Characterisation of genes associated with sheep growth and carcass traits

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A thesis
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by

G. Yang

Animal growth and carcass composition are of commercial importance for sheep meat production. Growth and fat deposition are affected by both environmental factors and genetic factors. Genetic selection to produce leaner, fast growing lambs has a long-term benefit for the sheep industry. Recent evidence supports the contention that lipolysis is important in the regulation of animal feed conversion efficiency and energy utilisation. In humans, genes encoding the β_3-Adrenergic Receptor (ADRB3), Hormone-Sensitive Lipase (HSL) and Uncoupling Protein 1 (UCP1) have been found to be associated with variation in growth-rate and body composition. Given these associations in humans, the aims of this thesis were to characterise variation in the ovine genes *ADRB3*, *HSL* and *UCP1*, and assess whether there is an association between variation in these genes and variation in sheep growth and carcass traits.

Using a Polymerase Chain Reaction-Single Strand Conformational Polymorphism (PCR-SSCP) method, variation in the three genes was investigated. Next, General Linear Mixed-effects Models (GLMMs) were used to investigate associations between variation in these genes and variation in various growth and carcass traits. The following was found:

The intron and the 3'untranslated region (3'UTR) of ovine *ADRB3* were screened for genetic variation in 808 NZ Suffolk sheep and 140 NZ Merino sheep. In the NZ Merino sheep, six 3'UTR variants (named *a-f*) were detected. Sequencing revealed three SNPs (g.*233A>C, g.*271C>G, g.*357A>T) and a single nucleotide deletion (g.*257delG) in the 3'UTR of the gene. Sixteen ovine *ADRB3* intron-3'UTR haplotypes were deduced (named *A-a*, *A-b*, *A-c*, *B-c*, *C-e*, *D-d*, *E-e*, *F-e*, *G-f, H-f, I-a, J-f, K-f, L-e, M-f*).

For the 808 Suffolk sheep investigated, in single variant model, the presence of intron variant *C* was associated with higher weaning-weight ($P = 0.021$) while the presence of *A* was associated with lower weaning-weight ($P < 0.001$). The presence of *C* was found to be associated with higher post-weaning growth-rate ($P = 0.002$) and higher Fat Depth above the eye Muscle (FDM) ($P= 0.033$). The presence of *B* was found to be associated with lower post-weaning growth-rate ($P = 0.002$).

The presence of 3'UTR variant *b* was associated with lower weaning-weight ($P = 0.001$). The presence of *a* was associated with higher post-weaning growth-rate ($P = 0.021$) and *c* was associated with lower post-weaning growth-rate ($P = 0.005$).

The presence of haplotype *A-b* was associated with a lower weaning-weight ($P = 0.001$). The presence of *C-a* was found to be associated with higher post-weaning growth-rate ($P = 0.008$), while the presence of *B-c* was associated with lower post-weaning growth-rate ($P = 0.005$).

Variation within three regions (exon 3-4, exon 5-6 and exon 9) of ovine *HSL* and its association with post-weaning growth (n = 538) and carcass traits (n = 262) was investigated in NZ Suffolk sheep. Four intron 5 variants (designated *A-D*) and two exon 9 variants (designated *a* and *b*) of ovine *HSL* were detected. In the single variant models, the presence of intron 5 variant *A* in a lamb's genotype was associated with lower Eye Muscle Depth (EMD) ($P = 0.036$) and Eye Muscle Width (EMW) ($P = 0.018$), whereas the presence of *C* was associated with higher EMD ($P < 0.001$), EMW ($P < 0.001$) and FDM ($P = 0.017$). The association of *C* with higher EMD ($P = 0.002$) and EMW ($P = 0.002$) persisted in the multi-variant model. No association between *HSL* intron 5 variants and post-weaning growth, or between *HSL* exon 9 variants, post-weaning growth or carcass traits, were found.

Variation within three regions (the promoter region, intron 2 and exon 5) of ovine *UCP1* and the association of this variation with variation in growth and carcass traits was investigated in 587 NZ Suffolk sheep and 236 NZ Romney sheep. Three promoter variants (designated *A-C*) and two intron 2 variants (designated *a* and *b*) of ovine *UCP1* were detected.

In the Suffolk sheep studied, promoter variant *C* was found to be associated with higher FDM in single variant model ($P = 0.033$). In the Romney sheep studied, the presence of variant *B* in a lamb's genotype was associated with lower subcutaneous carcass fat grade (VGR) ($P = 0.005$), whereas two copies of *C* was associated with higher VGR ($P < 0.001$), and a lower hind leg yield ($P = 0.032$).

The results suggest these genes could be used as gene-markers in sheep breeding to select for desirable growth and carcass quality characteristics.

Keywords: β_3-adrenergic receptor (ADRB3); post-weaning growth; carcass traits; PCR-SSCP; Hormonal-Sensitive Lipase (HSL), Uncoupling protein 1 (UCP1), variation; genetic selection.

Publications and conference presentations arising from this thesis

Yang G, Forrest RH. Zhou H, Hodge S, Hickford JG. (2014). Variation in the ovine Uncoupling Protein 1 Gene (*UCP1*) and its association with growth and carcass traits in New Zealand Romney and Suffolk sheep. *J. Anim. Breed. Genet.* 131:437-444.

Yang G, Forrest RH. Zhou H, Hickford JG. (2014). Variation in the ovine Hormone-Sensitive Lipase Gene (*HSL*) and its association with growth and carcass traits in New Zealand Suffolk sheep. *Mol. Bio. Rep.* 41: 2463-2469.

Yang G, Zhou H, Hu J, Luo Y and Hickford JG. (2013). Haplotypes of the ovine ADRB3 gene (*ADRB3*) and their association with post-weaning growth in New Zealand Suffolk sheep. *Mol. Bio. Rep.* 40:4805-4810.

Yang G, Hickford JG, Zhou H, Fang Q, Forrest RH. (2011). Extended haplotype analysis of ovine *ADRB3* using polymerase chain reaction single strand conformational polymorphism on two regions of the gene. *DNA Cell Biol.* 30:445-448.

Yang G, Zhou H, Hu J, Luo Y, Hickford JG. (2009). Extensive diversity of the ADRB3 gene in Chinese sheep identified by PCR-SSCP. *Biochem. Genet.* 47:498-502.

Conference proceedings

Yang G, Forrest R, Zhou H, Hickford JG. (2013). Brief Communication: Association of variation in the ovine ADRB3 gene with weaning weight and post-weaning growth in New Zealand Suffolk sheep. Proceeding of the New Zealand Society of Animal Production. 73: 155-157.

Presentations

Yang G. (2012) Role of variation in β_3-adrenergic receptor gene (*ADRB3*) in sheep production traits. Dalian, China. Second Annual World Congress of Agricultural Biotechnology (WCAB).

Yang G. (2012) Association of variation in β_3- adrenergic receptor gene (*ADRB3*) and lamb growth. Lincoln University, *Christchurch*, New Zealand. Post-graduates conference (PG-conference).

Yang G. (2010) Role of lipolytic genes in sheep production traits. Lincoln University, Christchurch, New Zealand. Proposal seminar.

Yang G. (2008) Variation in the 3'UTR of ovine β3- adrenergic receptor gene (ADRB3). NZSBMB (New Zealand Society for Biochemistry and Molecular Biology) mini conference.

Sequences submitted to the NCBI GenBank:

Ovine *ADRB3*
Ovis aries beta 3 adrenergic receptor (ADRB3) gene, *ADRB3-a* allele, 3' UTR
Accession: HM776668.1 GI: 309318671
Ovis aries beta 3 adrenergic receptor (ADRB3) gene, *ADRB3-b* allele, 3' UTR
Accession: HM776669.1 GI: 309318672
Ovis aries beta 3 adrenergic receptor (ADRB3) gene, *ADRB3-c* allele, 3' UTR
Accession: HM776670.1 GI: 309318673
Ovis aries beta 3 adrenergic receptor (ADRB3) gene, *ADRB3-d* allele, 3' UTR
Accession: HM776671.1 GI: 309318674
Ovis aries beta 3 adrenergic receptor (ADRB3) gene, *ADRB3-e* allele, 3' UTR
Accession: HM776672.1 GI: 309318675
Ovis aries beta 3 adrenergic receptor (ADRB3) gene, *ADRB3-f* allele, 3' UTR
Accession: HM776673.1 GI: 309318676
Ovis aries beta 3 adrenergic receptor (ADRB3) gene, *ADRB3-I* allele, intron
Accession: EU371403.1 GI: 166078293
Ovis aries beta 3 adrenergic receptor (ADRB3) gene, *ADRB3-J* allele, intron
Accession: EU371404.1 GI: 166078294
Ovis aries beta 3 adrenergic receptor (ADRB3) gene, *ADRB3-K* allele, intron
Accession: EU371405.1 GI: 166078295
Ovis aries beta 3 adrenergic receptor (ADRB3) gene, *ADRB3-L* allele, intron
Accession: EU371406.1 GI: 166078296
Ovis aries beta 3 adrenergic receptor (ADRB3) gene, *ADRB3-M* allele, intron
Accession: EU371407.1

Ovine *HSL*
Ovis aries Hormone-Sensitive Lipase gene (*HSL*), intron 5 variant *A*, Accession: KC610083
Ovis aries Hormone-Sensitive Lipase gene (*HSL*), intron 5 variant *B*, Accession: KC610084
Ovis aries Hormone-Sensitive Lipase gene (*HSL*), intron 5 variant *C*, Accession: KC610085
Ovis aries Hormone-Sensitive Lipase gene (*HSL*), intron 5 variant *D*, Accession: KC610086

Ovine *UCP1*
Ovis aries mitochondrial uncoupling protein 1 variant *A* (*UCP1*) gene, 5' UTR; nuclear gene for mitochondrial product Accession: KC243136
Ovis aries mitochondrial uncoupling protein 1 variant *B* (*UCP1*) gene, 5' UTR; nuclear gene for mitochondrial product Accession: KC243137
Ovis aries mitochondrial uncoupling protein 1 variant *C* (*UCP1*) gene, 5' UTR; nuclear gene for mitochondrial product Accession: KC243138

Abbreviations

%	percent	**α**	alpha
$	New Zealand dollar	**°C**	degree Celsius
β	beta	**µg**	microgram
µL	microlitre	**µM**	micromolar
µm	micrometre	**A**	adenine
ADRB3	beta-3 adrenergic receptor	**AMP**	adenosine 5'monophosphate
ANOVA	analysis of variance	**AR**	adrenergic receptor
ATP	adenosine 5'triphosphate	**BAT**	brown adipose tissue
bp	base pair	**C**	cytosine
cAMP	cyclic adenosine 3', 5' monophosphate	**cDNA**	complementary DNA transcript of an mRNA
C/EBPα	CCAAT/enhancer-binding protein alpha	**CNS**	central nervous system
COOH-terminus	carboxyl-terminal	**CRE**	cyclic AMP response element
CREB	cyclic AMP response element binding protein	**dATP**	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate	**dGTP**	deoxyguanosine triphosphate
DNA	deoxyribonucleic acid	**dNTP**	deoxynucleotide triphosphate
dTTP	deoxythymidine triphosphate	**EDTA**	ethylenediaminetetraacetic acid
EMA	eye muscle area	**EMD**	eye muscle depth
EMW	eye muscle width	**FDM**	fat depth above eye muscle
FFA	free fatty acids	**GDF8**	growth differentiation factor 8
GH	growth hormone	**GLMM**	general linear mixed model
GPCR	G protein-coupled protein	**G-protein**	guanine-nucleotide binding protein (subscript indicates class)
GRK	G-protein-coupled receptor kinase	**HCWT**	hot carcass weight
HSL	hormone-sensitive lipase	**IGF1R**	insulin-like growth factor 1 receptor
kb	kilobase	**M**	molar
MAPK	mitogen-activated protein kinase	**MAS**	marker-assisted selection
ME	metabolisable energy	**mg**	milligram
mL	millilitre	**mM**	millimolar
mRNA	messenger ribonucleic acid	**ng**	nanogram
NH_2-terminus	amino terminal	**nm**	nanometre
NST	non-shivering thermogenesis	**OAR**	Ovis aries chromosomes
PCR	polymerase chain reaction	**PI3K**	phosphoinositide 3-kinase
PKA	protein kinase A	**PKC**	protein kinase C
QTL	quantitative trait loci	**SE**	standard error
SNP	single nucleotide polymorphism	**SNS**	sympathetic nervous system

SSCP	single strand conformational polymorphism	**TAG**	triacylglycerol
TM	transmembrane	**Tris**	tris (hydroxymethyl) aminomethane
UCP	uncoupling protein	**UTR**	un-translated region
V	volt	**VGR**	VIAScan Fat Score
WAT	white adipose tissue		

Amino acid residue abbreviations

Amino Acid	3-Letter	1-Letter	Side-chain polarity	Side-chain charge (pH 7.4)
Alanine	Ala	A	nonpolar	neutral
Arginine	Arg	R	basic polar	positive
Asparagine	Asn	N	polar	neutral
Aspartic acid	Asp	D	acidic polar	negative
Cysteine	Cys	C	nonpolar	neutral
Glutamic acid	Glu	E	acidic polar	negative
Glutamine	Gln	Q	polar	neutral
Glycine	Gly	G	nonpolar	neutral
Histidine	His	H	basic polar	positive (10%) neutral (90%)
Isoleucine	Ile	I	nonpolar	neutral
Leucine	Leu	L	nonpolar	neutral
Lysine	Lys	K	basic polar	positive
Methionine	Met	M	nonpolar	neutral
Phenylalanine	Phe	F	nonpolar	neutral
Proline	Pro	P	nonpolar	neutral
Serine	Ser	S	polar	neutral
Threonine	Thr	T	polar	neutral
Tryptophan	Trp	W	nonpolar	neutral
Tyrosine	Tyr	Y	polar	neutral
Valine	Val	V	nonpolar	neutral

Acknowledgements

“宝剑锋自磨砺出，梅花香自苦寒来。”

“The great sword blade comes from innumerous times of sharpening and polishing, the fragrance of plum blossoms comes from the hard coldness.” - Chinese proverbs

It’s my great honor to do PhD with Professor Jon Hickford. Jon’s patience, and sense of humour have broken my jumpiness in facing every single trouble that resisted my ease feeling. His wisdom and openness has also taught me to focus on finding the best solution out of the mistakes I made. His presence everytime I need has impressed me that he is not only my great teacher, but also a very good friend of mine. Additionally, my deep appreciation is presented to Jon and his family for always invite me to celebrate for Chinese New Year. I am indebted to Jon for allowing me to learn a lot from him these years. I am sure that this thesis would not have been possible without his proofreading and strong supports.

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Chapter 1
General introduction

1.1 Background of this study

Sheep meat is at the core of New Zealand (NZ) red meat exports and the industry remains a very important component of the NZ economy. In 2011, the value of red meat exports was $6.2 billion and approximately $500 million higher than the previous year (Meat Industry Association of New Zealand, 2011). To maintain its position as the largest exporter of sheep meat, the industry is looking to double the value of sheep meat exports before 2025, from its 2011 value of approx. 7 billion to $14 billion (Meat Industry Association of New Zealand, 2011).

Strong returns and profitability per hectare for the dairy industry, has led to a significant land use shift away from sheep farming. This has been associated with a gradual decline in sheep numbers (Figure 1.1).

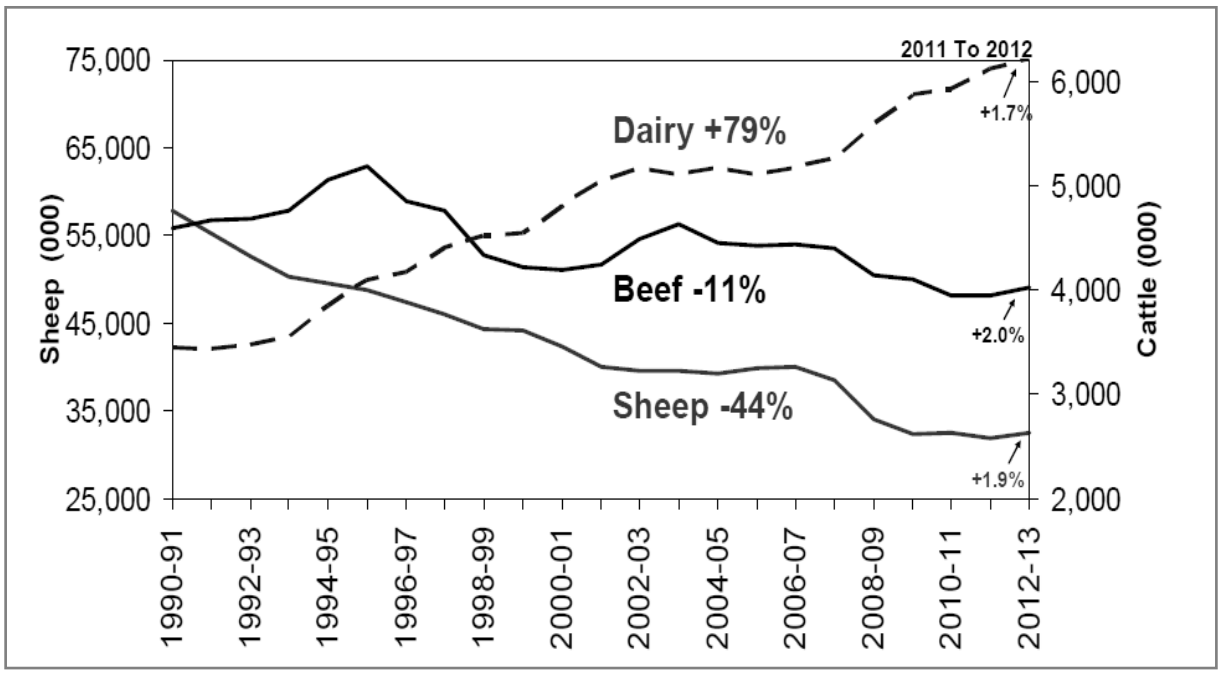


Figure 1.1 Changes in livestock numbers from 1990 to 2013. (Sourced from Beef + Lamb NZ Economic Service Statistics, NZ).

There is an increasing customer preference for lean meat because of the perception that dietary fat, in particular saturated fat, is linked to obesity and cardiovascular disease (Corley and Ward, 2013; Volk, 2007). This trend is unlikely to reverse and accordingly, some markets have been shown to financially reward the production of leaner sheep carcasses, while penalising the production of fatter carcasses (Bray, 1984).

In NZ, many abattoirs base their payments to farmers on a grading system that incorporates carcass weight and fat depth (Meat Industry Association of New Zealand, 2011). However, fewer than 50% of NZ lamb carcasses meet abattoir expectation (Gooch, 2007). Taken along with the decline in sheep numbers, the failure of some carcasses to meet expectations in the market, will continue to challenge the NZ sheep industry.

Carcass quality refers to the composition (proportions of lean, fat and bone) and shape or muscularity of slaughtered animals. In the short term, improvement in carcass quality could be achieved by changing management strategies; such as altering lamb slaughtering time, manipulating the composition of forage (Ball et al., 1997) and processing solutions for the slaughter. There are several disadvantages with these approaches: the early slaughtering of lamb potentially reducing the efficiency of meat output per unit of land or capital, because the lamb has not reached its full potential growth (Simm, 1992); the impracticality of manipulating feed quality and quantity in extensive pastoral farming systems and the high labour and equipment-cost associated with the trimming of fat.

In the longer-term, genetic selection has the potential to improve carcass traits and contribute to the profitability and sustainability of the sheep industry. Because of its size, the NZ sheep population has a large amount of variation for many traits and hence there is considerable potential to identify and select superior animals for breeding. This variation also provides resilience, as if there are any changes in markets, the diversity can provide sufficient variation to rapidly supply animals with the required characteristics.

According to Safari *et al.,*(2003), key production traits including growth rate, lean meat yield and carcass composition all show moderate heritability (0.20-0.35) and the use of gene Marker-Assisted Selection (MAS) could therefore have the potential to improve the accuracy of selection for these traits.

1.2 Aims of this study

Producing fast growing lambs with lean carcasses is of economic importance for the NZ sheep industry. Animal growth and fat deposition is affected by environmental and genetic factors.

Because both growth and fat deposition are dependent on energy partitioning, it is conceivable that genetic factors that impact on growth may also impact fat deposition and vice versa. Allowing genetic improvements to be made in both traits concurrently can serve the dual purpose of increasing lamb growth-rates while reducing the excessive fat content in carcasses.

Recent evidence supports the importance of lipolysis in regulating animal feed conversion efficiency and energy utilisation. Variation in the genes encoding the ovine β_3-Adrenergic Receptor (*ADRB3*) (Clement et al., 1995; De Luis et al., 2008; Evans et al., 2000; Forrest et al., 2003; Fujisawa et al., 1998; Horrell et al., 2009; Kurokawa et al., 2008; Mirrakhimov et al., 2011; Zhu et al., 2010), Hormone-Sensitive Lipase (*HSL*) (Carlsson et al., 2006; Hoffstedt et al., 2001; Lavebratt et al., 2002) and Uncoupling Protein 1 (*UCP1*) (Evans et al., 2000; Heilbronn et al., 2000; Jia et al., 2010; Labruna et al., 2009; Mori et al., 2001; Nagai et al., 2011; Schaffler et al., 1999; Shin et al., 2005) have been shown to be associated with growth and body composition in a variety of animal species, and thus they have the potential to provide insight into growth and carcass traits in sheep.

The sheep breeds chosen for study in this thesis were the NZ Suffolk and the NZ Romney. The Suffolk is a terminal-sire breed producing fast-growing lambs with good carcass conformation. The Romney is the most common breed of sheep in NZ and is a dual purpose (meat and wool) breed. Because of the popularity of these sheep breeds in the NZ, an understanding of the genes affecting the genetic potential for growth and variation in carcass traits in these sheep breeds could benefit the New Zealand sheep industry.

In this research, Polymerase Chain Reaction-Single Strand Conformational Polymorphism (PCR-SSCP) was used to identify variation in the ovine genes *ADRB3*, *HSL* and *UCP1*, and this was coupled with nucleotide sequencing to ascertain the nature and extent of the genetic variation. Association of the variation in the genes with variation in selected growth and carcass traits was subsequently investigated, and the potential of any associations as gene-marker for breeding explored. The aim is to contribute to the understanding of how variation in lipolytic genes may affect growth and carcass traits in sheep. An outline of the thesis structure is shown in Figure 1.2.

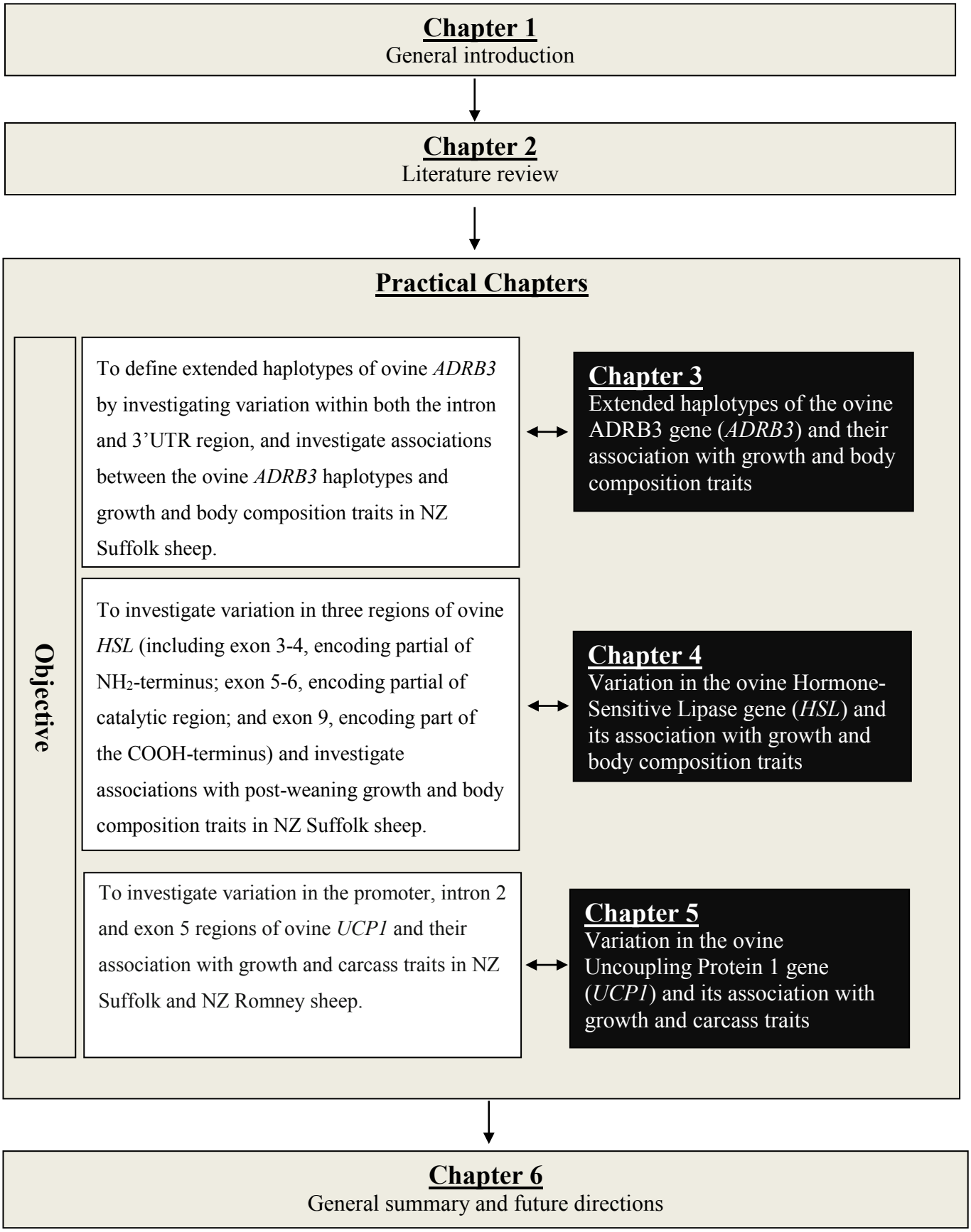


Figure 1.2 Outline of the chapters.

Chapter 2
Literature review

In the following literature review, the key factors that are known to underpin animal growth and carcass traits are described and the potential to exploit genetic factors to enable the breeding of better sheep is discussed.

2.1 The physiology of animal growth

In general, animal growth is a process that involves the realisation of an animal's genetic potential to increase in size and develop body functions under particular environmental influences. Cumulative weight plotted against animal age typically follows a sigmoid curve, which is composed of a pre-pubertal phase, a self-accelerating phase and a self-inhibiting phase. Although body size is genetically determined, it can be altered by nutritional and hormonal factors (Gluckman, 1986; Widdowson, 1980). Such factors may inhibit cell division and cause the mature body size to be less than the genetically determined maximum. Accordingly, animal nutrition is of critical importance to growth and hence in livestock production systems, feeding is said to "drive" growth.

The partitioning of gross energy in animals

In intensive livestock production systems, feed costs account for 50-70% of total production costs. The feed is also account for a large component of the productivity of extensive production systems (Moore et al., 2006). It is accordingly important to maximise the utilisation of the feed.

The energy derived from the feed consumed by animals is partitioned in different ways (Freer and Dove, 2002). Gross energy (GE) is the total energy intake derived from feed (Figure 2.1). When the un-utilised and indigestible energy is expelled as faeces, the "remaining energy" utilised in further metabolic processes is called "digestible energy". Of this component, energy loss occurs in the process of ruminant fermentation, including the release of combustible gases such as methane. This leaves metabolisable energy (ME), the energy effectively available for an animal's metabolism. Metabolisable energy is used for maintenance, heat production, muscle growth, pelage growth, lactation and reproduction, (see Figure 2.1 reviewed in Freer and Dove, 2002).

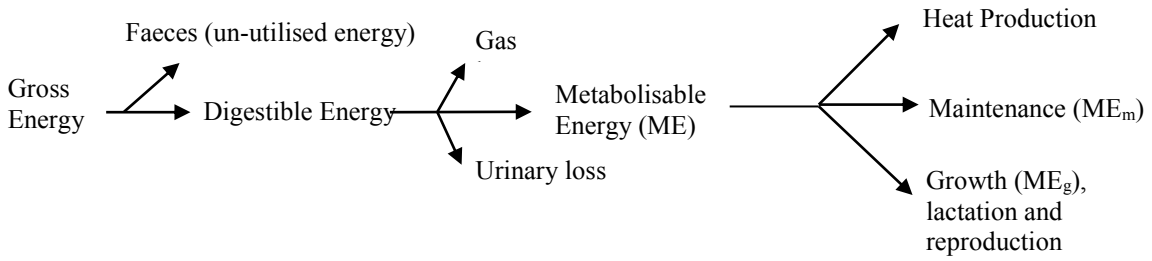


Figure 2.1 Partitioning of dietary energy. (Adapted from Freer and Dove, 2002).

The ME component utilised for maintenance (named ME_m) can be considered as an "overhead cost" to growth. As an animal's ME_m is determined by its size then the means for reducing feed costs to a minimum in growing animals, is to aim to maximise growth per unit of feed. This can be achieved by either providing nutrient rich feed, or by genetic selection to improve growth-rate, or both (reviewed in Lawrence and Fowler, 2002).

The equation: $EB = (ME_m\text{-}H)$ has been used to describe animal energy homeostasis (Verstegen and Henken, 1987). When Energy Balance (EB) is zero, it means that the ME intake equals the energy lost as heat, and the animal is said to be "at maintenance". In this state, an animal is stable in weight and in chemical composition over a period of time. When EB is positive and the ME intake is higher than the maintenance requirement (ME_m); an animal partitions energy into growth. In contrast, when EB is negative, ME is below the maintenance requirement, ME_m is then derived from catabolic processes within the body and the animal loses weight.

Animal maturity and growth efficiency

As a consequence of the different timing and rates of cell differentiation and proliferation, certain tissues, organs and body parts mature at different rates. An animal's degree of maturity is an important factor in determining animal growth and carcass-composition. Webster (1977) reported that the efficiency of Energy Retention (ER) reaches a maximum at around 25% of mature body weight and then declines dramatically. This is consistent with the work of Rattray *et al.,* (1974) who reported that in a growing animal the deposition efficiencies of protein and fat are extremely high in very young animals, and energetic efficiency is therefore higher than in older individuals. Hammond (1959) also described the sequential maturation of different tissues in growing animals. In this sequence, the last tissue to mature is fat.

Given that the last tissue to mature in growing animals is fat, and that the ratio of fat to protein in the body increases with age (Webster, 1980), leaner carcasses can be obtained by killing lambs after the majority of muscle growth has occurred, but before excessive fat is deposited. Additionally, given that animals with a higher mature body weight are of larger size during growth and require more energy for maintenance and also reach puberty later in life (Webster, 1980), a larger mature mass is not desirable in the context of feed conversion efficiency.

The utilisation of energy for growth

When the daily feed intake exceeds that required for maintenance (ME_m, Figure 2.1), then an animal can start using the additional energy for growth (ME_g, Figure 2.1). However, because there is an additional energy "cost" of growth from growth associated heat production, the energy retained by the animal in the growing tissues is less than ME_g. This increased growth associated heat production is due to the cumulative inefficiency of the biochemical reactions involved in the growth of the tissues (Lawrence and Fowler, 2002)(Figure 2.2).

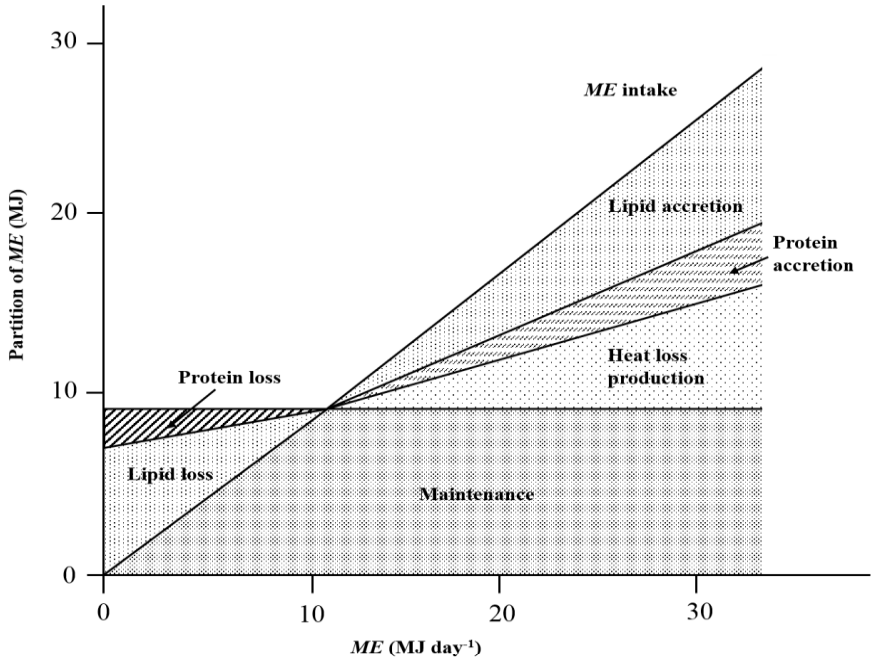


Figure 2.2 Partitioning and different rates of daily intake of Metabolisable Energy. Metabolisable Energy (ME) is partitioned at different rates for maintenance needs, lipid and protein accretions and heat loss. When ME is below maintenance then it can be compensated for by body protein and lipid loss via tissue catabolism (Adapted from Lawrence and Fowler, 2002).

Protein synthesis is linearly related to heat production, and that heat is mainly generated from the protein synthesis mechanisms (Webster, 1980). The greatest proportion of ME intake is therefore dissipated as heat (Figure 2.2) and according to Webster (1980), the maximum proportion of ME that can be retained as protein in meat is around 8%, even in intensively fed species such as broiler chickens. In semi-intensively fed species like cattle and sheep, the proportion of ME retained as protein in meat is below 3%. In comparison, the proportion detained as fat is more variable, but accounts for 5-20% of the ME in cattle and sheep (Webster, 1980). It can be concluded that protein accretion in meat is not very efficient in the context of energy demand.

Ørskov and McDonald (1970) found that the energy required for protein synthesis in sheep is higher than that for fat synthesis. The difference in the energy requirement of muscle and fat synthesis is due to the difference in their water content, as lean muscle tissue is approximately 75% water by weight and 20% protein, while body fat is approximately 30% water by weight (Warriss, 2010). Rattray *et al.,* (1974) confirmed that daily protein and fat deposition were highly correlated in sheep and confirmed that the energetic cost of protein deposition, was much greater than that of fat.

Gender also appears to affect the efficiency of energy use for growth. For example in sheep, Bull *et al.,* (1970) found that the net efficiency of the ME utilised for weight gain is 65.5% for ewes, and 57.6% for rams. The gains of the ewes contained significantly more fat.

The difference in the energetic efficiency of muscle and fat gain suggests that if there are changes in the ratio of fat to muscle resulting from either additional feeding, maturation processes or genetics, then the overall energetic efficiency of weight gain could also vary (Sutherland et al., 1974). The changes in energy partitioning may in turn cause increases in energy intake and body weight (Galgani and Ravussin, 2010).

Overall, in an animal production system, fat deposition in an animal's body costs a large amount of feed energy. Much of this fat may be removed and wasted after the animal is slaughtered especially if lean meat is desired, therefore the success of the NZ lamb industry has been strongly related to improvements in lean muscle growth-rates and in minimising excessive fat deposition in carcasses.

2.2 Fat deposition and meat value

Body fat deposition can occur in various depots including under the skin (known as subcutaneous fat), surrounding organs (known as visceral fat), between muscles (known as intermuscular fat) and between muscle fibre bundles (known as intramuscular fat). As a

consequence of the high levels of saturated fat and cholesterol in those fat depots, and these substance being considered to be associated with heart disease in humans (Volk, 2007), fat deposition is considered unacceptable by some customers.

While abdominal and subcutaneous fat are often considered to be unfavourable traits, intramuscular fat (or marbling) can be considered a favourable trait. In beef cattle, intramuscular fat contributes to sensory palatability including attributes like tenderness, juiciness, and flavour (Oddy et al., 2001). Nishimura *et al.,* (1999) reported that intramuscular fat in the *longissimus* muscle of cattle might physically alter connective tissue structures and thereby reduce the toughness of the meat. In this context, a challenge for the NZ sheep industry might be to reduce the levels of subcutaneous fat on carcasses, without leaving the meat dry and tasteless as a result of an accompanying drop in intramuscular fat levels.

In order to make best use of animal fats and the various fat depots, knowledge of such things as the hormonal pathways that affect growth, fat deposition and partitioning is essential.

2.3 Hormones that regulate growth and fat deposition

External factors such as nutrition are necessary to supply metabolisable energy for animal growth and body fat deposition. There are also many intrinsic factors involved in the regulation of growth and body fat deposition, including hormonal and genetic factors. A highly integrated neuro-hormonal system is known to be important for animal growth including hormones secreted by the pituitary, thyroid and parathyroid glands, and the ovaries and testes. This hormonal system minimises the impact of short-term fluctuations in energy balance.

The pituitary gland, for example, secretes Growth Hormone (GH), also called somatotropin. Growth hormone plays many roles in the body: it stimulates bone and muscle growth, maintains the normal rate of protein synthesis in all body cells, and it speeds up the release of fats as an energy source for growth.

The somatotropic axis is composed of multiple effectors and there are many points of regulation within the axis, including feedback loops. These function to maintain homeostasis in both physiological and patho-physiological situations. The axis is stimulated by secretion of GH in the anterior pituitary gland. The actions of GH on peripheral tissues occurs through interaction with specific Growth Hormone Receptors (GHR) and subsequent endocrine, paracrine and autocrine events. Many of these are mediated via the Insulin-like Growth Factor (IGF) system and its regulators. The insulin-like growth factor system, in turn, promotes

tissues growth by changing protein, carbohydrate and lipid metabolism (Ahmed and Farquharson, 2010).

2.4 The growth of different body tissues

When an animal has energy available for growth, the muscle, bone and fat tissues of body may then start growing under the effects of both hormonal and genetic factors. The growth of the various tissues affects the rate of weight gain, feed-conversion efficiency and carcass composition.

Carcass composition refers to the proportion of the major tissues: muscle, fat and bone in the carcass. These major tissues show differential growth during development. In early development: bone growth is described as having low growth impetus, muscle tissue shows intermediate growth impetus and fat tissue shows high growth impetus, particularly after the fattening phase begins (Berg and Butterfield, 1968).

The differential growth of tissues influences carcass composition and thus carcass value. Accordingly, factors influencing differential tissue growth should be understood in order that carcass-fat composition can be manipulated.

It is also necessary to understand the growth of different tissues in detail before the effect of genetic influences on growth and carcass composition can be fully understood.

The growth of bone: osteogenesis

The terms osteogenesis and ossification are often used synonymously to indicate the process of bone formation. Bone growth occurs both in the cartilage (endochondral growth) and in the membrane (intra-membranous growth). Endochondral ossification is responsible for the growth in bone length, while intra-membranous ossification deposits bone on the surface of pre-existing bone structures (reviewed in Lawrence and Fowler, 2002).

In the process of endochondral ossification, the future bones are first formed as hyaline cartilage “models”. The perichondrium that surrounds the hyaline cartilage models becomes infiltrated with blood vessels and osteoblasts and changes into a periosteum. The cartilage in the epiphyses continues to grow so the developing bone increases in length. Later, usually after birth, secondary ossification centres form in the epiphyses.

In the process of intra-membranous ossification, the bones are first formed as connective tissue membranes. These sheet-like connective tissue membranes are then replaced with bony tissue. These bones are called intra-membranous bones (Bruder et al., 1994).

Osteoblasts are the cells responsible for the formation and organisation of the extracellular matrix of bone cells and its subsequent mineralisation. They are derived from bone marrow mesenchymal precursor cells known as osteoprogenitor cells (Figure 2.3). Under the effect of growth factors, specifically Bone Morphogenetic Proteins (BMPs), osteoprogenitors are induced to differentiate into chondrocytes (Agata et al., 2007). Additionally, other growth factors including Fibroblast Growth Factor (FGF), Platelet-Derived Growth Factor (PDGF) and Transforming Growth Factor Beta (TGF-β), also promote the division of osteoprogenitors, and potentially increase osteogenesis (Lee et al., 2013).

Once osteoprogenitors start to differentiate into osteoblasts, they begin to express a range of genes including the osterix gene (*SP7*), constans-like 1 gene (*Col1*), bone sialoprotein gene (*BSP*), Macrophage Colony-Stimulating Factor gene (*M-CSF*), alkaline phosphatase gene (*ALP*), osteocalcin gene (*BGLAP*), osteopontin gene (*OPN*), and osteonectin gene (*SPARC*) (Ringe et al., 2008; Szulc et al., 2005).

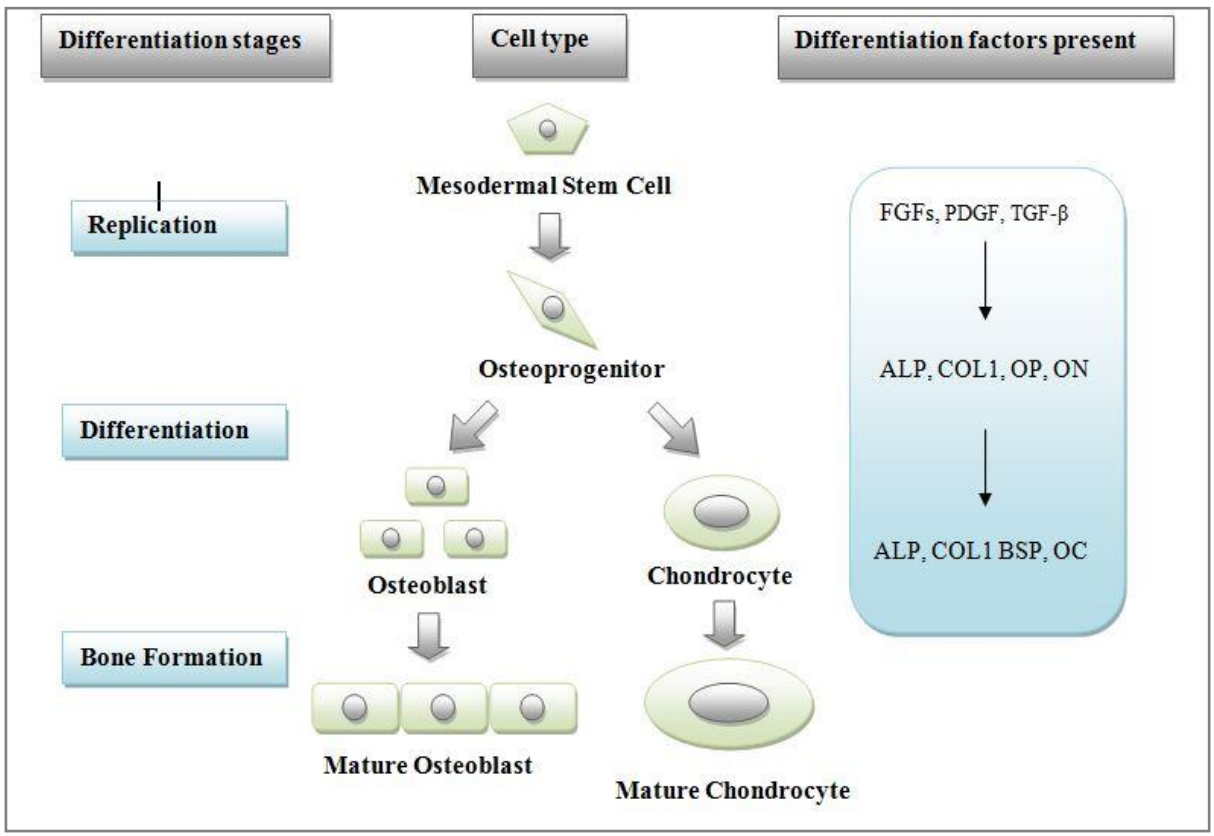


Figure 2.3 The process of osteogenesis. Bone formation is characterised by the replication of mesenchymal cells and the differentiation of osteoprogenitor cells into mature osteoblasts and mature chondrocytes. FGFs act through FGFRs to control several genes involved in osteoblast commitment and differentiation. FGF, Fibroblast Growth Factor; FGFR, Fibroblast Growth Factor Receptor; PDGF, Platelet-Derived Growth Factor; TGF-β, Transforming Growth Factor beta; ALP, Alkaline Phosphatase; COL1, Constans-Like 1; OP, Osteopontin; OC, Osteocalcin. (Adapted from Marie, 2003).

The growth of muscle: myogenesis

Development of skeletal muscle (myogenesis) starts at an early stage of embryonic development, with all muscle cells originating from the myotome and dermomyotome regions of the somite (Maltin et al., 2001) (Figure 2.4).

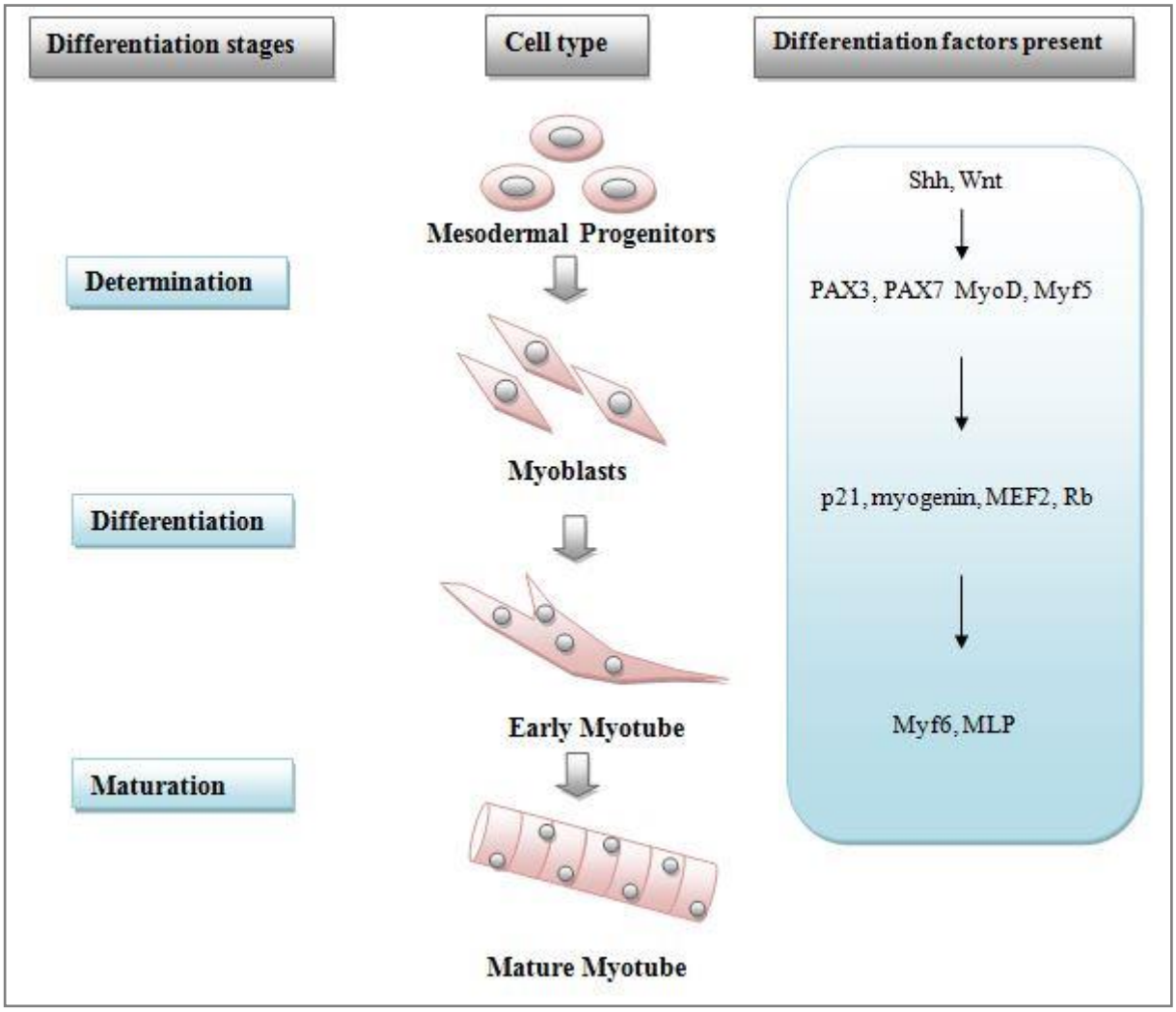


Figure 2.4 The process of myogenesis. During myogenesis, mesodermal stem cells become committed to the skeletal muscle cell lineage. This depends on myogenic transcription factors (such as PAX3, PAX7, MyoD and Myf5). Muscle precursor cells (myoblasts) remain in a proliferation state until they are "instructed" to differentiate. Differentiation is accompanied by cell-fusion and the expression of many muscle-specific genes including Shh, Sonic Hedgehog; PAX3, Paired box 3; PAX7, Paired box 7; MyoD, Myoblast determination protein 1; Myf5, Myogenic factor 5; p21, cyclin-dependent kinase inhibitor 1A; MEF2, Myocyte Enhancer Factor-2; Rb, Retinoblastoma; Myf6, myogenic factor 6; and MLP, Muscle LIM Protein. (Adapted from Hettmer and Wagers, 2010).

Myogenesis is initiated by protein factors including Wingless (Wnt) and Sonic hedgehog homolog (Shh) derived from the neural tubes (Wigmore and Evans, 2002). The protein factors of Wnt and Shh switch on the expression of the Paired box 3 gene (*PAX3*) and Paired box 7 gene (*PAX7*). Pax 3 and Pax 7 then activate the primary myogenic basic Helix-Loop-Helix (bHLH), transcription factors, Myogenic factor 5 (Myf5) and Myoblast determination protein

1 (MyoD), which leads to the development of the muscle-cell lineage (Maroto et al., 1997; Tajbakhsh et al., 1997). Myoblast determination protein 1 and Myf5 are therefore both considered to be the hallmarks of terminal specification to the muscle-cell lineage (Pownall et al., 2002). This muscle-cell lineage accordingly involves mesodermal progenitor cells becoming myoblasts (Brameld et al., 1998; Buttery et al., 2000).

Once differentiated, myoblasts migrate into the adjacent embryonic connective tissue or mesenchyme and express other muscle-specific genes such as the Myogenin gene (*MYOG*) and Myogenic Factor 6 gene (*MYF6*) (Knudsen, 1985; Knudsen et al., 1990). They proliferate until they encounter a shortage of Fibroblast Growth Factor (FGF). At this point, the precursor cells in different regions of the embryo can differentiate into skeletal, cardiac and smooth muscle (McKinsey et al., 2001).

After proliferation, myoblasts fuse into muscle "straps" to form multinucleated early myotubes (Yagami-Hiromasa et al., 1995). Once muscle cells have matured in response to myostatin signalling, a cyclin-dependent kinase inhibitor 1A (Cip1, p21) is up-regulated and it then inhibits cyclin-E·Cdk2 and retinoblastoma (Rb) protein activity. This subsequently can lead to the arrest of myoblasts in the G1 phase and hence, inhibit their proliferation (Thomas et al., 2000) and the proliferation of satellite cells (McCroskery et al., 2003). The activation of these satellite cells can result in the development of new fibres (Charge and Rudnicki, 2004).

In this process, a protein named myostatin (also known as Growth Differentiation Factor 8, GDF8) has been found to be important because it inhibits the differentiation of myoblasts into mature muscle fibres (Carnac et al., 2006). It binds to the activin type II receptor (Joulia-Ekaza and Cabello, 2007), and utilises Activin-Like Kinase Receptor-4 (ALK4) in myoblasts (Lee et al., 2005). These factors then induce myostatin-specific gene regulation.

It is concluded that myoblast number, myofibre number and subsequent muscle differentiation are regulated by myostatin (Thomas et al., 2000).

At the cellular level, the loss or malfunction of myostatin decreases the cytoplasmic phosphorylation of Smad2/3 and down-regulates p21 (Ohsawa et al., 2008). These inhibitory effects in-turn cause hyper-phosphorylation of the Retinoblastoma (Rb) protein, which binds to the transcription factor E2F-DP (Otto and Patel, 2010). This leads to progression of the cell cycle into the S phase. At the tissue level, elevated myoblast proliferation leads to an increase in the number of committed myoblasts, resulting in both muscle cell hyperplasia (an increase in the number of muscle fibres) and hypertrophy (an increase in muscle fibre size), and subsequent dramatically increased skeletal muscle mass (Thomas et al., 2000). This abnormal

phenotype has been called “double-muscling” (Kambadur et al., 1997; McPherron and Lee, 1997).

Double-muscling is characterised by dramatically increased muscle mass, specifically in the regions of the proximal forequarter and hindquarter muscles. This leads to prominent muscular protrusion and intra-muscular boundaries (Kambadur et al., 1997). Double-muscled cattle carcasses have a higher proportion of lean meat than normal cattle, as well as having an improved quality of lower-priced cuts of meat. This results in substantial improvement in total carcass value (Arthur, 1995). Double-muscled cattle therefore also have a higher efficiency in converting feed into lean muscle and a lower content of intra-muscular fat, connective tissue, bone and digestive tract (McPherron and Lee, 1997). This increased feed-conversion efficiency can benefit farmers because it can reduce production costs, give higher production yields and alleviate grazing pressure.

Although double-muscling in cattle may be beneficial to farmers, there are also some disadvantages, including reduced fertility in female cattle, delayed sexual maturation, an increased incidence of dystocia and an increased likelihood of bone breakages (Bellinge et al., 2005; Hanset, 1991; McPherron and Lee, 1997). Because of the higher content of white muscle fibres and a lower content of myoglobin, double-muscled meat is paler than normal meat (West, 1974) and this may affect consumer preferences. In addition, as a result of having reduced collagen and connective tissue content (Arthur, 1995), double-muscled meat has been reported to be more tender than normal meat (Bass et al., 1999; Uytterhaegen et al., 1994).

In sheep, a miRNA target site created by a nucleotide substitution in the 3’ untranslated region (3’UTR) of Myostatin gene (*MSTN*) (Callis et al., 2007) down-regulates *MSTN* translation. This decreases serum myostatin levels and ultimately results in muscle fibre hypertrophy and a disruption of muscle homeostasis (McCarthy and Esser, 2007). This nucleotide substitution has been found to commonly exist in the Texel sheep breed and together with some other variation in ovine *MSTN*, has been developed into gene-marker tests called MyoMAX® and MyoMAX$^{Gold®}$ by Zoetis NZ. A lamb that inherits one MyoMAX® allele has been shown to have 5% more muscle mass in the legs and loin, while also having 7% less carcass fat (Zoetis, Pfizer Animal Health, Dunedin, NZ).

The growth of adipose tissues: adipogenesis

In normally growing animals, the growth of adipose tissues accompanies skeletal and muscle growth. Beyond a certain body weight, fat gain becomes a large and constant portion of weight gain (Searle et al., 1972). The accretion of both fat and protein occur

simultaneously in early growth, while in later growth the rate of protein accretion slows down (Figure 2.5).

The rate of fat accretion is more closely related to the amount of energy available in excess of requirements for maintenance and lean growth, than to specific metabolic changes in adipose tissues (Sainz, 1990).

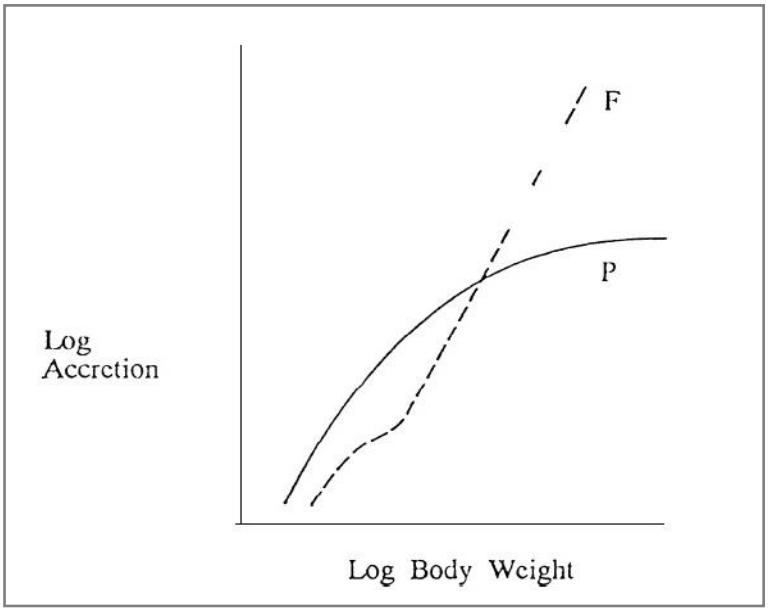


Figure 2.5 Idealised log-log plot of fat and protein accretion against body weight for sheep, rat, pig and cattle. (Adapted from Bergen, 1974).

In mature ruminants, when energy intake is in excess of current energy requirements, this energy can be converted to fatty acids which, after esterification to form triacylglycerol (TAG), are deposited as adipose tissue. When energy intake fails to meet current energy needs, the energy deficit is met by the degradation of adipose tissue through the hydrolysis of TAG. Accordingly, the amount of lipid stored in adipocytes reflects the cumulative supply of feed over time and the balance between energy intake and energy expenditure (Rosenbaum et al., 1997).

At the cellular level, fat deposition is determined by the relative rates of anabolism (lipogenesis) and catabolism (lipolysis) in adipocytes and it involves a variety of both lipogenic and lipolytic factors (Vernon et al., 1986).

Adipogenesis is the process by which mature adipocytes are formed from small, undifferentiated cells called adipoblasts. Adipoblasts contain no lipogenic enzymes or lipid droplets (Figure 2.6). They are derived from pluripotent mesenchymal cells and differentiate to pre-adipocytes that contain functional lipogenic enzymes and accumulated lipid droplets. A mature adipocyte is formed by the enlargement of these lipid droplets, through the actions of

insulin and other metabolic factors such as leptin and glucocorticoids (Hauner and Loffler, 1987).

Mature adipocytes can differentiate into two different types of adipoctyes: brown adipocytes and white adipocytes. These form two types of adipose tissues: Brown Adipose Tissue (BAT) and White Adipose Tissue (WAT).

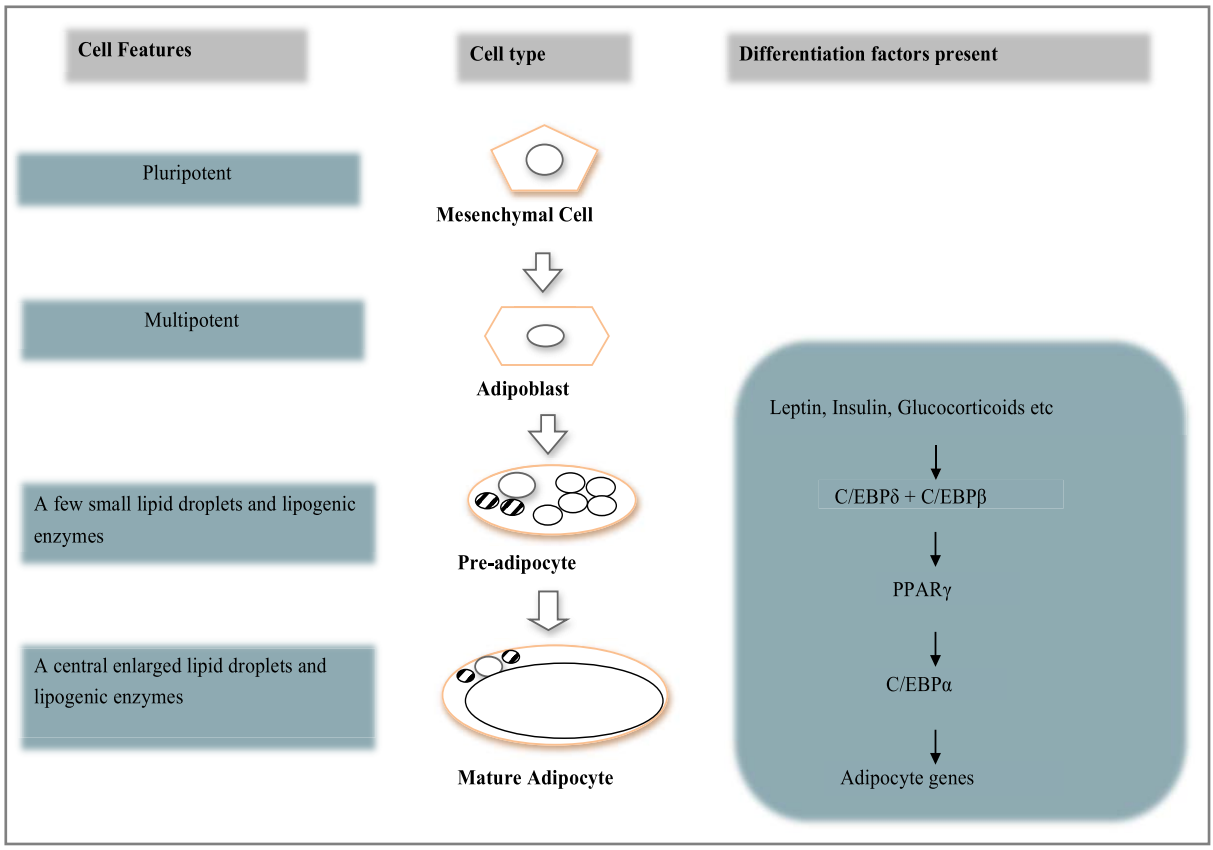


Figure 2.6 Adipocyte differentiation and adipogenesis. The nucleus is shown as a black circle, lipogenic enzymes are shown as circles with a diagonal pattern and small lipid droplets are shown as blank circles. During the process of adipogenesis, the pre-adipocytes are developed from pluripotent mesenchymal cells which differentiate into multipotent adipoblasts. The mature adipocytes are then formed by enlargement of the lipid droplets within the pre-adipocytes under the effects of insulin, and other metabolic factors such as leptin and glucocorticoids. These metabolic factors could assist the differentiation of adipocytes by activating many adipocyte genes via stimulation of C/EBPδ, C/EBPβ, PPARγ and C/EBPα. (Reviewed in Lawrence and Fowler, 2002).

White Adipose Tissue (WAT) and Brown Adipose Tissue (BAT)

White adipose tissue, the predominant type of adipose tissue in adult mammals, is the major storage site for energy. White Adipose Tissue (WAT) is less vascularised than BAT, nonetheless, it contains an extensive capillary bed (Ballard, 1978; Rosell and Belfrage, 1979). White adipocytes contain large unilocular lipid droplets (Cushman and Rizack, 1970).

Brown adipose tissue is considered a thermogenic tissue and contains more vascularisation and sympathetic innervations than WAT. Brown adipose tissue is present in most mammals during the pre-natal period and is responsible for diet- and cold-induced Non-Shivering Thermogenesis (NST) (Lowell et al., 2000). BAT contains a large number of mitochondria (Hull and Segall, 1966; Rosell and Belfrage, 1979; Sbarbati et al., 1987) and a tissue-specific protein called uncoupling protein 1 (UCP1) that stimulates NST (Klingenberg and Huang, 1999) by uncoupling oxidative respiration from ATP production (Girardier, 1977; Heaton et al., 1978), which results in heat production.

In cattle and sheep, almost all adipose tissue at birth is BAT at birth. However, within the first week of neonatal life, BAT is changed into WAT via trans-differentiation of brown adipocytes into white adipocytes (Casteilla et al., 1989b). Brown adipocytes are smaller and contain less lipid droplets than white adipocytes and TAG are stored as multilocular droplets in brown adipocytes, contrasting the unilocular droplets of white adipocytes (Himms-Hagen et al., 2000).

Some reports suggest the existence of a putative third type of adipocyte referred to as a "convertible white adipocyte" (Cousin et al., 1992; Himms-Hagen et al., 2000; Loncar, 1991). Recently, this third type of adipocyte has been characterised and named "brite" adipocytes. These adipocytes also have the capacity to express UCP1, but do not share some biological functions with brown adipocytes (Walden et al., 2012).

Although lipogenesis in white adipocytes has been well described, in brown adipocytes it is less well understood.

The degradation of lipid: lipolysis

When animals are exposed to cold-fasting or physical activity, a drop in blood glucose stimulates the Sympathetic Nervous System (SNS) to trigger the degradation of lipid stored in adipocytes. This is known as lipolysis (Figure 2.7) (Migliorini et al., 1997). The SNS innervates both WAT and BAT (Ballard et al., 1974; Slavin and Ballard, 1978; Wirsen and Hamberger, 1967), but because the sympathetic innervations are denser in BAT than WAT, the neural derived catecholamine norepinphrine is thought to play a greater role in BAT, whereas catecholamine derived from the circulation, plays a relatively greater role in WAT (Collins et al., 1996; Garofalo et al., 1996; Hausberg et al., 2002). In WAT, the adipocyte-derived hormone leptin increases energy expenditure by acting on hypothalamic centres that increase sympathetic outflow. Increased sympathetic tone stimulates lipolysis and the release of Fatty Acids (FAs) from WAT stores (Figure 2.7).

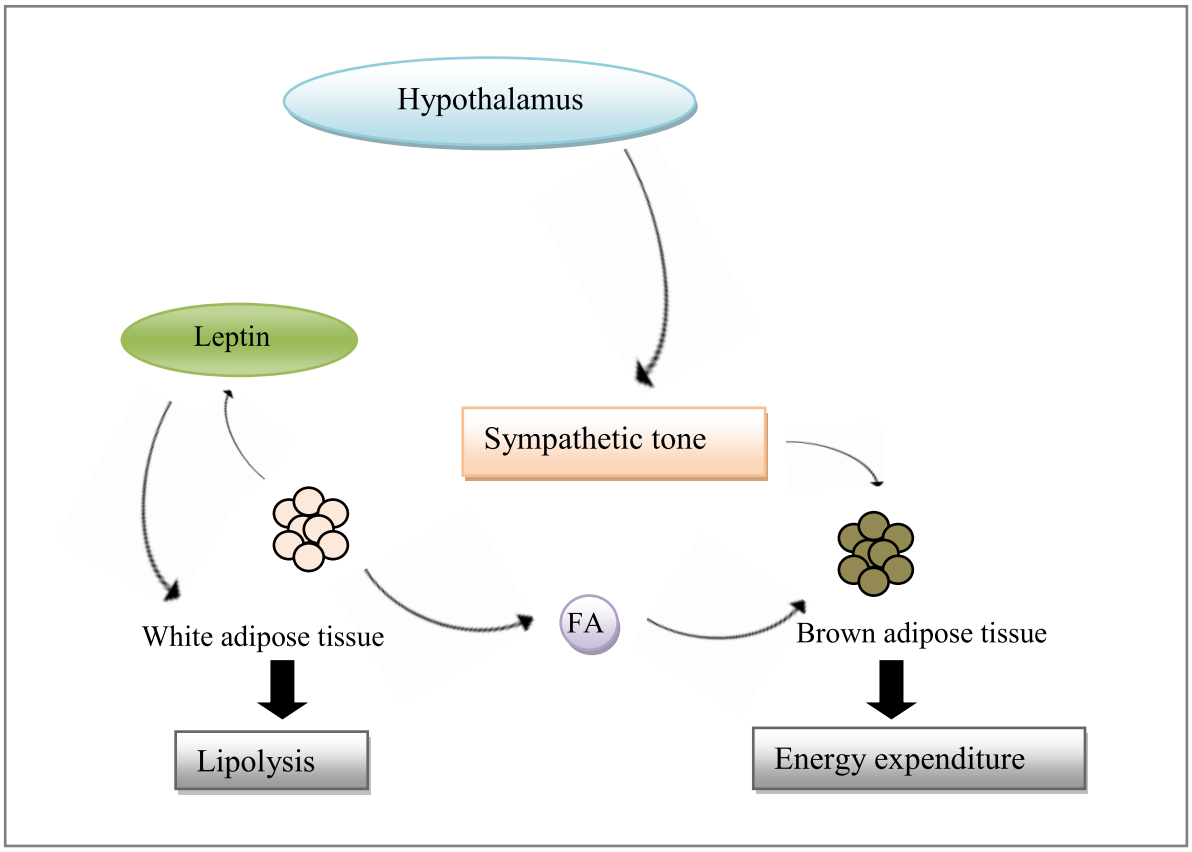


Figure 2.7 Hormonal regulation of lipolysis and energy expenditure. The adipocyte-derived hormone leptin increases energy expenditure by acting on hypothalamic centres and by affecting an animal's appetite for food intake. This increases sympathetic outflow. Increased sympathetic tone stimulates lipolysis and the release of fatty acids (FAs) from white adipose tissue stores. Circulating FAs are taken up and oxidised by brown adipose tissue. (Adapted from Altarejos and Montminy, 2011).

Studies suggest that the function of HSL in adipocytes likely depends on the translocation of HSL to the surface of lipid droplets (Brasaemle et al., 2000; Clifford et al., 2000; Egan et al., 1992) via the phosphorylation of perilipin (Miyoshi et al., 2006). Upon activation, perilipin then detaches from the surface of lipid droplets (Clifford et al., 2000; Clifford et al., 1997; Souza et al., 1998), which enables HSL to access its substrate.

Triacylglycerol is hydrolysed at a much slower rate than diacylglycerol (DAG), hence the hydrolysis of TAG by HSL is considered to be a rate-limiting step in lipolysis (Crabtree and Newsholme, 1972; Osterlund, 2001). Once Free Fatty Acids (FFA) are released, they are used as substrates for mitochondrial respiration in brown adipocytes (Fredrikson et al., 1986; Nedergaard et al., 2001).

Uncoupling protein 1 (UCP1) can uncouple respiration from ATP synthesis and trigger NST (Nicholls and Locke, 1984). Because UCP1 is found uniquely in brown adipocytes, BAT was thought to be the only tissue in the body known to be able to activate adipocyte-based

NST (Ashwell et al., 1983). Recent studies have also found there is muscle-based NST regulated by Sarcolipin (Bal et al., 2012). Given that both brown adipocytes and muscle cells originate from the same precursor cells (Walden et al., 2012), it is suggested that brown adipocytes and muscle cells may share the similarity in generating NST, due to an evolutionary constraint.

Some studies have suggested that HSL can also interact with Fatty Acid Binding Protein 4 (FABP4, also known as adipocyte Protein 2, aP2) (Shen et al., 1999), an intracellular carrier protein for fatty acids. The gene of *FABP4* is primarily expressed in adipocytes and macrophages (Baxa et al., 1989). Stimulation of FABP4 depends on the presence of FFA and it may be related to the activation of HSL in adipocytes (Shen et al., 1999). Mice that are *FABP4*-deficient exhibit reduced lipolytic efficiency (Scheja et al., 1999; Shen et al., 1999), as well as reduced insulin secretion (Shen et al., 1999).

Catecholamine not only stimulate lipolysis, but also show anti-lipolytic effects through binding to the α_2AR (ADRA2). This reduces cAMP levels in adipocytes (Limon-Boulez et al., 2001). Therefore, the α_2/β_3 Adrenergic Receptor (AR) ratio in adipocytes determines whether catecholamine is used for lipolysis or to inhibit lipolysis (Lafontan and Berlan, 1995).

Besides the AR-PKA cascade in adipocytes (Figure 2.8), the ARs also can trigger a PKA-independent pathway that involves stimulation of the Extracellular signal-Regulated Kinase 1/2 and Mitogen-Activated Protein Kinase (ERK1/2-MAPK) cascade in adipocytes (Cao et al., 2001; Nakata, 2004). This pathway occurs as a result of the ARs' coupling to the G-protein subtype G_i (Soeder et al., 1999) and appears to account for 15-25% of total lipolysis (Greenberg et al., 2001). Collins *et al.,* (2004) found that lipolysis is mainly activated by the PKA pathway when low catecholamine concentrations occur, whereas the ERK1/2 pathway may be more important at higher concentrations of epinephrine.

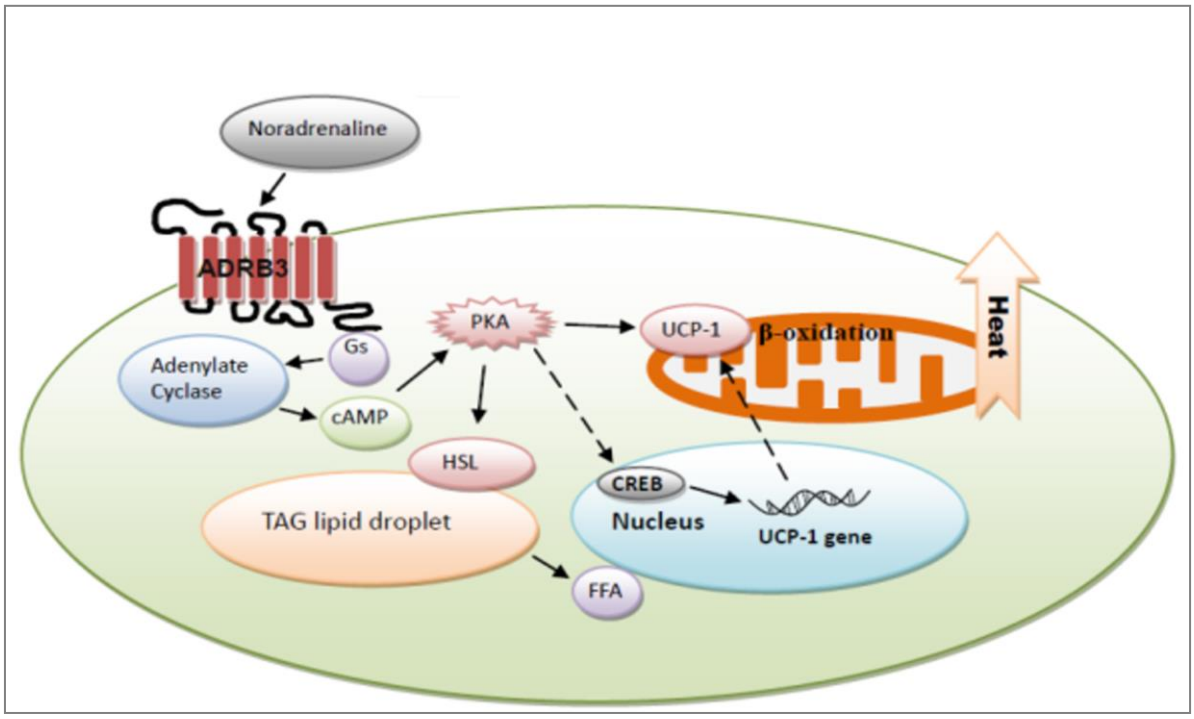


Figure 2.8 The process of lipolysis in adipocytes. This diagram illustrates some of the factors involved in the process of lipolysis in adipocytes. SNS, Sympathetic Nervous System; ADRB3, β_3-adrenergic receptors; PKA, Protein Kinase A; HSL, Hormone-Sensitive Lipase; TAG, triacylglycerols; FFA, Free Fatty Acids; UCP1, Uncoupling protein-1; CREB, cAMP Response Element-Binding Protein. (Adapted from Langin, 2006).

2.5 Manipulation of body composition and minimising fat deposition

The flow of energy repartitioned into parts of the body that are growing has been investigated using β-agonists. After stimulation with β-agonists, the fatty acids that are released in adipose tissue appear to be diverted towards the provision of energy for protein synthesis (Ghorbani, 2012; McDowell and Annison, 1991). They lead to the repartitioning of energy to support both protein synthesis and a higher basal metabolic rate. Subsequently, muscle growth is enhanced and adipose tissue growth is reduced. This ultimately results in an increase in lean mass and a reduction in fat deposition. The effect may be enhanced by a simultaneous reduction in protein degradation and an increase in growth hormone levels (Ghorbani, 2012; McDowell and Annison, 1991; Yue, 2011). Beta-agonists have been approved for use in livestock production. Ractopamine (Paylean®; Elanco Animal Health, Greenfield, IN) was approved for use in swine in 1999, and later for cattle (Optaflexx®, Elanco Animal Health, Greenfield, IN) in 2003. Zilpaterol (Zilmax®; Merk Corp., Summit, NJ) was approved for use in cattle in South Africa and Mexico, and received FDA approval for use in cattle in the United States in 2006.

The effects of β-agonists on animal growth and carcass traits has been found to involve the binding of the agonists to ADRBs and the trigger of various regulatory cascades, hence the ADRBs are thought to play an important role in the regulation of body composition and growth.

2.6 ADRB3-mediated lipolysis and fat deposition and growth

In adipose tissues, the major effects of β-agonists on adipose tissue are an increase in lipolysis and reduction in lipogenesis (Yang and McElligott, 1989). Recent studies have emphasised the important role that ADRB3-mediated lipolysis plays in the degradation of fat and repartitioning of the energy into protein gain. In humans, impaired lipolysis could lead to excessive ectopic lipid deposition in non-adipose tissues, which leads to lipo-toxicity, and it is associated with metabolic diseases such as type-2 diabetes (DeFronzo, 2004; Lelliott and Vidal-Puig, 2004; Slawik and Vidal-Puig, 2006; Unger et al., 2010).

The lipolytic genes *ADRB3*, *HSL* and *UCP1*, and fat deposition and growth

The genes encoding ADRB3, HSL and UCP1, which are all the factors in ADRB3-mediated lipolysis have been reported to be associated with variation in human growth and body composition (Klannemark et al., 1998; Shin et al., 2005; Silver et al., 1999).

The important role of *ADRB3*, *HSL* and *UCP1* in growth and fat catabolism has become evident from the analysis and examination of mice made deficient in *ADRB3* (Revelli et al., 1997; Susulic et al., 1995), *HSL* (Fortier et al., 2004; Haemmerle et al., 2002; Osuga et al., 2000; Voshol et al., 2003) and *UCP1* (Surwit et al., 1998) and human patients with mutations in the *ADRB3* (Kurokawa et al., 2008; Silver et al., 1999), *HSL* (Hoffstedt et al., 2001; Klannemark et al., 1998; Lavebratt et al., 2002; Magre et al., 1998; Nieters et al., 2002; Qi et al., 2004) and *UCP1* (Kotani et al., 2007; Shin et al., 2005).

In sheep, variation in *ADRB3* has been reported to be able to cause the differences in pre-weaning growth (Forrest et al., 2003; Horrell et al., 2009), carcass composition (Forrest et al., 2003) and survival (Forrest et al., 2007; Forrest et al., 2003; Forrest et al., 2006).

Because greater variation than that already described is suspected in the *ADRB3* and variation in *HSL* and *UCP1* has not been investigated in sheep. Variation in these gene could potentially be used as gene markers for production traits if such associations exist. In this study, further investigations of these genes and their roles in ovine growth and carcass-composition are performed.

Chapter 3
Extended haplotypes of the ovine β_3-adrenergic receptor gene (*ADRB3*) and their association with growth and body composition traits in sheep

3.1 Introduction

The ADRB3 is a member of the G-protein coupled receptor (GPCR) superfamily. To date, at least nine subtypes of AR have been identified, including six α- and three β-subtypes (called α_{1A} AR (ADRA1A), α_{1B} AR (ADRA1B), α_{1D} AR (ADRA1D), α_{2A} AR (ADRA2A), α_{2B} AR (ADRA2B), α_{2C} AR (ADRA2C), ADRB1, ADRB2 and ADRB3, respectively). Some studies have suggested the presence of a putative β_4AR (ADRB4) in mice (Kaumann and Lynham, 1997), but it has also been suggested to be a novel ADRB3 isoform (Kaumann et al., 1998). The ADRB3 is present on BAT and WAT (Arch et al., 1984), while ADRB1 and ADRB2 are mainly present in heart and skeletal muscle, respectively (Kim et al., 1991; Williams et al., 1984).

The ARs can selectively couple to different G-protein subtypes, especially to G_s and G_i, and stimulate different downstream pathways in different tissues (Dascal, 2001; Mirshahi et al., 2002; Steinberg, 2000).

The ovine ADRB3 protein

Sheep ADRB3 is 405 amino acids in length. It is comprised of seven transmembrane (TM) α-helices that form three extracellular loops including an NH_2-terminus and three intracellular loops including a COOH-terminus (Figure 3.1).

The structure of ovine ADRB3 is stabilised by two disulphide bonds that are formed between cysteine residues in the second and the third extracellular loops (aa 110-196 and aa 189-195). A ligand binding site and a G-protein coupling site have been located at the centre of three extracellular domains and the third intracellular loop of ADRB3, respectively (Kobilka et al., 1988). Ligand-binding causes conformational changes in the intracellular loops of ADRB3 and this leads to uncovering of the previously blocked G-protein binding-site. This in turn, activates the coupling of ADRB3 with G_s or G_i protein (Filipek et al., 2004; Meng and Bourne, 2001).

At the NH_2-terminus, two putative glycosylation sites (Guillaume et al., 1994) have been found in ovine ADRB3 (Figure 3.1). Unlike ADRB2 which contains many potential

phosphorylation sites for PKA, PKC and G-protein-coupled Receptor Kinases (GRKs) within its third intracellular domain and COOH-terminus, the intracellular domains of ADRB3 contain no potential phosphorylation sites for PKA or PKC, and only one putative phosphorylation site for GRKs (Strosberg and Gerhardt, 2000). This lack of phosphorylation sites for PKA and PKC, suggests ADRB3 is more resistant to rapid agonist-stimulated desensitisation than ADRB2 (Liggett and Raymond, 1993; Nantel et al., 1993).

The ovine ADRB3 amino acid sequence (Figure 3.1) shares 80-90% homology with the human, monkey, bovine, dog, mouse, rat, guinea pig and hamster sequences (Strosberg, 1997). However, the homology between ADRB1, ADRB2 and ADRB3 from the same species is 40-50% (Strosberg, 1997). The residues conserved between the ADRBs are mainly located in the seven transmembrane domains (Gros et al., 1998; Guan et al., 1995; Jockers et al., 1996; Nantel et al., 1993; Nantel et al., 1994).

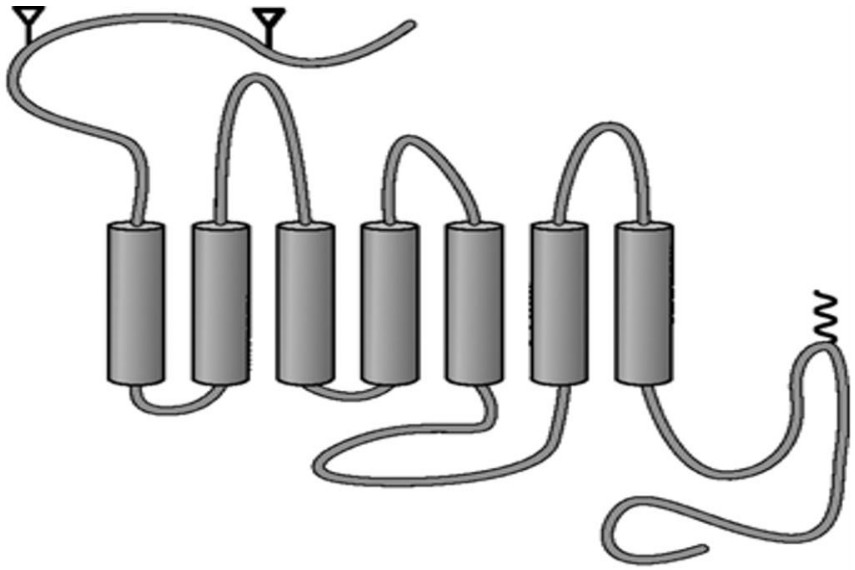


Figure 3.1 The structure of human β_3-adrenergic receptor (ADRB3). (Adapted from Gerald and Dorn, 2010).

The ovine ADRB3 gene (*ADRB3*)

To date, the ADRB3 gene (*ADRB3*) has been identified and cloned in mice (Nahmias et al., 1991), rats (Granneman et al., 1991), dogs (Lenzen et al., 1998), humans (Emorine et al., 1989), monkeys (Walston et al., 1997), domestic pigs (McNeel and Mersmann, 1995, 1999), cattle (Forrest and Hickford, 2000; Pietri-Rouxel et al., 1995), goats (Forrest and Hickford, 2000) and sheep (Forrest and Hickford, 2000). It has been mapped to chromosome 8 in humans (GenBank, Gene ID: 155), chromosome 8 in mice (GenBank, Gene ID: 25645), chromosome 16 in rats (GenBank, Gene ID: 25645), chromosome 15 in pigs (GenBank, Gene

ID: 397356), chromosome 27 in cattle (GenBank, Gene ID: 281606) and chromosome 26 in sheep (GenBank, Gene ID: 100294559).

Ovine *ADRB3* is approximately 2.5kb in length. It is composed of a large first exon of approximately 1.1 kb that encodes the first 398 amino acid residues, and a small second exon of approximately 21 bp that encodes the seven consistency COOH-terminal residues (Figure 3.2). An intron only exists in *ADRB3*, but not in *ADRB1* and *ADRB2* (Bensaid et al., 1993; Granneman et al., 1993; van Spronsen et al., 1993). In rats, the intron of *ADRB3* contains potential binding sites for transcription factors involved in tissue-specific expression (Granneman et al., 1993).

Figure 3.2 Structure of ovine β$_3$-adrenergic receptor gene (*ADRB3*).

ADRB3 "knockout" mouse phenotypes

An understanding of *ADRB3* is important as any variation in the intact gene may cause structural and functional changes in the protein. Studies have shown that *ADRB3* "knockout" mice have reduced lipolysis and decreased muscle mass (Revelli et al., 1997; Susulic et al., 1995). Lowell *et al.,* (2000) found that *ADRB3*-deficient mice also showed impaired thermoregulatory activity and decreased *UCP1* mRNA levels in responses to cold-exposure. The results from these gene "knockout" studies indicated that the *ADRB3* is critical for animal energy homeostasis and variation in this gene may also cause changes in the expression and function of other genes and subsequently result in changes in growth performance (Lowell et al., 2000).

Associations between variation in *ADRB3* and variation in phenotypic traits

In humans, a missense mutation in codon 64 of *ADRB3*, leading to the replacement of tryptophan by arginine (Trp64Arg) in the protein, has been reported to be associated with increased body weight, early development of type 2 diabetes mellitus, and clinical features of insulin resistance in several populations (Silver et al., 1999). In these studies, the Arg 64 allele of the *ADRB3* was associated with Metabolic Syndrome (MS) components such as obesity and decreased High-Density Lipoprotein Cholesterol (HDL-C) levels (Mirrakhimov et al., 2011).

However, such findings have not been consistent among studies. Kurokawa *et al.,* (2008) reported association between the Trp64Arg polymorphism and variation in Body Mass Index (BMI) in East Asians, but not in Europeans, by genotyping a large number of individuals (n = 44 833). The Arg 64 allele had a frequency of 32.7% in the East Asian populations. In contrast, only 14.5% of Europeans carry the Arg 64 allele. The Arg 64 allele was also associated with increased fasting insulin levels in Asians, but not Caucasians (Zhan and Ho, 2005).

In Duroc pigs, a nucleotide insertion/deletion of a thymine base in exon 2 of porcine *ADRB3* has been described and associated with variation in loin Eye-Muscle Area (EMA) (Hirose et al., 2009; Tanaka et al., 2007). No association was detected between this variation and growth-rate or fat deposition (back-fat thickness and intramuscular fat content) (Hirose et al., 2009). A similar study failed to detect this polymorphism in other pig breeds (Polish Landrace, Polish Large White and a Polish synthetic line) (Cieslak et al., 2009). This suggests that the variation in porcine *ADRB3* may be breed-specific.

In cattle, variation within the intron of *ADRB3* has been reported (Hu et al., 2010). A comparative analysis of human chromosome 8 and bovine chromosome 27 suggests that bovine *ADRB3* may play an important role in partitioning body fat deposition in dairy cattle and be associated with the "dairy form" trait, an important indicator of a dairy cow's capacity to utilise energy into milk production, rather than in fat storage (Connor et al., 2006).

In sheep, variation in both coding and non-coding regions of *ADRB3* has been reported in breeds that include traditional European/English breeds and crossbred sheep (Byun et al., 2008; Forrest et al., 2003) and some Chinese sheep breeds (Yang et al., 2009). Among all of the ovine *ADRB3* variants identified to date, the putative polypeptide encoded by variant *D* shows two amino acids substitutions (Val52Ala and Leu322Val, Forrest et al., 2003), which occur in positions that are normally conserved in all ADRBs and that are thought to be involved in ligand-binding (reviewed in Strosberg, 2000).

It is notable that most of the variation detected in the *ADRB3* are in the non-coding region. In the *ADRB* family, an intron only exists in *ADRB3* and not in *ADRB1* or *ADRB2* (reviewed in Strosberg, 2000). This unique existence of intron in *ADRB3* suggests that this region may play important role in regulation of *ADRB3* expression and be important for the function of ADRB3. Forrest *et al.,* (2007) found an association between variation in the ovine *ADRB3* intron and lamb cold-survival by genotyping 13427 lambs from 13 different breeds born on 22 different farms throughout the South Island of NZ. These results underpin the use of ovine *ADRB3* as a gene-marker for breeding more cold-tolerant lambs, and a gene-marker

test for lamb cold-tolerance is now commercially available from the Gene-Marker Laboratory, Lincoln University, NZ. Forrest *et al.,* (2003) also reported the association between variation in ovine *ADRB3* intron and variation in birth weight, pre-weaning growth-rate and carcass traits.

In the NZ Romney sheep, an association between variation in the ovine *ADRB3* intron and variation in post-weaning growth-rate has been reported (Horrell et al., 2009), but no association between intron variants and birth weight were detected. In Horrell's study (2009), the presence of intron variant *A* was found to be associated with pre-weaning growth-rate. It is noteworthy that ninety-four percent of those Romney lambs studied possessed intron variant *A*. In addition, in previous studies (Byun et al., 2008; Forrest and Hickford, 2000; Forrest et al., 2007; Forrest et al., 2003; Forrest et al., 2006; Horrell et al., 2009), intron *A* was found to be a common variant in some sheep breeds. However, because of the ambiguities in the sequence data derived from the Polymerase Chain Reaction-Single Strand Conformational Polymorphism (PCR-SSCP) banding pattern of variant *A*, greater sequence variation within the extended *ADRB3 A* sequence has been suggested (Forrest et al., 2003). Therefore, it would be helpful for using ovine *ADRB3* as a gene-marker if extended variation and haplotypes in the gene could be identified.

Research reveals that the 3'UTR of *ADRB2* mRNA may have roles in post-transcriptional regulatory mechanisms that are involved in the stability and degradation of various cellular RNAs, sub-cellular targeting and translation of many transcripts (Tholanikunnel and Malbon, 1997; Yang et al., 1997). Deletion of the 3'UTR sequences resulted in 2-2.5 fold increases in *ADRB2* expression (Subramaniam et al., 2011).

The similarities of the ADRB2 3'UTRs from humans and rodents were calculated to be 73% and 79%, respectively (Nakada et al., 1989), thus, it is presumed that this regions contain genetic elements that are required to regulate receptor expression and therefore have been subject to selective pressure to preserve their sequence (Nakada et al., 1989). The ADRB1 mRNA has also been reported to be post-transcriptionally regulated at the level of mRNA stability and undergo accelerated agonist-mediated degradation via interaction of its 3'UTR with RNA binding proteins (Nakada et al., 1989). To date, little has been revealed about characteristics of ovine *ADRB3* 3'UTR.

Given the important role of variation in *ADRB3* and its association with sheep growth, in this chapter, extended haplotypes of ovine *ADRB3* were defined by investigating variation within both the intron and 3'UTR region. Next associations between ovine *ADRB3* haplotypes and selected growth and body composition traits in the NZ Suffolk sheep were investigated.

3.2 Material and methods

Sheep analysed and blood collection

There were 948 lambs studied in total, including 808 NZ Suffolk lambs from 38 sire-lines and eight studs (see Appendix A), and 140 NZ Merino sheep from eight sire-lines. The latter were known to include sheep possessing the thirteen previously described *ADRB3* intron variants (*A-M*) (Byun et al., 2008; Forrest et al., 2003; Yang et al., 2011).

Blood was collected from the ear using side-cutters onto FTA® cards (Whatman, Middlesex, UK). This sampling approach did not require ethical approval as ear-clipping is considered a standard sheep management practice.

Sheep investigated to define haplotypes of *ADRB3*

The 140 NZ Merino ewes and their lambs that were known to have the 13 previously described intron variants of *ADRB3*, were investigated for variation in the 3'UTR and haplotypes of ovine *ADRB3*.

Sheep investigated for growth traits analyses

The Suffolk breed was used for growth analyses. This breed has been widely used as a terminal sire throughout the world because of its excellent growth-rate and lean meat production.

Because lambs birth weights were not available for dataset, the pre-weaning growth rate were not investigated in this study. The weights of the NZ Suffolk lambs from different farms were recorded at weaning (approximately 12 weeks of age), and at six months of age. Sire, gender and birth rank (whether the lamb was born single, twin, triplet or quad) of the lambs were recorded at birth. The post-weaning growth-rate of the NZ Suffolk lambs was calculated as the average weight gain per day (gram/day), from weaning to 6-months of age.

Sheep investigated for body composition traits analyses

The 225 NZ Suffolk ram lambs were chosen at 6-8 months of age for ultrasound scanning to estimate body composition. Traits including loin Eye Muscle Width (EMW), Eye Muscle Depth (EMD) and Fat Depth above the Eye Muscle (FDM) were measured.

DNA purification

Genomic DNA was purified from sheep studied using a two-step method described by Zhou *et al.,* (2006). In this method, a 1.2mm blood disc was punched from each blood spot

and was transferred into a 200 µL PCR tube. Solution of 200 µL of 20mM NaOH was added to the tube and it was incubated for 30 min at room temperature. The solution was then discarded, and the disc was equilibrated in 200 µL of 1 × TE buffer (10mM Tris-HCl, 0.1mM EDTA, pH 8.0). After removal of the TE buffer, the disk was air-dried, prior to its use in PCR.

PCR primer design

Two sets of primers were designed based on a published ovine *ADRB3* sequence (GenBank accession number: DQ269497) to amplify the intron and the 3'UTR region of ovine *ADRB3* (see Figure 3.3).

PCR amplification and SSCP analysis

Multiplex PCR amplification of both the intron and 3'UTR fragments was carried out in a single 15 µL reaction, containing the genomic DNA on a 1.2mm diameter punch of FTA paper, 0.5µM of intron and 3'UTR primer pairs for ovine *ADRB3*, 150µM of each dNTP (Eppendorf, Hamburg, Germany), 0.5 U of Taq DNA polymerase (Qiagen, Hilden, Germany) and 1 × reaction buffer supplied with the polymerase enzyme (containing 1.5mM $MgCl_2$). After initial denaturation at 94°C for 2 min, 35 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 30 s were utilised, followed by a final 5-min extension at 72°C.

A 0.7 µL aliquot of the PCR products was mixed with 7 µL of loading dye (98% formamide, 10mm EDTA, 0.025% bromophenol blue, 0.025% xylene-cyanol). After denaturation at 94°C for 5 min, samples were rapidly cooled on wet ice and then loaded on 16 × 18 cm, 12% acrylamide/bisacrylamide (37.5:1) (Bio-Rad) gels. Electrophoresis was performed using Protean II xi cells (Bio-Rad), at 290 V for 18 h at 25°C in 0.5 × TBE buffer. Gels were silver-stained according to the method of Byun *et al.,* (2009). In this method, gels were fixed and stained in a solution containing 10% ethanol, 0.5% acetic acid, and 0.2% silver nitrate (for anywhere between 3 and 20 min). The gels were rinsed with distilled water once and then developed with a solution of 3% NaOH and 0.1% HCOH (pre-warmed to 55 °C) until dark staining bands appear on the yellow background of the PCR-SSCP gels (varies between 5 and 10 min). Development was then stopped with a solution containing 10% ethanol and 0.5% acetic acid. SSCP banding patterns then could be observed directly from the gels.

Amplicons representative of the previously reported intron variants (variants *A-M*) (Yang et al., 2009) and the 3'UTR variants identified in this study were also included in each

polyacrylamide gel and their banding patterns were used as PCR-SSCP pattern standards for determining intron and the 3'UTR genotypes of the individual sheep.

Sequencing of ovine *ADRB3* variants and sequence analysis

Amplicons representative of different PCR-SSCP patterns from sheep that were homozygous for the each region of the ovine *ADRB3* were directly sequenced at the Lincoln University DNA Sequencing Facility. For rare variants, only found in heterozygous sheep, a band corresponding to the rare variant was excised as a gel slice from the first PCR-SSCP gel. This gel slice was macerated, then used as a template for re-amplification with the original primers to produce a PCR-SSCP gel pattern equivalent to a sheep homozygous for that rare variant. This second amplicon was then directly sequenced. Sequence alignments, translations and comparisons were carried out using DNAMAN (version 5.2.10, Lynnon Biosoft, Vaudreuil, Canada). The BLAST algorithm was used to search the NCBI GenBank databases (http://blast.ncbi.nlm.nih.gov/) for homologous sequences.

Haplotype determination

For progeny that typed as homozygous in either of the regions, haplotype could be directly inferred based on the co-inheritance of sequences. For example, for an animal presenting with intron genotype *AA* and the 3'UTR genotype *ab*, the presence of haplotypes *A-a* and *A-b* could be confirmed.

For progeny that typed as heterozygous in both regions, the sire haplotypes could be deduced by comparison with previously defined haplotypes and comparison with other progeny from the same sire. Any given half-sib progeny's diplotype consists of one haplotype common to both its sire and to half of its siblings, along with a maternally derived haplotype. For example, for a sire having a diplotype of *A-b*/*B-c*, approximately half of its offspring will have the haplotype *A-b*, while the other half will be *B-c*.

Statistical analyses

Analysis of association between ovine ADRB3 intron and 3'UTR variants and selected traits in the NZ Suffolk sheep

General Linear Mixed-effects Models (GLMMs) were used to explore the effect of the presence of a particular intron or 3'UTR variant in a sheep's genotype on growth-rate and body composition traits.

All statistical analyses were performed using SPSS version 19 (SPSS Inc., Chicago, IL, USA). General Linear Mixed-effects Models (GLMMs) were used to assess the effects of the presence/absence of a particular sequence variant in a lamb's genotype, on post-weaning growth-rate (n = 538 lambs), and various body composition traits (n = 262 ram lambs) including EMW, EMD and FDM. Gender (for growth-rate analysis only, as body composition traits were only assessed in ram lambs) and birth rank were fitted as fixed factors and sire was fitted as a random factor in order to correct for sire effects as well as farm effects such as management and environmental effects (each sire was used exclusively on one farm).

The generalized statistical model used to test the sequence variant (or genotype – see below) effects was as follows: $Y_{ijk} = \mu + \tau_i + \beta_j + \alpha_k + \epsilon_{ijk}$; where Y_{ijk} = traits evaluated on the i_{th} level of the fixed factor genotype (τ_i), the j_{th} level of the fixed factor birth rank (β_j) and the k_{th} sire of random effect (α_k); where μ = overall mean for each trait and ϵ_{ijk} is the random error for ijk.

Each variant was coded as present (1) or absent (0) for each animal's genotype. Initially, single-variant models were performed to ascertain which variants should be included in subsequent multi-variant models and to assess whether the effect of the variants were independent of the other variants. The multi-variant models included any variant that had a variant-trait association in the single-variant models with a *P* value of less than 0.200 (and which could thus potentially impact on the trait of interest). For variants that had homozygous forms higher than 5% of all genotypes (thus providing an adequate sample size) a second set of analyses was performed, this time with the number of variant copies present in the animal's genotype included (i.e. 0, 1 or 2) in place of presence/absence of coding, followed by planned orthogonal contrasts to ascertain whether additive, dominant or recessive effects were present.

For genotypes with a frequency greater than 5% (thus having adequate sample size per group), a GLMM (fixed effect: genotype, gender [not for body composition analysis, and birth rank; random effect: sire) and multiple pair-wise comparisons with a Bonferroni correction were used to ascertain the effect of genotype on weaning weight, growth rate and body composition traits.

Analysis of association between ovine ADRB3 haplotypes and selected traits in the NZ Suffolk sheep

The association of the variants from these two regions were then investigated by assessing the effect of the presence of a particular intron-3'UTR haplotype in a sheep's diplotype on growth-rate and body composition traits.

Birth rank and gender were fitted as fixed factors. Sire-group was fitted as a random factor in order to correct for farm-specific management and environmental effects and because no sire was used on more than one property. Single-haplotype present/absent models were performed to ascertain which haplotype should be included in subsequent multi-haplotype models and such that we could assess whether the effect of the single haplotypes was independent of the other haplotypes in the diplotype. The multi-haplotype models included any haplotype that had associations with post-weaning growth-rate in the single-haplotype models with a *P* value of less than 0.200.

Each haplotype was coded as present (1) or absent (0) for each animal's diplotype. For haplotypes that had homozygous forms greater than 5%, a second set of analyses was performed; this time with the number of haplotype copies present in the animal's diplotype included (i.e. 0, 1 or 2) in place of the presence/absence of the haplotype. This was followed by planned orthogonal contrasts to ascertain whether additive, dominant or recessive effects could be observed.

For diplotypes with a frequency greater than 5%, a GLMM (fixed effect: diplotype, gender and birth rank; random effect: sire) and multiple pair-wise comparisons with a Bonferroni correction, were used to estimate the association between diplotype, weaning-weights, post-weaning growth-rates and body composition traits.

3.3 Results

Correlations between lamb growth and body composition traits in the NZ Suffolk sheep

Pearson correlation coefficients between the lamb growth data and various body composition traits evaluated are listed in Table 3.1. Lamb post-weaning growth-rate was correlated with EMD ($R = 0.433$, $P < 0.010$), EMW ($R = 0.179$, $P < 0.010$) and FDM ($R = 0.287$, $P < 0.010$). Weaning-weight was correlated with EMD ($R = 0.566$, $P < 0.001$), EMW ($R = 0.593$, $P < 0.001$) and FDM ($R = 0.508$, $P < 0.001$), but not post-weaning growth-rate ($R = 0.098$, $P > 0.050$).

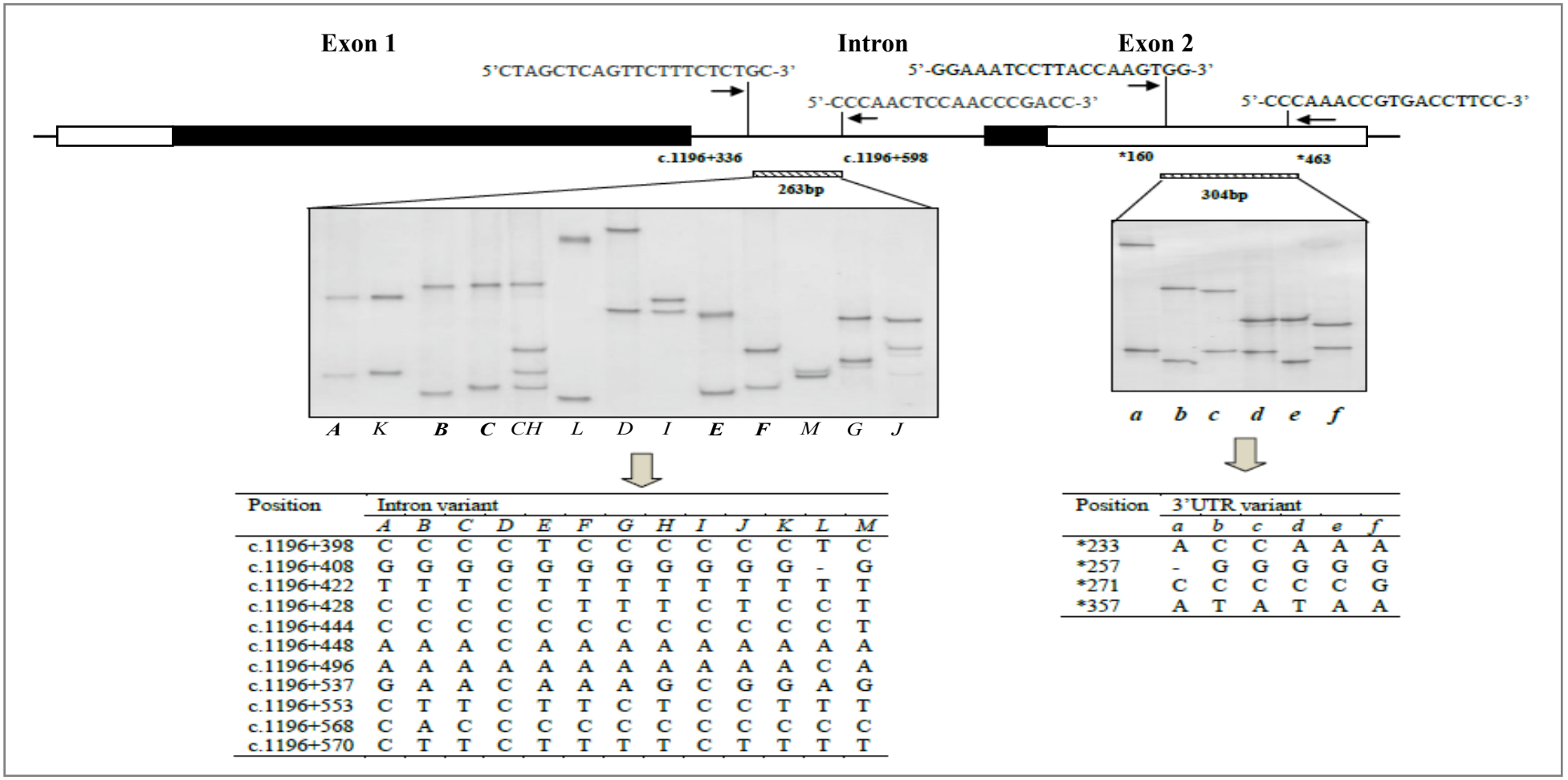
Table 3.1 Correlation coefficients for comparisons between lamb growth and body composition traits in the NZ Suffolk sheep

	Weaning-Weight (WWT)	Eye Muscle Depth (EMD)	Eye Muscle Width (EMW)	Fat Depth above the eye Muscle (FDM)
Post-weaning Growth-rate	0.098	0.433**	0.179**	0.287**
Weaning-Weight (WWT)		0.566**	0.593**	0.508**
Eye Muscle Depth (EMD)			0.630**	0.561**
Eye Muscle Width (EMW)				0.574**

** Correlation is significant at the 0.010 level (2-tailed).

Variation within the 3'UTR of ovine *ADRB3*

Six unique PCR-SSCP banding patterns were observed for the approximately 304 bp 3'UTR amplimers of ovine *ADRB3* (Figure 3.3).



Position	Intron variant												
	A	*B*	*C*	*D*	*E*	*F*	*G*	*H*	*I*	*J*	*K*	*L*	*M*
c.1196+398	C	C	C	C	T	C	C	C	C	C	C	T	C
c.1196+408	G	G	G	G	G	G	G	G	G	G	G	-	G
c.1196+422	T	T	T	C	T	T	T	T	T	T	T	T	T
c.1196+428	C	C	C	C	C	T	T	T	C	T	C	C	T
c.1196+444	C	C	C	C	C	C	C	C	C	C	C	C	T
c.1196+448	A	A	A	C	A	A	A	A	A	A	A	A	A
c.1196+496	A	A	A	A	A	A	A	A	A	A	A	C	A
c.1196+537	G	A	A	C	A	A	A	G	C	G	G	A	G
c.1196+553	C	T	T	C	T	T	C	T	C	C	T	T	T
c.1196+568	C	A	C	C	C	C	C	C	C	C	C	C	C
c.1196+570	C	T	T	C	T	T	T	T	C	T	T	T	T

Position	3'UTR variant					
	a	*b*	*c*	*d*	*e*	*f*
*233	A	C	C	A	A	A
*257	-	G	G	G	G	G
*271	C	C	C	C	C	G
*357	A	T	A	T	A	A

Figure 3.3 The PCR-SSCP patterns and the single nucleotide variations detected in the intron and 3'UTR of ovine *ADRB3*. Two regions of ovine *ADRB3* amplified were shown in diagonal lines pattern; The 5'UTR in exon 1 and 3'UTR in exon 2 of the gene are shown in blank boxes. The primer coordinates are defined by a sequence of ovine *ADRB3* (DQ269497); The PCR-SSCP patterns observed in NZ Suffolk sheep are shown in bold italics; The positions of single nucleotide variations in the intron and 3'UTR of ovine *ADRB3* are described relative to ovine *ADRB3* coding sequence (DQ269497) and named according to sequence variation nomenclature (http://www.hgmd.cf.ac.uk/docs/mut_nom.html).

Sequencing confirmed that each pattern represented a novel 3'UTR variant of *ADRB3* (designated *a-f*, GenBank accession numbers: HM776668-HM776673, respectively).

Three SNPs and a single-nucleotide deletion in the 3'UTR of ovine *ADRB3* were identified by comparison of the six 3'UTR variant sequences. They are c.*233A>C, c.*271G>C, c.*357A>T and c.*257delG, respectively (Figure 3.3).

Frequencies of the ovine *ADRB3* intron and 3'UTR variants in the NZ Suffolk Sheep

Results from PCR-SSCP analysis of the 808 Suffolk sheep revealed five previously reported variant sequences (*A*, *B*, *C*, *E* and *F*) in the intron and four variant sequences (*a*, *b*, *c* and *e*) in the 3'UTR of ovine *ADRB3*. The variant sequences of the intron of ovine *ADRB3* were identified with the following frequencies: *A* (33.1%), *B* (25.5%), *C* (15.7%), *E* (14.9%) and *F* (10.8%), and in the 3'UTR the frequencies were: *a* (9.5%), *b* (25.1%), *c* (26.4%) and *e* (39.0%).

Association between ovine *ADRB3* intron variants, 3'UTR variants and weaning-weights in the NZ Suffolk sheep

In the single- and multi-variant models (Table 3.2), the presence of *A* was associated ($P < 0.001$ and $P = 0.009$, respectively) with lower mean weaning-weight. Variant *C* was associated ($P = 0.021$, Table 3.2) with higher mean weaning-weight in the single-variant model, but this effect did not persist in the multi-variant model suggesting the effect was not an independent effect. Variant *b* was associated with lower mean weaning-weight in the single- and multi-variant models ($P = 0.001$ and $P = 0.012$, respectively), suggesting this is an independent allele effect.

The frequency of the variant *A*, *B*, *E*, *b*, *c*, and *e* homozygous form was greater than 5% and so additive, dominant and recessive effects were assessed. Lambs possessing the heterozygous *A* variant were found to have lower mean weaning-weights than those that did not have variant *A* in their genotype ($P < 0.050$, Table 3.3). Lambs heterozygous for variant *b* had lower mean weaning-weights ($P < 0.050$, Table 3.3) than those did not have variant *b* in their genotype.

The genotypes *AA*, *AB*, *AC*, *AE*, *BB*, *BC*, *BE*, *BF* and *CE* in the intron and *ab*, *ac*, *ae*, *bb*, *bc* , *be*, *cc*, *ce* and *ee* in the 3'UTR occurred at a frequency of greater than 5%.

The marginal means for weaning-weights are listed in Table 3.4 for each genotype. Those lambs with the intron *CE* genotype had higher weaning-weight than those with *AA* ($P = 0.003$), *AB* ($P < 0.001$), *AC* ($P = 0.0012$), *AE* ($P < 0.001$), *AF* ($P = 0.001$), *BB* ($P = 0.001$) and

BE ($P = 0.005$). Those sheep with the *BF* genotype had higher weaning-weight than *AB* ($P = 0.046$) and *AE* ($P = 0.023$).

The 3'UTR genotype was also found to effect weaning-weight (Table 3.4). Those animals with the *ae* genotype had higher weaning-weight than those with *ab* ($P = 0.012$), *ac* ($P = 0.022$), *bb* ($P = 0.014$), *bc* ($P < 0.001$), *be* ($P < 0.001$), *cc* ($P = 0.005$) and *ce* ($P = 0.036$). Those sheep with the *ee* genotype had higher weaning-weight than *bc* ($P = 0.004$), *be* ($P = 0.002$) and *cc* ($P = 0.036$). Those sheep with the *ce* genotype had higher weaning-weight than those with *bc* ($P = 0.021$) and *cc* ($P = 0.014$).

Associations between the ovine *ADRB3* intron variants, 3'UTR variants and post-weaning growth-rates

In the single- and multi-variant models, the presence of *B* or *C* was associated with variation in post-weaning growth-rate, with *B* being independently associated ($P = 0.017$, Table 3.2) with lower post-weaning growth and *C* being independently associated ($P = 0.018$, Table 3.2) with higher in post-weaning growth-rate. Variant *c* was associated with lower post-weaning growth-rate in both the single- and multi-variant models ($P = 0.005$ and $P = 0.012$, respectively), while the increase in post-weaning growth-rate associated with *a* in the single-variant model ($P = 0.021$, Table 3.2), only persisted as a trend ($P = 0.054$, Table 3.2) in the multi-variant model.

The frequency of homozygous *A, B, E, b, c* and *e* was greater than 5% of all genotypes, and so additive, dominant or recessive effects could be ascertained (Table 3.3). The homozygous and heterozygous forms of *B* were found to be associated with a significant decrease in post-weaning growth-rate (Table 3.3). The homozygous and heterozygous forms of *c* were associated with a significant decrease in post-weaning growth-rate (Table 3.3). No other associations were detected ($P > 0.050$).

Those marginal means for post-weaning growth-rates are listed in Table 3.4 for each genotype occurred at a frequency of greater than 5%. Those animals with the *BB* genotype had lower post-weaning growth-rates than those with *AC* ($P = 0.036$), *AE* ($P = 0.034$), *BC* ($P = 0.021$) and *CE* ($P = 0.029$).

Those animals with the *ab* genotype had higher post-weaning growth-rates than those with *bb* ($P = 0.037$), *bc* ($P = 0.004$), *cc* ($P = 0.007$) and *ce* ($P = 0.038$). Those sheep with the *ee* genotype had higher growth-rates than *bb* ($P = 0.032$), *bc* ($P = 0.002$), *cc* ($P = 0.003$) and *ce* ($P = 0.014$).

Association between ovine *ADRB3* intron variants, 3'UTR variants and selected body composition traits in the NZ Suffolk sheep

In the single variant models, the presence of *C* was associated with higher Fat Depth above the eye Muscle (FDM) ($P = 0.033$, Table 3.5). No other associations were detected ($P > 0.050$).

The frequency of *CC* was greater than 5% of all genotypes in 225 Suffolk sheep analysed, and so additive, dominant or recessive effects could be ascertained. The heterozygous forms of *C* were found associated with higher FDM ($P = 0.040$) suggesting a dominant effect of *C*.

The marginal means for FDM for each genotype listed in Table 3.6 occurred at a frequency of greater than 5%. Those animals with the *AA* genotype had lower FDM than those with *AC* ($P < 0.001$), *AE* ($P = 0.049$), *BC* ($P = 0.005$) and *BE* ($P = 0.005$). Those animals with the *AC* genotype had higher FDM than those with *AB* ($P = 0.002$), *AE* ($P = 0.016$), *AF* ($P = 0.004$), *BF* ($P = 0.001$) and *CE* ($P = 0.001$).Those animals with the *CE* genotype had lower FDM than *BC* ($P = 0.039$) and *BE* ($P = 0.039$).

Those animals with the *ac* genotype had higher FDM than those with *bb* ($P = 0.037$). Those sheep with *be* genotype also had higher growth-rates than *bb* ($P = 0.003$).

No association ($P > 0.050$) was detected between intron, 3'UTR genotypes, EMD and EMW.

Table 3.2 Association between *ADRB3* intron, 3'UTR variants, weaning-weights and post-weaning growth-rates in the NZ Suffolk sheep

Variant	Weaning-weight (kg) Marginal Mean [1] ± Std Error				*P* value[1]	Post-weaning Growth-rate (g/day) Marginal Mean [1] ± Std Error				*P* value[1]
	Absent	n	Present	n		Absent	n	Present	n	
Intron: single model										
A	33.3 ± 1.19	354	31.5 ± 1.19	454	**< 0.001**	175 ± 12	354	172 ± 12	454	0.575
B	32.6 ± 1.19	435	32.1 ± 1.21	373	0.221	179 ± 11	435	165 ± 12	373	**0.002**
C	32.0 ± 1.18	594	33.2 ± 1.23	214	**0.021**	169 ± 11	594	185 ± 12	214	**0.002**
E	32.2 ± 1.19	532	32.8 ± 1.23	276	0.266	172 ± 11	532	177 ± 12	276	0.436
F	32.1 ± 1.18	668	33.2 ± 1.25	140	0.067	172 ± 11	668	178 ± 12	140	0.354
Multi model (A,C and F fitted as fixed factors in weaning-weight analysis; B and C fitted as fixed factors in post-weaning growth-rate analysis)										
A	33.5 ± 1.19	354	32.0 ± 1.23	454	**0.009**	Not in model				
B	Not in model					181 ± 11	435	170 ± 12	373	**0.017**
C	32.3 ± 1.18	594	33.2 ± 1.24	214	0.082	169 ± 11	594	181 ± 12	214	**0.018**
F	32.4 ± 1.18	668	33.2 ± 1.26	140	0.182	Not in model				
3'UTR: single model										
a	32.2 ± 1.18	671	33.3 ± 1.27	137	0.089	172 ± 11	671	186 ± 12	137	**0.021**
b	33.2 ± 1.19	373	31.5 ± 1.20	435	**0.001**	176 ± 12	373	173 ± 12	435	0.497
c	32.6 ± 1.19	425	32.1 ± 1.20	383	0.285	180 ± 11	425	167 ± 12	383	**0.005**
e	31.9 ± 1.21	362	32.7 ± 1.19	446	0.097	172 ± 12	362	176 ± 11	446	0.359
Multi model (a, b and e fitted as fixed factors in weaning-weight analysis; a and c fitted as fixed factors in post-weaning growth-rate analysis)										
a	32.1 ± 1.18	671	33.0 ± 1.28	137	0.209	171 ± 11	671	183 ± 12	137	0.054
b	33.3 ± 1.20	373	31.8 ±1.22	435	**0.012**	Not in model				
c	Not in model					183 ± 11	425	171 ± 12	383	**0.012**
e	32.3 ± 1.20	362	32.8 ± 1.21	446	0.339	Not in model				

[1]*P* value associated with the variant being assessed. Estimated marginal means and *P* values were derived from GLMMs with variant presence/absence, gender and birth rank fitted as fixed factors and sire-line fitted as a random factor ($P < 0.050$ in **bold**).

Table 3.3 Association of ovine *ADRB3* intron, 3'UTR variant copy number with weaning-weights and post-weaning growth-rates in the NZ Suffolk sheep

Variant	Weaning-weight (kg) Marginal Mean [1, 2] ± Std Error						*P* value[1]	Post-Weaning Growth-rate (gram/day) Marginal Mean [1, 2] ± Std Error						*P* value[1]
	Absent	n	1 copy	n	2 copies[3]	n		Absent	n	1 copy	n	2 copies[3]	n	
Intron														
A	33.2 ± 1.19[a]	354	31.2 ±1.20[b]	369	32.4 ± 1.33[ab]	85	**0.001**	176±12	354	174±12	369	168±13	85	0.616
B	32.6 ± 1.19	435	32.1 ± 1.21	332	31.4 ± 1.55	41	0.368	180±11 [a]	435	167±12 [b]	332	154±15[b]	41	**0.005**
E	32.2 ± 1.19	532	32.9± 1.23	252	31.3 ± 1.78	24	0.272	172±11	532	176±12	252	179±17	24	0.728
3'UTR														
b	33.2 ± 1.19[a]	367	31.2 ±1.21[b]	357	32.3 ± 1.34[ab]	84	**0.001**	176±12	367	173±12	357	169±13	84	0.689
c	32.6 ± 1.19	425	32.2 ± 1.21	340	31.3 ±1.54	43	0.412	179±11[a]	425	168±12[b]	340	154±15[b]	43	**0.012**
e	31.9 ± 1.20	362	32.5 ± 1.19	369	33.7 ± 1.37	77	0.079	170±12[a]	362	173±12[a]	369	189±13[b]	77	0.069

[1]*P* value associated with intron and 3'UTR variants being assessed. Estimated marginal means and *P* values were derived from GLMMs with variant copy number, gender and birth rank fitted as fixed factors and sire-line fitted as a random factor ($P < 0.050$ in **bold**).
[2]Means within rows with superscripts in common are not significantly different (multiple pair-wise comparisons with a Bonferroni correction, $P > 0.050$).

Table 3.4 Association between ovine *ADRB3* intron, 3'UTR genotypes and key growth traits in the NZ Suffolk sheep

Genotype	n	Weaning-weight (kg) Marginal Mean[1, 2] ± Std Error	Post-Weaning Growth-rate (g /day) Marginal Mean[1, 2] ± Std Error
Intron			
AA	86	32.4 ± 1.36^{ac}	168 ± 13^{ef}
AB	148	31.2 ± 1.31^{a}	166 ± 12^{ef}
AC	83	32.0 ± 1.40^{ac}	181 ± 13^{e}
AE	99	30.7 ± 1.35^{a}	180 ± 13^{e}
AF	54	31.3 ± 1.43^{ac}	177 ± 14^{ef}
BB	41	31.2 ± 1.56^{ac}	155 ± 15^{f}
BC	63	32.0 ± 1.41^{abc}	185 ± 13^{e}
BE	86	32.6 ± 1.40^{ac}	165 ± 13^{ef}
BF	43	33.4 ± 1.55^{bc}	162 ± 15^{ef}
CE	47	36.1 ± 1.51^{b}	185 ± 14^{e}
3'UTR			
ab	38	31.9 ± 1.49^{cd}	191 ±14^{eh}
ac	38	32.1 ±1.51^{bcd}	183 ± 14^{efh}
ae	45	35.2 ±1.47^{a}	175 ± 14^{efgh}
bb	76	32.3 ± 1.34^{cd}	168 ± 13^{fg}
bc	129	31.2 ± 1.29^{d}	162 ± 12^{fg}
be	164	31.1 ± 1.25^{cd}	175 ± 12^{efgh}
cc	42	31.3 ± 1.53^{d}	154 ± 15^{g}
ce	151	32.9 ± 1.26^{c}	169 ± 12^{fg}
ee	75	33.9 ± 1.36^{ab}	190 ± 13^{h}

[1]Estimated marginal means were derived from GLMMs with genotype, gender and birth rank fitted as fixed factors and sire-line fitted as a random factor.
[2] Means in each column for both intron and 3'UTR variants with superscripts in common are not significantly different (multiple pair-wise comparisons with a Bonferroni correction, $P > 0.050$).

Table 3.5 Association between ovine *ADRB3* intron, 3'UTR variants and selected body composition traits in the NZ Suffolk sheep

Trait[2]	Variant being assessed	Marginal Mean [1] ± Std Error				*P* value[1]
		Variant absent	n	Variant present	n	
EMD (mm)	*A*	30.4 ± 0.6	116	29.7 ± 0.5	109	0.101
	B	30.0 ± 0.5	124	30.0 ± 0.6	101	0.972
	C	30.0 ± 0.5	153	30.6 ± 0.6	72	0.072
	E	30.0 ± 0.6	156	30.1 ± 0.5	69	0.632
	F	30.0 ± 0.5	173	30.2 ± 0.6	52	0.616
	a	30.0 ± 0.5	193	30.8 ± 0.7	32	0.160
	b	30.4 ± 0.5	116	30.0 ± 0.5	109	0.101
	c	30.3 ± 0.5	124	30.0 ± 0.6	101	0.972
	e	29.6 ± 0.6	83	30.2 ± 0.5	142	0.135
EMW (mm)	*A*	75.8 ± 0.8	116	75.2 ± 0.8	109	0.415
	B	75.5 ± 0.8	124	75.5 ± 0.9	101	0.940
	C	75.4 ± 0.8	153	75.8 ± 0.9	72	0.578
	E	75.4 ± 0.8	156	75.6 ± 0.8	69	0.749
	F	75.4 ± 0.8	173	75.7 ± 0.9	52	0.683
	a	75.4 ± 0.8	193	75.8 ± 1.1	32	0.670
	b	75.8 ± 0.8	116	75.2 ± 0.8	109	0.415
	c	75.5 ± 0.8	124	75.5 ± 0.9	101	0.940
	e	75.2 ± 0.9	83	75.6 ± 0.8	142	0.480
FDM (mm)	*A*	3.4 ± 0.2	116	3.4 ± 0.1	109	0.911
	B	3.3 ± 0.1	124	3.4 ± 0.2	101	0.800
	C	3.3 ± 0.1	153	3.5 ± 0.2	72	**0.033**
	E	3.4 ± 0.2	156	3.3 ± 0.2	69	0.898
	F	3.4 ± 0.1	173	3.3 ± 0.2	52	0.403
	a	3.3 ± 0.1	193	3.6 ± 0.2	32	0.084
	b	3.4 ± 0.2	116	3.3 ± 0.1	109	0.911
	c	3.3 ± 0.1	124	3.4 ± 0.2	101	0.800
	e	3.3 ± 0.2	83	3.4 ± 0.1	142	0.517

[1]*P* value associated with intron and 3'UTR variants being assessed. Estimated marginal means and *P* values were derived from GLMMs with variant copy number, gender and birth rank fitted as fixed factors and sire-line fitted as a random factor ($P < 0.050$ in **bold**).
[2]EMD: Eye Muscle Depth; EMW: Eye Muscle Width; FDM: Fat Depth above the eye Muscle.

Table 3.6 Association between ovine *ADRB3* intron, 3'UTR genotypes and Fat Depth above the eye Muscle (FDM) in the NZ Suffolk sheep

Genotype	n	Fat Depth above the eye Muscle (FDM) (mm) Marginal Mean [1, 2] ± Std Error
Intron		
AA	26	2.8 ± 0.2^{a}
AB	27	3.2 ± 0.2^{ab}
AC	19	4.0 ± 0.2^{b}
AE	21	3.3 ± 0.2^{ab}
AF	16	3.2 ± 0.2^{ab}
BC	25	3.5 ± 0.2^{b}
BE	17	3.6 ± 0.2^{b}
BF	22	3.1 ± 0.2^{ab}
CE	17	3.0 ± 0.2^{a}
3'UTR		
ac	13	3.5 ± 0.3^{d}
ae	12	3.2 ± 0.3^{de}
bb	26	2.9 ± 0.2^{e}
bc	27	3.1 ± 0.2^{de}
be	49	3.5 ± 0.2^{d}
ce	51	3.3 ± 0.2^{de}
ee	30	3.2 ± 0.2^{de}

[1]Estimated marginal means were derived from GLMMs with genotype, gender and birth rank fitted as fixed factors and sire-line fitted as a random factor.
[2]Means in each column with superscripts in common are not significantly different (multiple pair-wise comparisons with a Bonferroni correction, $P > 0.050$).

Haplotypes of ovine ADRB3

In the 140 Merino sheep investigated, sixteen intron-3'UTR haplotypes of ovine *ADRB3* were deduced: *A-a, A-b, A-c, B-c, C-a, C-e, D-d, E-e, F-e, G-f, H-f, I-a, J-f, K-e, L-e and M-f* (Figure 3.4).

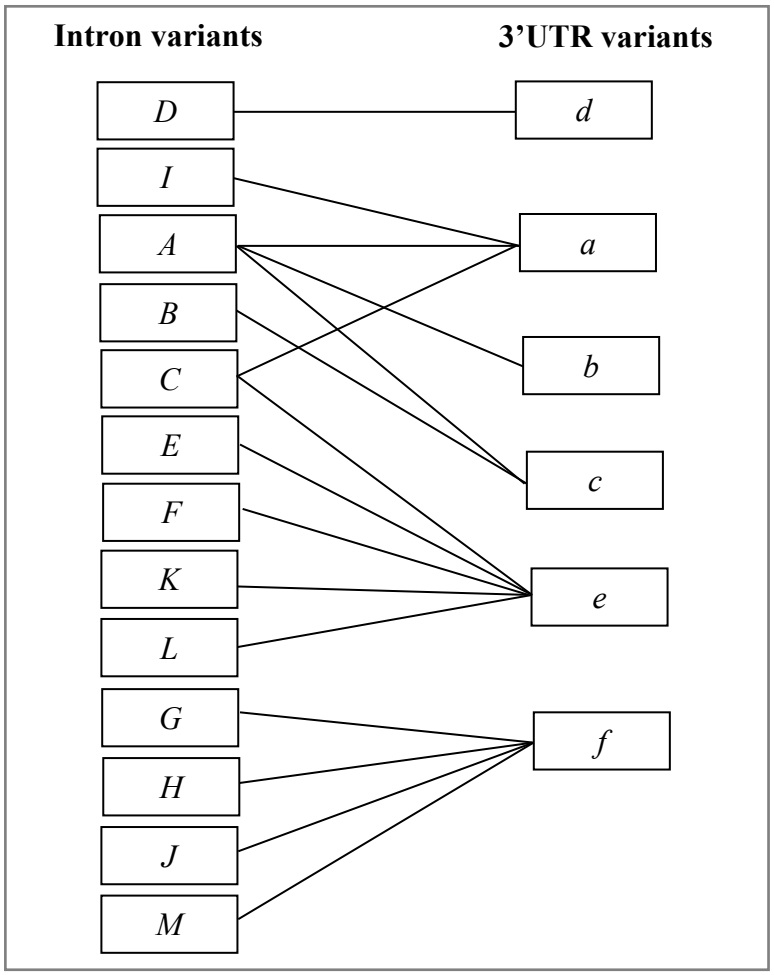


Figure 3.4 Diagram of sixteen potential haplotypes within ovine *ADRB3*

Frequencies of the ovine *ADRB3* haplotypes and diplotypes in the NZ Suffolk Sheep

In the 808 Suffolk sheep investigated, seven *ADRB3* haplotypes were found and these were composed of the five previously reported intron variants (*A*, *B*, *C*, *E* and *F*; GenBank accession numbers: AF314200-AF314202, AF314204 and AF314205, respectively) and four 3'UTR variants (*a*, *b*, *c* and *e*; GenBank accession numbers: HM776668-HM776670 and HM776672, respectively). The frequencies of the individual haplotypes ranged from 0.8% to 32.5% and various diplotypes ranged from 0.1% to 17.6% (Table 3.7 and Table 3.10, respectively).

Association between ovine *ADRB3* haplotypes and weaning-weights in the NZ Suffolk sheep

In the single-haplotype models, the presence of haplotype *A-b* was associated with lower weaning-weight ($P = 0.001$, Table 3.7). The associations between haplotype *A-b* and weaning-weight persisted in the multi-haplotype models ($P = 0.003$), with *C-a* and *F-e* fitted as fixed factors (Table 3.7). No associations of the other haplotypes and variation in weaning-weights were detected in the multi-haplotype model (Table 3.7).

The frequency of the haplotype *A-b, B-c* and *E-e* homozygous form was greater than 5% and so additive, dominant and recessive effects were assessed. Lambs possessing the heterozygous *A-b* haplotype were found to have a lower mean weaning-weight than those that did not have haplotype *A-b* in their diplotype ($P < 0.050$, Table 3.8).

Those marginal means for weaning-weights are listed in Table 3.10 for each diplotype occurred at a frequency of greater than 5%. Lambs with the *B-c/F-e* diplotype had a higher mean weaning-weight than those with the diplotype of *A-b/B-c* ($P < 0.050$) and *A-b/E-e* ($P < 0.050$). No other significant difference between diplotypes were detected (Table 3.9).

Table 3.7 Association between *ADRB3* haplotypes, weaning-weights and post-weaning growth rates in the NZ Suffolk sheep

Haplotype	Frequency (%)	Weaning-weight (kg) Marginal Mean [1] ± Std Error				*P* value[1]	Post-weaning Growth-rate (g/day) Marginal Mean [1] ± Std Error				*P* value[1]
		Absent	n	Present	n		Absent	n	Present	n	
Single haplotype model											
A-b	32.5	33.2 ± 1.19	367	31.5 ± 1.20	441	**0.001**	176 ± 12	367	172 ± 12	441	0.414
A-c	0.8	32.4 ± 1.17	796	30.7 ± 2.20	12	0.366	174 ± 11	796	185 ± 22	12	0.271
B-c	26.0	32.6 ± 1.19	433	31.1 ± 1.20	365	0.221	179 ± 11	433	165 ± 12	365	**0.002**
C-a	9.1	32.2 ± 1.18	669	33.3 ± 1.27	139	0.064	171 ± 11	669	186 ± 12	139	**0.008**
C-e	4.8	32.3 ± 1.18	729	33.5 ± 1.36	79	0.129	173 ± 11	729	183 ± 13	79	0.167
E-e	18.3	31.1 ± 1.19	471	32.9 ± 1.21	337	0.082	173 ± 12	471	176 ± 12	337	0.556
F-e	8.5	32.5 ± 1.18	756	31.8 ± 1.41	52	0.413	173 ± 11	756	178 ± 14	52	0.612
Multi-haplotype model (A-b, C-a and E-e fitted as fixed factors for weaning-weight analysis; B-c, C-a and C-e fitted as fixed factors for post-growth rate analysis)											
A-b	32.5	33.3 ± 1.25	367	31.7 ± 1.26	441	**0.003**	Not in model				
C-a	9.1	32.1 ± 1.21	669	32.8 ± 1.31	139	0.265	173±12	669	187±13	139	**0.020**
E-e	8.5	32.6 ± 1.19	756	32.4 ± 1.42	52	0.802	Not in model				
B-c	26.0	Not in model					186±12	433	175±12	365	**0.017**
C-e	4.8	Not in model					176±11	729	185±14	79	0.257

[1]*P* value associated with the haplotype being assessed. Estimated marginal means and *P* values were derived from GLMMs with haplotype presence/absence, gender and birth rank fitted as fixed factors and sire-line fitted as a random factor ($P < 0.050$ in **bold**).

Table 3.8 Association between ovine *ADRB3* haplotype copy numbers and weaning-weights and post-weaning growth-rates in the NZ Suffolk sheep

Haplotype	Weaning-weight Marginal Mean [1, 2] ± Std Error (kg)						*P* value[1]	Post-Growth-rate Marginal Mean [1, 2] ± Std Error (g /day)						*P* value[1]
	Absent	n	1 copy	n	2 copies	n		Absent	n	1 copy	n	2 copies	n	
A-b	33.2 ± 1.19[a]	362	31.2 ± 1.20[b]	355	32.4 ± 1.34[ab]	81	**0.001**	176 ± 12	362	172 ± 12	355	168 ± 13	81	0.622
B-c	32.7 ± 1.19	433	32.1 ± 1.21	326	31.4 ± 1.55	39	0.368	180 ± 11[a]	433	167 ± 12[b]	326	154 ± 15[b]	39	**0.005**
E-e	32.1 ± 1.19	461	33.0 ± 1.21	313	31.4 ± 1.77	24	0.107	173 ± 12	461	175 ± 12	313	178 ± 17	24	0.823

[1]*P* value associated with the haplotype being assessed. Estimated marginal means and *P* values were derived from GLMMs with haplotype copy number, gender and birth rank fitted as fixed factors and sire-line fitted as a random factor ($P < 0.050$ in **bold**).
[2]Means within rows with superscripts in common are not significantly different (Pair-wise comparisons with Bonferroni corrections, $P > 0.050$).

Table 3.9 Association between ovine *ADRB3* diplotypes and key growth traits in the NZ Suffolk sheep

Diplotype	n	Frequency (%)	Weaning-weight (kg) Marginal Mean[1, 2] ± Std Error	Post-weaning Growth-rate (g /day) Marginal Mean[1, 2] ± Std Error
A-b/A-b	81	10.3	32.6 ± 1.45^{ab}	172 ± 13^{de}
A-b/B-c	141	17.6	31.3 ± 1.39^{a}	169 ± 12^{de}
A-b/C-a	46	5.8	32.2 ± 1.60^{ab}	194 ± 14^{c}
A-b/E-e	88	11.0	30.8 ± 1.44^{a}	179 ± 13^{ce}
A-b/F-e	48	6.2	31.5 ± 1.52^{ab}	177 ± 14^{ce}
B-c/B-c	39	5.1	31.6 ± 1.66^{ab}	156 ± 15^{e}
B-c/C-a	41	5.0	31.4 ± 1.61^{ab}	186 ± 15^{c}
B-c/E-e	84	10.4	32.9 ± 1.50^{ab}	167 ± 13^{de}
B-c/F-e	39	4.9	33.7 ± 1.63^{b}	164 ± 15^{de}

[1]Estimated marginal means were derived from GLMMs with diplotype, gender and birth rank fitted as fixed factors and sire-line fitted as a random factor.
[2]Means in each column with superscripts in common are not significantly different (Pair-wise comparisons with Bonferroni corrections, $P > 0.050$).

Table 3.10 Association between ovine *ADRB3* diplotypes and Fat Depth above the eye Muscle (FDM) in the NZ Suffolk sheep

Diplotype	n	Fat Depth above the eye Muscle (FDM) (mm) Marginal Mean [1, 2] ± Std Error
A-b/A-b	26	2.8 ± 0.2[a]
A-b/B-c	27	3.1 ± 0.2[ac]
A-b/C-e	12	3.8 ± 0.3[bd]
A-b/E-e	21	3.2 ± 0.2[abc]
A-b/F-e	16	3.1 ± 0.3[ac]
B-c/C-a	13	3.7 ± 0.3[b]
B-c/C-e	12	3.4 ± 0.3[bc]
B-c/E-e	17	3.6 ± 0.3[b]
B-c/F-e	22	3.0 ± 0.2[ac]

[1]Estimated marginal means were derived from GLMMs with diplotype, gender and birth rank fitted as fixed factors and sire-line fitted as a random factor.
[2]Means in each column with superscripts in common are not significantly different (Pair-wise comparisons with a Bonferroni correction, $P > 0.050$).

Association between ovine *ADRB3* haplotypes and post-weaning growth-rates in the NZ Suffolk sheep

In the single-haplotype models, the presence of haplotype *C-a* was associated with higher post-weaning growth-rate ($P = 0.008$; Table 3.7) and the presence of the haplotype *B-c* was associated with lower post-weaning growth-rate ($P = 0.002$; Table 3.7). The associations between haplotypes *C-a*, *B-c* and post-weaning growth-rates persisted in the multi-haplotype models (*C-a*: $P = 0.020$ and *B-c*: $P = 0.017$, Table 3.7). No associations of the haplotypes *A-b*, *A-c*, *C-e*, *E-e* and *F-e* with variation in the post-weaning growth-rates were detected ($P > 0.050$, Table 3.7).

Lambs possessing the heterozygous and homozygous *B-c* haplotype were found to have lower mean post-weaning growth-rates than those that did not have haplotype *B-c* in their diplotype ($P < 0.050$, Table 3.8).

For those diplotype frequencies over 5%, lambs (Table 3.9) with the *A-b/C-a* diplotype had a higher mean post-weaning growth-rate than those with the diplotypes of *A-b*/*A-b* ($P = 0.017$), *A-b/B-c* ($P = 0.003$), *B-c/B-c* ($P < 0.001$), *B-c/E-e* ($P = 0.003$) and *B-c/F-e* ($P = 0.003$). Lambs with the *B-c/B-c* diplotype had a lower mean post-weaning growth-rate than *A-b/C-a* ($P < 0.001$) and *B-c/C-a* ($P = 0.003$).

Association between ovine *ADRB3* haplotypes and selected body composition traits in the NZ Suffolk sheep

Of 225 Suffolk sheep analysed, no association ($P > 0.050$) was found between *ADRB3* haplotypes and EMD, EMW and FDM.

Those marginal means for FDM for each diplotype occurred at a frequency of greater than 5% were calculated (Table 3.10). Those animals with the *A-b/A-b* diplotype had lower FDM than those with *A-b/C-e* ($P < 0.001$), *B-c/C-a* ($P = 0.006$), *B-c/C-e* ($P = 0.047$) and *B-c/E-e* ($P = 0.006$). Those animals with the *A-b/C-e* diplotype had higher FDM than those with *A-b/B-c* ($P = 0.009$), *A-b/F-e* ($P = 0.014$) and *B-c/F-e* ($P = 0.003$). Those animals with the *B-c/F-e* diplotype had FDM lower than *B-c/C-a* ($P = 0.031$) and *B-c/E-e* ($P = 0.038$).

No association ($P > 0.050$) was detected between *ADRB3* diplotypes and EMD, and EMW.

3.4 Discussion

Association between intron, 3'UTR variant and selected traits

In this chapter, variation in both the intron and the 3'UTR of ovine *ADRB3* was found to be associated with weaning-weight and post-weaning growth in the NZ Suffolk sheep. The 3'UTR variant *a* was associated with higher weaning-weight and post-weaning growth-rate while variant *b* was associated with lower weaning-weight. Similarly, the 3'UTR variant *c* also tended to be associated with lower weaning-weight and was significantly associated with reduced a post-weaning growth-rate.

The presence of intron variant *C* was associated with higher mean weaning-weight and post-weaning growth-rate. Variant *A* was associated with lower mean weaning-weight. Variant *B* was associated with lower mean post-weaning growth-rate. This appears to contrast half-sib analyses in NZ Merino (Forrest et al., 2003) and NZ Romney (Horrell et al., 2009) sheep where progeny inheriting *A* had significantly higher weaning-weight than their littermates inheriting *C*. However, the frequency of *A* (33.1%) was notably lower in the NZ Suffolk sheep, when compared to its frequency in NZ Romney (73.9%) (Horrell et al., 2009) and NZ Merino cross-bred sheep (60%) (Byun et al., 2008). Forrest *et al.,* (2003) speculated that the *A* variant probably represented more than one variant. This was subsequently confirmed by identification of three intron-3'UTR haplotypes (*A-a*, *A-b* and *A-c*) associated with *A* in the NZ Merino sheep. Therefore, it is conceivable that the growth traits association previously seen with the Merino and Romney sheep is the result of a different intron *A*-3'UTR haplotype than that present in the Suffolk population and/or the effect is moderated by some other genetic influence or environment effects.

Extended haplotypes of ovine *ADRB3*

Variation was identified in the 3'UTR and intron of ovine *ADRB3*. Sixteen haplotypes were deduced in the sheep studied based on the variation in the 3'UTR and intron region of gene. This extended variation is of note as the *ADRBs* have often been thought of as "house-keeping genes".

It is notable that the previously described ovine *ADRB3* variant *D*, which has been associated with reduced cold-tolerance in lambs (Forrest et al., 2006), also appears to have a unique sequence in the 3'UTR (designated *d*), that is not associated with any of the other intron variants studied. Given that the variation in this *D-d* haplotype may alter the binding of ADRB3 with hormones and association between the intron variant *D* with decreased cold-

survival, it suggests that the *D-d* haplotype may be also associated with lamb survival traits. This needs further work to confirm.

The majority of the sequence variation within ovine *ADRB3* has been reported to be silent (Byun et al., 2008; Forrest et al., 2003; Yang et al., 2011). However, silent variation within genes may be in linkage disequilibrium with other variation in the gene (or flanking the gene), and this may affect gene expression or protein structure and function. Recently, it has also been suggested that naturally occurring silent variation within non-coding sequences can affect *in vivo* protein folding, and consequently function. This silent variation may create codons in the mRNA for which tRNAs are less available and this may slow the rate of translation and protein function (Kimchi-Sarfaty et al., 2007; Komar, 2007). This would require further functional studies with *ADRB3* to be undertaken to see whether an effect is observed.

Association of ovine *ADRB3* haplotypes and key growth traits in the NZ Suffolk Sheep

This study suggests that ovine *ADRB3* has a role in the variation of the growth traits which may be of economic importance to sheep farmers and provides a further insight into the association between ovine *ADRB3* and growth.

In total, seven previously defined haplotypes were detected, in the NZ Suffolk lambs studied. This is somewhat less than the sixteen haplotypes described in the NZ Merino sheep breed. Furthermore, the "cold mortality-associated" haplotype *D-d* (Forrest et al., 2007) described previously, was not found in these NZ Suffolk lambs.

This lower level of *ADRB3* diversity in the NZ Suffolk sheep compared to the NZ Merino sheep may be because the NZ Suffolk sheep have historically been selected for growth and survival (Barwick et al., 1990) and thus they may contain fewer "negative growth-associated" haplotypes.

It is notable that haplotype *C-a*, which was associated with higher post-weaning growth, has a 3'UTR deletion [*257 delA or loss of an adenine at position 1603 of the ovine *ADRB3* sequence (GenBank accession number: AF314200)]. Whether this deletion or its associated variation in the gene affects the binding of a microRNA, or in some other way affects mRNA stability, however, is still unknown.

However, of the 808 NZ Suffolk sheep analysed, the frequency of the higher growth-rate associated haplotype *C-a* was considerably lower (9.1%) than other common haplotypes such as *A-b* (32.5%) and *B-c* (26.0%). Having diplotype *A-b/C-a* could notionally increase post-

weaning growth-rate by up to 28.8 ± 9.1% relative to having diplotype *B-c*/*B-c* (estimated from Table 3.9).

Interestingly, intron variant *C* has previously been associated with higher birth weight in both NZ Merino-cross (× Coopworth and Dorset Down) sheep (Forrest et al., 2003) and NZ Romney sheep (Horrell et al., 2009). It has also been associated with decreased perinatal survival across several breeds (Forrest et al., 2007). The lower frequency of *C-a* in the NZ Suffolk sheep may therefore indicate that the haplotype may be associated with compromised perinatal survival in this breed as well, and has been selected against by ongoing selection for improved survival.

Given that the overall frequencies of the *A*-3'UTR haplotypes (33.3%, sum of frequency of *A-a*: 0%, *A-b*: 32.5% and *A-c*: 0.8%) was notably lower in the NZ Suffolk sheep than in the NZ Romney sheep (73.9%) (Horrell et al., 2009) and the NZ Merino sheep (60%) (Byun et al., 2008), it is conceivable that the previous association of intron variant *A* with higher pre-weaning growth-rate found in NZ Romney and NZ Merino sheep may because of higher frequency of either or both of haplotype *A-a* or *A-c*; haplotypes that either were not found at all in the NZ Suffolk sheep (*A-a*) or that are only found at a very low frequency (*A-c*). Further studies will be needed to confirm these associations, to examine whether any of the *ADRB3* haplotypes such as *A-b* and *A-c* are breed-specific or sire-specific haplotypes and to ascertain if extended variation and haplotypes can be identified in more sheep breeds.

Association of ovine *ADRB3* haplotypes and selected body composition traits in the NZ Suffolk Sheep

The *ADRB3* variants and haplotypes were found to not strongly be associated with the body composition traits EMD, EMW and FDM. Variant *C* was found to be associated with higher FDM. Given that *C* was associated with higher wool staple strength (Forrest et al., 2009), it is suggested that this variant may be associated with varied *ADRB3* function..

In NZ, variation in ovine *ADRB3* is currently used in marker-assisted selection for improved cold tolerance. The finding of extended *ADRB3* haplotypes and their association with post-weaning growth and body composition traits obtained in this work might allow for further development of this breeding technology.

Chapter 4
Variation in the ovine Hormone-Sensitive Lipase gene (*HSL*) and its association with growth and body composition traits

4.1 Introduction

Hormone-Sensitive Lipase (HSL) is a multifunctional enzyme involved in many metabolic processes including lipolysis, FFA mobilisation, BAT-mediated thermogenesis, steroidogenesis and male spermatogenesis (Osuga et al., 2000). It is predominantly found in WAT and BAT, but also can be found in several non-adipose tissues such as skeletal muscle and the testis (Liu et al., 1995). The lipolytic activity of HSL is under the hormonal control of catecholamine and insulin (Anthonsen et al., 1998; Frayn et al., 1995; Holm, 2003; Holm et al., 1988; Schwartz and Jungas, 1971; Yeaman, 1990).

The HSL protein

Protein structure of HSL contains at least four functional domains: an NH_2-terminus, a catalytic domain, a regulatory domain and a potential lipid-binding domain (Figure 4.1) (Contreras et al., 1996). In the regulatory domain of rat HSL, two phosphorylation sites named site 1 and site 2 have been identified (Smith et al., 1996). The phosphorylation of site 2 can cause conformational changes in HSL that renders the regulatory domain susceptible to proteolysis.

In the COOH-terminus, a catalytic triad and a regulatory module (Figure 4.1) are thought to be important for the activity of the lipase. The catalytic triad in rat HSL involves three residues: a serine residue at amino acid position 423 (Ser-423), an aspartate residue at amino acid position 703 (Asp-703) and a histidine residue at amino acid position 733 (His-733). This catalytic triad is responsible for the hydrolysis of ester bonds in TAG (Marchler-Bauer et al., 2011).

In the catalytic domain, a "GXSXG" motif is highly conserved in almost all lipases and is considered the catalytic core of the enzyme (Holm et al., 1994; Tsujita et al., 1989). In rat HSL, the substitution of Ser-423 in the "GXSXG" motif, can lead to the complete ablation of esterase and lipase activity (Holm et al., 1994).

Additionally, two regions (aa 1-300 and aa 300-767) of rat HSL are able to interact with the full length HSL peptide. This suggests that a "nose to tail" structure may be involved in the regulation of HSL activity (Yeaman, 2004).

Protein of HSL has different forms in different tissues, and they are generated by alternative exons usage in the HSL gene (*HSL*) (Figure 4.2). In humans, at least six HSL forms have been identified to date. One adipocyte HSL form is encoded by a short non-coding exon named exon B located 1.5 kb upstream of exon 1, and the second adipocyte HSL form is produced by a further alternative splicing of the HSL pre-mRNA that skips over exon 6 and generates a catalytically inactive, but phosphorylatable form. Two different testicular HSL forms are encoded by exon T1 and exon T2 respectively, and the two HSL forms found in the adenocarcinoma cell line HT29, contain different 5' ends, and are encoded by exon A which is located approximately 12.5 kb upstream of exon 1 (Blaise et al., 1999; Blaise et al., 2001; Grober et al., 1997; Langin et al., 1993).

To date, our knowledge of ovine HSL is poor. Recently, two different ovine *HSL* forms (*ovHSL-A* and *ovHSL-B*) have been identified (Lampidonis et al., 2008). These two forms are homologous in their Open Reading Frames (ORFs) of 2.089 Kb and 2.086 Kb respectively, with *ovHSL-B* lacking the 688th triplet coding for glutamine (ΔQ688) (Lampidonis et al., 2008). Both *ovHSL-A* and *ovHSL-B* exhibit homology with *HSL* from cattle (88%), humans (76%), mice (74%), rats (76%) and domestic pigs (79%) (Lampidonis et al., 2011). This homology among the mammalian *HSL*s suggests functional constraint over evolutionary time.

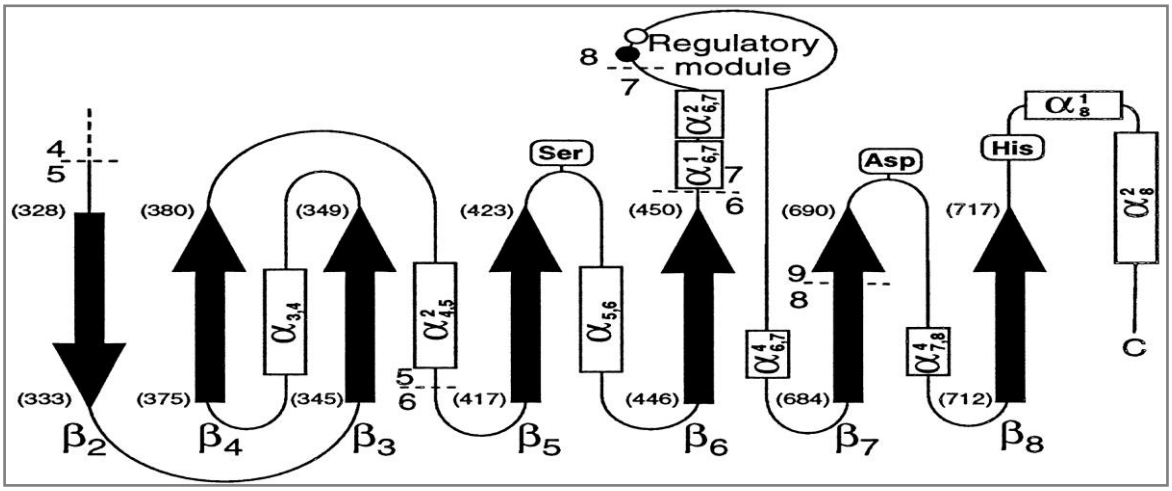


Figure 4.1 The structure of human Hormone-Sensitive Lipase (HSL). The exon limits are indicated with dashed lines and the numbers of the corresponding exons. Numbers in parentheses indicate the residue position (in the human HSL sequence) of the N- and C-terminal residues of each β-strand. The catalytic triad is shown, and the phosphorylation sites are also indicated. (Adapted from Contreras *et al.,* 1996).

The HSL gene (*HSL*)

The HSL gene (*HSL*) has been mapped to chromosome 19 in humans (Holm et al., 1988; Levitt et al., 1995), chromosome 7 in rats (Wang et al., 1994), chromosome 6 in domestic pigs (Gu et al., 1992), chromosome 18 in cattle (Cordle et al., 1986; Yonezawa et al., 2008) and chromosome 14 in sheep (GenBank Gene ID: 100169699).

HSL is a "mosaic gene", in which every exon encodes a distinct domain (Langin et al., 1993). Human *HSL* is comprised of nine exons that are interrupted by eight introns (Figure 4.2). Exon 6 encodes the catalytic serine motif (GXSXG), exon 8 encodes the two serine phosphorylation sites and exon 9 encodes a hydrophobic region containing the putative lipid-binding region (Holm et al., 1994).

Human *HSL* lacks a "TATA-box" within its 5'-regulatory region (proximal promoter) (Grober et al., 1997). However, an AT-rich "TTTAT" sequence is found in the promoter of mouse *HSL* (Laurin et al., 2000; Sztrolovics et al., 1997). This "non-canonical" TATA-box has also been reported in the porcine *HSL* promoter, where two "TATA-like" sequences and a reverse "CCAAT-box" have been described (Harbitz et al., 1999).

Ovine *HSL* does not appear to have a "TATA-box" within the regulatory region (Lampidonis et al., 2008). Instead, several *cis*-regulatory elements have been described, including binding sites for Stimulating Protein 1 (SP1), "CCAAT-box" Binding Factors (CBFs) and other *trans*-acting proteins (Lampidonis et al., 2008; Smale, 1997).

The full genome sequence of ovine *HSL* is still unknown, although the 5'regulatory region and the cDNA sequence of ovine *HSL* have been cloned and sequenced (Lampidonis et al., 2008). In contrast to bovine *HSL* which encodes a polypeptide of 756 amino acids (Langin et al., 1993), the putative ovine *HSL* polypeptide has been found to be shorter and in two forms of 695 and 694 amino acids length, encoded by *ovHSL-A* (GenBank accession number: DQ647325) and *ovHSL-B* (GenBank accession number: DQ647326), respectively (Lampidonis et al., 2008).

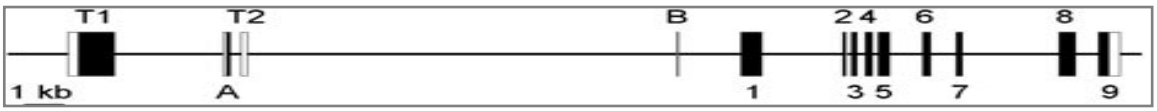


Figure 4.2 Genomic organisation of human Hormone-Sensitive Lipase gene (*HSL*). Coding sequences are shown as filled boxes and untranslated regions as open boxes. Exons T1 and T2 are used in testis. Exons A and B are used in adenocarcinoma cell line HT29 and adipose tissue, respectively. Exons 1 to 10 are used in all tissues expressing HSL. (Adapted from Osuga *et al.,* 2000).

HSL "knockout" mouse phenotype

HSL "knockout" mice have decreased circulating fatty acid levels and altered TAG storage in adipose tissues. This results in impaired lipid and glucose metabolism (Fortier et al., 2004; Haemmerle et al., 2002; Osuga et al., 2000; Voshol et al., 2003). In addition, these mice also have a reduced quantity of WAT and diminished expression of lipolytic associated proteins, but a significantly enlarged BAT reservoir and an increased number of macrophages in their WAT (Kraemer and Shen, 2006). The *HSL* knockouts are found to be resistant to high-fat diet induced obesity, have increased thermogenesis and increased expression of many adipose specific proteins (Kraemer and Shen, 2006). A recent study demonstrated *HSL* knockout mice also have increased growth-rates and higher bone densities during aging compared to normal mice (Shen et al., 2011). In addition, male knockout mice are sterile, due to oligospermia (Osuga et al., 2000). These knockout studies reinforce the important role of HSL in fat metabolism and growth.

Association between variation in *HSL* and variation in phenotypic traits

In humans, several variant forms of *HSL* have been described and associated with obesity (Hoffstedt et al., 2001; Klannemark et al., 1998; Lavebratt et al., 2002; Magre et al., 1998; Nieters et al., 2002; Qi et al., 2004). A nucleotide substitution in the *HSL* promoter (-60C/G) has been reported to be associated with altered *HSL* promoter activity *in vitro* (Talmud et al., 1998). The rare *G* variant of this polymorphism has been associated with increased insulin sensitivity in women, decreased FFA levels in men and increased waist circumference in non-obese subjects (Carlsson et al., 2006). A recent study reported that this polymorphism is also associated with infertility in men (Vatannejad et al., 2011).

In dairy goats, variation in *HSL* has been associated with various milk traits including milk yield, C18:3 n-6g content, trans-10 cis-12 Conjugated Linoleic Acid (CLA) content and C12:0 milk content (Zidi et al., 2010). These results suggest that HSL may play an important role in the mobilisation of medium-chain and unsaturated fatty acids.

In pigs, polymorphism in exon I of porcine *HSL* has been reported and associated with variation in Eye Muscle Area (EMA) (Knoll et al., 1998).

To date, little is known about variation in ovine *HSL*, or of any association with phenotypic traits. A recent study reported the mRNA level of *HSL* is negatively related to the amount of Intra-Muscular Fat (IMF) in Kazak and Xinjiang fine wool sheep (Qiao et al., 2007).

Given the important role that HSL plays in fat metabolism, this suggests that variation within ovine *HSL* may cause variation in the function of the lipase and result in variation in growth or body composition traits in sheep. In this chapter, three potentially important regions of *HSL* (exon 3-4, encoding part of the NH_2-terminus; exon 5-6, encoding part of the catalytic region; and exon 9, encoding part of the COOH-terminus) were investigated to see nucleotide variation existed and whether variation if found was associated with post-weaning growth and body composition traits in NZ Suffolk sheep.

4.2 Materials and methods

Suffolk Sheep analysed and blood collection

538 NZ Suffolk lambs from 13 sires from different farms in NZ were investigated. The process of lamb weight recording is described in Chapter 3.

262 Suffolk ram lambs were randomly chosen at 6-8 months of age for ultrasound scanning to estimate body composition following standard guidelines. Traits measured included Eye Muscle Width (EMW), Eye Muscle Depth (EMD) and Fat Depth above the Eye Muscle (FDM).

DNA purification

See chapter 3.

PCR amplification, SSCP analysis of exon 3-4, exon 5-6 and exon 9 of ovine *HSL*

Three regions of ovine *HSL* were investigated in this study: region 1 is approximately 261 bp in length covering a portion of exon 3, all of intron 3 and a portion of exon 4, region 2 is approximately 351 bp in length covering a portion of exon 5, all of intron 5 and a portion of exon 6 and region 3 is approximately 385 bp in length covering a portion of intron 8 and a portion of exon 9. Three pairs of primers for amplifying these regions were designed based on the published bovine *HSL* gene sequence (ENSBTAT00000043890) (Figure 4.3).

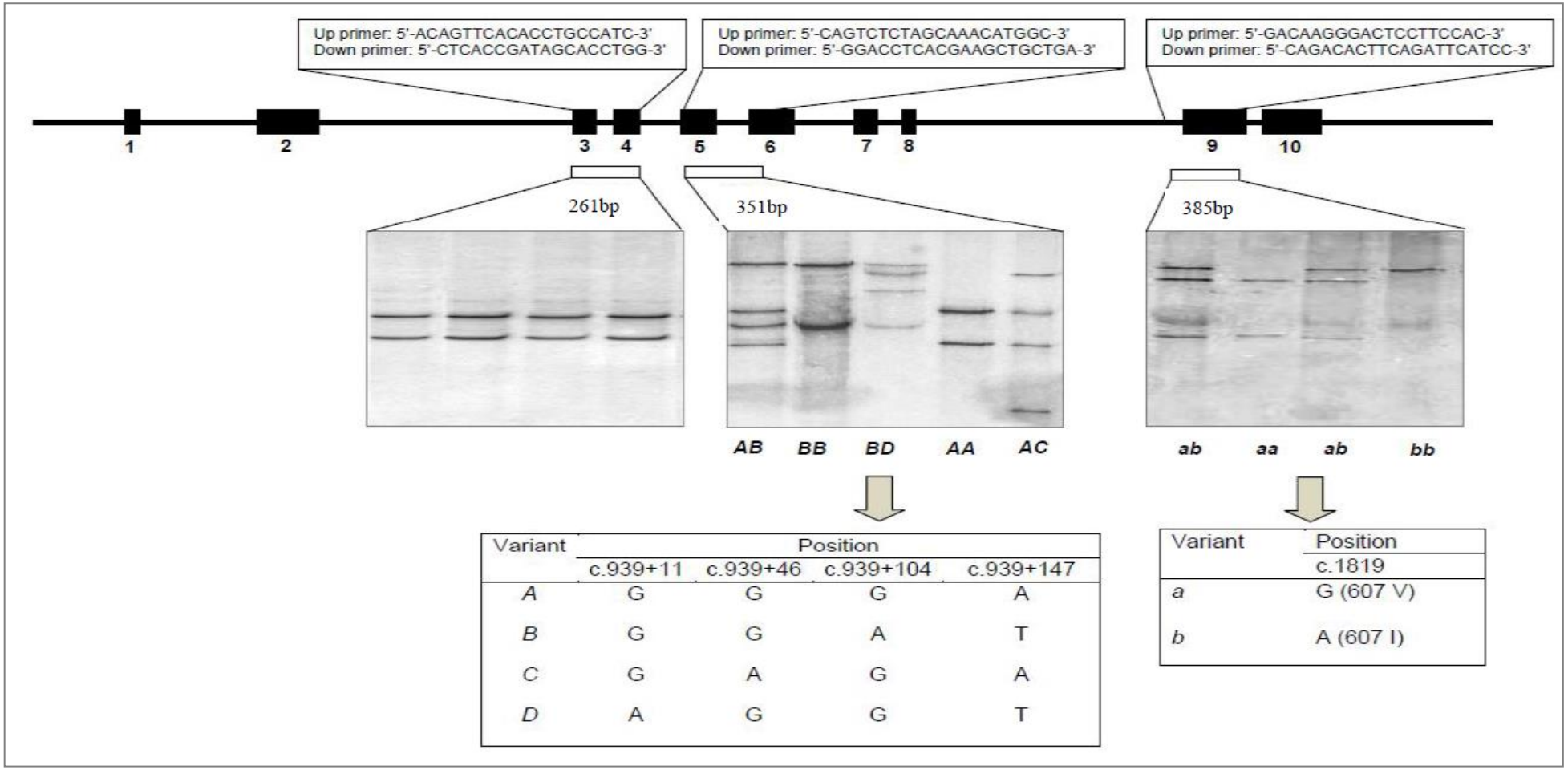


Figure 4.3 The PCR-SSCP patterns and single nucleotide variations detected in three regions of ovine *HSL*. The three regions of ovine *HSL* amplified are shown. The exons of ovine *HSL* are shown as black boxes with their numbers under the boxes. The PCR-SSCP pattern names are shown in bold italic. At times weaker bands are observed on the gels and these likely represent other less-stable conformers. The positions of the nucleotide substitutions in intron 5 and exon 9 are described relative to the ovine *HSL* coding sequence (GenBank accession number: DQ647326). The position of the putative amino acid substitution is defined relative to the amino acid sequence of ovine *HSL* (GenBank accession number: ABG49111.1), named according to sequence variation nomenclature (http://www.hgmd.cf.ac.uk/docs/mut_nom.html).

Amplifications were performed in a 15 µL reaction containing the DNA on one 1.2mm punch of FTA paper. 0.25µM of each primer, 150µM of each of dATP, dCTP, dGTP, and dTTP (Eppendorf, Hamburg, Germany), 2.5mM Mg^{2+}, 0.5 U Taq DNA polymerase (Qiagen, Hilden, Germany) and 1 × the reaction buffer supplied with the enzyme. The thermal profiles for the three regions amplified were composed of 2 min at 94°C, followed by 35 cycles of 30 s at 94°C, 30 s at an optimised annealing temperature for each amplicon (region 1 at 58°C, region 2 at 60°C and region 3 at 60°C) and 30 s at 72°C, with a final extension of 5 min at 72°C. Amplification was carried out in an iCycler (Bio-Rad Laboratories, Hercules, CA, USA). Amplicons were visualized by electrophoresis in 1% agarose (Quantum Scientific, Queensland, Australia) gels, using 1 × TBE buffer (89mM Tris, 89mM boric acid, 2mM Na_2EDTA) containing 200 ng/ml of ethidium bromide.

An aliquot of 0.7 µL of each amplicon was mixed with 7 µL of loading dye (98% formamide, 10mM EDTA, 0.025% bromophenol blue and 0.025% xylene-cyanol). After denaturing at 95ºC for 5 min, samples were cooled rapidly on wet ice and then 7.5 µL was loaded onto 16 × 18 cm, 14% acrylamide / bisacrylamide (37.5:1) (Bio-Rad) gels. Electrophoresis was performed using Protean II xi cells (Bio-Rad). The SSCP conditions were optimised for each amplicons. The amplicons of region 1, region 2 and region 3 were run at 250 V, 18°C; 280 V, 22°C; and 250 V, 25°C, respectively for 18 h in 0.5 × TBE buffer. Gels were silver-stained according to the method of Byun *et al.,* (2009).

Sequencing of the ovine *HSL* variants and sequence analysis

PCR amplicons representative of different PCR-SSCP patterns from sheep that were homozygous at *HSL* were directly sequenced at the Lincoln University DNA Sequencing Facility. For the variants that were only found in heterozygous sheep, a band corresponding to the rare allele was excised as a gel slice from the polyacrylamide gel, macerated, and then used as a template for re-amplification with the original primers. This produces a PCR-SSCP pattern equivalent to a sheep homozygous for that rare allele. This second amplicon was then directly sequenced. Sequence alignments, translations and comparisons were carried out using DNAMAN (version 5.2.10, Lynnon BioSoft, Canada). The BLAST algorithm was used to search the NCBI GenBank databases (http://blast.ncbi.nlm.nih.gov/) for homologous sequences.

Statistical analyses

All statistical analyses were performed using SPSS version 19 (SPSS Inc., Chicago, IL, USA). General Linear Mixed-effects Models (GLMMs) were used to assess the effects of the

presence/absence of a particular sequence variant in a lamb's genotype, on post-weaning growth-rate (n = 538 lambs), and various body composition traits (n = 262 ram lambs) including EMW, EMD and FDM. Gender (for growth-rate analysis only, as body composition traits were only assessed in ram lambs) and birth rank were fitted as fixed factors and sire was fitted as a random factor in order to correct for sire effects as well as farm effects such as management and environmental effects (each sire was used exclusively on one farm).

The generalized statistical model used to test the sequence variant (or genotype – see below) effects was as follows: $Y_{ijk} = \mu + \tau_i + \beta_j + \alpha_k + \epsilon_{ijk}$; where Y_{ijk} = traits evaluated on the i_{th} level of the fixed factor genotype (τ_i), the j_{th} level of the fixed factor birth rank (β_j) and the k_{th} sire of random effect (α_k); where μ = overall mean for each trait and ϵ_{ijk} is the random error for ijk.

Initially, single-variant models were performed to ascertain which variants should be included in subsequent multi-variant models, and such that we could assess whether the effect of any given variant was independent of the other variants. The multi-variant models included any variant that had associations with post-weaning growth-rate and body composition traits in the single-variant models with $P < 0.200$ (and which could thus potentially impact on the traits). For variants that had homozygous forms greater than 5% of all genotypes (thus providing an adequate sample size) a second set of analyses was performed, this time with the number of variant copies present in the animal's genotype included (i.e. 0, 1 or 2) in place of presence/absence of coding, followed by planned orthogonal contrasts to ascertain whether additive, dominant or recessive effects were present.

For genotypes with a frequency greater than 5% (thus having adequate sample size per group), a GLMM (fixed effect: genotype, birth rank and gender if appropriate; random effect: sire) and multiple pair-wise comparisons with Bonferroni corrections were used to ascertain the association between genotype and post-weaning growth-rate and body composition traits.

4.3 Results

Variation within ovine *HSL*

Of the 538 NZ Suffolk sheep investigated, four PCR-SSCP patterns were found for region 2 amplicons and two PCR-SSCP patterns for region 3 amplicons were observed (Figure 4.3). Only one PCR-SSCP banding pattern was observed for the amplicons from region 1 (exon 3-4) suggesting there was no sequence variation within this region of ovine *HSL* in the sheep studied. Further sequence analysis of this region was not undertaken.

After sequencing, these patterns were confirmed as novel variant sequences of ovine *HSL* and deposited into GenBank with accession numbers as follows: intron 5; *A*-*D*, GenBank accession numbers KC610083-KC610086, and exon 9; *a*-*b*, GenBank accession numbers KC585035-KC585036). The SNPs detected in the intron 5 and exon 9 regions of ovine *HSL* are shown in Figure 4.3.

Frequencies of the ovine *HSL* variants in NZ Suffolk sheep

In the 538 Suffolk sheep investigated, intron 5 genotypes *AA* (27.1%), *AB* (22.1%), *AC* (14.1%), *AD* (3.0%), *BB* (18.2%), *BC* (7.2%), *BD* (2.6%), *CC* (4.2%), *CD* (1.3%) and *DD* (0.2%) were found with individual variant frequencies of *A* (46.8%), *B* (34.3%), *C* (15.4%) and *D* (3.5%). In exon 9, genotypes *aa* (49.0%), *ab* (31.5%) and *bb* (19.5%) were found with individual variants frequencies of *a* (64.8%) and *b* (35.2%).

Association of ovine *HSL* variants with body composition traits in NZ Suffolk sheep

In the single-variant models, the presence of intron 5 *A* was associated with reduced EMW ($P = 0.031$). The presence of *C* was associated with increased EMW ($P = 0.006$), EMD ($P < 0.001$) and FDM ($P < 0.001$) (Table 4.1). The associations between *C*, and EMW and EMD persisted in the multi-variant models (Table 4.1).

The frequency of homozygous *AA* (27.1%) and *BB* (18.3%) sheep were greater than 5% and so additive, dominant and recessive effects were assessed for these variants (Table 4.2). In the single-variant models, lambs homozygous for *A* had lower EMW than those that did not have *A* in their genotype ($P = 0.014$). Similarly, lambs homozygous for *A* had lower EMD than those that did not have *A* in their genotype ($P = 0.047$) (Table 4.2). For both traits, the estimated marginal means for those animals heterozygous for variant *A* was intermediate to those homozygous for variant *A* and those without variant *A*, but it was not significantly different to either, thus additive effects could not be confirmed (Table 4.2).

The intron 5 genotypes *AA*, *AB*, *AC*, *BB* and *BC* occurred at a frequency of greater than 5% in the sheep studied. Marginal means for selected body composition traits for sheep possessing each genotype are given in Table 4.3. Sheep with the *AA* genotype had lower mean EMW than those with the *AC* ($P = 0.007$), and tended to have a lower mean EMW than those with the *BC* ($P = 0.098$) genotype (Table 4.3). Sheep with the *AC* genotype had higher mean EMW than those with the *AB* ($P = 0.013$) genotype. Similarly, sheep with the *AA* genotype had lower mean EMD than those with the *AC* ($P = 0.017$) (Table 4.3).

No significant association of ovine *HSL* intron 5 variants or genotypes with post-weaning growth-rate were detected ($P > 0.050$) and no associations between ovine *HSL* exon 9 variation and growth or body composition traits were detected ($P > 0.050$).

Table 4.1 Analysis of association between ovine *HSL* intron 5 variants and various body composition traits in NZ Suffolk sheep

Trait[2]	Variant being assessed	Other variants in model	Mean [1] ± Std Error				*P* value[1]
			Absent	n	Present	n	
EMD (mm)	*A*	None	30.8 ± 0.3	86	30.3 ± 0.2	176	0.128
	B	None	30.6± 0.3	132	30.3 ± 0.3	130	0.519
	C	None	29.9 ± 0.2	190	31.5 ± 0.3	72	**< 0.001**
	D	None	30.5 ± 0.2	255	31.2 ± 1.0	7	0.504
	A	*C*	30.7 ± 0.4	86	30.6 ± 0.3	176	0.712
	C	*A*	29.9 ± 0.3	190	31.5 ± 0.4	72	**< 0.001**
EMW (mm)	*A*	None	76.7 ± 0.6	86	75.3 ± 0.4	176	**0.031**
	B	None	75.9 ± 0.4	132	75.6 ± 0.5	130	0.609
	C	None	74.7 ± 0.4	190	77.7 ± 0.6	72	**< 0.001**
	D	None	75.8 ± 0.4	255	75.7 ± 1.8	7	0.983
	A	*C*	76.7 ± 0.5	86	75.9 ± 0.4	176	0.243
	C	*A*	74.9 ± 0.4	190	77.7 ± 0.6	72	**< 0.001**
FDM (mm)	*A*	None	3.5 ± 0.1	86	3.3 ± 0.1	176	0.228
	B	None	3.4 ± 0.1	132	3.3 ± 0.1	130	0.458
	C	None	3.3 ± 0.1	190	3.6 ± 0.1	72	**0.006**
	D	None	3.4 ± 0.7	255	3.1 ± 0.3	7	0.344

[1]*P* value associated with the variant being assessed. Estimated marginal means and *P* values were derived from GLMMs with variant presence/absence and birth rank fitted as fixed factors and sire-line fitted as a random factor ($P < 0.050$ in **bold**).
[2]EMD: Eye Muscle Depth; EMW: Eye Muscle Width; FDM: Fat Depth above the Eye Muscle.

Table 4.2 Association between the copy numbers of ovine *HSL* intron 5 variants and various body composition traits in the NZ Suffolk sheep

Trait	Variant being assessed	Other variants in model	Mean [1, 2] ± Std Error					
			Absent	n	1 copy	n	2 copies	n
EMD[3]	*A*	None	30.9 ± 0.3[a]	86	30.7 ± 0.3[ab]	99	29.8 ± 0.3[b]	77
(mm)	*B*	None	30.6 ± 0.3	132	30.4 ± 0.3	82	30.3 ± 0.5	48
EMW[3]	*A*	None	76.7 ± 0.6[a]	86	75.9 ± 0.5[ab]	99	74.6 ± 0.6[b]	77
(mm)	*B*	None	75.9 ± 0.4	132	75.4 ± 0.6	82	75.9 ± 0.8	48
FDM[3]	*A*	None	3.5 ± 0.1	86	3.4 ± 0.1	99	3.3 ± 0.1	77
(mm)	*B*	None	3.4 ± 0.1	132	3.3 ± 0.1	82	3.4 ± 0.1	48

[1]*P* value associated with the variant being assessed. Estimated marginal means and *P* values were derived from GLMMs with variant copy number and birth rank fitted as fixed factors and sire fitted as a random factor ($P < 0.050$ in **bold**).
[2]Means with superscripts in common are not significantly different (multiple pair-wise comparisons with a Bonferroni correction, $P > 0.050$).
[3] EMD: Eye Muscle Depth; EMW: Eye Muscle Width; FDM: Fat Depth above the Eye Muscle.

Table 4.3 Association between *HSL* intron 5 genotype and assessments of some body composition traits in the NZ Suffolk sheep

Trait[3]	Mean[1, 2] ± Std Error				
	AA (77)	*AB* (61)	*AC* (36)	*BB* (48)	*BC* (19)
EMW (mm)	74.4 ± 0.6^{a}	74.4 ± 0.7^{a}	77.7 ± 0.8^{b}	75.6 ± 0.8^{ab}	77.6 ± 1.1^{ab}
EMD (mm)	29.7 ± 0.3^{a}	29.9 ± 0.4^{ab}	31.5 ± 0.5^{b}	30.0 ± 0.5^{ab}	31.4 ± 0.6^{ab}
FDM (mm)	3.3 ± 0.1	3.2 ± 0.1	3.6 ± 0.1	3.4 ± 0.1	3.6 ± 0.2

[1]Estimated marginal means were derived from GLMMs with genotype and birth rank fitted as fixed factors and sire fitted as a random factor.
[2]Means with superscripts in common are not significantly different (multiple pair-wise comparisons with a Bonferroni correction, $P > 0.050$).
[3]EMD: Eye Muscle Depth; EMW: Eye Muscle Width; FDM: Fat Depth above the Eye Muscle.

4.4 Discussion

This is the first report describing variation in ovine *HSL* and its association with sheep growth and body composition traits. Three single nucleotide substitutions in intron 5 and one non-synonymous substitution in exon 9 of ovine *HSL* were identified. The failure to find variation in region 1 may be because this region is under greater functional constraint and hence be more conserved than other regions of ovine *HSL*; or may due to PCR-SSCP has not detected all potential variaitons in this region of the gene.

In the 538 NZ Suffolk sheep investigated, it is notable that exon 9 *a*, which would putatively encode a valine at the position 607 (GenBank accession number ABG49111.1) as opposed to the isoleucine encoded by *b*, was common (64.8%). However, no association was found between exon 9 variation and growth or body composition traits in the sheep studied. This may be because the variation is in an amino acid whose substitution does not affect gene expression. However, given that exon 9 of rat *HSL* encodes a hydrophobic region containing a putative lipid-binding region (Holm et al., 1994) and this is likely important for the function of HSL, it is never-the-less tempting to speculate that the amino acid substitution (V607I) detected in this study, may be associated with some lipid metabolism-related traits by altering the structure and function of ovine HSL. This effect of this variation certainly warrants further investigation in other sheep of a variety of breeds.

It is notable that the frequencies of both the intron 5 and exon 9 genotypes of *HSL* were not in Hardy-Weinberg Equilibrium (HWE). The principle of HWE states that the genetic variation in a population will remain constant from one generation to the next in the absence of disturbing factors.This could be due to the sheep having been "selected" and being derived from related rams and thus the frequency of the *HSL* variants may be different between the sires and dams of these lambs. It may also be the result of some other effect that changes

genotype frequency. For example,Vatannejad et al. (2011) found that the variation in human *HSL* is associated with inferitility in men. Their study suggested that some of the *HSL* variants may be sex-dependant which causes the differences in the frequencies of *HSL* variants inmales and females. This could be true of sheep too. It could also be that the genotyping of ovine *HSL* in this study failed in someway and couldn't accurately detect all the *HSL* variants. In effect, sheep that appeared honmozygous, might actually be heterozygous for one undetectable variant and possibly as a result of variation in the primer binding regions affecting the ability to amplify that variant.

The statistical modelling approaches used suggest that the presence of intron 5 *A* may be associated with lower EMA, while the presence of intron 5 *C* may be associated with increased EMA and fat content in body. This suggests it is an independent effect. Interestingly, variation within human *HSL* has been reported to be associated with impaired fat metabolism and varied body composition (Hoffstedt et al., 2001). In addition, a study in pigs (Knoll et al., 1998) has reported associations between variation in *HSL* and various body composition traits including EMA (Knoll et al., 1998). Given the role HSL plays in lipolysis, it could be suggested that the association observed here between variation within intron 5 *HSL* and variation in EMA is because the genetic variation affects the expression of the gene. This impaired function of *HSL* may ultimately affects EMA by altering the intramuscular fat content, and this needs further investigation.

No associations were detected between intron 5 variants, exon 9 variants of *HSL*, and post-weaning growth-rate. This may be because the variation in *HSL* does not directly affect the overall weight gain of an animal after weaning, instead altering the subcutaneous and intramuscular fat composition of the body, but not so much as to affect overall weight.

In humans, significant associations have been found between variation in *HSL* and variation in subcutaneous fat deposition including an increased BMI, increased waist circumference and increased risk of obesity (Carlsson et al., 2006). However, in this study, only a slight association was found between ovine *HSL* variants and variation in subcutaneous fat depth. This might be because only the Suffolk sheep breed was investigated and the small range of variation in FDM (ranged from 2 to 5mm). NZ Suffolk sheep have been widely used for breeding lean lambs, so further work will need to focus on investigating other Suffolk sheep and other "fatter" sheep breeds to confirm the association between ovine *HSL* variation and fat traits.

In this study, only small numbers of lambs were studied. Future work will be needed to investigate larger numbers of NZ Suffolk lambs and lambs from other breeds, and including

both ewe and ram lambs. Further work is also needed to characterise the full extent of variation within the gene and confirm these preliminary associations between variation within intron 5 of ovine *HSL* and key carcass traits.

Chapter 5

Variation in ovine Uncoupling Protein 1 gene (*UCP1*) and its association with growth and carcass traits

5.1 Introduction

Uncoupling Protein 1 (UCP1) belongs to a family of mitochondrial integral Membrane Carrier Proteins (MCPs) (Ricquier and Bouillaud, 2000) and is a distinguishing characteristic of BAT because of its role in thermogenesis (Cannon and Nedergaard, 2004). Protein of UCP1 has also been found in WAT which could cause energy expenditure in the tissue (Flachs et al., 2013). UCP1 dissipates energy as heat by generating a "leak" that enables protons to cross the inner mitochondrial membrane. This ultimately uncouples the electron transport chain from ATP synthesis leading to NST. The generation of NST through the protonophoric function of UCP1 prevents energy being stored as fat promoting its loss as heat. Uncoupling Protein 1 is therefore also considered an anti-obesity factor (Nedergaard et al., 2005).

Besides UCP1, four other UCPs; UCP2, UCP3, UCP4 (also known as StUCP) and UCP5 (also known as AtUCP or BMCP-1) have been identified to date (Krauss et al., 2005; Ricquier and Bouillaud, 2000). Because UCP4 and UCP5 phylogenetically belong to a different group (Borecky et al., 2001), the UCP family is often considered to contain only UCP1, UCP2 and UCP3. Despite it sharing structural similarities with the other UCPs, UCP1 is thought to be the only protein that mediates thermogenesis (Nedergaard et al., 2001).

The UCP1 protein

The structure of UCP1 has been described in many studies (Kim et al., 2011). It is composed of six transmembrane (TM) α-helices (Figure 5.1) (Garlid et al., 2000; Hoang et al., 2013). It shares homology with other members of the greater MCP family, including a tripartite structure that is thought to be conserved (Borecky et al., 2001; Garlid et al., 2000). In rat UCP1, three prolines (P32, P132 and P231) have been found with one in each part of the tripartite structure, and these are thought to be important for the function of UCP1. Two amino acid sequences and a GDP-binding domain, found to be conserved across mammals, have been found in UCP1. But they are not found in any other MCPs, not even the closely related UCP2 and UCP3 (Borecky et al., 2001; Garlid et al., 2000). These sequences are found at the middle of the central loop and in the last part of the COOH-terminus. The actual function of these conserved sequences is still unclear (Echtay et al., 1998; Urbankova et al.,

2003), but their unique and consistent presence in UCP1 from different species, suggests that they could be of importance for the function of this protein.

The GDP-binding site of UCP1 has been proposed to be involved in mediating UCP1-dependent thermogenesis through its competitive binding to FFA and GDP in mitochondria (Huang and Klingenberg, 1996). This binding can cause conformational changes in UCP1.

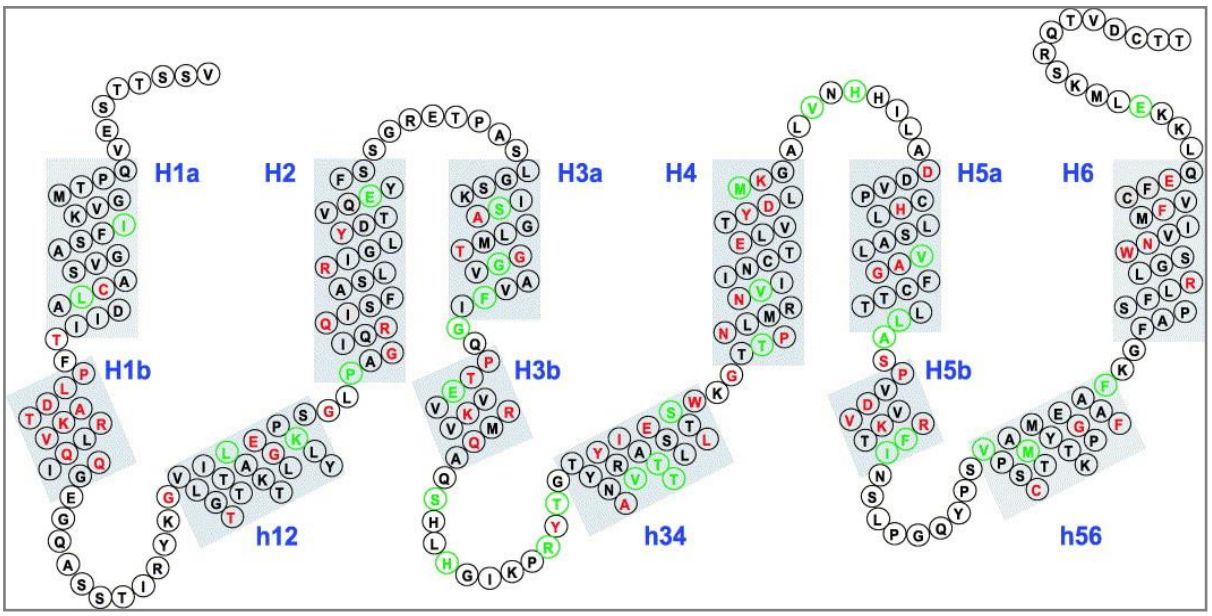


Figure 5.1 The structure of rat Uncoupling Protein 1 (UCP1). Two-dimensional representation of the folding of rat UCP1. Shaded boxes correspond to a-helices and they are numbered following the nomenclature described by Pebay-Peyroula *et al.,* (2003). Residues conserved in all the analysed UCPs (UCP1, UCP2, UCP3 and plant UCPs) are shown in red. Amino acid residues that are shared across species in UCP1 and that have consistency indexes above 50% are shown in green. (Adapted from Jimenez-Jimenez *et al.,* 2006; Hoang *et al.,* 2013).

The UCP1 gene (*UCP1*)

The UCP1 gene (*UCP1*) has been mapped to chromosome 4 in humans (GenBank, Gene ID: 7350), chromosome 8 in mice (GenBank, Gene ID: 22227), chromosome 19 in rats (GenBank, Gene ID: 24860), chromosome 17 in cattle (GenBank, Gene ID: 281561), chromosome 19 in dogs (GenBank, Gene ID: 403574), chromosome 8 in pigs (GenBank, Gene ID: 751863) and chromosome 17 in sheep (GenBank, Gene ID: 494434). It has been cloned from humans and sequenced (Cassard et al., 1990), and sequences have been revealed for mice (Kozak et al., 1988), rats (Bouillaud et al., 1988), domestic dogs (Ishioka et al., 2002), cattle (Casteilla et al., 1989a; Sonstegard and Kappes, 1999) and sheep (Yuan et al., 2012).

Two regulatory regions have been described in the human and rat genes: a proximal promoter and a more distal enhancer region (del Mar Gonzalez-Barroso et al., 2000; Yubero et al., 1994). The promoter region contains TATA and CCAAT elements, two C/EBP sites

and other potential binding sites (Yubero et al., 1994). An enhancer region at around -2.5kb in rodent *UCP1* and -3.5kb in human *UCP1* has also been described (del Mar Gonzalez-Barroso et al., 2000). This region contains a multipartite response element (del Mar Gonzalez-Barroso et al., 2000) and many smaller response elements including CREs (Kozak et al., 1994; Rim and Kozak, 2002), Retinoic Acid Response Elements (RAREs) (Alvarez et al., 1995, 2000), Peroxisome Proliferator-Activated Receptor Response Elements (PPREs) (Barbera et al., 2001; Sears et al., 1996) and thyroid or T3 Response Elements (TREs) (Cassard-Doulcier et al., 1994; Rabelo et al., 1995, 1996). These sites can amplify the effects of norepinephrine on *UCP1* transcription (Bianco et al., 1988; Hernandez and Obregon, 2000).

The *UCP1* "knockout" mouse phenotype

Monemdjou *et al.,* (2000) described increased mitochondrial proton leakage in the skeletal muscle mitochondria of *UCP1* "knockout" mice. The mice are found to be sensitive to cold, due to impaired thermogenesis in BAT. *UCP1* knockout mice also have a higher risk of obesity than normal mice (Bachmanov et al., 2001). Conversely, transgenic mice over-expressing *UCP1* have been reported to have elevated UCP1 levels and reduced adiposity (Hofmann et al., 2001).

Associations between variation in *UCP1* and variation in phenotypic traits

In humans, variation within *UCP1* has been associated with variation in body fat mass and waist-to-hip ratios in Korean females (Shin et al., 2005). A large scale study in a Caucasian population suggested -3826A/G does not play a major role in the pathogenesis of obesity and diabetes (Schaffler et al., 1999). Nevertheless, the G variant has been suggested to be able to either act together with, or in interaction with, variation in other lipolytic genes such as *ADRB3* and Adiponectin gene (*Adipoq*) to accelerate the development of obesity (Evans et al., 2000; Heilbronn et al., 2000), accumulation of visceral fat (Tsunekawa et al., 2011) and predisposition to severe liver steatosis (Labruna et al., 2009).

Uncoupling Protein 1 gene is disrupted in some pig breeds and these piglets appear to lack BAT (Berg et al., 2006). In these pigs, the sequence spanning exons 3 to 5 is eliminated by a deletion. In the exons remaining in these pigs, three additional disrupting mutations are also detected (Berg et al., 2006). In comparison with *UCP1* sequences of humans, cattle and mice, the rate of non-synonymous substitutions is higher in pig *UCP1*, suggesting that this gene has been disrupted during their evolution (Berg et al., 2006). This may explain why piglets are poor at thermoregulation.

To date, only a few studies have attempted to detect variation within ovine *UCP1*. In a recent report, variation in the coding and non-coding region of ovine *UCP1* has been described (Yuan et al., 2012), but no association with any traits was reported. The gene was located on OAR17 (Yuan et al., 2012), and the coding sequence of ovine *UCP1* is similar to many species with a high similarity to cattle (95%), horse (88%), human (84%), macaque (84%), dog (81%) and rat (81%) sequences (Yuan et al., 2012). The gene is approximately 6.6 kb in length with a coding sequence of approximately 1.6 kb, and is composed of six exons and five introns. To date, variation has been found in ovine *UCP1* and this variation appears to affect the mRNA level of ovine *UCP1* in different tissues (Yuan et al., 2012). Whether variation in ovine *UCP1* is associated with variation in sheep traits, including growth and carcass traits, has not been reported.

In humans, variation were found in the UCP1 promoter region (-3826A>G, -1766A>G), exon 2 p.Ala64Thr (Shin et al., 2005) and exon 5 p.Met229Leu (Mori et al. 2001), which were associated with fat metabolism, obesity and diabetes (Jia et al., 2010; Nagai et al., 2011). The promoter region plays an important role in transcription regulation through interacting with a spectrum of regulatory elements. Variation in the promoter region of the gene could alter the expression level of mRNA and thus, function of the protein. Variation in the exons could lead to a substitution in amino acids sequence, this could also affect the function of protein.

Therefore, given the important role that variation in *UCP1* plays in the function of UCP1 and fat metabolism, in this chapter variation in the promoter, intron 2 and exon 5 regions of the ovine *UCP1* was investigated and its association with growth and carcass traits studied in NZ Suffolk and NZ Romney sheep.

5.2 Materials and methods

Sheep Studied and Data Collection

In total 823 sheep, including 587 NZ Suffolk sheep from 30 independent sires and six studs, and 236 NZ Romney sheep from 12 independent sires were investigated.

NZ Suffolk lamb blood samples were supplied by the Gene-Marker Laboratory, Lincoln University. NZ Romney lamb samples were collected from the "Ancare-Merial Romney NZ Saleable Meat Yield Trial". This trial was carried out at "Gleneyre", Oxford, North Canterbury, NZ and "Osborne" farm, Ashhurst, NZ.

The method of lamb live-weight calculation and collection of body composition data are described in Chapter 3.

For the 236 NZ Romney sheep, carcass traits: including fat composition scores (VGR), lean meat yield in the hind-leg (leg yield), loin (loin yield) and shoulder (shoulder yield), and total yield (the sum of the leg, loin and shoulder yields for any given carcass), were estimated at slaughter using VIAscan ® (VIAScan® Sastek, Hamilton, QLD Australia). The VIAscan® technology developed by Meat and Livestock Australia and described in Hopkins *et al.,* (2004), is based on video imaging. Loin yield, shoulder yield and leg yield is the percentage of lean tissues as a proportion of the Hot Carcass Weight (HCWT). Total yield is the sum of the leg, loin and shoulder yield for any given carcass.

DNA purification

Blood samples were collected from individual animals (for method, see chapter 3) and genome DNA was purified (for method see Chapter 3).

Primer design

Three regions of ovine *UCP1* were investigated in this study: region 1 is approximately 350 bp in length covering a portion of the promoter, region 2 is approximately 325 bp in length covering a portion of intron 2 and region 3 is approximately 233 bp in length covering the whole of exon 5 (see Figure 5.2). Three pairs of primers were designed based on a published ovine *UCP1* gene sequence (GenBank accession number: JN604985.1) (Figure 5.2).

PCR amplification of the promoter, intron 2 region and exon 5 region of ovine *UCP1* and PCR-SSCP analysis

Amplifications were performed in a 15 µL reaction containing the DNA on one 1.2mm punch of FTA paper. 0.25µM of each primer, 150µM of dATP, dCTP, dGTP, and dTTP (Eppendorf, Hamburg, Germany), 2.5mM Mg^{2+}, 0.5 U Taq DNA polymerase (Qiagen, Hilden, Germany) and 1 × the reaction buffer supplied with the enzyme. The thermal profiles for the three regions amplified were 2 min initial denaturation at 94°C, followed by 35 cycles of 30 s at 94°C, 30 s at an optimised annealing temperature for each region (promoter at 62°C, intron 2 at 61°C and exon 5 at 60°C) and 30 s at 72°C, and with a final extension of 5 min at 72°C. Amplification was carried out in an iCycler (Bio-Rad Laboratories, Hercules, CA, USA).

Amplicons were visualized by electrophoresis in 1% agarose (Quantum Scientific, Queensland, Australia) gels, using 1 × TBE buffer (89mM Tris, 89mM boric acid, 2mM Na_2EDTA) and containing 200 ng/ml of ethidium bromide.

An aliquot of 0.7 µL of each amplicon was mixed with 7 µL of loading dye (98% formamide, 10mM EDTA, 0.025% bromophenol blue and 0.025% xylene-cyanol). After being denatured at 95 ºC for 5 min, samples were cooled rapidly on wet ice and then 7.5 µL were loaded onto 16 × 18 cm, 14% acrylamide / bisacrylamide (37.5:1) gels. Electrophoresis was performed using Protean II xi cells (Bio-Rad). The amplicons from the promoter, intron 2 and exon 5 regions were run at 350 V, 22°C; 280 V, 21°C; and 250 V, 25°C, respectively for 18 h in 0.5 × TBE buffer. Gels were silver-stained according to the method described by Byun *et al.,* (2009).

Sequencing of ovine *UCP1* variants and sequence analysis

Amplicons representative of different PCR-SSCP patterns from sheep that were homozygous for any given region were directly sequenced at the Lincoln University DNA Sequencing Facility. For the rarer variants that were only found in heterozygous sheep, a band corresponding to the rare variant was excised as a gel slice from the polyacrylamide gel, macerated and then used as a template for reamplification with the original primers. This produces a PCR-SSCP pattern equivalent to a sheep homozygous for that rare allele. This second amplicon was then directly sequenced. Sequence alignments, translations and comparisons were carried out using DNAMAN (version 5.2.10, Lynnon BioSoft, Canada). The BLAST algorithm was used to search the NCBI GenBank databases (http://blast.ncbi.nlm.nih.gov/) for homologous sequences.

Statistical analyses

All statistical analyses were performed using SPSS version 19 (SPSS Science Inc., Chicago, IL, USA). General linear mixed-effects models (GLMMs) were used to assess the effect of the presence of a particular sequence variant in a sheep's genotype on post-weaning growth-rate and carcass traits: including subcutaneous fat depth (VGR), hind-leg yield, loin yield, shoulder yield and total carcass yield. Gender and birth rank were fitted as fixed factors in these models and sire of each lamb were fitted as a random factor. As each sire was only used on a single farm, sire effects were therefore confounded with farm-specific management or environmental effects, and thus only sire was corrected for in the models and as a proxy for

other farm-related effects. Only male Romney lambs were slaughtered, so gender was not fitted to carcass trait analyses.

The generalized statistical model used to test the sequence variant (or genotype – see below) effects was as follows: $Y_{ijk} = \mu + \tau_i + \beta_j + \alpha_k + \epsilon_{ijk}$; where Y_{ijk} = traits evaluated on the i_{th} level of the fixed factor genotype (τ_i), the j_{th} level of the fixed factor birth rank (β_j) and the k_{th} sire of random effect (α_k); and where μ = overall mean for each trait and ϵ_{ijk} is the random error for ijk.

Each variant was coded as either present (1) or absent (0) for each animal's genotype. Initially, single-variant models were performed to ascertain which variants should be included in subsequent multi-variant models, and such that we could assess whether the effect of a given variant was independent of the other variants. The multi-variant models included any variant that had associations with post-weaning growth-rate and carcass traits in the single-variant models with a *P* value less than 0.200 and which could thus potentially have impact on the growth-rate and carcass traits.

For variants that had homozygous forms greater than 5% of all genotypes (thus providing an adequate sample size) a second set of analyses was performed, this time with the number of variant copies present in the animal's genotype included (i.e. 0, 1 or 2) in place of variant presence/absence, followed by planned orthogonal contrasts to ascertain whether additive, dominant or recessive effects were present.

For genotypes with a frequency greater than 5%, a GLMM (Fixed effects: variant/genotype, gender and birth rank. Random effect: sire) and multiple pair-wise comparisons with Bonferroni corrections were used to ascertain the association of genotype with post-weaning growth-rate and carcass traits.

5.3 Results

Variation within ovine *UCP1*

Amplicons of approximately 350 bp for the promoter, 325 bp for intron 2 and 233 bp for exon 5 were obtained from ovine genomic DNA using the methods described above.

Only one PCR-SSCP banding pattern was observed for the exon 5 amplicons in the NZ Suffolk and NZ Romney sheep studied. Accordingly, further sequencing and association analysis with exon 5 was not undertaken.

Three PCR-SSCP banding patterns (named *A*-*C*) (Figure 5.2) were detected for the promoter amplicons in both the NZ Suffolk and NZ Romney sheep. After sequencing, these patterns were confirmed to be produced by three novel sequences and these were deposited into GenBank with accession numbers (KC243136 - KC243138). Two PCR- SSCP patterns were observed for intron 2 amplicons (these were named *a* and *b*) in the NZ Suffolk sheep, but were not found in the Romney sheep which were exclusively of the *a* variant. Nucleotide sequence variation in the two amplicons is summarised in Figure 5.2. Two SNPs were revealed in the promoter region and one SNP in the intron 2 region.

Frequencies of promoter and intron 2 variants in NZ Romney and NZ Suffolk sheep

Of the 587 NZ Suffolk sheep investigated, promoter genotypes *AA* (15.1%), *AB* (36.7%), *AC* (11.4%), *BB* (21.3%), *BC* (13.2%) and *CC* (2.3%) were found, with individual variant frequencies of *A* (38.8%), *B* (46.6%) and *C* (14.6%). Two intron 2 genotypes, *aa* (93.3%) and *ab* (6.7%), with individual variants frequencies of *a* (96.7%) and *b* (3.3%), were observed.

Of the 236 NZ Romney sheep investigated, promoter genotypes *AA* (24.6%), *AB* (26.3%), *AC* (30.5%), *BB* (3.0%), *BC* (7.2%) and *CC* (8.4%) were found, with individual variant frequencies of *A* (53.0%), *B* (19.7%) and *C* (27.3%).

The *b* variant of intron 2 observed in the Suffolk sheep was not observed in the Romney lambs. Thus, further association analysis between intron 2 and growth and carcass traits could not be undertaken in this breed.

Correlations between lamb growth and carcass traits in the NZ Romney sheep

Pearson correlation coefficients between the sheep growth data and various carcass traits evaluated are shown in Table 5.1.

Sheep loin yield was correlated with post-weaning growth-rate ($R = 0.147$, $P = 0.021$). Total lean meat yield was correlated with post-weaning growth-rate ($R = 0.125$, $P = 0.050$).

Sheep birth weight was correlated with weaning-weight ($R = 0.392$, $P < 0.001$) and post-weaning growth-rate ($R = 0.213$, $P = 0.001$). Weaning-weight was correlated with hot carcass weight ($R = 0.476$, $P < 0.001$), VGR ($R = 0.467$, $P = 0.001$), loin yield ($R = 0.176$, $P = 0.006$), shoulder yield ($R = 0.236$, $P < 0.001$) and post-weaning growth-rate ($R = 0.729$, $P < 0.001$).

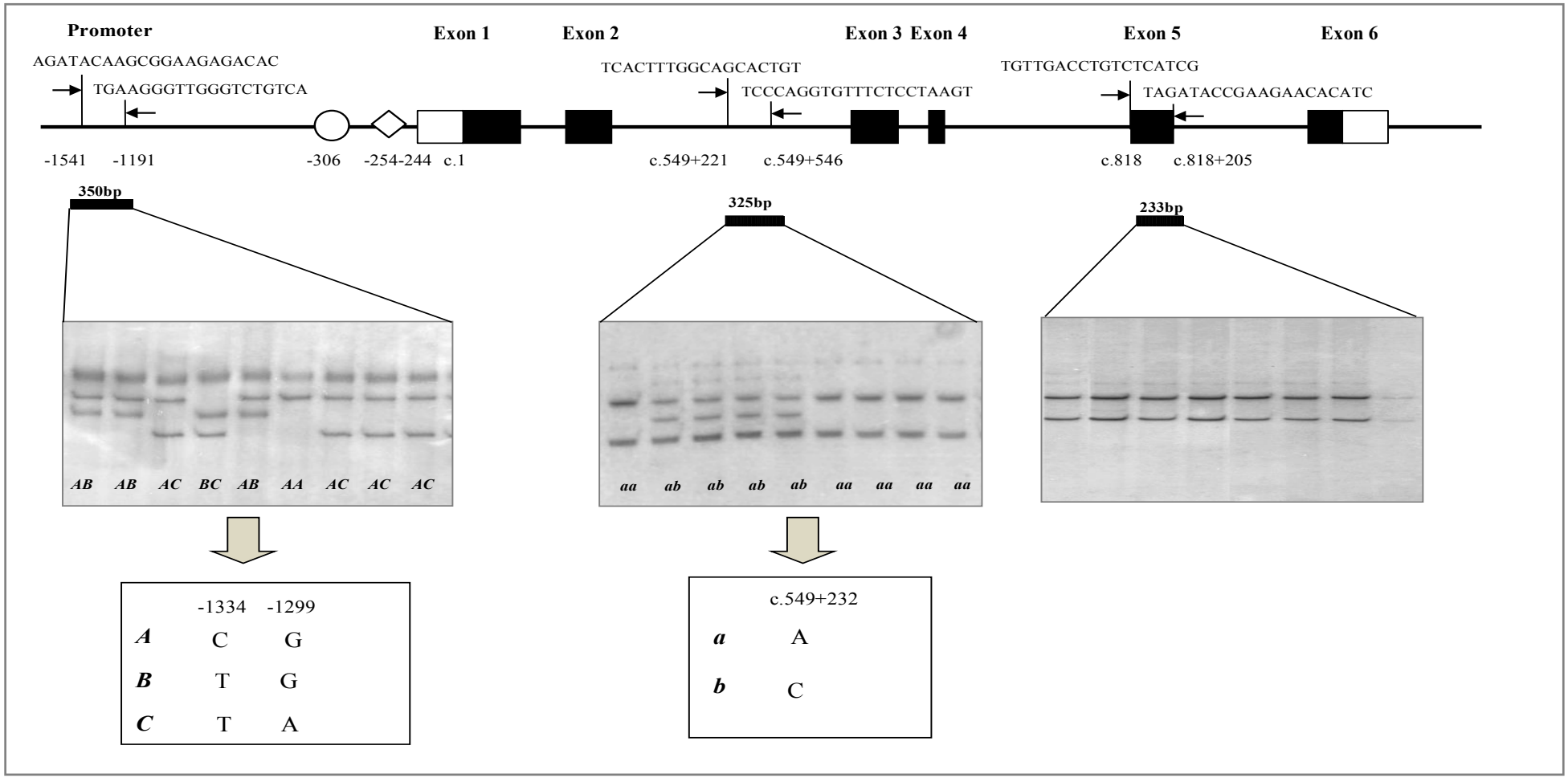


Figure 5.2 The PCR-SSCP patterns and single nucleotide variations detected in the promoter, intron 2 and exon 5 regions of ovine *UCP1*. The three regions of ovine *UCP1* amplified are shown in a diagonal line pattern. The 5'UTR in exon 1 and 3'UTR in exon 6 of the gene are shows in blank boxes. The cis-regulatory elements found in the promoter region of the ovine *UCP1* are shown in blank diamond shape (TATA box) and blank circle (CAAT box). The SSCP pattern names are shown in bold italic. The PCR-SSCP pattern names are shown in bold italic. The coordinates of primer and single nucleotide variations of ovine *UCP1* are defined relative to a known sequence of ovine *UCP1* (GenBank JN604985.1), and named according to sequence variation nomenclature (http://www.hgmd.cf.ac.uk/docs/mut_nom.html).

Table 5.1 Correlation coefficients for comparisons between lamb growth and carcass traits in the NZ Romney sheep

	Weaning-weight (kg)	VGR	Leg yield	Loin Yield	Shoulder yield	Total yield	Post-weaning growth-rate (g/day)
Birth weight (kg)	0.392**	0.105	-0.066	0.006	-0.048	-0.046	0.213**
Weaning-weight (kg)		0.467**	-0.058	0.176**	0.236**	0.112	0.729**
VGR			-0.440**	-0.014	0.135*	-0.173**	0.227**
Leg yield				0.667**	0.473**	0.878**	0.079
Loin Yield					0.623**	0.883**	0.147*
Shoulder yield						0.785**	0.105
Total yield							0.125*

* $0.010 < P \leq 0.050$, ** $0.001 < P \leq 0.010$, *** $P \leq 0.001$.

Association between ovine *UCP1* variation and carcass traits in the NZ Romney sheep

In the single-variant (presence/absence) models, the presence of promoter variant *B* was associated with lower VGR ($P = 0.005$, Table 5.2). The associations with *B* detected in the single-variant models persisted in the multi-variant models (Table 5.2). The presence of *A* was associated with lower VGR ($P = 0.041$) in the multi-variant model only. The frequency of genotype *BB* was not greater than 5% so its association was not analysed.

Lambs inheriting two copies of *C* (homozygous for *C*) had a higher mean VGR than those with one copy of *C*, or those that did not have *C* in their genotype (Table 5.3), whereas lambs inheriting two copies of *C* had a lower mean lean hind-leg meat yield than those with one copy of *C* and those that did not have *C* in their genotype (Table 5.3).

Five promoter genotypes *AA* (n =58), *AB* (n = 62), *AC* (n = 72) and *BC* (n = 17) and *CC* (n= 20) occurred at a frequency of greater than 5%. The *UCP1* genotype was found to have a significant effect on mean VGR (Table 5.4).

Association of ovine *UCP1* variants with body composition traits in the NZ Suffolk sheep

Of the 225 NZ Suffolk sheep analysed, no association ($P > 0.050$) was found between promoter variant and EMD and EMW.

In the single-variant (presence/absence) models, the presence of promoter variant *C* was associated with higher FDM ($P = 0.033$) (Table 5.5). No other associations were detected.

Those marginal means for FDM for each genotype occurred at a frequency of greater than 5% were listed in Table 5.6. Those animals with the *BB* genotype had lower FDM than those with *AB* ($P = 0.019$), *AC* ($P = 0.003$) and *BC* ($P = 0.010$). No other associations were detected.

Association of ovine *UCP1* variants with post-weaning growth-rate in the NZ Suffolk and Romney lambs

No association ($P > 0.050$) was found between ovine *UCP1* variation and mean birth weight, weaning-weight and post-weaning growth-rate in the NZ Suffolk and the NZ Romney sheep studied.

Table 5.2 Association between presence/absence of ovine *UCP1* promoter variants and various carcass trait measures in the NZ Romney sheep

Trait	Variant being assessed	Other variant in the model	Mean [1] ± Std Error				*P* value[1]
			Absent	n	Present	n	
VGR[2]	*A*	None	8.0 ± 0.40	44	7.2 ± 0.18	192	0.062
	B	None	7.7 ± 0.21	150	6.6 ± 0.31	86	**0.005**
	C	None	7.1 ± 0.23	127	7.7 ± 0.25	109	0.084
	A	*B, C*	7.9 ± 0.41	44	7.0 ± 0.20	192	**0.041**
	B	*A, C*	8.1 ± 0.29	150	6.8 ± 0.33	86	**0.004**
	C	*A, B*	7.5 ± 0.32	127	7.5 ± 0.28	109	0.917
Hind-leg yield (%) [3]	*A*	None	21.5 ± 0.19	44	21.5 ± 0.08	192	0.806
	B	None	21.4 ± 0.10	150	21.7 ± 0.15	86	0.067
	C	None	21.6 ± 0.11	127	21.4 ± 0.12	109	0.271
Loin yield (%) [3]	*A*	None	14.7 ± 0.14	44	14.8 ± 0.06	192	0.304
	B	None	14.8 ± 0.07	150	14.8 ± 0.11	86	0.687
	C	None	14.8 ± 0.08	127	14.8 ± 0.09	109	0.631
Shoulder yield (%) [3]	*A*	None	18.0 ± 0.13	44	18.0 ± 0.06	192	0.987
	B	None	17.9 ± 0.07	150	18.0 ± 0.10	86	0.505
	C	None	18.0 ± 0.08	127	18.0 ± 0.08	109	0.822
Total yield (%) [4]	*A*	None	54.1 ± 0.39	44	54.3 ± 0.18	192	0.691
	B	None	54.1 ± 0.21	150	54.5 ± 0.31	86	0.375
	C	None	54.4 ± 0.23	127	54.2 ± 0.25	109	0.574

[1]*P* value associated with the variant being assessed. Estimated marginal means and *P* values were derived from GLMMs with variant (absent and present) and birth rank fitted as fixed factors and sire fitted as a random factor ($P < 0.050$ in **bold**).
[2]VGR represents VIAScan Fat Score.
[3]Lean meat yield expressed as a percentage of hot carcass weight.
[4]Total yield is the sum of the hind-leg, loin and shoulder yield.

Table 5.3 Association between ovine *UCP1* promoter variant copy numbers and various assessments of yield in the NZ Romney sheep

Trait	Variant being assessed	Other variant in the model	Mean [1, 2] ± Std Error						*P* value[1]
			Absent	n	1 copy	n	2 copies	n	
VGR[3]	*A*	None	8.0 ± 0.40	44	7.1 ± 0.22	134	7.4 ± 0.34	58	0.137
	C	None	7.1 ± 0.22[a]	127	7.3 ± 0.26[a]	89	9.7 ± 0.55[b]	20	**< 0.001**
	A	*C*	7.4 ± 0.41	44	8.1 ± 0.32	134	9.0 ± 0.52	58	0.127
	C	*A*	6.7 ± 0.29[a]	127	7.4 ± 0.33[a]	89	10.3 ± 0.67[b]	20	**< 0.001**
Hind-leg yield (%) [4]	*A*	None	21.5 ± 0.19	44	21.5 ± 0.10	134	21.5 ± 0.16	58	0.970
	C	None	21.6 ± 0.11[a]	127	21.5 ± 0.13[a]	89	20.8 ± 0.27[b]	20	**0.032**
Loin yield (%) [4]	*A*	None	14.6 ± 0.14	44	14.8 ± 0.07	134	14.9 ± 0.12	58	0.289
	C	None	14.8 ± 0.08	127	14.8 ± 0.09	89	14.4 ± 0.19	20	0.074
Shoulder yield (%) [4]	*A*	None	18.0 ± 0.13	44	18.0 ± 0.07	134	17.9 ± 0.11	58	0.922
	C	None	18.0 ± 0.08	127	18.0 ± 0.09	89	18.0 ± 0.19	20	0.965
Total yield (%) [5]	*A*	None	54.1 ± 0.40	44	54.3 ± 0.21	134	54.4 ± 0.33	58	0.885
	C	None	54.4 ± 0.23	127	54.4 ± 0.27	89	53.3 ± 0.56	20	0.192

[1]*P* value associated with the variant being assessed. Estimated marginal means and *P* values were derived from GLMMs with variant (absent, one copy and two copies) and birth rank fitted as fixed factors and sire fitted as a random factor ($P < 0.050$ in **bold**).
[2]Means across rows with different superscripts are significantly different (multiple pair-wise comparisons with a Bonferroni correction, $P > 0.050$).
[3]VGR represents VIAScan Fat Score.
[4]Lean meat yield expressed as a percentage of hot carcass weight.
[5]Total yield is the sum of the hind-leg, loin and shoulder yield.

Table 5.4 Association of *UCP1* promoter genotype with various carcass measures in the NZ Romney sheep

Trait	Mean [1, 2] ± Std Error				
	AA (58)	*AB* (62)	*AC* (72)	*BC* (17)	*CC* (20)
VGR[3]	7.5 ± 0.32[a]	6.7 ± 0.34[a]	7.4 ± 0.29[a]	6.8 ± 0.58[a]	9.6 ± 0.55[b]
Hind-leg yield[4]	21.5 ± 0.16	21.6 ± 0.17	21.4 ± 0.14	21.8 ± 0.29	20.8 ± 0.27
Loin yield[4]	14.9 ± 0.12	14.7 ± 0.12	14.8 ± 0.10	14.9 ± 0.21	14.4 ± 0.20
Shoulder yield[4]	17.9 ± 0.11	18.0 ± 0.12	17.9 ± 0.10	18.1 ± 0.21	18.0 ± 0.19
Total yield[5]	54.4 ± 0.33	54.3 ± 0.36	54.2 ± 0.31	54.9 ± 0.61	53.3 ± 0.57

[1]*P* value associated with the variant being assessed. Estimated marginal means were derived from GLMMs with genotype presence/absence and birth rank fitted as fixed factors and sire fitted as a random factor.
[2]Means across rows with different superscripts are significantly different (multiple pair-wise comparisons with a Bonferroni correction, $P > 0.050$).
[3]VGR represents VIAScan Fat Score.
[4]Lean meat yield expressed as a percentage of hot carcass weight.
[5]Total yield is the sum of the leg, loin and shoulder yield.

Table 5.5 Association of *UCP1* promoter variant with assessments of body composition traits in the NZ Suffolk sheep

Trait[2]	Variant being assessed	Other variant in model	Mean [1] ± Std Error				*P* value[1]
			Absent	n	Present	n	
EMD (mm)	*A*	None	30.4 ± 0.6	116	29.7 ± 0.5	109	0.101
	B	None	30.0 ± 0.5	124	30.0 ± 0.6	101	0.972
	C	None	30.0 ± 0.5	153	30.6 ± 0.6	72	0.072
EMW (mm)	*A*	None	75.8 ± 0.8	116	75.2 ± 0.8	109	0.415
	B	None	75.5 ± 0.8	124	75.5 ± 0.9	101	0.940
	C	None	75.4 ± 0.8	153	75.8 ± 0.9	72	0.578
FDM (mm)	*A*	None	3.4 ± 0.2	116	3.4 ± 0.1	109	0.911
	B	None	3.3 ± 0.1	124	3.4 ± 0.2	101	0.800
	C	None	3.3 ± 0.1	153	3.5 ± 0.2	72	**0.033**

[1]*P* value associated with intron and 3'UTR variants being assessed. Estimated marginal means and *P* values were derived from GLMMs with variant copy number, gender and birth rank fitted as fixed factors and sire-line fitted as a random factor ($P < 0.050$ in **bold**).
[2]EMD: Eye Muscle Depth; EMW: Eye Muscle Width; FDM: Fat Depth above the Eye Muscle.

Table 5.6 Association of *UCP1* promoter genotype with Fat Depth above the eye Muscle (FDM) in the NZ Suffolk sheep

Genotype	Fat depth above the eye Muscle (FDM) (mm) Mean [1, 2, 3] ± Std Error
AA	3.3 ± 0.2[ab]
AB	3.4 ± 0.1[a]
AC	3.7 ± 0.2[a]
BB	2.9 ± 0.2[b]
BC	3.6 ± 0.2[a]

[1] *P* value associated with the variant being assessed. Estimated marginal means were derived from GLMMs with genotype presence/absence and birth rank fitted as fixed factors and sire fitted as a random factor.
[2] Means across rows with different superscripts are significantly different (multiple pair-wise comparisons with a Bonferroni correction, $P > 0.050$).
[3] FDM: Fat Depth above the eye Muscle.

5.4 Discussion

This is the first report describing a relationship between variation in *UCP1* and carcass traits in sheep. Sequencing of portions of the gene revealed two new SNPs in the promoter and one that has been reported previously in intron 2 (Yuan et al., 2012). No variation was detected in the exon 5 amplicon. Together these results suggest that the promoter of ovine *UCP1* may be subject to less functional constraint, than other regions of the gene.

Previously, Yuan *et al.,* (2012) reported variation in exon 5 and promoter region of ovine *UCP1* in Chinese Fat Tail sheep, while in this study no variation was found in exon 5 and two new SNPs were found in the promoter region of gene. This difference in the frequency of the *UCP1* variants may due to different genetic selection programs being used in different countries, or because some of the *UCP1* variants are breed-specific, or simply because insufficient sheep were studied to fully reveal the variation that exists in sheep. These genetic differences need further clarification by investigating more sheep samples and breeds, from different areas or countries.

The promoter sequence of the *UCP1* has been reported to be important for gene expression through its interaction with the CCAAT/enhancer binding proteins (C/EBP) α and C/EBPβ (Yubero et al., 1994). These elements, can amplify the effect of norepinephrine on *UCP1* transcription and therefore affect fat metabolism and deposition (Bianco et al., 1988; Hernandez and Obregon, 2000). In humans, variation within the promoter region of *UCP1* has been reported to be associated with abnormal fat metabolism phenotypes, including an increased risk of hypertension, obesity and type 2 diabetes mellitus (Kieć-Wilk et al., 2002; Kotani et al., 2007) and varied body fat mass and waist-to-hip ratio (Shin et al., 2005). In addition, variation in the promoter region of human *UCP3* has also been found to be associated with risk of obesity and variation in cholesterol levels in Japanese population (Hamada et al., 2008). In sheep, we found a few regulatory elements in the promoter of ovine *UCP1*. These elements including TATA box, CAAT box are shown in Figure 5.2. It is therefore notable that in this study, the variation detected within the promoter of ovine *UCP1* was associated with carcass fat composition (VGR). This suggests that the effects of variation in the promoter sequence on the regulation of fat metabolism may be similar in sheep to that in humans.

Interestingly, a recent morphometric study in mice (Almind et al., 2007) has located UCP1 in brown adipocytes that are interspersed in the perimuscular and inter-muscular adipose tissues of the leg. Additionally, in obesity-resistant mice, dramatically increased

levels of UCP1 mRNA, UCP1 protein and mitochondrial uncoupling were detected in hind-limb skeletal muscle (Li et al., 2000). It is therefore conceivable that the variation observed within the promoter of ovine *UCP1* could affect the expression of UCP1 mRNA in both perimuscular and intermuscular adipose tissues. This might then lead to varied energy expenditure and variable fat content in skeletal muscles and consequently affect the lean meat yield of hind-leg, shoulder and loin. This requires further investigation, especially to confirm whether the leg meat does actually contain more intramuscular fat. This increased intramuscular fat may be desirable with respect to meat eating-quality, as has been suggested by Dodson *et al.,* (2010).

Of the 823 NZ sheep typed in this study, it is noteworthy that *B* had a higher frequency in NZ Suffolk sheep (46.6%) than NZ Romney sheep (19.7%). Given that *B* was associated with leaner carcasses in this study, it could be argued that the majority of NZ Suffolk sheep appear to be more genetically predisposed to being leaner when compared to NZ Romney sheep. This too needs confirmation, both in the NZ Romney and the NZ Suffolk breeds and in other breeds that differ in growth and carcass characteristics.

Variant *b* was not present in NZ Romney sheep (0%) and rare in NZ Suffolk sheep (3.3%). This might suggest that variant *b* may negatively associate with a trait or traits that have been selected against historically.

The *C* variant of *UCP1* was found to be the least common variant in both breeds (14.6% in NZ Suffolk and 27.3% in NZ Romney). The small number of the lambs inheriting *C,* the variant that was associated with higher VGR, might suggest that NZ Romney and NZ Suffolk sheep have been selected against having fatter carcasses. Over-fatness is a common cause of meat being deemed unacceptable or less desirable in some markets, and because of the connection between the consumption of high levels of saturated fat, obesity, cancer, diabetes and heart disease (Volk, 2007). Given that having two copies of *B* is associated with lower fat composition score, genetic selection for variant *B* of ovine *UCP1* could benefit breeders in future by allowing them to select leaner lambs.

In this chapter and previous chapter (Chapter 4), because the NZ Suffolk studied were stud sheep and the NZ Romney studied were commercial sheep, the carcass traits of these two breeds were assessed differently. The carcass characteristics of the NZ Romney sheep were measured using VIAscan® and different from the NZ Suffolk sheep in chapter 3 which were measured using Ultrasound scanning.

Ultrasound estimation of genetic merit for carcass traits is based on scanning in the width and depth of the loin at the 12th rib of live animals (Figure 5.3). Body composition indices of

EMD, EMW, FDM together with live weight at scanning and weaning were measured to calculate the genetic merit (Estimated Breeding Values, eBVs) for lean weight, fat weight and EMA for the NZ Suffolk sheep. Ultrasound scanning is relatively inexpensive, economic to measure a large number of animals in a flock and having moderately accurate predictor of weight of muscle and fat in the carcass.

VIAscan® estimation of sheep carcass traits is based on the video imaging analyses of animal's carcass. VIAscan® technology provides measurements reflecting whole carcass yield (Figure 5.4). It has been used to predict eBVs for yield of hind-leg, loin and shoulder cuts. In comparison with Ultrasound scanning, VIAscan® provides the key advantages of greater accuracy and improved practicability (Cannell et al., 1999).

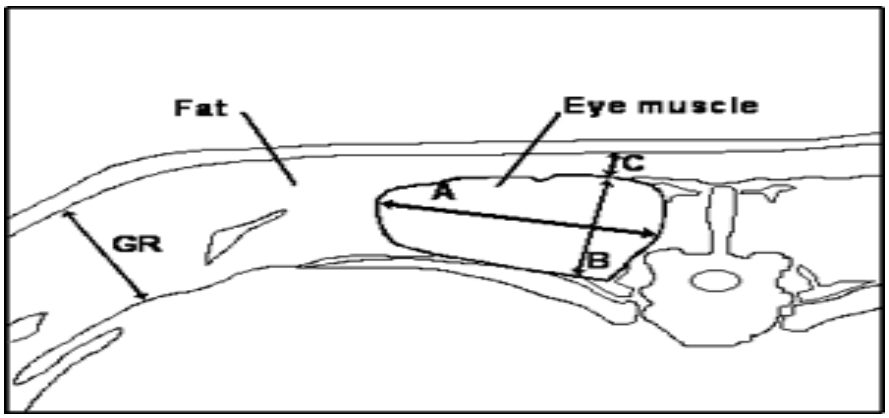


Figure 5.3 Ribeye areas of lamb carcass scanned by ultrasound. A: Eye Muscle Width (EMW); B: Eye Muscle Depth (EMD); C: Fat Depth above the eye Muscle (FDM)

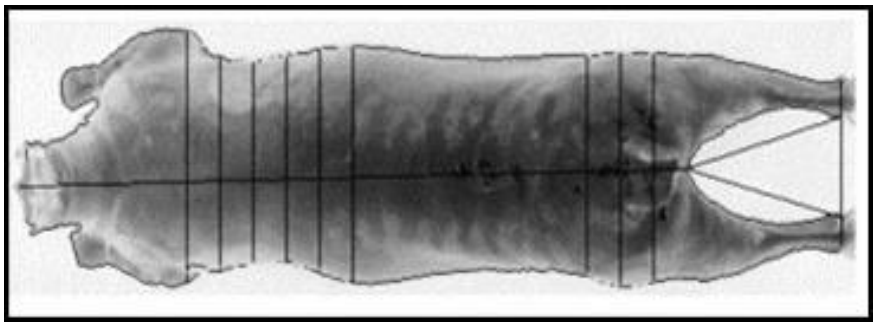


Figure 5.5 Whole lamb carcass scanned by VIAscan®

Chapter 6
General Summary and Future Directions

This thesis focused on the identification of variation within the ovine genes *ADRB3*, *HSL* and *UCP1*, and the potential for association between this variation and variation in post-weaning growth and carcass traits.

The results from chapter 3 suggest that *ADRB3* is highly variable in sheep. This work confirmed that ovine *ADRB3* could be used as a gene-marker for lamb growth. Variation in both intron and 3'UTR of the gene was found to be associated with variation in weaning-weight and post-weaning growth-rate. Haplotype analysis of the gene gave us a further understanding of these associations. According to the statistical modelling results, a single copy of *ADRB3* haplotype *C-a* could deliver an increase in daily weight gains. Therefore, it is tempting to suggest these associations could underpin development of a tool for selecting faster growing lambs.

Of the sixteen *ADRB3* haplotypes defined, seven were detected in NZ Suffolk sheep. The rarity of some *ADRB3* haplotypes in NZ Suffolk sheep could be due to reduced genetic diversity in this breed relative to the NZ Romney sheep, and that it might have come about through historical selection for production traits. Further studies will need to investigate whether the associations found in this study are consistent across different sheep breeds. Given the extended intron variation identified previously in ovine *ADRB3* in Chinese breeds (Yang et al., 2009), it is also suggested that more variation and haplotypes of the ovine *ADRB3* may exist, if more sheep breeds from around the world are investigated. This will further expand our understanding of the association between variation in this gene and key animal traits.

It is notable that variation in the intron of ovine *ADRB3* has also been found to be associated with other economic traits in sheep such as wool staple strength, cold-tolerance and carcass traits as described by Forrest *et al.,* (2003; 2006 and 2007). Because the haplotype covers a larger region of the gene than an individual fragment, the haplotyping method developed in this work may help the further understanding of those associations by investigating the association between haplotypes and those traits. More accurate haplotypes will potentially allow for the detection of stronger associations, especially for the *D-d* haplotype which has been found to be associated with impaired lamb cold-tolerance and was absent in the breeds studied here.

Recently, a comparative analysis of human chromosome 8 and bovine chromosome 27 suggests that bovine *ADRB3* is located at Quantitative Trait Locus (QTL) that is associated with "dairy form", an estimated index for cows efficiency to mobilise energy stored as fat into milk production (Connor et al., 2006). This suggests that haplotypes of *ADRB3* may also be associated with key milk traits in milking sheep, dairy cows, and other milking animals. Therefore, the extended variation now revealed in sheep, suggests greater effort should be made to investigate *ADRB3* variation in cattle, goat, sheep and other livestock.

This is the first research reporting the variation in ovine *HSL* and its association with lamb carcass traits. The results in chapter 4 suggest that ovine *HSL* is moderately genetically variable and that variation in this gene is associated with variation in EMA and fat deposition. Since EMA represents a primal cut area of a carcass and is considered an important trait for quality carcasses, the breeding for lambs that have the *C* variant could therefore potentially benefit the lamb industry by the production of more lambs with larger EMAs.

However, further work is needed to confirm the associations found in this work by investigating the association between variation in *HSL* and carcass traits in some other "fatter" sheep breeds than the NZ Suffolk breeds.

Although a non-synonymous nucleotide substitution was detected in exon 9 of ovine *HSL*, no association was found between this variation and growth or carcass traits. This may once again be because the NZ Suffolk lambs studied have been selected for growth and carcasses traits for many years and therefore contain less genetic diversity and a reduced frequency of some genetic variants.

This is also the first research reporting an association between variation in ovine *UCP1* and variation in lamb carcass traits. The results suggest that ovine *UCP1* is moderately genetically variable. Variation in ovine *UCP1* is possibly associated with variation in both carcass subcutaneous fat and hind-leg intramuscular fat deposition. This result is once again promising for sheep farmers and could potentially be used in sheep breeding. The future use of *UCP1* testing could possibly help farmers to identify those lambs with greater genetic potential to produce leaner carcasses. However, further studies are once again needed to investigate the variation in more sheep breeds and confirm the associations revealed between ovine *UCP1* variation and variation in key carcass traits such as fat content.

Given the association found between the promoter variants of ovine *UCP1* and variation in fat composition in lamb carcass, it could be suggested that the promoter region of ovine *UCP1* may be important for the regulation of UCP1 function and that the variation in this

region may have similar effects on fat metabolism in humans and sheep. However, the exact mechanism of how this variation affects the expression of *UCP1* is still unclear.

No association was found between variation in ovine *HSL*, ovine *UCP1* and variation in post-weaning growth. This may be because that these genes are encoding "down-stream" lipolytic factors which are mainly involved in the degradation of fat deposits and thus have less effects on growth than on the fat traits. Once again, further study is required to better understand this association through investigating variation within more regions of the gene and in more sheep breeds.

The majority of the sequence variation within the genes investigated in this work is located in non-coding region and is expected to be silent. However, given the association found between these "silent variants" and sheep traits, the variation within ovine *ADRB3*, ovine *HSL* and ovine *UCP1* may be in linkage disequilibrium with other variation in the gene (or flanking the gene). Recently, it has also been suggested that naturally occurring silent variation within non-coding sequences can affect *in vivo* protein folding, and consequently function (Kimchi-Sarfaty et al., 2007). Silent variation in coding region may create codons in the mRNA for which tRNAs are less available and this may slow the rate of translation and protein function (Kimchi-Sarfaty et al., 2007; Komar, 2007). This may affect gene expression or protein structure and function, which ultimately cause the changes in animal growth and carcass traits. This would require further functional studies with these genes to be undertaken to see whether an effect is observed.

Given that the genes for ADRB3, HSL and UCP1 are located on different chromosomes (*ADRB3* located at OAR chromosome 26, *HSL* located at OAR chromosome 14 and *UCP1* located at OAR chromosome 17), a direct interaction between these genotypes is unlikely to occur. Therefore, the effects of these variants on post-weaning growth and carcass traits are expected to be additive and the combination of these additive effects may be able to improvement sheep breeding approaches to produce lambs having leaner carcasses and faster post-weaning growth-rates. For example, one approach may be utilising these identified genetic variants for "multi-gene assisted selection" as shown in Figure 6.1.

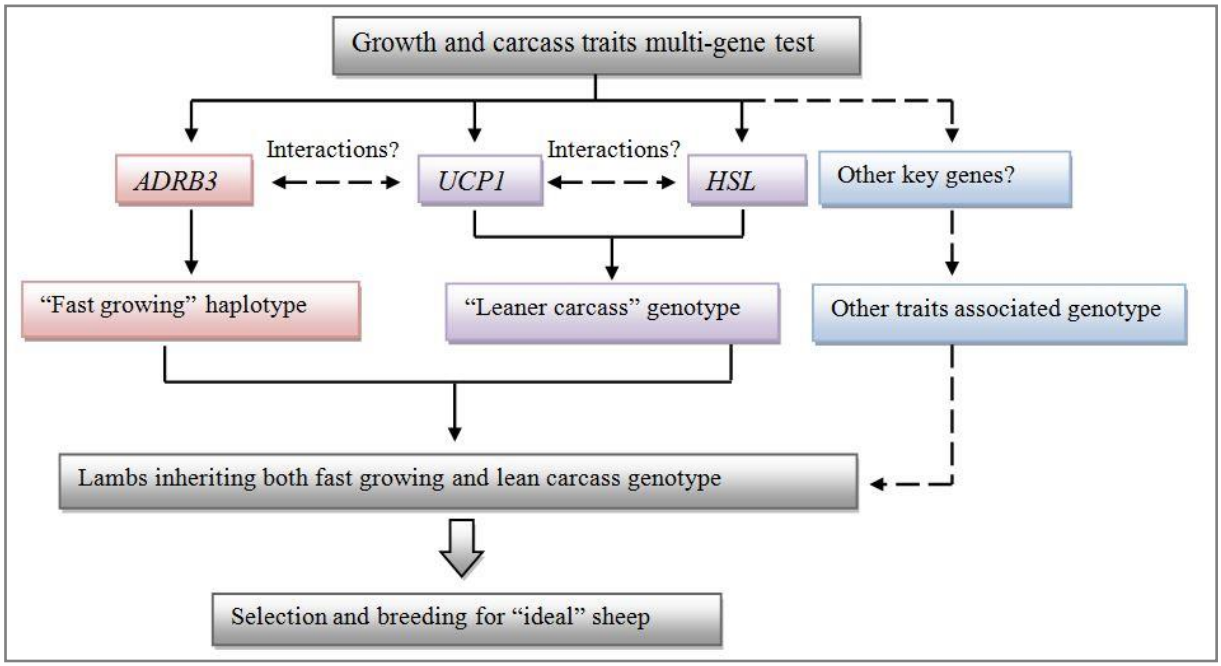


Figure 6.1 Process of multi-gene assisted selection for ideal sheep.

This approach would provide more accurate criteria for breeding lambs with better growth and carcass traits.

As mentioned before, caution is still needed if breeding for those "better genotypes" in other sheep breeds due to the need to confirm the consistency of these associations. Further work is also required to ascertain if the same effects are seen in other breeds.

It is well known that quantitative traits like growth and carcass traits, which vary continuously, tend to be affected by the combined effects of one or more genes and the environments. Therefore, future work needs to investigate more genes derived from key energy metabolism pathways such as the myostatin gene (*MSTN*), MyoD gene (*MYOD1*), Myf5 gene (*MYF5*), myogenin gene (*MYOG*), and Myf6 gene (*MYF6*). The associations of those genes with growth and carcass traits could be combined into a multi-gene assisted selection approach to increase the accuracy of genetic selection.

Appendices

Appendix A: The NZ Suffolk sires used in Chapter 3 and their *ADRB3* diplotypes

Stud	Sire ID[1]	Sire haplotype	Number of progeny
1	14/07	*C-a/E-e*	11
	399/07	*B-c/E-e*	13
	56/05	*A-b/C-a*	10
	70/01	*A-b/C-e*	8
	9/07	*A-b/F-e*	6
	94/06	*B-c/E-e*	4
	95/06	*B-cE-e*	14
2	Lindsay01	*C-a/E-e*	5
	Beechbank 206.04	*A-b/B-c*	2
	Coleford 213.06	*C-a/E-e*	15
	Phoebe 89.05	*A-b/A-b*	9
	Stonylea 156.02	*E-e/C-a*	10
	Te Takutai 228.2	*B-c/C-e*	8
	Waterton 507F.06	*B-c/C-a*	3
3	Clifton JM 148.03	*A-b/E-e*	4
	Coleford 381.03	*A-b/E-e*	2
	Goldstream 192.02	*A-b/A-b*	5
	Omagh 131.07	*A-b/E-e*	13
	Studholme 20.07	*A-b/B-c*	10
	Waimotu 934.07	*B-c/C-e*	11
4	GS254/04	*A-b/A-b*	26
	PB120/06	*A-c/B-c*	18
	Tyanee 110/06	*A-b/E-e*	13
	Kyle01	*A-b/B-c*	15
	Kyle02	*A-b/C-e*	37
	Windsor 2/04	*B-c/C-e*	45
5	Punchbowl 41/04	*B-c/E-e*	60
6	Co 83/06	*E-e/E-e*	31
	Gs 275/04	*A-b/E-e*	74
	VA118/07	*A-b/E-e*	31
	VA155/06	*A-b/F-e*	26
	VA203/07	*B-c/F-e*	18
7	BR 106/06	*A-b/E-e*	59
	PA 42/06	*A-b/B-c*	36
	PA 65/03	*A-b/B-c*	45
	PA 76/05	*A-b/C-a*	67
8	Gardiner 01	*C-a/E-e*	17
	Gardiner 02	*A-b/F-e*	16

[1]Sires in the same stud might be related.

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