, electrophoresed in triplicate on different northern blots (Section 3.6.1.3). Unfortunately, the standard error between identical samples on different northerns may have contributed to the lack of significant differences in expression of all keratin genes examined. It is difficult to ascertain if this variability is inherent to the northern blot technique, because experiments of skin and follicle expression are limited and often have little replication of samples (for example, Fratini et al, 1994).

Triest et al (1995) documented the intra-assay variation (the same RNA sample within one northern blot) and inter-assay variation (the same sample on two different northern blots) of rat liver RNA samples probed with various gene transcripts and 28S rRNA. The variation ranged significantly between the different genes examined, the different volumes of RNA loaded into the northern gels, and within (intra) and between (inter) different northerns. The average intra-assay coefficient of variation was 29% (range 8% to 59%) and the average inter-assay coefficient of variation was 15% (range 10% to 20%). The approach used in the current experiment was to remove any triplicate sample outside a coefficient of variation of 30%, therefore the variability described in the current work was similar to that previously reported (Triest et al, 1995). Given the high expression levels of keratin genes in the follicle, the northern blot technique was appropriate, but more sensitive approaches such as Real-Time RT-PCR or Poly-A+ northerns, may be required for work examining expression of lower level transcripts, such as the amino acid transporter, oASCT2.

6.4.6 Normalisation of RNA concentration across samples

Northern blot analysis permits the quantification of mRNA transcript expression, but differential loading of the RNA sample to the gel and transfer of the electrophoresed samples to the membrane for example, may influence the accuracy of the technique. Contamination of the RNA sample with protein or DNA following extraction can lead to variable spectrophotometry readings and miscalculation of RNA concentration. Inaccuracies when loading the gel and variability in electrophoretic efficiencies between different samples, as well as variable efficiency and consistency of RNA transfer to the membrane, are other possible sources of error. It becomes apparent that an appropriate control or reference sequence to indicate RNA concentration is required. This sequence indicates the total quantity of RNA in a sample on a northern blot or may act as an internal standard, assuming constant expression levels (eg. a 'house-keeping' gene), thus allowing for normalisation of differential RNA loading and RNA transfer, and improved accuracy of signal quantification.

Relative total amounts of RNA per sample on a northern blot may be determined by staining the gel or the membrane (following transfer), commonly with ethidium bromide. A photograph or scan is taken under UV light and relative 28S and/or 18S rRNA bands quantified using computer assisted densitometry (Correa-Rotter et al, 1992; Fratini et al, 1994; Eykholt et al, 2000). However, detection and quantification of small transcriptional changes may be difficult with this approach, particularly when transcript expression levels are low (Spiess and Ivell, 1998).

'House-keeping' genes are commonly used for quantitative assays including northern blots, assuming a constant and steady-state expression level of these genes. The most common transcripts utilised include β-actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and albumin. GAPDH is an important enzyme in the glycolytic pathway and evidence suggests that its use as an internal standard is not always appropriate, as levels vary considerably between individual animals (de Leeuw et al, 1989; Bustin et al, 1999), within tumorigenic cell lines (Bhatia et al, 1994) and when animals are fed differently (Yamada et al,

1997). GAPDH has been used in our laboratory for standardisation of sheep skin RNA concentration (Nattrass, 2000) and has been used for normalisation of skin RNA in other species (Mitsui et al, 1998; Sato et al, 1999) but a number of reports suggest caution with this approach (Bhatia et al, 1994; Yamada et al, 1997; Bustin, 2000). β-actin is an essential structural protein of the cytoskeleton used to standardise skin RNA concentrations (Rufaut et al, 1999) but the levels of expression, as with GAPDH, can vary with cell proliferation and differentiation (de Leeuw et al, 1989; Spanakis, 1993; Yamada et al, 1997). Given that the sheep in the present experiment were fed different diets and different levels of intake, the explicit use of 'house-keeping' genes is cautioned and alternative approaches were explored.

Ribosomal RNA (rRNA) concentrations do not appear to vary considerably compared to mRNAs and as such, the use of rRNAs for normalisation of total RNA levels was considered most appropriate (Barbu and Dautry, 1989). Studies comparing the accuracy of RNA concentration standardisation using rRNAs (18S and 28S) and various 'house-keeping' genes have concluded that rRNAs are comparatively superior (de Leeuw et al, 1989; Bhatia et al, 1994; Yamada et al, 1997; Thellin et al, 1999). The rRNAs comprise approximately 90% of the total cellular RNA and are detected in far greater levels than any of the target mRNA transcripts investigated, thus oligonucleotide probes are necessary to overcome excessive signal when probing northern blots (Barbu and Dautry, 1989). Given that feeding different diets at different levels of intake effects the expression of 'house-keeping genes' (Yamada et al, 1997), 28S rRNA was considered the most appropriate sequence for normalising RNA concentration in northern blots and was used throughout the current work.

Liu et al (2000) used 28S rRNA to normalise for total loading of sheep skin RNA samples but ideally an indicator of the total follicle RNA levels would be most appropriate. There was no significant difference in the average total number of follicles between +W and –W sheep, thus the total amount of follicle RNA compared to whole skin RNA was assumed to be similar. However, the determination of a follicle-specific RNA sequence for standardising would eliminate the need for this assumption and is even more appropriate,

given that the sequences investigated in the current experiment were all follicle specific. Trichohyalin is found in large quantities in the cells of the follicle IRS and therefore has been utilised as a standardising sequence by some researchers (Bawden et al, 1998; Lui et al, 2000). However, in light of earlier discussion (Section 6.4.4) concerning the possible variation in proportioning of bulb cells to the IRS and the fibre between +W and –W sheep, the use of trichohyalin was not appropriate in this work. Another possible indicator of total follicle RNA yet to be utilised in this context is the protease inhibitor Nexin 1, produced by dermal papilla cells of the follicle, with no expression detected in any other follicle cells (Yu et al, 1995). The utilisation of Nexin 1 could allow quantification of follicle RNA concentrations for the normalisation of whole skin RNA samples in northern blot analysis, therefore may eliminate some of the current assumptions necessary in this work.

6.5 Summary

The expression of the HS (KAP 2.12), UHS (KAP 4.2), HGT (KAP 6.1) and IF (K2.10) genes in the skin of sheep from selection groups (+W and –W) that produced divergent quantities of wool was similar, at the start of the trial and after feeding a HS or a LS diet. The HS and IF genes independently accounted for approximately 5% of the variation in WGR between selection groups but more than 75% of the variation was related to dietary treatment and selection group. Gene expression (all genes examined) was not related to wool S concentration. This suggests that differential gene expression was not the source of divergent wool growth and composition in this experiment, but follicle cellular processes, such as bulb cell turnover rate and cortical cell production, may be the cause. Any differences in the expression of the oASCT2 amino acid transporter in the skin of +W and –W sheep were not quantifiable using the northern blot technique but a more sensitive technique, for example real-time RT-PCR, is recommended for low-level transcripts. Finally, the standardisation of gene expression levels in the skin relative to 28S rRNA concentration was the most accurate method of RNA normalisation across samples, compared with using GAPDH and TRN sequences.

Chapter 7

General discussion

7. General discussion

This thesis describes a series of novel experiments designed to enhance our understanding of nutrient utilisation for growth of wool and the whole body. Strategic use of nutritional, endocrinological and molecular variation in Merino sheep that differed phenotypically and genotypically in wool and body growth provided good models to study mechanisms involved in growth. Experiment 1 (Chapter 4) examined the association of insulin-like growth factors (IGFs) and different levels of feed intake, with nitrogen partitioning between the skin and the rest of the body in Merino sheep divergent in wool and body traits. Experiment 2 (Chapter 5 and 6) investigated the effect of diets differing in sulphur (S) content on the utilisation of sulphur-containing amino acids (SAAs) for wool growth in Merino sheep genetically divergent in wool growth. The expression of wool keratin and other follicle genes, in sheep used in Experiment 2 was examined in the final chapter. The implications and applications of the results from these studies have been addressed throughout this thesis and prospective directions for follicle biology research in the future are now discussed.

The selection approach used in the experiments described here, particularly the use of breeding values combining phenotypic and genetic information, allows for the investigation of physiological, metabolic and molecular variation that exists between sheep used in commercial practice. Results from this work suggest that follicle rate processes and cellular mechanisms, and the uptake of SAA into the follicle, differ between sheep producing divergent quantities of wool, notwithstanding the possibility that differences in keratin gene expression may still exist. Therefore, to further develop our understanding of cellular and molecular activities in the follicle, it may be necessary to step outside the realms of what are considered 'agriculturally' to be viable and practical wool growth phenotypes. That is, to take a 'big picture' approach to follicle biology by examining cellular mechanisms in animals more

divergent in fibre characteristics than was investigated in this thesis and to utilise techniques in molecular biology to identify genes responsible for these differences in production.

The development of laser capture microdissection enables the isolation of specific cell types within tissues from histological sections and has been applied successfully to isolate cells of the follicle (Jahoda et al, 2001). Once cells are isolated, RNA can be extracted and molecular techniques could be used to compare gene expression between individuals that differ in follicle morphology or activity, or the growth or composition of the fibre. For example, comparisons of follicle cells from normal mice with transgenic or mutant mice that produce wavy hair (Luetteke et al, 1994; Crew, 1933), or sheep with extreme phenotypes, such as the overtly curved follicles of fine wool Merinos compared to the generally straight follicles of strong wool Merinos, could provide useful and diverse animal models when searching for genes that contribute to phenotypic variation.

Follicle efficiency and functioning is thought to be set-up during fetal follicle induction, thus an approach using laser capture could also be used to identify key signalling molecules involved in follicle initiation, by isolating pre-papilla cells in fetal skin. Microarrays of gene expression in these cells, compared with fibroblasts that are incapable of initiation of follicles, would provide a powerful tool for determination of major genes involved in primary follicle initiation or secondary follicle branching for example. The development of DNA microarrays of sheep skin, to comparatively 'scan' DNA from biologically distinct animals such as sheep producing extremely high and low quantities of wool, would allow detection of any sequence differences and could be related to variation in fibre properties or follicle function. Application of such technologies indicate exciting times ahead in follicle biology research, eventually influencing how we manage and select sheep in the future for optimum fibre production and quality.

Appendix 1

1.1 Histology

1.1.1Tissue Fixation, Processing, Embedding and Sectioning

10% Buffered Formalin

18L MiilQ water, 2L Formalin (AnalaR, BDH), 130g $NaHPO_4$, 80g NaH_2PO_4.

Processing Program

(Automatic Tissue Processor SE 400; Shandon Scientific Co. Ltd. London)

70% ethanol	60 minutes
80% ethanol	60 minutes
95% ethanol	30 minutes
95% ethanol	90 minutes
Absolute ethanol	120 minutes
Absolute ethanol	120 minutes
Histolene / ethanol	60 minutes
Histolene	120 minutes
Histolene	120 minutes
Paraffin wax (Histoplast)	120 minutes
Paraffin wax (Histoplast)	120 minutes

Embedding and Sectioning procedure

Samples were embedded in paraffin wax using Tissue-Tek II embedding machine (Lab-Tek Division, Miles Lab. Inc, Naperville, II) and 7μm sections cut using a microtome (Lietz 1512, Ernst Leitz, Wetzlar). Sections were fixed onto poly-lysine (Sigma Diagnostics, St. Louis) coated slides and oven dried at 60°C for up to 24 hours prior to staining.

1.1.2 Staining protocols

Bonney's Triple stain modification (for follicle density measurement)

Histoclear	15 minutes
Absolute ethanol	2 minutes
80% ethanol	2 minutes
30% ethanol	2 minutes
Bi-distilled water	2 minutes
Methyl Violet / Pyronin[A]	15 minutes
Running tap water	1 minute
Orange G / Acetone[B]	5-7 dips
Acetone	2 seconds
Histoclear	2-5 minutes

* Differential staining shows fibres as bright violet colour and the remaining tissue as orange.

[A] *Methyl Violet / Pyronin*

25 ml 1% aqueous methyl violet 6B (Jensen), 10ml 10% aqueous pyronine, 65ml RO water

[B] *Orange G / Acetone*

To 100ml acetone add 2% aqueous orange G drop by drop, until flocculent precipitate redissolves and solution goes clear (approximately 10 ml Orange G per 100 ml acetone)

1.2 Wool washing procedure

Midside wool samples were exposed to air (20±2°C; 65%±2% relative humidity) and greasy fleece weight determined 24 hours later. Samples were washed in pre-weighted fine weave calico bags as follows; 5 minutes at 50°C in 0.36% Lissapol solution, 5 minutes at 45°C in 0.13% Lissapol solution, 5 minutes at 30°C in RO water and 10 minutes at room temperature in RO water, all with gentle agitation. Excess water was removed and samples dried at 60°C for 48-60 hours. Once dry, samples were placed in a desiccator for 20 minutes then the clean, dry sample was weighed.

1.3 Molecular techniques: general chemicals and solutions, plasmids, enzymes, radiochemicals and molecular biology kits.

1.3.1 General chemicals

Sigma : agarose, dithiothreitol (DTT), ethylenediaminetetra acetic acid (EDTA), mineral oil, β-mercaptoethanol, salmon sperm DNA, sodium dodecyl sulphate (SDS), sarkosyl (sodium dodecyl sarcosinate), Tris (Tris (hydroxymethyl) aminomethane), Tween 20, Diethyl pyrocarbonate (DEPC)

BioRad, USA : Zetaprobe GT

Boehringer Mannheim, Germany : glycogen (molecular biology grade), dNTPs

Amersham- Pharmacia Biotech, UK : Sepharose CL-6B

Life Technologies, USA : guanidinium isothiocyanate

1.3.2 Solutions

RNA extraction Solution D

4M Guanidinium isothiocyanate (Life Technologies), 25mM Sodium Citrate (pH=7.0), 0.5% Sarkosyl, 100mM β-mercaptoethanol

20 x TAE buffer

800mM Tris-acetate, 20mM EDTA

Formaldehyde Running Buffer (5 x FRB)

100mM MOPS (pH=7.0), 40mM Sodium Acetate, 5mM EDTA

Agarose Load Buffer (10 x ALB)

50% Glycerol (v/v), 100mM EDTA, 0.1%SDS, 0.1% Bromophenol Blue, 0.1% Xylene Cyanol

Formaldehyde Gel Load Buffer (10 x FGLB)

50% Glycerol (v/v), 1mM EDTA (pH=8.0), 0.25% Bromophenol Blue, 0.25% Xylene Cyanol

Formamide Load Buffer (2 x FLB)

95% Formamide (v/v), 20mM EDTA, 0.05% Bromophenol Blue,

0.05% Xylene Cyanol

Hybridisation solution (and pre-hybridisation)

50% Formamide (v/v), 120mM Na_2HPO_4 (pH=7.2), 250mM NaCl, 7% (w/v) SDS

20 x SSC (10 x SSC diluted in MQ water1:1 with 20 x SSC)

150mM Sodium Chloride, 20mM Sodium Citrate, 1mM EDTA ; pH=7.0

PCR solution

25mM $MgCl_2$	1.5µl
5mM dNTPs	1.0µl
10 x buffer (Super-Dooper)	2.5µl
10µM Primer/ oligo A	0.25µl
10µM Primer/ oligo B	0.25µl
Sterile water	13.5µl
DNA template	1.0µl (approx. 50pg)

Super Dooper Buffer (10x)

330mM Tris-Acetate (pH 7.8), 625 mM Potassium Acetate, 100mM Magnesium Acetate, 40mM Spermidine, 5mM Dithiothreitol

1.3.3 Plasmids

pBluescript I-KS (Stratagene, USA)

pGEM-2 (Promega, USA)

pGEM7-Zf(+)(Promega, USA)

pGEM3-Zf(+) (Promega, USA)

1.3.4 Enzymes

Restriction endonuclease (Promega, USA)

Proteinase K (Merck, Germany)

T4 polynucleotide kinase (PNK) (New England Biolabs Inc., Genesearch, Australia)

Taq DNA polymerase (Applied Biosystems, Melbourne)

1.3.5 Oligonucleotides

SP6 (Geneworks, South Australia)

T7 (Geneworks, South Australia)

RSP (Geneworks, South Australia)

1.3.6 Radiochemicals

Oligolabelling : γ-^{32}P-rATP (specific activity, 3000Ci/mmole; Geneworks, South Australia)

Northern probes : α-^{32}P-dCTP (specific activity, 3000Ci/mmole; Geneworks, South Australia)

1.3.7 Molecular biology kits

Megaprime oligolabelling kit (Amersham, UK)

UltraClean GelSpin DNA purification kit (MoBio Laboratories Inc., Geneworks, Adelaide)

1.3.8 Northern Blot molecular markers

SPP1- EcoRI DNA Markers

Fragment No.	Size (kb)	Approx. amount (ng)
1	8.51	96
2	7.35	83
3	6.11	69
4	4.84	55
5	3.59	40
6	2.81	32
7	1.95	22
8	1.86	21
9	1.51	17
10	1.39	16
11	1.16	13
12	0.98	11
13	0.72	8
14	0.48	5
15	0.36	4

pUC19-HpaII DNA Markers

Fragment No.	Size (bp)	Approx. amount (ng)
1	501	93
2	489	91
3	404	75
4	331	62
5	242	45
6	190	36
7	147	27
8	111	20
9	110	20
10	67	12
11	34	6
12	34	6
13	26	5

0.24 – 9.5 Kb RNA ladder (Life Technologies)

Fragment No.	Size (kb)
1	9.49
2	7.46
3	4.40
4	2.37
5	1.35
6	0.24

Appendix 2

2.1 Chapter 4

2.1.1 Experiment 1 problems

During weeks nine and ten of Experiment 1, six experimental animals developed urethral blockages due to formation of calcium-phosphorus calculi. The direct cause of this problem was unknown, although mineral imbalances from past grazing history or experimental diets, the constricted nature of the male urinary tract, and stress from housing indoors may have been contributing factors. All six sheep were euthanased following veterinary advice and replacement animals, that had been housed concurrently but hadn't developed calculi, were brought in to the experiment. A visual inspection of all 48 rams found no obvious signs of affliction and despite the possibility of sub-clinical disease, the trial proceeded albeit in a shortened time frame and with less extreme dietary treatments than originally planned. That is a 0.6 x maintenance diet was increased to a 0.9 x maintenance level to minimise stress to the animals.

The composition of the diet was altered to correct for any imbalances in the ratio of calcium to phosphorus (2:1 ratio recommended) and all animals were monitored twice daily to ensure early detection of any further problems. The new diet (Section 4.2.1.3) was supplied at maintenance levels for four weeks prior to experimental intake levels (0.9 x maintenance; 1.8 x maintenance) being given. Probable disease symptoms were diagnosed in 2 more individuals at the end of the trial, hence they were removed from the data analysis.

2.1.2 Nitrogen intake and balance, per kg body weight.

There was no significant difference between selection groups in the average amount of nitrogen consumed or retained, however, sheep fed the high level of intake consumed and retained significantly more nitrogen per kg body weight compared to sheep consuming the low intake level ($p<0.05$; Table 1).

Table 1: Mean nitrogen (N) intake and retention (mg N / kg BW) for each selection group and level of intake for sheep in Experiment 1.

	Intake	+W +BW	+W -BW	-W -BW	-W +BW	Average for each intake level
N intake (mg/ kg BW/ d)	Low	341	352	358	329	345 [a]
	High	624	627	625	574	612 [b]
N retained (mg/ kg BW/ d)	Low	76	76	90	81	81 [a]
	High	225	214	279	210	232 [b]

Maximum standard error of mean 19.08 (N intake) and 20.75 (N retained).
Different superscripts differ at $p<0.05$ *within* selection group and *within* intake level.

2.1.3 Nitrogen partitioning to wool and other body components

The total amount of nitrogen (g/d) partitioned to wool or partitioned to other body components was similar for all selection groups. The amount of nitrogen partitioned to wool increased by more than 35% when comparing sheep fed the low intake to sheep fed the high intake level (2.6 vs 3.6 g/d), and the amount of nitrogen that was partitioned to the body increased by more than 400% when comparing sheep fed the low to the high level of intake (3.2 vs 14.9 g/d) (Table 2).

Table 2: The average quantity of nitrogen (g/d) partitioned to wool growth or other body components for each selection group and level of intake.

	Intake	+W +BW	+W -BW	-W -BW	-W +BW	Average for each intake level
Total nitrogen to wool (g/d)	Low	2.99	2.31	2.18	2.91	2.60 [a]
	High	4.05	3.51	3.50	3.38	3.61 [b]
Total nitrogen to body components other than wool (g/d)	Low	3.08	2.88	3.38	3.62	3.24 [a]
	High	17.24	12.49	15.60	14.17	14.88 [b]

Maximum standard error of mean 0.335 (N to wool) and 1.508 (N to other).
Different superscripts differ at $p<0.05$ *within* selection group and *within* intake level.

2.1.4 Mean plasma IGF-I and IGF-II concentrations

Table 3: The mean plasma concentrations of IGF-I and IGF-II for each selection group and dietary treatment measured in Experiment 1.

	Intake	+W +BW	+W -BW	-W -BW	-W +BW	Average for each intake level
Plasma IGF-I concentration (ng/ml)	Low	354 [b]	308 [ab]	288 [a]	360 [b]	328 [a]
	High	517 [b]	381 [a]	341 [a]	385 [a]	406 [b]
Plasma IGF-II concentration (ng/ml)	Low	136	131	155	125	137 [b]
	High	102	115	112	113	111 [a]

Maximum standard error of mean 63.59 (IGF-I) and 24.46 (IGF-II).
Different superscripts differ at $p<0.05$ *within* selection group and *within* intake level.

2.2 Chapter 5

2.2.1 Average values for S balance

Table 4: Mean values for S balance in Experiment 2, for each selection group and dietary treatment

	HS diet	LS diet	+W group	-W group	Pooled SEM
S consumed	5012[b]	2699[a]	3918	3793	127.3
Faecal DM (g/d)	453	442	462[b]	433[a]	28.34
Faecal S (mg/d)	1598	1111	1378	1331	137.6
Urine volume (ml/d)	8894[b]	4904[a]	8133	5666	1363
Urine S (mg/d)	3141[b]	1511[a]	2270	2381	74.59
S balance (mg/d)	273	27	219	81	79.14

Different superscripts differ at $p<0.05$ *within* selection group and *within* intake level.

2.3 Chapter 6

2.3.1 Principal Components analysis

Principal Component 1: equal loadings of all genes

Principal Component 2: disproportionate negative KAP 6.1, and positive KAP 2.12 and K2.10

Principal Component 3: disproportionate positive KAP 4.2 and negative KAP 6.1

Principal Component 4: disproportionate positive K2.10 and negative KAP 2.12

Table 5: Latent vectors (loadings) for the generation of four Principal Components to represent the four keratin genes

	PC1	PC2	PC3	PC4
K2.10	-0.528	0.318	-0.167	0.769
KAP 2.12	-0.500	0.546	-0.251	-0.624
KAP 4.2	-0.514	-0.195	0.830	-0.093
KAP 6.1	-0.454	-0.750	-0.469	-0.104

Table 6: The percentage variation in WGR (g/d), wool S concentration (%S) and wool S output (mg/d) accounted for by each Principal Component, using a Simple Linear Regression

	Percentage variation accounted for by each Principal Component		
	WGR	Wool S	Wool S output
PC 1	ns	ns	ns
PC 2	ns	21 (p=0.05)	ns
PC 3	ns	ns	ns
PC 4	ns	ns	ns

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