Genetic Independence of Fat Depots in Cattle

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Abbreviations

BTA	cattle chromosome
dATP	2’ deoxyadenosine 5’-triphosphate
dCTP	2’ deoxycytosine 5’-triphosphate
dGTP	2’ deoxyguanosine 5’-triphosphate
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide-triphosphate
dTTP	2’ deoxythymidine 5’-triphosphate
EBV	Estimated breeding value
EMA	Eye muscle area (*Longissimus dorsi*)
emaam	Eye muscle area (*Longissimus dorsi*)
hscw	hot standard carcass weight
HRM	High Resolution Melt
IMF	Intramuscular fat
marbam	AUS-MEAT marble score
mbms / msamb	Meat Standards Australia marble score
mbusms	USDA marble score
RFI	Residual Feed Intake
TAE	tris acetate ethylenediaminetetra-acetic acid
w/v	weight per volume

Abstract

The amount and distribution of adipose tissue is important to cattle production. Fat influences the animal's reproductive efficiency and determines its carcass value. As a cow's reproductive efficiency is associated with a level of overall fatness, not just a particular fat depot, being able to re-partition fat to a more valuable depot while reducing fat in less valuable depots would be advantageous. Most previous research involving fat deposition in cattle focussed on subcutaneous and intramuscular fat, and usually evaluated these in relation to total fat or carcass weight rather than the relationship between individual fat depots. The hypothesis that there is a genetic basis for variation in fat distribution in cattle and a weak relationship between fat depots independent of anatomical site was tested. The principal aim of this research was to gain a better understanding of the mechanisms controlling fat deposition in cattle, including any relationship between fat depots.

Marbling features (e.g. shape and orientation) and seam (intermuscular) fat area were quantified using image analysis. The seam fat area and other carcass fat measurements were used to examine the relationship between fat depots. Candidate genes for fat deposition traits were identified and sequenced in Jersey – Limousin mapping sires to find single nucleotide polymorphisms (SNPs). In all, 33 SNPs from 11 candidate genes for fat deposition were selected for association studies in the sire progeny.

There was large variation in all of the measures but the variation was largely independent of other marbling factors. The seam fat area data were used to identify a quantitative trait locus on chromosome 19, and subsequently identify candidate genes for seam fat area. In general, there were low correlations between fat traits suggesting the relationship between the depots was not strong. The fixed effects of cohort, breed

and *myostatin* variant affected general fat deposition. However, sire affected fat distribution, as no sire had progeny consistently higher or lower for all fat traits. These results suggest there is only a weak genetic link between the fat depots.

The size of effect was small for most of the SNPs associated with fat deposition, although there were some candidate genes with sizeable effects, for example, *tyrosine kinase, endothelial (TEK1)* (channel fat, 28%) and *ß, ß-carotene 15, 15'-monooxygenase (BCMO1)* (subcutaneous fat, 20%). Moreover, the combined effect of all SNPs affecting a single trait explained 38% (channel fat), 26% (seam fat and subcutaneous fat) and 23% (omental fat) of the phenotypic variation. Interestingly, although some genes were associated with variation in more than one fat trait, no one gene was associated with all fat traits or overall fatness.

The major conclusion from the research described herein is that there is genetic influence on fat deposition in addition to the effects of age, breed and management, the deposition of fat into the various adipose sites is controlled in an independent manner genetically and there appears to be no one gene that affects deposition in all sites. There were four principal results that support this conclusion; 1) there were low correlations between fat traits, 2) there were no sires with progeny consistently high or low for all fat traits, 3) the QTL for the various fat depots did not overlap with each other, and 4) no SNP was associated with all fat traits. These results indicate that there is large scope for selecting for and against individual fat traits without altering other fat depots.

Declaration

I, Andrew Egarr certify this work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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Andrew R. Egarr
October 2011

Dedication

I dedicate this work to my wife Bronwyn, the love of my life.

Acknowledgements

The time spent on this project has been interesting, enlightening and fulfilling, if at times frustrating. However, this would not have been possible on my own, and I am indebted to many people who have been part of the process.

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This project utilised data from three cattle herds. I am grateful to the J.S. Davies Bequest that provided the funds necessary to establish the Davies Gene Mapping herd, and to those who have gone before me, who measured, sampled and analysed this valuable resource. These people have paved the way for the mining of much information in cattle genetics by many students. I also thank Dr Neil Cullen, Dr Chris Morris and others at AgResearch (NZ) who supplied data from the AgResearch Gene Mapping herd; to Dr Robert Herd, Dr John Thompson and the NSW Department of Industry and Investment for making samples available from steers of the Trangie Residual Feed Intake herd, Jason Siddell who organised the collection of the Trangie samples and the commercial feedlot for their cooperation.

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It may surprise those around me, but underlying all of this has been my faith. I have often questioned my situation but as I look back it all becomes clear, and I am sure that with the passage of time it will all make perfect sense.

Andrew Egarr

October 2011

What appears as a thoroughly systematic piece of scientific work is actually the final product: a cleanly washed offspring that tells us very little about the chaotic mess that fermented in the mental womb of its creator.

Auner Treinin.

Chapter 1 Introduction and literature review

1.1 Background

Adipose tissue has been the subject of much research over recent years due not only to the role it plays in sustaining the body's energy balance (Robelin, 1986), but also in response to the ever increasing incidence of obesity (Rosen *et al.*, 2000, MacDougald and Mandrup, 2002, Phan *et al.*, 2004, Gesta *et al.*, 2006). Previously, adipose tissue had been accepted as a rather passive tissue that absorbs excess fatty acids and glycerol and stores these in the form of triacylglycerols (TAG). TAG is then released from these reserves as required, such as during periods of mild fasting (Hu *et al.*, 1996, Mandrup and Lane, 1997, Boone *et al.*, 2000, Ntambi and Kym, 2000, Gregoire, 2001). Interest in the role of adipose tissue has increased since its correlation to an elevated incidence of cardiovascular disease, stroke and non insulin-dependent diabetes mellitus (type 2 diabetes) in humans was established (Hausman *et al.*, 2001, Lehrke and Lazar, 2005, Gesta *et al.*, 2006). Adipose tissue is now considered a dynamic metabolic and endocrine organ that not only has a role in energy balance but also a role in immunological responses (Gregoire, 2001, Phan *et al.*, 2004, Fu *et al.*, 2005, Laviola *et al.*, 2006, Anghel and Wahli, 2007).

Interestingly, the role of adipose tissue in the above mentioned diseases is depot specific, with increased risk of these diseases correlated to excess upper body fat in humans (Jensen, 1997, Després *et al.*, 2001, Laviola *et al.*, 2006), the so called apple shape, and not lower body fat, or pear shape. Furthermore, there is a correlation between insulin resistance and internal fat, but not subcutaneous fat (Adams *et al.*, 1997, Zierath *et al.*, 1998, Torriani and Grinspoon, 2005, Rosen and MacDougald, 2006). In general, the distribution of fat is gender specific, with men storing more abdominal fat and women more subcutaneous fat, particularly in the gluteal-femoral region (Mauriege *et al.*, 1991, Cooke and Naaz, 2004, Klaus and Keijer, 2004).

The depot specific effect of adipose tissue is not restricted to disease. It has been reported (Djian *et al.*, 1985) that cultured adipocyte precursors isolated from epididymal fat of rats have less capacity for differentiation and replicate slower than perirenal pre-adipocytes from the same animal. Also, the cells within the adipocyte precursor population are heterogeneous for replication and differentiation capacity. The authors suggested that an individual's predisposition to obesity could be the result of an increased frequency of pre-adipocytes with a greater capacity for replication and differentiation. There is evidence from human studies to support this supposition. Roncari *et al*. (1981) demonstrated that in obese humans, the precursor cell populations from omental fat replicate faster than those from humans of normal weight and this, in turn, may be related to abnormal lipoprotein lipase activity.

However, the interest in adipose tissue and how it is distributed is not restricted to human disease; it is also of great interest to the cattle industry. Where fat is deposited determines, to a large part, the grade of the carcass, and hence, its value (Robelin, 1986). The distribution of fat also determines the animal's ability to store and use fat, which in turn, influences the animal's productive life and resistance to metabolic diseases (Sonstegard *et al.*, 2000). This has significant financial impact for both the beef and dairy industries. As there appears to be significant variation in fat distribution between cattle, even those with the same level of total body fat, it is important to investigate both the genetic and environmental factors involved in the partitioning of fat to the various adipose depots (Kempster *et al.*, 1976). The aim of this work was to gain a better understanding of the mechanisms controlling fat deposition traits in cattle by identifying the genes involved and examining their biological interactions. This should firstly, lead to the identification of molecular markers that will allow reliable marker assisted selection, and secondly, facilitate ways to manipulate the amount of fat and the distribution of that fat in cattle.

1.2 White adipose tissue versus brown adipose tissue

Adipose tissue exists in two forms, white and brown. These differ morphologically in that white adipocytes have a single large lipid vacuole and brown adipocytes have numerous lipid vacuoles, mitochondria and greater vascularisation, which gives the cells the brown colour. Brown adipose tissue produces a non-shivering thermogenesis via an uncoupling protein (UCP1) in the mitochondria, which allows energy to be consumed without generating ATP (Flier, 1995, Bonet *et al.*, 2003, Rosen and MacDougald, 2006). This is particularly important for heat regulation in newborn mammals. White adipose tissue is the most common form in adult animals, making up approximately 90% of the total fat content of the animal, functioning in the storage and release of fatty acids as required, as well as releasing various signalling molecules (Bonet *et al.*, 2003). The discussion of deposition and distribution of fat herein will focus on white adipose tissue only, as the major form in mature animals.

1.3 White fat depots

White adipose tissue develops in distinct sites (depots) within the body. The fat at these depots serve particular functions such as insulation, provision of a ready supply of fatty acids for surrounding muscle, and protection of internal organs, as well as the established role of energy storage (Vernon, 1992). The amount and distribution of fat in the carcass varies both between and within breeds of cattle, and as such, it is arguably the most variable tissue in the body (Berg and Butterfield, 1976). This variation indicates that more investigation is warranted to determine the genetic and environmental components of fat distribution (Kempster *et al.*, 1976).

There are four generally accepted white fat depots in cattle: the removable fat depots of internal, subcutaneous and intermuscular fat, and intramuscular fat. Internal fat

includes visceral, omental, abdominal, kidney and channel fat. Fat in this depot is likely to serve as cushioning for the internal organs (Bone, 1988). Subcutaneous fat includes rump (measured at position 8) and rib fat, and provides insulation (Eckert *et al.*, 1988). Intermuscular fat is the fat between muscles and includes the fat inside the rib cage and brisket (Cianzio *et al.*, 1982). This fat also occurs in high value meat cuts where it is often referred to as seam fat. Intermuscular fat can be as much as 40% of the total fat in cattle (Robelin, 1986) (Figure 1.1), and approximately 60% of the removable fat in retail beef (Christensen *et al.*, 1991). Seam fat is difficult to remove without altering the characteristic shape of the cut that consumers recognise (Kempster, 1981, Christensen *et al.*, 1991), and therefore, intermuscular fat, particularly seam fat, can have a significant adverse effect on carcass quality.

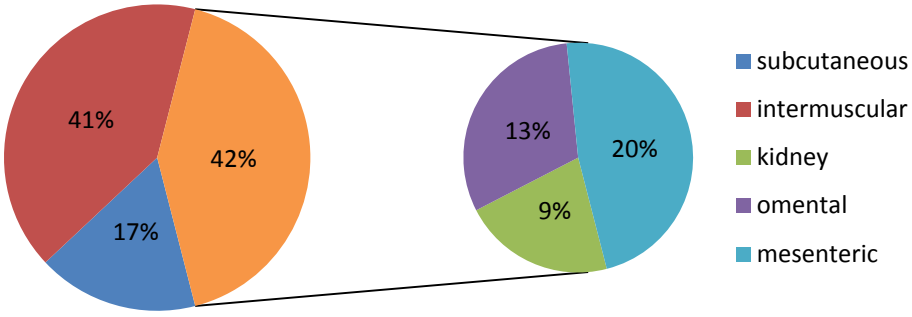


Figure 1.1: Percentage of fat in each depot of mature Friesian cattle.
Data taken from Robelin, 1986.

The fourth fat depot is the non-removable intramuscular fat (IMF). This is described as "taste fat" while the other fat depots are "waste fat" (Rouse and Wilson, 2001) and is often referred to as marbling. Marbling increases the tenderness and palatability of beef (Sasaki *et al.*, 2006) and is highly valued, particularly in the Australian export markets such as Japan and Korea (Bindon, 2004).

Marbling is intramuscular fat, but intramuscular fat is not necessarily marbling. Marbling is the term generally used for the fat seen as streaks or flecks between the muscle

fibres in a cross section of the *Longissimus dorsi* and other skeletal muscles (Harper and Pethick, 2004, Sasaki *et al.*, 2006). Intramyocellular lipid, fat stored in myocytes, is not visible to the naked eye but does contribute to the total intramuscular fat content, although this contribution is small (Pethick *et al.*, 2004). Intramuscular fat is important in humans because of its correlation with obesity and insulin resistance (Hulver *et al.*, 2003). Also intramuscular fat is concentrated near the mitochondria in skeletal muscle and therefore, is the first of the fat reserves to be utilised as an energy source (Dagenais *et al.*, 1976).

The order in which fat is deposited in a young animal is accepted as internal, intermuscular, subcutaneous and then intramuscular. As the animal grows, the amount of fat deposited increases relative to muscle gain and therefore, the intramuscular fat will appear as a late maturing fat depot (Pethick *et al.*, 2004). However, as these authors noted, Johnson *et al*. (1972) found that after the initial development phase, intramuscular fat (and the other fat depots) stayed constant as a percentage of total side fat throughout subsequent growth of the animal. Cianzio *et al.* (1982) also found that intramuscular fat stayed constant as a percentage of total fat. Pethick *et al.* (2000) reported that intramuscular fat remained constant relative to subcutaneous and intermuscular fat even with a considerable increase in total fat deposition. Bruns *et al*., (2004) concluded that marbling increases linearly with growth, and Oddy *et al*., (2000) showed that the percentage of intramuscular fat in cattle at entry to the feedlot was related to the percentage of intramuscular fat at the end of the feeding period. This would suggest that although the expression of the marbling phenotype is associated with mature animals, the deposition of intramuscular fat is not. Consequently, for marbled beef production, fast growth will produce the marbled phenotype at an earlier age because the animal will reach its maximum size sooner, allowing a longer period for the expression of marbling (Pethick *et al.*, 2000, Pethick *et al.*, 2004).

In addition to the differences in the function of each depot and the stage of maturity at which fat is deposited to the individual depots, fat depots differ in other features. For example, there is variation between depots in the structure and density of vascularisation (Hausman and Thomas, 1986, Crandall *et al.*, 1997), in adipocyte cell size (Meade and Ashwell, 1980, Hausman and Thomas, 1986, Crandall *et al.*, 1997) and in hormone secretion and response (Arner, 1995, Bornstein *et al.*, 2000, Lafontan and Berlan, 2003). All of these differences suggest that the fat depots are physiologically and functionally distinct from one another and therefore, the deposition of fat at each site may be also controlled independently.

1.4 Cattle breed differences in fat distribution

Cattle breeds vary in body composition. In the results reported by Marshall (1994), there was large variation in backfat depth (6.4 to 14.2mm) and marble score (444 – 614) between breeds, but less variation in kidney fat% (2.2 – 4.6%), with European breeds generally leanest and British breeds fattest (Table 1.1). Similar results were reported by Schenkel *et al.* (2004), with backfat ranging from 2.58 to 6.29mm and intramuscular fat % from 2.78 to 4.45%, and by Pitchford *et al.* (2002), where fat depth varied from 8 – 14mm and intramuscular fat percent from 3 – 4.8%. European breeds are consistently leaner than British breeds (Table 1.1). When adjusted to a constant endpoint (slaughter age or time in feedlot), British breeds have a higher marble score than *Bos indicus* and many European breeds (Marshall, 1994). Charolais steers have less carcass fat than Friesian, which in turn have less than Angus and Shorthorn, and this trend is generally consistent in European and British breeds (Robelin, 1986). Dairy breeds deposit more internal but less subcutaneous fat than beef breeds (Berg and Butterfield, 1976, Charles and Johnson, 1976, Kempster *et al.*, 1976, Truscott *et al.*, 1976). Fortin *et al.* (1981) reported Holstein animals deposit more internal fat than

Angus, but found no difference in subcutaneous fat deposition between Holstein and Angus. Also, there appears to be no difference in intermuscular fat deposition between dairy and beef breeds (Charles and Johnson, 1976, Truscott *et al.*, 1976, Fortin *et al.*, 1981).

Table 1.1: Breed comparisons of carcass composition measurements.

Breed	fat depth (mm)	carcass weight (kg)	eye muscle area (cm2)	marble score	IMF (%)	Kidney Fat (%)	Adapted from
Jersey	9.7	262	71.3	614	-	4.6	Marshall *et al.*
Jersey	10.7	236	-	-	4.8	-	Pitchford *et al.*
Holstein	8.6	292	74.3	521	-	3	Marshall *et al.*
Angus	6.06	-	73.55	-	4.45	-	Schenkel *et al.*
Angus	14.2	288	76.1	564	-	3	Marshall *et al.*
Angus	14.3	283	-	-	4.6	-	Pitchford *et al.*
Red Angus	11.7	285	75.4	574	-	2.9	Marshall *et al.*
Hereford	6.29	-	70.32	-	3.57	-	Schenkel *et al.*
Hereford	12	268	-	-	3.7	-	Pitchford *et al.*
Hereford	13.2	288	75.6	519	-	2.8	Marshall *et al.*
Shorthorn	10.9	309	76.2	562	-	2.9	Marshall *et al.*
South Devon	9.8	284	-	-	3.8	-	Pitchford *et al.*
South Devon	11.2	293	77.8	550	-	3.3	Marshall *et al.*
Santa Gertrudis	13	298	72.1	534	-	-	Marshall *et al.*
Beefmaster	13.7	-	77.6	518	-	-	Marshall *et al.*
Brahman	11.7	291	74.2	475	-	2.8	Marshall *et al.*
Simmental	4.07	-	86.45	-	3.31	-	Schenkel *et al.*
Simmental	8.3	302	82	506	-	2.8	Marshall *et al.*
Gelbvieh	8.4	299	82.2	503	-	2.7	Marshall *et al.*
Maine Anjou	8.1	308	84.1	496	-	2.6	Marshall *et al.*
Salers	8.9	309	81.9	511	-	2.8	Marshall *et al.*
Piedmontese	6.4	298	89.8	506	-	2.6	Marshall *et al.*
Belgian Blue	8	289	-	-	3	-	Pitchford *et al.*
Limousin	3.55	-	88.39	-	3.15	-	Schenkel *et al.*
Limousin	8.7	293	84	477	-	2.7	Marshall *et al.*
Limousin	9.9	278	-	-	3.1	-	Pitchford *et al.*
Charolais	3.75	-	84.52	-	3.25	-	Schenkel *et al.*
Charolais	10	310	84	471	-	2.8	Marshall *et al.*
Chianina	6.6	302	84.9	444	-	2.2	Marshall *et al.*
Blonde d'Aquitaine	2.58	-	92.26	-	2.78	-	Schenkel *et al.*
Wagyu	11.8	244	-	-	4.5	-	Pitchford *et al.*

Adapted from Marshall *et al.* 1994, Schenkel *et al.* 2004, and Pitchford *et al.* 2002. Intramuscular fat % was calculated as an actual percentage of *L. dorsci* muscle (Pitchford *et al*.) and predicted from ultrasound scan information (Schenkel *et al.*).

1.5 Adipogenesis

Adipogenesis is the differentiation of pre-adipocytes to adipocytes (Rosen and Spiegelman, 2000). Approximately one third of adipose tissue consists of adipocytes, with pre-adipocytes, fibroblasts, blood vessels and nerve tissue constituting the remainder (Ntambi and Kym, 2000). This makes the isolation of pre-adipocytes difficult, and when coupled with the fact that the pre-adipocytes are in various stages of development, the difficulty of investigating adipogenesis *in vivo* is increased. The lack of preadipocytic molecular markers has also hindered *in vivo* studies (Rosen *et al.*, 2000). Thus, most of the work on adipogenesis has been done *in vitro* using pre-adipocyte cell cultures. This is considered valid as it replicates most of the main features of adipogenesis *in vivo* (Macdougald and Lane, 1995, Rosen *et al.*, 2000, Salma *et al.*, 2006). One of the most reliable cell lines for this purpose is the mouse pre-adipocyte 3T3-L1 cell line because it exhibits many of the biochemical and metabolic functions of the *in vivo* pre-adipocytes (Novikoff *et al.*, 1980) and the development of fat droplets is identical to that in live adipose tissue (Green and Meuth, 1974, Green and Kehinde, 1975).

There are disadvantages with using an established cell line. Firstly, pre-adipocytes from different fat depots have different adipogenic potential (Djian *et al.*, 1985). Also, cell culture does not allow for investigation of the depot specific aspects of the adipose tissue (Rosen *et al.*, 2000), which, as mentioned above, have an impact for both human health and the beef and dairy industries. Secondly, culturing removes the cell from the normal extra cellular matrix (ECM) and supporting structures (Rosen *et al.*, 2000), and the ECM degrading proteases are required for regulating adipogenesis (Selvarajan *et al.*, 2001). Thirdly, the aneuploid status of the pre-adipocyte cell line may compromise the cell's ability to undergo differentiation (Rosen *et al.*, 2000).

Primary pre-adipocyte culture has been investigated, but this method also has inherent problems. A large amount of adipose tissue is required because pre-adipocytes only make up a small proportion of adipose tissue. It is also difficult to isolate the pre-adipocytes from the fibroblast-like cells and lastly, cultured primary pre-adipocytes have a limited life span (Ntambi and Kym, 2000). However, Boone *et al*., (2000) have noted that although they are harder to culture, primary pre-adipocytes have been isolated from various animals, not just the mouse 3T3-L1 cell line. These primary pre-adipocytes are euploid and therefore, may replicate *in vivo* conditions more faithfully. Consequently, cells can be cultured from different fat depots within the animal or from different animals within a breed to compare ages and physical conditions.

Adipogenesis is an intricate process, highly regulated both positively and negatively via a range of stimuli, including transcription factors (Anghel and Wahli, 2007), various hormones and nutritional signals (Tseng *et al.*, 2005). Fat cells are derived from mesenchymal stem cells, multipotent cells that have the ability to differentiate into myocytes (muscle), osteocytes (bone), chondrocytes (cartilage) or pre-adipocytes (Cornelius *et al.*, 1994, Mandrup and Lane, 1997, Ntambi and Kym, 2000). Initially, the mesenchymal cells proliferate (clonal expansion) and some of these cells differentiate into pre-adipocytes. The trigger(s) for this first differentiation is unknown (Ntambi and Kym, 2000, Gregoire, 2001, Tseng *et al.*, 2005). Although these cells are committed to the adipocyte fate at this stage, they can still become either white or brown fat cells (MacDougald and Burant, 2005). These committed pre-adipocytes then proliferate and go into a growth arrest, at which point, there is expression of *c-fos, c-jun, junB, c*-myc and the transcription factors, CCAAT/enhancer binding proteins (*C/EBP*) *β* and *δ* (Ntambi and Kym, 2000). In cultured cell lines, this is in response to the addition of an adipogenic mixture MDI, methylisobutylxanthine (MIX), insulin and dexamethasone (DEX). MIX is a cAMP-phosphodiesterase inhibitor used to stimulate the cAMP-

dependent protein kinase pathway and DEX is a synthetic glucocorticoid agonist that stimulates the glucocorticoid receptor pathway (Ntambi and Kym, 2000). Following this, there is another one or two rounds of clonal expansion prior to the second, permanent, growth arrest which corresponds to the expression of two other transcription factors, peroxisome proliferator-activated receptor gamma (*PPARG*) and *C/EBPα* (Ntambi and Kym, 2000, Rosen *et al.*, 2000). Although this implies that mitosis is required to allow differentiation to proceed, Entenmann and Hauner (1996) showed that inhibition of mitosis does not impede development of adipocytes. This permanent growth arrest occurs when the cells have completed the post-confluent mitosis, and it is assumed that mitosis allows the DNA to be unwound to enable access to regulatory response elements by transcription factors (Ntambi and Kym, 2000). The adipose cells begin to accumulate lipid molecules, initially in small droplets (multilocular) which then eventually fuse into one large droplet (unilocular), and take on the characteristic rounded shape. Once the cells start to accumulate lipid, they are terminally differentiated (Martin *et al.*, 1999, Ntambi and Kym, 2000, Rosen *et al.*, 2000, MacDougald and Burant, 2005) and the expression of insulin receptors, fatty acid synthase (FAS) and glucose transporter-4 (Glut-4) is observed (Tseng *et al.*, 2005). It has been demonstrated that for a short time after the initiation of differentiation, exposing cells to retinoic acid (vitamin A) or methylisobutylxanthine can cause the cells to “dedifferentiate” (Cornelius *et al.*, 1994).

1.6 Transdifferentiation

A small number of mesenchymal stem cells exist within the skeletal muscle mass with the number staying relatively constant through asymmetric division, where after mitosis, one daughter cell remains multipotent while the other continues on its committed fate (Harper and Pethick, 2004). At this point, the stem cells are still able to

differentiate into muscle, bone, cartilage or fat cells. However, even when the stem cells have become committed to a myogenic fate, they still can differentiate into an adipocyte. When grown in culture and exposed to the correct adipogenic stimuli, muscle satellite cells can undergo adipogenesis (Asakura *et al.*, 2001, Wada *et al.*, 2002). Wada *et al*. (2002) also found that myogenic cells express some of the markers for osteogenesis (Runx2) and adipogenesis (*PPARG*) as well as myogenesis (MyoD). This implies that marbling adipocytes can develop from different uncommitted stem cells within the skeletal muscle (Harper and Pethick, 2004).

Although transdifferentiation has for a long time been doubted, there is considerable evidence that it is in fact a real phenomenon (Slack and Tosh, 2001). One example of normal (i.e. not pathogenic) transdifferentiation in animals is the reversible, adipocyte to endothelium and endothelium to adipocyte transdifferentiation that occurs in the mammary glands of mice during pregnancy, lactation and weaning (Morroni *et al.*, 2004). Peroxisome proliferator-activated receptor gamma (*PPARG*) is a major factor in the differentiation of adipocytes and is implicated in the transdifferentiation of myocytes to adipocytes (Hu *et al.*, 1995, Holst *et al.*, 2003, Yu *et al.*, 2006). *PPARG* expression is correlated with adiposity (Anghel and Wahli, 2007), and as the deposition of intramuscular fat increases, so too does the expression of *PPARG*. Potentially, this may lead to transdifferentiation of myocytes to adipocytes and therefore, increased marbling.

1.7 Hyperplasia versus hypertrophy

Adipose tissue increases in mass through hyperplasia (adipogenesis) and hypertrophy. Hyperplasia is the proliferation (clonal expansion or mitotic division) of adipocyte cells from pre-adipocytes whereas hypertrophy is the deposition of lipids into pre-adipocytes

or adipocytes. The filling of the adipocytes with lipid is a marker of their terminal differentiation.

It had been thought that all growth through hyperplasia occurs during fetal growth and during early life and that any increase in adipose tissues in the mature animal is the result of hypertrophy. However, adipose tissue contains a percentage of pre-adipocytes that are able to proliferate and differentiate. Therefore, this tissue retains a permanent capacity for hyperplasia (Wood, 1984, Adams *et al.*, 1997, Bonet *et al.*, 2003). Martin *et al*., (1999) found that there is terminal differentiation of pre-adipocytes in weaned calves (approx. 220 days). This supports the theory that there are periods of hyperplasia and terminal differentiation of pre-adipocytes in growing cattle. It also suggests that there may be options for hormonal and dietary manipulations to influence the development of adipose tissue in maturing cattle (Martin *et al.*, 1999).

1.8 Manipulation of fat distribution by diet

As intramuscular fat is highly valued in certain markets (Bindon, 2004), altering how the total fat is distributed by increasing intramuscular fat without increasing other fat depots would be advantageous. Adjusting the diet may be a cost effective and efficient method to maximise intramuscular fat, without relying solely on long term breeding programs. Furthermore, this may enable beef producers to target opportunity markets, without committing to breeding high marbling cattle.

It has been observed that rats eat to maintain muscle and when fed a diet deficient in amino acids, consumed more total food in order to fulfil this need, thereby becoming fatter (Pethick *et al.*, 2004). Pigs fed reduced amino acid, short term diets resulted in a smaller eye muscle area, increased intramuscular fat percent, but no effect on marble score, and a trend to increased subcutaneous fat (Cisneros *et al.*, 1996). When pigs are fed a diet that has a low protein to digestible energy ratio, they have increased

intramuscular fat content and reduced carcass dressing percentage with no effect on subcutaneous fat depth (D'Souza *et al.*, 2003). A similar result has also been suggested when lambs are fed a low protein diet (Pethick *et al.*, 2004). However, altering the amount of protein available in a grain based diet did not have a significant effect on intramuscular fat content in cattle (Oddy *et al.*, 2000). Suess *et al.* (1969) found that altering the level of nutrition during growth changed the intramuscular fat percent without altering muscle growth in cattle, but maintaining a constant high or medium level of nutrition throughout growth had no significant effect on intramuscular fat percent in Holstein steers. Smith and Crouse (1984) found a high energy diet increased subcutaneous and internal fat but did not significantly alter marble score in cattle. Bonet *et al.*, (2003) reported that diets low in vitamin A, carotenoids and other fat-soluble vitamins tended to increase marbling, although Montgomery *et al.* (2005) found no difference in marble score, internal fat or fat thickness in relation to vitamin E supplementation. Pigs fed a vitamin A deficient diet had increased intramuscular fat percent (D'Souza *et al.*, 2003). Cattle supplemented with vitamin A have a decreased marble score (Oka *et al.*, 1998), and cattle and sheep on vitamin A restricted diets have increased intramuscular fat percent, although this did not alter subcutaneous fat depth (Siebert *et al.*, 2006). Furthermore, cell cultures from Angus steers showed that subcutaneous fat incorporates more acetate and lactate than glucose, whereas more glucose than acetate and lactate is incorporated into intramuscular fat (Smith and Crouse, 1984).

These results indicate that altering the diet may provide a method to preferentially increase intramuscular fat (Siebert *et al.*, 2006) or decrease subcutaneous fat without altering intramuscular fat (Smith and Crouse, 1984). However, there appears to be no information on how altering the diet will affect either internal fat or intermuscular fat, an area that should be addressed. Furthermore, altering diet may not always be practical

or affordable (e.g. large alteration to energy content) or may have adverse effects on animal health (e.g. vitamin A deficiency). Therefore, identifying the genes involved in fat distribution is more likely to be the better long term strategy, and these methods should be viewed as complementary rather than alternatives (Bindon, 2004).

1.9 Genes/proteins involved in adipogenesis

Adipogenesis, as with the differentiation of any other cell type, is essentially a change in gene expression patterns. Those genes whose expression dictates the initial state of the cell are down-regulated or overtaken by other gene products that alter and determine the final phenotype of the cell (Rosen *et al.*, 2000).

1.9.1 Transcription factors

Clonal expansion and differentiation of cells, as well as the control of specialised cell function, are affected through an alteration of the expression of genes within that cell. This is generally controlled at the transcription level via interactions between specific transcription factors and regulatory sequences within the promoters and enhancer regions of these genes (Zhang *et al.*, 1997, Ramji and Foka, 2002).

1.9.1.1 CCAAT/enhancer binding proteins (C/EBP)

C/EBPs are transcription factors that have a conserved basic-leucine zipper domain. All isoforms share greater than 90% homology in this region yet the N-terminal region is comparatively divergent (Ramji and Foka, 2002). There are at least six isoforms of C/EBP and within each of these isoforms, there is further variation due to multiple translation start sites, regulated proteolysis and alternative promoters and splicing (Ramji and Foka, 2002). All of these proteins have structural and functional homologies and all act as homo- or heterodimers to enable DNA binding (Lekstrom-Himes and

Xanthopoulos, 1998, Rosen *et al.*, 2000). These proteins are found in a number of tissues including granulocytes, liver (hepatocytes), adrenal gland, lung, intestine and adipose. They also have a role in resistance to infections and injury response (Zhang *et al.*, 1997, Lekstrom-Himes and Xanthopoulos, 1998, Rosen *et al.*, 2000).

There are various methods by which C/EBPs are regulated, including transcriptionally, post-translationally, and through interactions with other C/EBP family members (Rosen *et al.*, 2000, Salma *et al.*, 2006). As an example, the alternative translation initiation site of *C/EBPA* produces a 42kDa protein which is a stronger transcriptional activator than the alternative 30kDa protein. The 42kDa protein prevents cell proliferation. The 30kDa isoform is still functional, but has no inhibitory effect on cell proliferation. Therefore, the 12kDa N-terminal sequence is likely to contain an antimitotic region capable of blocking cell proliferation (Lin *et al.*, 1993).

At least three C/EBPs are implicated in the regulation of adipogenesis along with *PPARG* (*C/EBPA, C/EBPB* and *C/EBPD)* (Clarke *et al.*, 1997). *C/EBPB* and *C/EBPD* are expressed in response to insulin, cAMP and glucocorticoid (Ramji and Foka, 2002) within one hour of the start of differentiation (Salma *et al.*, 2006) and are likely to be involved in directing the differentiation (Ntambi and Kym, 2000). Following this, the pre-adipocytes go into growth arrest and *C/EBPA* is expressed (Ramji and Foka, 2002). *C/EBPA* and *PPARG* expression is induced by the binding of *C/EBPB* and *C/EBPD* (Clarke *et al.*, 1997, Ramji and Foka, 2002, Salma *et al.*, 2006). *C/EBPA* is upregulated in adipose cells and operates in fully differentiated cells that are non-proliferating, playing an important role in adipocyte differentiation. When *C/EBPA* is expressed, differentiation is induced and when *C/EBPA* is inhibited, differentiation is blocked (Zhang *et al.*, 1997).

As stated above, *C/EBPB* and *C/EBPD* induce expression of *C/EBPA* and *PPARG*. However, when expression of these two early expressed proteins diminishes, *C/EBPA* and *PPARG* are able to induce and regulate each other's expression, most likely via cross regulation (Ntambi and Kym, 2000).

Most of the genes expressed in differentiating adipocytes are induced by *C/EBPA*, *C/EBPB* and *C/EBPD* as well as *PPARG* (Salma *et al.*, 2006). These genes include resistin, adiponectin and leptin, which are all adipocyte secreted peptides that regulate body mass and are implicated in obesity (Miner, 2004, Salma *et al.*, 2006). MacDougald and Mandrup (2002) and Fu *et al.* (2005) reported that *C/EBPA* is required for the cell to achieve insulin sensitivity. However, Rosen *et al.,* (2000) attributed this role to *PPARG*.

Another C/EBP protein involved in adipogenesis is the murine C/EBP homologous protein (*CHOP*) or growth arrest DNA damage protein 153 (*gadd153*) (hamster). This protein can dimerize with the other C/EBP proteins, but has no DNA binding domain. When it dimerizes with another C/EBP protein, DNA binding will not occur and differentiation will not proceed (Batchvarova *et al.*, 1995, Clarke *et al.*, 1997). *CHOP* may act act as a dominant-negative inhibitor of C/EBP transcription to modulate adipocyte differentiation (Batchvarova *et al.*, 1995, Clarke *et al.*, 1997, Rosen *et al.*, 2000). Expression of *CHOP* is increased in response to low glucose and cellular stress (toxins, metabolic inhibitors and low nutrient conditions) (Batchvarova *et al.*, 1995).

1.9.1.2 Peroxisome proliferator-activated receptor gamma (PPARG)

PPARs regulate the transcription of genes involved in lipid metabolism (Clarke *et al.*, 1997). There are 48 transcription factors in the nuclear receptor family that are regulated by steroid and thyroid hormones as well as lipid metabolites and xenobiotics (Lehrke and Lazar, 2005). *PPARG* is a nuclear hormone receptor that uses alternative

promoters and alternative splicing to express two isoforms, PPARG1 and PPARG2 (Clarke *et al.*, 1997, Rosen *et al.*, 2000). PPARG1 is expressed in the liver and other organs (Clarke *et al.*, 1997) and at low levels, in adipose tissue as well as the colon, breast, prostate, bladder, macrophages and type II pneumocytes (Rosen *et al.*, 2000). PPARG2 is 30 amino acids longer than PPARG1 and is selectively expressed in adipose tissue. It is the dominant *PPARG* isoform found in adipose cells and regulates adipogensis (Clarke *et al.*, 1997, Rosen *et al.*, 2000, Tsai and Maeda, 2005).

In order to bind DNA, *PPARG* must heterodimerize with the retinoid X receptor (RXR), another nuclear hormone receptor (Rosen *et al.*, 2000, Lehrke and Lazar, 2005). Like other nuclear hormone receptors, *PPARG* is a ligand activated transcription factor (Rosen *et al.*, 2000), but in the absence of ligand can silence a gene upon binding (Lehrke and Lazar, 2005). *PPARG* is activated by thiazolidinediones (TZDs), synthetic compounds used for anti-diabetic treatment (Lehmann *et al.*, 1995). Activation by *PPARG* leads to increased lipid uptake through an increase in the differentiation of adipocytes, increasing in their number but not their size (Okuno *et al.*, 1998, Lehrke and Lazar, 2005). No endogenous ligand has been determined for *PPARG* (Rosen *et al.*, 2000). *PPARG* is activated by micromolar concentrations of compounds such as long chain fatty acids (Lehmann *et al.*, 1995) and eicosanoids (compounds containing 20 carbon atoms). Oxidized alkyl phospholipids and oxidized low-density lipoproteins (Rosen *et al.*, 2000, Lehrke and Lazar, 2005) have been shown to activate *PPARG* to some degree as well. The binding affinity of all these is very low (Rosen *et al.*, 2000), and it is unclear if *PPARG* has a specific ligand or if it acts as a sensor responding to a combined concentration of weakly activating agents (Lehrke and Lazar, 2005).

PPARG regulates genes involved in the lipid accumulation of adipocytes, such as lipoprotein lipase, fatty-acid transport protein, oxidized LDL receptor, glycerol kinase, phosphoenolpyruvate carboxykinase and glycerol transporter aquaporin 7. This

suggests *PPARG* has a role in regulating the lipid metabolism of adipocytes (Lehrke and Lazar, 2005). Morphological changes, lipid accumulation and the acquisition of insulin sensitivity are all effects on mature adipocytes due to *PPARG* (Rosen *et al.*, 2000). The role of *PPARG* in inducing adipogenesis was demonstrated by the transdifferentiation of myoblasts to adipocytes when coexpressed with *C/EBP* (Hu *et al.*, 1995).

1.9.1.3 Adipocyte determination and differentiation factor-1 (*ADD1*)

ADD1, also known as sterol regulatory element binding protein-1 (*SREBP-1*), belongs to the basic helix-loop-helix-leucine zipper family of transcription factors (Ericsson *et al.*, 1997, Rosen *et al.*, 2000). *ADD1/SREBP1c* has a similar expression profile to that of *PPARG*, but its actual role is unclear. It is suggested that *ADD1/SREBP1c* is required for adipogenesis, but is not adipogenic in its own right (Fu *et al.*, 2005). It may produce a ligand that enhances *PPARG* production or activity. What has been determined is that *ADD1/SREBP1c* has a role in lipogenesis, stimulating genes such as lipoprotein lipase, fatty acid synthase and glycerol phosphate acyltransferase (Ericsson *et al.*, 1997) and is regulated by insulin (Rosen *et al.*, 2000).

1.9.1.4 Fos, jun and c-myc

Fos and jun proteins are likely to have mitogenic roles rather than be involved in any differentiation-specific events (Ntambi and Kym, 2000). *c-myc* has a role in cell proliferation and can be regulated by the *C/EBPA* promoter, which contains a *c-myc* binding site (Cornelius *et al.*, 1994). The expression of *c-myc* may be the branch point where cells are either terminally differentiated or return to a normal cell cycle (Ntambi and Kym, 2000).

1.9.2 Extracellular agents

1.9.2.1 Glucocorticoids

Glucocorticoids are a class of steroid hormones. These induce the expression of *C/EBPD* and extra cellular matrix components that are essential for the differentiation of adipocytes (Boone *et al.*, 2000). Glucocorticoids also regulate the function and distribution of adipose tissue (Masuzaki *et al.*, 2001). The glucocorticoid re-amplifying enzyme *11β-hydroxysteroid dehydrogenase type 1* (*11β-HSD-1*), has been associated with regulation of adipose tissue (Man *et al.*, 2011) although the effect is greater in visceral fat than subcutaneous fat (Morton *et al.*, 2004).

1.9.2.2 Thyroid hormones

Thyroid hormones have been shown to increase the activity of adipogenic enzymes, such as fatty acid synthase and glycerol 3-phosphate dehydrogenase, but only to a small extent. These are unlikely to be adipogenic on their own, and probably work indirectly by modulating other factors. They have also shown an additive effect with insulin (Boone *et al.*, 2000).

1.9.2.3 Fatty Acids

Long chain fatty acids (C14 – C20) have a role in adipocyte differentiation, acting as transcriptional regulators although short, medium and very long chain fatty acids have little effect (Boone *et al.*, 2000). Long chain fatty acids bind directly to *PPARG* and activate *PPARG* to enhance adipogenesis (Grimaldi *et al.*, 1999). Polyunsaturated fatty acids reduce expression of *PPARG* and *C/EBPA*, as well as suppressing insulin stimulation of fatty acid synthase transcription, thereby inhibiting adipogenesis (Fukuda *et al.*, 1999, Boone *et al.*, 2000).

1.9.2.4 Insulin

Insulin is released in response to high blood sugar content, to initiate the storage of glucose as glycogen and fat (Tilg and Moschen, 2006), and also inhibits the release of glucagon, which prevents lipolysis as an energy source (Elliott and Elliott, 1977). Insulin probably has the most adipogenic effect of all the extracellular agents, either enhancing adipose conversion without other agents or working in concert with glucocorticoids to cross regulate each other's action (Boone *et al.*, 2000).

1.9.2.5 Growth hormone

Growth hormone enhances adipogenesis by stimulating transcription of the *IGF-1* gene, and then, sensitising the adipocytes to the mitogenic effect of *IGF-1* by stimulating expression of the *IGF-1* receptor which leads to cell proliferation (Green *et al.*, 1985, Zezulak and Green, 1986, Doglio *et al.*, 1987). Growth hormone also causes pre-adipocytes to enter the differentiation program and upregulates cytoskeleton proteins to change the cell shape necessary for differentiation (Boone *et al.*, 2000).

1.9.2.6 Other growth factors

Epidermal growth factor (EGF) and transforming growth factors α and β (*TGFA, TGFB*) are associated with inhibiting adipose conversion. EGF inhibits both differentiation of pre-adipocytes *in vitro* and adipose development *in vivo* (Serrero *et al.*, 1993). *TGFA* most likely inhibits the differentiation of pre-adipocytes (Luetteke *et al.*, 1993). *TGFB* may increase the synthesis of fibronectin and collagen and reduce the expression of adipose genes in mature adipocytes, a type of dedifferentiation. Tumor necrosis factor α (*TNFA*) inhibits adipose gene expression in mature adipocytes, strongly reduces adipose conversion, increases lipolysis, decreases lipogenesis and fatty acid uptake,

interferes with pre-adipocyte proliferation (Boone *et al.*, 2000) and has a role in the regulation of leptin (Sethi and Hotamisligil, 1999).

1.9.2.7 Retinoids

Diets with high vitamin A (retinoic acid) content suppress both adipogenesis and lipogenesis. This occurs through the interference of the transcriptional activity of C/EBPs and down-regulation of RXR expression (Bonet *et al.*, 2003), a component essential to allow *PPARG* to bind DNA (described above), which is required for both adipogenesis and lipogenesis (Rosen *et al.*, 2000). Thus, increased levels of retinoic acid inhibit pre-adipocyte differentiation (Kuriharcuch, 1982). It has been suggested that because of these effects, a decreased dietary vitamin A concentration would lead to increased fat deposition (D'Souza *et al.*, 2003). However, low vitamin A increases growth hormone production (Bedo *et al.*, 1989) and growth hormone enhances adipogenesis (Green *et al.*, 1985, Zezulak and Green, 1986, Doglio *et al.*, 1987, Boone *et al.*, 2000) (Section 1.8). Nevertheless, low vitamin A diets increase adipogenesis and fat deposition (Oka *et al.*, 1998, Bonet *et al.*, 2003, Siebert *et al.*, 2006). Bonet *et al*., (2003) also noted that vitamin A reduces the secretion of leptin, which normally causes an increase in body fat and this seemingly contradictory effect may be part of a feedback loop that serves to prevent an excessive depletion of fat.

1.9.3 Adipokines

Adipokines are cell to cell signaling proteins produced predominantly in the adipose tissue (Fantuzzi, 2005). Although prostaglandins are produced in many other cell types as well as adipocytes, and therefore not strictly adipokines, they have been included in this section because they are derived from lipids and have autocrine and paracrine functions (Bigler *et al.*, 2007).

1.9.3.1 Prostaglandins

Adipocytes and pre-adipocytes produce large amounts of prostaglandins (Hyman *et al.*, 1982) which can inhibit adipose conversion (e.g. *PGF2a*), act as adipogenic agents (e.g. *PGI2*) or may act as a ligand for *PPARG* (e.g. *PGD2*). The *PGI2* receptor is mostly present in the pre-adipocytes, and therefore, *PG12* increases cAMP concentration in these cells, thereby stimulating differentiation. As *PGE2* receptors are mainly found in mature adipose cells, *PGE2* would decrease cAMP concentration and lipolysis. Together these actions increase adipose tissue mass through enhanced hyperplasia and hypertrophy (Boone *et al.*, 2000).

1.9.3.2 Leptin

Leptin is a hormone encoded by the obese (*OB* or *LEP*) gene and is expressed in the adipose tissue to inhibit appetite when a functional level of energy stores is reached (Friedman and Halaas, 1998, Kershaw and Flier, 2004). Plasma leptin concentration is positively correlated with the percentage of body fat (Friedman and Halaas, 1998, Yamada *et al.*, 2003, Yang *et al.*, 2003). Yamada *et al.* (2003) did not examine leptin protein levels in intramuscular fat but found that leptin expression was not depot specific in crossbred steers. Similarly, Geary *et al.* (2003) reported significant correlations between serum leptin levels and measures of subcutaneous fat, internal fat and marble scores in cattle, but did not measure intramuscular fat. However, Altmann *et al.* (2006) reported in lambs that subcutaneous fat, intermuscular fat and internal fat were all correlated to serum leptin concentration but intramuscular fat was not correlated. Furthermore, Yang *et al.* (2003) demonstrated that levels of leptin mRNA and adipocyte size varied between fat depots, with intramuscular fat having significantly lower leptin mRNA levels and adipocyte sizes than the other fat depots.

They concluded that leptin levels are correlated with adipocyte size. This correlation has also been reported by Fu *et al.* (2005).

1.9.3.3 Adiponectin

Adiponectin is an adipocyte derived cytokine (Sato *et al.*, 2001, Morsci *et al.*, 2006), which enhances pre-adipocyte proliferation and differentiation, and increases lipid accumulation and insulin sensitivity (Fu *et al.*, 2005). The increase in insulin sensitivity occurs because adiponectin increases the expression of glucose transporter 4 (GLUT4) and the number of these transporters in the plasma membrane in response to insulin (Fu *et al.*, 2005). The expression of adiponectin is highly regulated during adipocyte differentiation (Hu *et al.*, 1996), enhanced by insulin (Scherer *et al.*, 1995) and reduced in obese mice and humans (Hu *et al.*, 1996, Arita *et al.*, 1999, Wiecek *et al.*, 2002). It is likely that adiponectin is an important component in energy homeostasis (Scherer *et al.*, 1995).

1.10 Quantitative Trait Loci

It is evident that there are many genes, proteins and extracellular agents involved in adipogenesis, lipogenesis and lipolysis and therefore, fat deposition, despite some of these effects not being immediately intuitive (e.g. prostaglandins). DNA polymorphisms in these genes and proteins may contribute to the variation in fat deposition observed between both breeds of cattle and individuals within each breed. While there are qualitative traits controlled by a single gene that have a distinct phenotype such as flower colour, most traits are controlled by multiple genes. This is particularly likely for quantitative traits such as size, milk production, weight gain and fat content. These traits have a continuous distribution and are known as polygenic and multifactorial. Areas of the genome that are identified as linked to these traits are known as quantitative trait loci (QTL) (Jones *et al.*, 1997, Kearsey, 1998, Burrow *et al.*, 2001,

Andersson and Georges, 2004, Collard *et al.*, 2005, Williams, 2005). Finding QTL can lead to either molecular markers that can be used for selection of the trait or identification of the genes controlling the trait. Identifying QTL requires locating molecular markers that can be used to differentiate between individuals and then testing for association with a particular phenotype.

1.10.1 Molecular markers

The identification of DNA polymorphisms that can be used as molecular markers has only emerged because of recent advances in DNA technology (Collard *et al.*, 2005). There are two common polymorphisms used for genotyping, microsatellites and single nucleotide polymorphisms (SNPs). SNPs are located at small intervals throughout the mammalian genome, estimated at between 100bp (Wang *et al.*, 2006b) and 1000bp (Taillon-Miller *et al.*, 1998, Armstrong *et al.*, 2000, Miller and Kwok, 2001). Although SNPs are less informative than microsatellites, they occur at greater frequency than microsatellites which can minimise, or overcome, the difference in information content (Lindholm *et al.*, 2004, Thalamuthu *et al.*, 2005). Furthermore, SNPs are easier to genotype, leading to automation and therefore, higher throughput (Lindholm *et al.*, 2004, Sellner *et al.*, 2007), and most importantly, they are stably inherited (Armstrong *et al.*, 2000). Therefore, SNPs are very useful as DNA markers for QTL mapping and trait selection.

The identification of markers enables the production of linkage maps, where markers are allocated firstly to a chromosome, and then a region of the chromosome. The genetic distance between markers is calculated via recombination frequency analysis (Jones *et al.*, 1997). When a molecular marker or markers are shown to segregate with a particular trait at a high frequency, that is the marker and trait are linked, a QTL is established in that region (Jones *et al.*, 1997).

The question that arises is what is actually being controlled within that locus? It may be a gene involved in a biochemical or metabolic pathway, a cell structure, or possibly a gene regulator such as an enhancer, promoter, transcription factor, micro RNA or an alternative splice site. There may also be epistatic or imprinting effects within these genes. The answer to this lies with identifying the genes responsible for the effect. However, identifying the genes is made difficult because, firstly, the QTL usually covers a large region (20 – 50Mb) of the chromosome and therefore, the exact location of the gene is unknown. Secondly, the effect of individual genes on a quantitative trait is usually small, unlike a single gene effect on a qualitative trait. Thirdly, there are often environmental factors that contribute to the final phenotype (Andersson, 2001, Mackay, 2001, Andersson and Georges, 2004).

When a gene that lies within a QTL has a function that may be allied to the trait of interest, that gene becomes a 'candidate' for controlling the phenotype. The list of candidate genes within a QTL is further refined via sequencing individual genes to determine if any SNPs are located within a particular gene. SNPs, or other variants within a candidate gene, allow better analysis of linkage or association with a trait or phenotype. Any variant that alters the action of the gene or the protein provides strong indication that the biological activity of that gene should be studied in order to clarify the mode of action and any interaction with other genes that may have an effect on the phenotype. Without a functional variation within the gene, or strong linkage of the gene to the trait, functional studies are not justified (Morsci *et al.*, 2006).

1.10.2 Comparative genomics

Comparative genomics has been used to identify variations in gene sequence and function between species, and mice models have been used to identify genes affecting cattle traits (Burrow *et al.*, 2001). However, comparing breeds within a species,

particularly those selected for different traits such as beef and dairy breeds, should be more successful for discovering sequence variants associated with, or causing, different phenotypes (Andersson and Georges, 2004). The majority of the work herein utilises a within species comparison. A double backcross from two dissimilar cattle breeds, the Limousin beef breed and the Jersey dairy breed was used to identify a number of QTL for fat traits (Esmailizadeh, 2006).

1.10.3 Candidate genes

Quantitative trait loci are used with the linkage map to identify candidate genes for the various traits (e.g. fat deposition). As there is significant conservation of genes between species, information from the human genome can be used via the cattle/human comparative map. This involves determining a region around the QTL and comparing that region to the corresponding human chromosome. The human sequence is used because although other species sequence data is generally good, the human sequence is better annotated, therefore providing more information. Examining the function of the genes in this region of the human chromosome enables the selection of genes that may be involved in fat deposition, either directly or indirectly. The next step is to align the sequence of the human gene against the cattle genome which will identify any homologous sequences. If these sequences are located near the markers associated with the QTL, these genes become the candidates. The candidate genes are then sequenced to identify SNPs that either act as more informative markers or possibly change the function of the gene in some animals. Once SNPs or other polymorphisms are confirmed, a wider population is genotyped to determine if any of these SNPs are linked to the QTL or associated with the trait.

1.11 Marker Assisted Selection

Natural selection will always favour disease resistance and the ability to reproduce successfully (i.e. fitness), but selection by breeders for particular traits will often be at the expense of other traits, sometimes with major deleterious effects (Sonstegard *et al.*, 2000, Williams, 2005). Marker assisted selection allows selection for a number of unlinked traits to overcome this problem (Jones *et al.*, 1997, Sonstegard *et al.*, 2000, Williams, 2005). It also makes the selection of breeding stock more objective and will increase the rate at which the herd is improved (Schaeffer, 2006). In traditional breeding programs, the selection is subjective and there is considerable time between mating and determining if the offspring express the desired traits. Using marker assisted selection, animals carrying markers linked to particular phenotypes can be identified any time after birth and kept as breeding stock, while those that do not have the desired trait can be culled. Furthermore, marker assisted selection enables traits that are sex specific, such as milk production, to be estimated more accurately.

Alternatively, markers may be used for management decisions. For example, in a beef production system that included lot feeding for marbled beef, unless an animal is genetically likely to express a high marbling phenotype, it would not be included in the lot feeding program, but could be grown on pasture for the domestic market.

Marker assisted selection relies on the trait being either directly or indirectly associated with a DNA polymorphism, and both the direct and indirect methods have inherent problems. Although more powerful (Williams, 2005), the direct method necessitates the long and difficult process of identifying the causative mutation, which involves locating candidate genes, sequencing these genes for polymorphisms and confirming the causative mutation. The indirect method only requires the generation of a dense marker map and associating markers with the trait. However, it relies on a marker

being in strong linkage disequilibrium with the causative mutation (Kruglyak, 1999) and this linkage may be lost through recombination in successive generations. Nevertheless, the indirect method, through whole genome selection, may prove ultimately to be more effective, with the possibility of selecting an unlimited number of markers for cattle production traits (Hayes and Goddard, 2001, Sellner *et al.*, 2007). However, identification of causative genes may lead to methods of manipulating the system to improve the trait.

1.11.1 Whole genome selection

Whole genome selection (WGS) involves the use of thousands of DNA markers spread across the entire genome. As this produces a dense marker map, each quantitative trait loci, and presumably each gene, should be in linkage disequilibrium with at least one marker (Goddard and Hayes, 2007). This method of selection is especially useful for predicting the breeding value of each animal with regards to quantitative traits, as all markers associated with variation in each trait will be genotyped. Once the effect of each marker on a trait is calculated, the total effect on that trait can be estimated (Goddard and Hayes, 2007). However, this requires a large number of cattle from multiple breeds to be phenotyped. This information is currently available in the dairy industry, but would require a large input from the beef industry to become viable for traits such as internal and intermuscular fat that are not routinely measured at slaughter. Whole genome selection also relies on DNA markers remaining in strong linkage disequilibrium with the trait, and this may be lost over successive generations. Nevertheless, continual monitoring of the accuracy of selection should prevent or overcome this potential problem if it is possible within the structure of the industry.

1.12 Hypothesis

Leptin, the product of the obese (*OB* or *LEP*) gene, plays an important role in appetite regulation (Friedman and Halaas, 1998, Kalra *et al.*, 1999), with levels of leptin correlated with body fat percent in mammals in general (Friedman and Halaas, 1998) and cattle in particular (Yamada *et al.*, 2003, Yang *et al.*, 2003). Mutations in the obese gene lead to obesity in mice (Coleman, 1978, Friedman and Leibel, 1992). This would suggest that there is a 'fat gene' and that overall fatness increases and decreases with level of feed, and therefore, there is a strong genetic relationship between all fat depots.

However, a simple observation of the wide variation in the body shape of humans, and the breed differences evident in cattle (Section 1.4), suggest that this might not be the case. There is evidence that there may be no relationship, or only a weak relationship between fat depots. Firstly, adipocyte precursor cells cultured from different fat pads vary in differentiation potential and rate of replication (Djian *et al.*, 1985) (Section 1.1). Secondly, fat distribution varies both between and within cattle breeds (Berg and Butterfield, 1976) (Section 1.3). Thirdly, fat depots develop in a specific order, not concurrently (Section 1.3). Fourthly, although leptin is associated with fatness, levels of leptin expression vary between fat depots (Yamada *et al.*, 2003) (Section 1.9.3.2). Finally, Siebert *et al.* (2006) found that reducing vitamin A in cattle diets increased intramuscular fat percent but did not affect subcutaneous fat (rib fat), suggesting a repartitioning of fat induced by diet. Added to this is the low to moderate genetic correlations between subcutaneous fat and intramuscular fat. Subcutaneous fat and marble score correlations have been reported as 0.12 (rib) and 0.24 (rump) (Reverter *et al.*, 2003), and 0.35 (backfat) (Koots *et al.*, 1994). Subcutaneous fat and intramuscular fat percent correlations have been reported as 0.21 (rib) and 0.34 (rump)

(Reverter *et al.*, 2003), 0.36 (rump) (Pitchford *et al.*, 2002), and 0.45 (rib) and 0.48 (rump) (Robinson and Oddy, 2004). These genetic correlations suggest that between 75 and 99% (r^2 = 0.01 – 0.25) of the genetic variation in intramuscular fat is independent of the genetic variation in subcutaneous fat. However, this only accounts for the relationship between the subcutaneous and intramuscular fat depots, which raises the question of how these depots are related to seam and internal fat. There is evidence above, to suggest that all of these depots will act independently to some extent, which is preferable as this would allow the high value intramuscular fat to be increased in beef cattle without increasing the other adipose depots, particularly seam fat. The hypothesis being tested herein is that there is a genetic basis for variation in fat distribution in cattle and the genetic relationship between fat depots is not strong.

Chapter 2 General methods

2.1 Cattle

The aim of this research was to gain a better understanding of the genetics influencing, and mechanisms controlling, fat deposition in cattle. To achieve this, three cattle herd resources were utilised, the Davies Gene Mapping Herd, the AgResearch Gene Mapping Project and the NSW Department of Industry and Investment Trangie Residual Feed Intake (RFI) selection line. All herds were measured for a number of muscle, fat and growth traits during growth and post slaughter. As methods of measurement varied between herds, animals were compared within herds not between herds.

This research was initially focussed on the Davies herd for measurement of fatness traits and DNA sequence analysis. The AgResearch herd was included firstly, to compare overall fat distribution results between herds of the same breed but different ages and feeding regimes. Secondly, the AgResearch herd sires were half brothers to the sires used in the Davies herd, and therefore, this herd could be used to confirm any gene association with variation in fat traits. The Trangie herd was included for image analysis as the Davies herd images were not suitable for analysis of marbling flecks. As these animals had been grain fed for a long period they were likely to be a good resource of highly marbled meat and provided another cattle breed for fat distribution analysis. Unfortunately, genotype data were not available from the Trangie herd so the DNA variant associations could not be confirmed using this resource.

2.1.1 Davies Gene Mapping Herd

The Davies Gene Mapping herd was a Jersey – Limousin double backcross herd established to identify DNA markers associated with quantitative trait loci (QTL) for carcass composition traits in cattle. The Jersey (dairy) and Limousin (beef) are two

dissimilar *Bos taurus* breeds. These animals were crossed to form a F1 generation from which three bulls were selected to be backcrossed over both parental breeds, forming a double backcross generation totalling 366 progeny born over three years. Calves were reared on their dams, grown out on pasture until approximately two years of age, and finished on an approximately 65% grain ration in a feedlot for at least 180 days and slaughtered at ages between 34 and 40 months (Esmailizadeh *et al.*, 2008).

Table 2.1: Cohort details, Davies Gene mapping herd.

Year	Sex	Count	Ave HSCW
96	H	49	346
96	S	28	381
97	H	69	295
97	S	84	345
98	H	63	298
98	S	73	366
Total		**366**	

2.1.2 AgResearch Gene Mapping Project

The AgResearch Gene Mapping Project was also a Jersey – Limousin double backcross herd using bulls that were half brothers to the bulls used in the Davies Gene Mapping herd. There were 424 calves born over two years. The Jersey backcross calves were born to Jersey dams and reared by hand as is the standard dairy practice. The Limousin backcross calves were born to Hereford Friesian cross dams by embryo transplant in the first year of the project, while the second year calves were born to Limousin dams. All Limousin calves were reared on their dams until approximately 180 days and grown out on pasture. Calves were allocated to groups based on breed, sex and year of birth and slaughtered between ages 22 and 28 months (Esmailizadeh *et al.*, 2008).

Table 2.2: Slaughter date, sex and number in each slaughter group, AgResearch Gene Mapping herd.

Sl Gp	Sl Date	Sex	Count	Ave Pre Kill Weight	Ave HCW
1	20/07/1998	H	15	366	197
2	27/07/1998	H	15	366	182
3	10/08/1998	H	15	365	189
4	17/08/1998	H	15	372	194
5	24/08/1998	H	15	381	201
6	31/08/1998	S	15	414	211
7	07/09/1998	H	14	378	193
8	14/09/1998	S	15	425	218
9	21/09/1998	H	15	414	216
10	28/09/1998	S	15	441	222
11	05/10/1998	H	15	419	224
12	12/10/1998	S	15	457	236
13	19/10/1998	H	15	420	226
14	02/11/1998	S	15	475	249
15	09/11/1998	S	15	480	254
16	16/11/1998	S	14	492	255
17	23/11/1998	S	15	499	267
18	30/11/1998	S	16	507	265
19	06/09/1999	H	15	391	206
20	13/09/1999	H	15	401	210
21	20/09/1999	H	15	400	212
22	27/09/1999	H	15	413	217
23	04/10/1999	H	15	428	223
24	11/10/1999	S	17	487	247
25	18/10/1999	S	16	471	248
26	01/11/1999	S	15	493	254
27	08/11/1999	S	16	492	263
28	15/11/1999	S	16	498	268
Total			**424**		

2.1.3 Trangie Residual Feed Intake (RFI) trial herd

Two hunded and eight Angus steers from the NSW Department of Industry and Investment, Trangie RFI selection line with a large divergence in mid-parent residual feed intake estimated breeding values (RFI EBVs) were used in a feedlot trial conducted at a commercial feedlot in southern NSW. This herd was included in this research as the breed and length of time on supplementary feed meant that this was an ideal source of highly marbled carcasses for fat distribution studies. The steers were progeny of 26 sires with numbers of progeny ranging from 1 to 21. The trial comprised 3 pens, with steers allocated to the pens based on mid-parent RFI EBV (low RFI EBV = -0.85 to -0.52, n = 68; medium RFI EBV = -0.29 to 0.14, n = 72; high RFI EBV = 0.16 to

0.98, n = 68) and fed for 250 days in a large commercial feedlot in NSW. The steers were managed together from birth until they entered the feedlot. Age at feedlot entry ranged from 13 to 16 months. Each pen was supplied with the same ration, *ad libitum,* adjusted weekly for under- or over-feeding. All steers were slaughtered on the same day with ages varying from 21 to 24 months.

2.1.4 Data

2.1.4.1 Carcass measurements

The animals were slaughtered and processed at a licensed abattoir. Carcasses were weighed, cut into sides and stored in a chiller overnight at close to 0°C. The carcasses were quartered at the $10^{th}/11^{th}$ rib (Davies) or $5^{th}/6^{th}$ rib (Trangie), approximately 18 or 24 hours post slaughter, respectively. AUS-MEAT and Meat Standards Australia chiller assessments were carried out by accredited graders.

2.1.4.2 Traits

The carcass traits measured varied between herds as follows:

Davies Gene Mapping herd

Hot standard carcass weight, cross sectional area of the *Longissimus dorsi* (eye muscle area) at the quartering point, marble score, subcutaneous fat depth at the $10^{th}/11^{th}$ rib, fat depth at position 8 (p8) over the rump, trimmable fat weight, channel (kidney) fat weight and omental fat weight were measured at slaughter. Omental fat was not measured in the 1996 heifer and steer cohorts. The percentages of fat, muscle and bone in the carcass were calculated using prediction equations determined previously (Ewers *et al.*, 1999). The fat measured was trimmable fat as a percentage of

the carcass weight. The resulting percentages were used to calculate fat to bone and meat to bone ratios. In addition, samples of the *Longissimus dorsi* were taken for intramuscular fat extraction and analysis of fatty acid composition, and images of the cut site were recorded for image analysis (described in section 3.2).

AgResearch Gene Mapping herd

Hot standard carcass weight, cross sectional area of the *Longissimus dorsi* (eye muscle area) at the quartering point, channel (kidney) fat weight, omental fat weight and pericardial fat weight, rib fat depth, and weight of subcutaneous fat at the flank, topside, silverside, porterhouse, forequarter and hindquarter were measured at slaughter. Samples of the *Longissimus dorsi* were taken for intramuscular fat extraction and analysis of fatty acid composition and intramuscular fat in the eye muscle was calculated using video image analysis.

Trangie RFI herd

Hot standard carcass weight, cross sectional area of the *Longissimus dorsi* (eye muscle area) at the quartering point, marble score, subcutaneous fat depth at the $10^{th}/11^{th}$ rib were measured at slaughter. In addition, samples of the *Longissimus dorsi* were taken for intramuscular fat extraction, and images of the cut site were recorded for image analysis.

2.1.4.3 Intramuscular fat extraction

Intramuscular fat content (IMF%) was measured according to the protocol described by Siebert et al. (2006). Approximately 60g of *Longissimus dorsi* trimmed of all subcutaneous and intermuscular fat was finely cut and blended in a food processor

(*Braun Multipimer; model MR 5550MCA*) to an even paste (approx. 30 seconds). An accurately weighed sub-sample of approximately 2g was placed in a 50ml polypropylene tube. 27ml of a chloroform and methanol mixture (2:1 vol/vol) was added before homogenising for approx. 15 – 20 seconds. The homogenate was filtered under vacuum through Whatman No. 1 filter paper (42.5mm) using a Buchner flask and funnel. The residue was returned to the tube and homogenised again in another 27ml of 2:1 chloroform – methanol and filtered as before. The combined filtrates were transferred to a 100ml measuring cylinder and 15ml (approximately equal to one quarter of the volume of the filtrate) of 0.88% potassium chloride (KCl) was added, the mixture was shaken vigorously and allowed to settle into two layers (30 – 60 minutes). The upper layer (water) was aspirated and the remaining layer which contained the lipid fraction was placed into a labelled 50ml polypropylene tube and centrifuged at 3000 RPM (1700g) for 5 minutes using a Sorvall® RT6000D (*DuPont*™) centrifuge. The upper layer, containing any remaining water or solids, was aspirated.

The volume was reduced to a few millilitres using a rotary evaporator with a 40°C water bath, and transferred to an accurately weighed 100ml borosilicate glass culture tube, washing the flask 3 times with chloroform. This volume was then reduced to approximately 0.5ml using a stream of CO_2 while being heated in a 40°C water bath, then dried completely in a desiccator for at least 24 hours. The tubes containing the intramuscular fat were then weighed and the result was recorded as intramuscular fat content as a percentage of the initial accurately weighed sample.

2.1.4.4 Melting point of intramuscular fat

Following the measurement of intramuscular fat, each sample was heated until the fat melted. A capillary tube (Hirschmann® melting point determination tube, *Hirschmann Laborgeräte GmbH & Co*) was inserted into the liquid fat sample until the fat had

moved approximately 25mm up the tube. This was cooled at 4°C overnight. Once the fat re-solidified, the capillary tube was marked level with the top of the meniscus of the fat. A thermal cycler (*Perkin Elmer Cetus*) was used to heat the fat samples. Wells were filled with MiliQ water to improve temperature conduction, and capillary tubes put into individual wells so that the fat was level with the top of the well. The thermal cycler increased in temperature in 1°C steps, remaining at each temperature for 60 seconds. Each tube was visually inspected after each temperature step had been reached for at least 20 seconds. The melting point of a sample was deemed to be when the level of the fat 'slipped' at least 1mm above the original level mark.

2.2 Polymerase chain reaction

2.2.1 Primer design

Primers were designed using Oligo® version 6.71 (*Molecular Biology Insights*). Primers were selected on the basis of closely matched (usually less than 2°C difference) forward and reverse Tm (nearest neighbour method) and Td (GC%). Primer dimers formed within and between the primers had ΔG values between zero and -10kcal/mol, and hairpin loops formed within the primers had a melting temperature <30°C.

2.2.2 Polymerase Chain Reaction

2.2.2.1 Reaction mix

Four different polymerases were used throughout this work. Selection depended on the enzyme in common use in the laboratory at the time and also the relative difficulty encountered when optimising new primers. All PCR mixes were as specified by the manufacturer (Appendix A.1).

2.2.2.2 Thermal cycling

PCR reactions were amplified using a Palmcycler (*Life Science Research*) heated lid thermal cycler. Generally a 'Touchdown' program was used, where the annealing temperature was reduced by 1°C each cycle for 11 cycles, with the initial annealing temperature being 60°C (TD2) or 70°C (TD1). The duration of each stage of the program varied to suit the requirements of the polymerase being used (Appendix A.2).

2.2.2.3 Reaction optimisation

Touchdown program TD2 was generally used for the first reaction (Appendix A.2). If a reaction produced a strong single band upon gel electrophoresis, no further optimisation was required. Where there was no product, a weak product, multiple products or strong smearing occurred, the first option was to use touchdown program TD1 (Appendix A.2). If this failed to produce a good result, the reaction was adjusted using one or more of the following methods:

No or weak product. The primer concentration was increased to 5pmol, 10pmol and 25pmol or the $MgCl_2$ was increased to reduce the stringency of the reaction. Alternatively, a different polymerase and associated buffer was used.

Multiple products. The primer concentration was reduced or a temperature gradient was used to identify the optimal annealing temperature. Alternatively, the polymerase concentration or DNA template concentration was reduced. If the extra product was unavoidable and the product was required for sequencing, the desired product was removed from an agarose gel and cleaned directly from the gel (section 5.2.2.2). If the primers were for high resolution melt genotyping, new primers were designed.

Strong smears. The polymerase and/or DNA template concentration was reduced or the number of amplification cycles was reduced from 35.

2.2.2.4 Agarose gel electrophoresis

PCR products were separated using gel electrophoresis of a 2% w/v standard agarose (*AppliChem*) gel. The electrophoresis unit used was a 15 lane 'mini-sub cell™' or a 30 lane 'wide mini-sub cell™' (*Biorad*) with 1 x TAE buffer. Samples were resolved at 6.5 – 8V/cm, depending on the unit used and the expected product size. Typically, 5µl of PCR product with 1.5µl of loading dye (*Promega*) was loaded into each well. The product size was estimated using either a pGEM® (*Promega*) or HyperLadder™11 (*Bioline*) DNA marker. Gels were stained in a 0.5µg/ml ethidium bromide solution for 15 minutes, rinsed briefly with clean water and examined under UV illumination using a Gel Doc™ 1000 (*Bio-Rad*). Images were captured and printed on plain paper with a Hewlett Packard 890C inkjet printer.

2.3 Contributions to this project

The Davies Gene Mapping herd and the AgResearch Gene Mapping Project were slaughtered prior to the commencement of this project. Therefore, all of the phenotypic measurements of these herds were recorded by others, except the seam fat measurements of the Davies herd. The photographs used for image analysis were provided to me in hard form. I scanned all of the photographs, performed all manipulations and saved them in digital form. The software used for the image analysis was provided by Associate Professor Murk Bottema (School of Informatics and Engineering, Flinders University, South Australia). The Trangie Residual Feed Intake trial herd was slaughtered during the course of this project. I was part of the team that attended the slaughter and took samples for this and other projects. The digital photographs used for the image analysis were taken by Dr. Zbigniew Kruk, with some assistance provided by me. I completed all manipulations and image analysis of these

photographs. With initial instruction from Dr Kruk, I extracted the intramuscular fat from the Trangie samples to calculate the percentage and melting point.

The microsatellites used for QTL mapping of the Davies herd were identified prior to the commencement of this project, as were all QTL with the exception of the seam fat QTL that I identified herein (Section 3.3.1.4). All of the sequencing of the candidate genes listed in Table 5.1 and verification of the single nucleotide polymorphisms (SNPs) listed in Table 5.3 were my work, except for four SNPs in TEK1 that were previously identified by members of this research group. Fourteen of the SNPs listed in Table 6.1 were genotyped by me, and the remaining 18 SNPs were genotyped by other members of the research group or by the Department of Primary Industries, Victoria. All data analyses described herein were performed by me.

Chapter 3 Image analysis

3.1 Introduction

Visually observable intramuscular fat is commonly known as marbling and the amount of marbling (marble score) is an important aspect of beef quality (Yang *et al.*, 2006). Increased marbling improves the tenderness and palatability of the meat (Sasaki *et al.*, 2006), and can increase the value of the carcass (Bindon, 2004). Marble score is assessed visually by accredited graders during the bone out process, 12 – 24 hours post slaughter. The number and spread of marble flecks on the *Longissimus dorsi* (eye muscle) are important aspects of marbling (Gerrard *et al.*, 1996, Yoshikawa *et al.*, 2000), and these are visually assessed against reference standards to determine the marble score (Yoshikawa *et al.*, 2000, Harper and Pethick, 2004).

While visual assessment is the accepted method for evaluating the amount of marbling within the muscle, it can be both subjective (Yoshikawa *et al.*, 2000) and inaccurate (Gerrard *et al.*, 1996). Also, as a tool to help improve the understanding of the molecular mechanisms involved in this trait, visual assessment is limiting. In order to better understand the marbling phenotype, it is necessary to better describe the features of marble flecks; the number and area of the fat flecks, how this combination is related to the visually assessed marble score, the position of the flecks in the muscle (e.g. concentrated in one area or distributed evenly), and the shape of the flecks (round, elongated or branched). Assessing these features, and any interrelationship, may help to advance the understanding of factors involved in the marbling phenotype. For example, if the fat flecks are generally following lines, it may be that the fat is deposited near blood vessels. This would be likely if intramuscular fat is utilised as a ready supply of metabolisable energy and fat is transported through the blood stream before being deposited. Alternatively, if the fat flecks are concentrated in one area, it

may indicate that there is some factor, genetic or biochemical, which is causing cells to differentiate into fat rather than muscle.

Intermuscular fat is another important fat depot in terms of carcass quality. Intermuscular fat forms the majority of removable fat on the carcass including the fat inside the ribcage and brisket (Cianzio *et al.*, 1982) (Section 1.3). When intermuscular fat is situated between the muscles of a higher value cut (e.g. scotch fillet, T bone), it is commonly referred to as seam fat. Generally, consumers prefer smaller areas of seam fat and although it is classed as a removable fat, seam fat is impossible to remove from these higher value cuts during processing. Therefore, large areas of seam fat result in a reduction in the value of the cut.

As marble score and seam fat have such important, yet opposite, effects on the value of beef carcasses, it is important to improve the understanding of both depots. The purpose of this investigation was to use image analysis, firstly, to improve the description of the marbling phenotype by better assessing the marble fleck features, and secondly, to quantify the area of seam fat in similar animals to identify any variation that may exist. This information was subsequently used to assess how these depots relate to other fat depots, and identify candidate genes for association with marbling and seam fat.

3.2 Methods

3.2.1 Cattle

Images were obtained from the Davies gene mapping herd and the Trangie residual feed intake (RFI) selection line (NSW Department of Industry and Investment) commercial feedlot trial (described in sections 2.1.1 and 2.1.3, respectively).

3.2.2 Image analysis – Davies Gene Mapping Herd

3.2.2.1 Image capture

Photographs, including a ruler and identification tag, were taken of the *Longissimus dorsi* at the $10^{th}/11^{th}$ rib, 24 hours after slaughter. The photographs were taken of the cut site, in the chiller (i.e., the muscle was not removed from the carcass for photography). The photographs were converted to jpg files using a Scanjet 4400C (*Hewlett Packard*) 1200dpi 48-bit colour scanner, and stored as separate files less than 75kb.

3.2.2.2 Image processing

The images were manipulated using Photoshop® CS2 (*Adobe*®) to remove the background, bone, subcutaneous fat and intermuscular (seam fat). The remaining muscle image was placed on a plain green background (R = 36, G = 206, B = 45) with the ruler, to be used as a scale, and saved as a jpg file (Figure 3.1). Images of seam fat were saved in the same manner. All images were stored as separate files of less than 185kb.

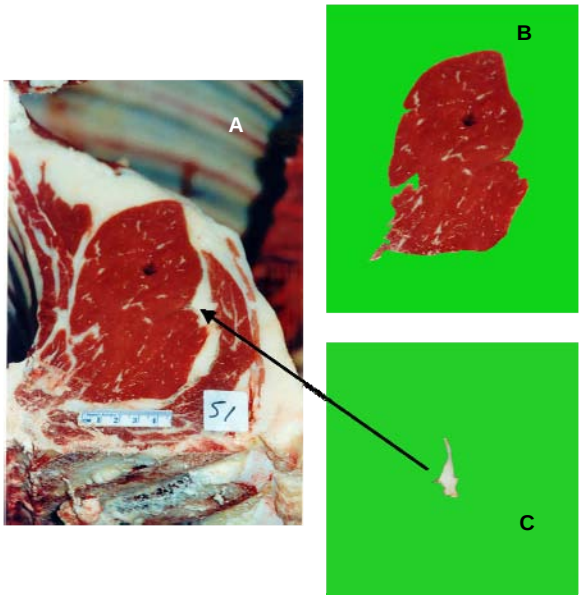


Figure 3.1: Method used for processing images, Davies Gene Mapping herd.
Image (A), original photograph of steak, (B), area used to calculate marbling score and (C), area used to calculate intermuscular (seam) fat.

3.2.3 Image analysis – Trangie Residual Feed Intake Herd

An approximately 15mm thick steak was taken from the 5th/6th rib incorporating the *Longissimus dorsi, Spinalis dorsi and Semi-spinalis dorsi* muscles (Figure 3.2) of the left side of the carcass at the feedlot abattoir during bone out. 207 of the 208 steers were sampled and the steaks vacuum sealed in cryovac bags and stored at 4°C prior to transport, on ice, to the Roseworthy Campus of the University of Adelaide where they were frozen and stored at -20°C approximately 36 hours after bone out.

3.2.3.1 Image capture

Each steak was removed from its storage bag, brushed with 70% ethanol to remove moisture and any surface fragments, and immediately photographed using a Canon G7 10 megapixel digital camera mounted in a light box at a constant distance. Two, horizontally opposed, tungsten lamps (150W) provided a constant light source. A green sheet of paper was placed beneath the steak to provide a contrast background. The camera used a focal length of 7.4mm, aperture at F4 and an exposure time of 1/500th

or 1/640th second. A 10mm wide piece of laminated white paper was included in each image to act as a scale for the subsequent image analysis. Also included was the label indicating the "bone out order" (used for identifying the steak for analysis) and a tag to indicate if the image was of the anterior end (head or H) or posterior end (rump or R). The steaks were photographed on both sides (H or R), all in the same orientation with subcutaneous fat at the top of the final image. Immediately following photography, the steaks were again vacuum sealed and returned to the freezer. Once all of the frozen steaks had been photographed, they were thawed and both sides were photographed again using the same method to ensure no differences were observed between fresh and frozen samples. The steaks were photographed both sides as an aid to differentiating between seam fat and intramuscular fat (Section 3.3.1.2). All images were stored as separate jpeg files with a 180dpi resolution and file size of greater than 1.3MB.

3.2.3.2 Image processing

The stored image was manipulated using Adobe® Photoshop® CS2 to remove the seam fat and eye muscle (*Longissimus dorsi,*) and each image was placed separately on to a green background (R = 0, G = 214, B = 0), 1800 x 1800 pixels with resolution of 180dpi (70.87 pixels per cm) and stored as jpeg files of approximately 150KB (Figure 3.2).

3.2.3.3 Image analysis

A software program was developed using Matlab® 7.4 (R2007a) (*The Mathworks*™) by M. Bottema to calculate either muscle area, intermuscular (seam) fat area or marbling parameters; area (mm^2), eccentricity, ellipticity, normalised ellipticity, orientation, major axis length, minor axis length, x coordinate and y coordinate of each fleck. Eccentricity is the ratio of the length of the major to minor axis to give a measure of the roundness

of the fleck (1 = round fleck). Ellipticity measures the number of fleck pixels outside of the ideal ellipse and the percentage of fleck pixels outside of the ideal ellipse. Therefore, ellipticity indicates the irregularity of flecks (i.e., from smooth to branched). The X and Y coordinates were used to indicate the position of flecks on the steak. The average distance from the centre of the eye muscle was calculated ($\sqrt{[X^2 + Y^2]}$) and the standard deviation of this provides a measure of the dispersal of the flecks.

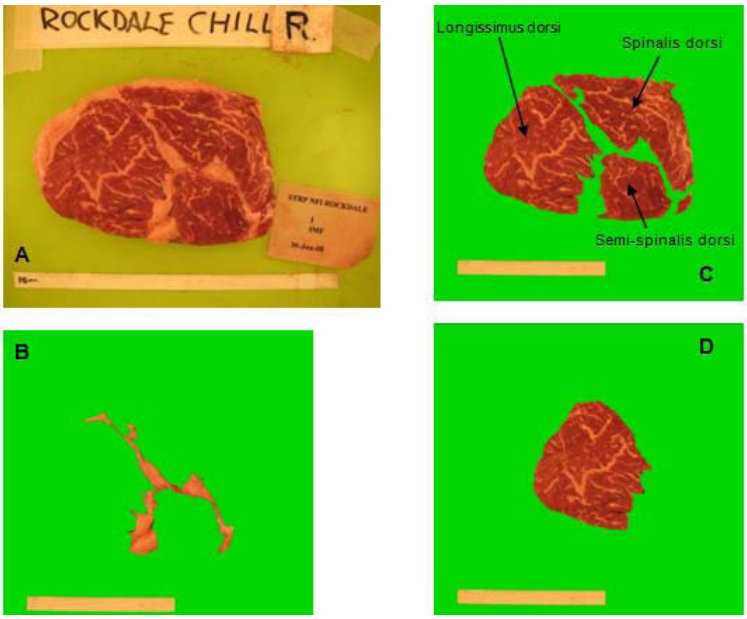


Figure 3.2: Method used for processing images, Trangie RFI selection line.
Original image (A) with line demonstrating the delineation of seam and subcutaneous fat, (B) seam fat used for calculation of seam fat area, (C) image with seam fat removed, showing the three muscles of the cube roll, $5^{th}/6^{th}$ rib, (D), image of eye muscle only (*L. dorsi*), for muscle area and marbling analysis and (E) alternate delineation of seam and subcutaneous fat.

3.2.4 Quantitative Trait Loci analysis

Quantitative trait loci were mapped using QTLExpress (http://qtl.cap.ed.ac.uk/) (Seaton *et al.*, 2002) half-sib regression interval mapping. The first model included fixed effects of sire, breed of dam and cohort. The second model included these, plus myostatin genotype as fixed effects. Chromosome-wide significance was accepted at F > 4 in the sire family analysis.

3.3 Results

Images of the *Longissimus dorsi* from two herd resources were used for the image analysis experiments, the Davies Gene Mapping herd and the Trangie Residual Feed Intake (RFI) selection line. The aim was to assess features of intermuscular (seam) fat and intramuscular fat (marble score). However, it must be noted that the original photographs of the Davies herd were taken not for this specific purpose, with the added challenge of working within a commercial slaughter. Consequently, the general quality of these images was low, and this adversely affected the analyses. As a result, the images of the Trangie herd were taken under more controlled conditions with a corresponding increase in image quality.

3.3.1 Intermuscular (seam) fat

3.3.1.1 Davies Gene Mapping Herd

Differentiating the intermuscular (seam) fat from the subcutaneous fat was problematic in the images from the Davies Gene Mapping herd. In some images, there were obvious lines between layers of fat and these could be used as the intermuscular/subcutaneous fat border (Figure 3.3A), but in other images, this was not the case and no definite line was visible (Figure 3.3B). Initially, the fat between the *Longissimus dorsi* (eye muscle) and the *Spinalis dorsi* (including between the *Spinalis dorsi* and the *Trapezius thoracis*, extending around between the *Longissimus dorsi* and the *Multifidi dorsi* to the *Semispinalis capitis*) was selected for measurement of intermuscular fat (Figures 3.4 and 3.5A). However, because of the circumstances under which the photography was conducted, there were problems encountered such as identification numbers and scale bars inadvertently placed over the intermuscular fat, as well as fat, bone and muscle residue from the quartering. Therefore, this method

was impractical and an alternative method was investigated. The small area of fat that made up the roughly shaped triangle between the *Longissimus dorsi* and the *Spinalis dorsi* was selected (Figure 3.5B). For consistency, the area selected was from the top of the *Spinalis dorsi* to the lowest point of the indentation in the *Longissimus dorsi*. There was a high correlation (r = 0.76) between these methods and as the 'reduced' intermuscular fat was considered more accurate, this was used as the measured area of intermuscular fat.

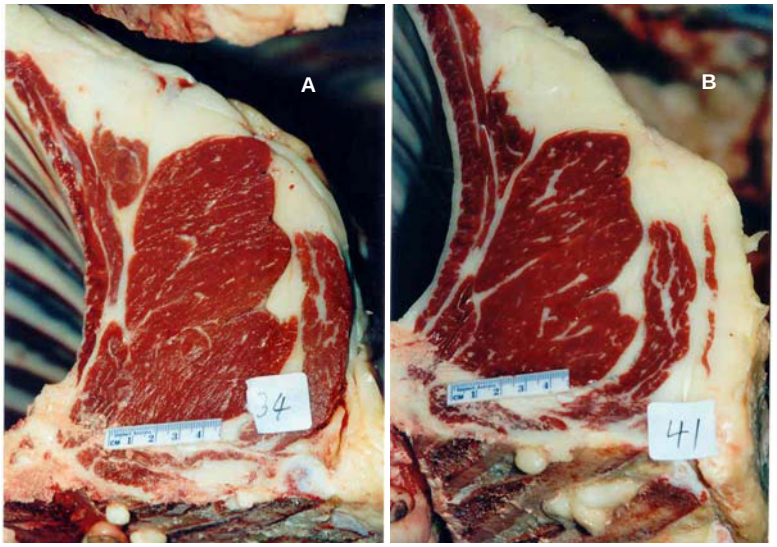


Figure 3.3: Images of steaks illustrating the difficulty in delineating intermuscular fat in the Davies Gene Mapping herd.

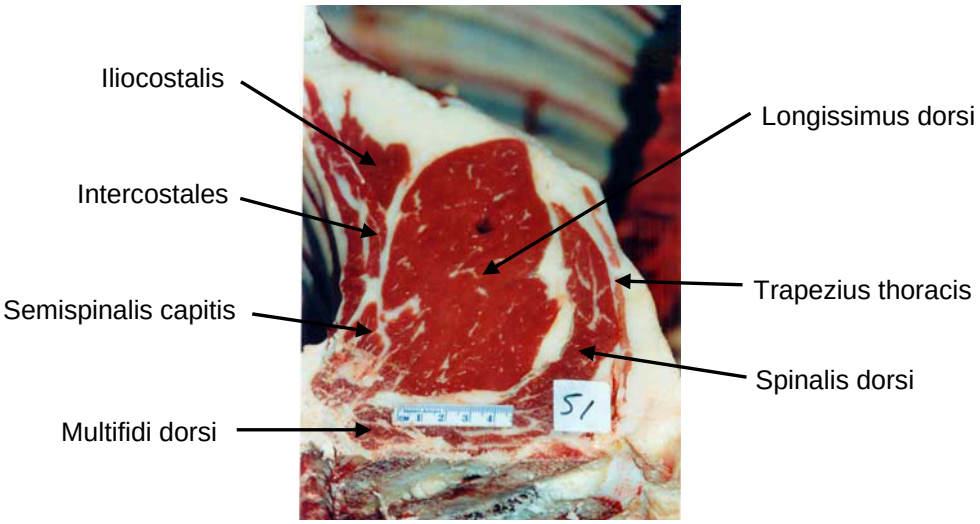


Figure 3.4: Image of steak indicating the muscles at the 10th/11th rib site, Davies Gene Mapping herd.

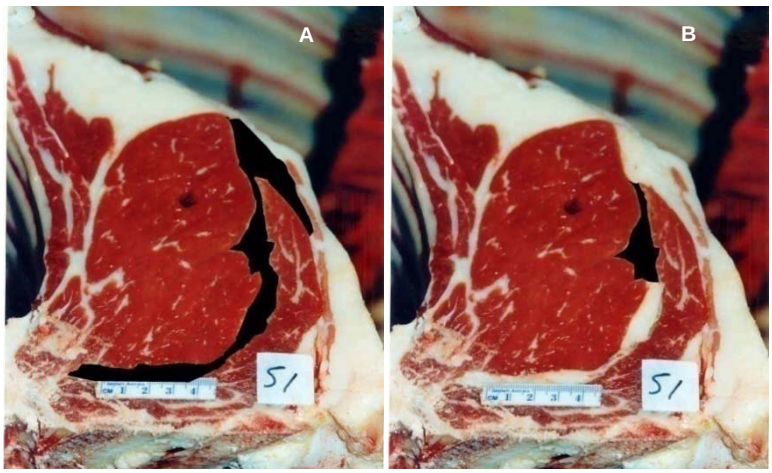


Figure 3.5: images of steak indicating the areas used to calculate intermuscular fat area. Black area indicates the Initial 'complete' area (A) and reduced area (B).

3.3.1.2 Trangie Residual Feed Intake Selection Line

In the images from the Trangie residual feed intake (RFI) selection line, seam fat area was defined as the fat between the three muscles of the original image, *Longissimus dorsi* (eye muscle), *Spinalis dorsi* and *Semi-spinalis dorsi*, but not including the subcutaneous fat. Fat outside of these muscles was classed as subcutaneous. In general, seam fat was cut off at a point where the line of the muscles would have continued (figure 3.6A). However, on some steaks, it was clear that the seam fat extended beyond this usual limit, and in these cases, the boundary of seam fat was extended along this line (figure 3.6B).

The delineation of fat between seam fat and marbling is somewhat subjective. The accepted definition of marbling fat is that it is not connected to either intermuscular or subcutaneous fat (Harper and Pethick, 2004). In general, the large areas of fat were designated as seam fat. However, when the fat extended well into the muscle area, such that the shape of the muscle appeared altered from the 'norm', the reverse side of the steak was inspected. It was assumed that seam fat would extend along the muscle (head end to rump end), whereas marbling would be not as extensive, i.e., not extend through the steak. Although there is some conjecture that marbling may also extend

along the muscle, this has not yet been investigated thoroughly and for the purposes herein, if the fat was only on one side of the steak and not the other, it was deemed to be marbling, and conversely, when the fat extended from one side to the other, it was accepted as seam fat.

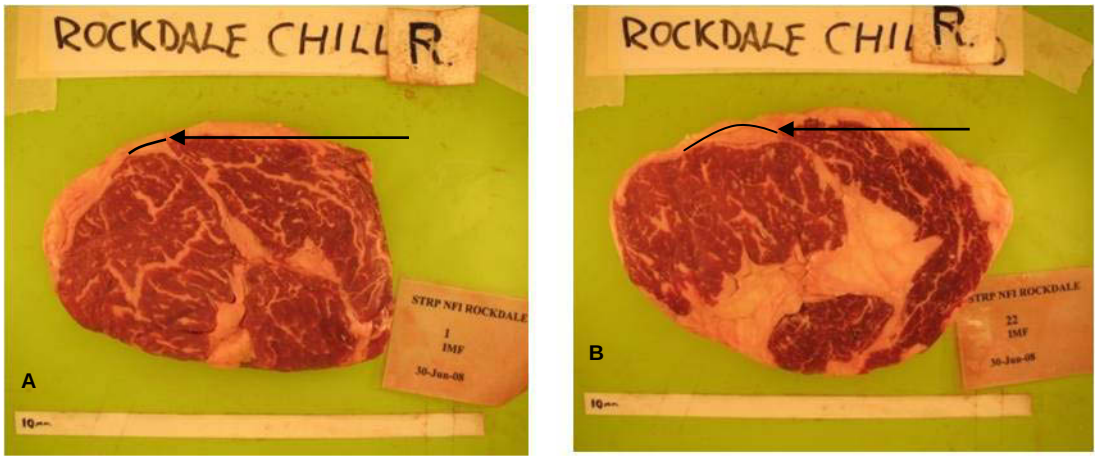


Figure 3.6: Alternative delineation of the intermuscular and subcutaneous fat border, Trangie RFI Selection line.

3.3.1.3 Intermuscular fat area variation

There was large variation in intermuscular fat area of the carcasses from both herds (Table 3.1). In the Davies herd, the average intermuscular fat area was 28% greater in the steers than the heifers within each year. Although the maximum of the 1998 steer cohort was much larger than all of the others, this was influenced by one very large result, 1180mm^2, with the next largest area being 872mm^2. Removing the largest seam fat area reduced the average (353 to 337), the standard deviation (211 to 178) and the coefficient of variation (0.60 to 0.50).

The intermuscular fat areas were much larger in the Trangie herd, due to methods of measurement used in the two herds (Figures 3.2 and 3.5). However, the variation in the Trangie herd was less as demonstrated by the coefficient of variation in each herd (Table 3.1). The Trangie herd consisted of all Angus steers, raised in the same year and of similar age, and grain fed in one of three pens. The main difference within the

herd was a variation in daily feed of 0.7kg per steer (i.e. 1.4kg from lowest to highest pen). Conversely, the Davies herd was a Limousin – Jersey double backcross, including steers and heifers, raised in three consecutive years, and therefore introducing variation in breed, sex and environment.

Table 3.1: Intermuscular (seam) fat area (mm^2) results, Davies Gene Mapping herd and Trangie RFI Selection line.

	min	max	ave	SD	CV
Davies gene mapping herd					
96H	88	848	306	146	0.48
96S	205	673	384	144	0.38
97H	45	868	286	149	0.52
97S	92	745	330	136	0.41
98H	25	731	246	141	0.57
98S	56	1180	353	211	0.60
Trangie RFI selection herd					
high RFI	967	3611	2212	563	0.25
med RFI	1380	4422	2564	613	0.24
low RFI	1174	4123	2466	638	0.26

Although low, the raw correlations between intermuscular fat area and carcass weight, marble score and intramuscular fat % were consistent in both herds (Table 3.2). There was no correlation between intermuscular fat area and eye muscle area in the Davies herd. This is likely due to the large variation in eye muscle area in the Davies herd associated with the breed differences, the Jersey being a smaller and generally fatter breed. Nevertheless, this is interesting as it was expected that the influence of the smaller and fatter Jersey compared with the larger and leaner Limousin would result in a negative correlation between intermuscular fat area and eye muscle area. The results suggest that intermuscular fat does not increase as eye muscle area decreases; rather it is more likely to be independent of size, as measured by carcass weight and eye muscle area. However, it must be noted that these correlations were generated from the intermuscular fat areas without the inclusion of any fixed effects (i.e. cohort, breed, sire) which is addressed later (Chapter 4).

Table 3.2: Raw correlations between intermuscular (seam) fat area and other measured traits.

Seam fat (mm)	hscw	ema	p8am	ribfat	marb*	IMF%
Davies	0.16	-0.02	0.10	0.12	0.14	0.23
Trangie	0.13	0.16	n/a	-0.05	0.12	0.30

Correlations between intermuscular fat area (seam fat) and carcass weight (hscw), eye muscle area (ema), rump fat at P8 (p8am), rib fat (ribfat), marble score (marb) and intramuscular fat % (IMF%).
*** Davies marble score = mbusms, Trangie marble score = msamb.**

3.3.1.4 Quantitative Trait Loci

The intermuscular fat data from the Davies Gene mapping herd were used to search for quantitative trait loci (QTL) for this fat deposition trait across the three sire families. One very large QTL was identified on bovine chromosome (BTA) 2 at approximately four centimorgans (cM) (Figure 3.7). *Myostatin*, which is situated in this region, has a large effect on muscle mass and fat deposition (McPherron *et al.*, 1997, Rodgers and Garikipati, 2008, Martinez *et al.*, 2010). Furthermore, the *myostatin* F94L DNA variant has been associated with increased muscle mass in the Davies Gene Mapping herd (Sellick *et al.*, 2007), and therefore, it was an obvious candidate gene for this QTL. Subsequent analyses, which included the *myostatin* F94L genotype as a fixed effect, indicated that the *myostatin* F94L variant was indeed responsible for this QTL. When the analysis was repeated with the *myostatin* F94L genotype included as a fixed effect, there was only one novel QTL identified on BTA19 at approximately 4cM and the QTL on BTA2 was eliminated (Table 3.3, Figure 3.8).

The QTL on BTA19 had a reasonable size of effect of 86mm^2. No other QTL for fat traits have been identified on BTA19 in the Davies Gene Mapping Herd (Esmailizadeh, 2006). There were other putative QTL for seam fat area located on BTA1 and BTA3, but these were just below the 'across sire family' significance threshold of 4.0. Therefore, only BTA19 was examined for seam fat candidate genes. Two candidate genes within this QTL on BTA19 were selected for sequencing to identify

polymorphisms for association analysis with variation in intermuscular fat area and other fat deposition traits (see Section 5.3.1, Table 5.1).

Table 3.3: Intermuscular (seam) fat quantitative tait loci from the across sire family linkage analyses, without (A) and with (B) myostatin F94L genotype as a fixed effect.

	A		B	
BTA	**Region**	**F value**	**Region**	**F value**
1	8cM	2.23	8cM	3.58
2	4cM	**7.03**	0cM	2.18
3	20cM	3.57	16cM	3.62
4	0cM	1.89	0cM	1.88
5	96cM	2.04	12cM	2.16
6	100cM	2.94	92cM	3.26
7	84cM	1.60	84cM	1.97
8	12cM	2.88	12cM	2.28
9	88cM	0.66	92cM	0.40
10	84cM	2.35	84cM	1.84
11	0cM	2.04	0cM	1.87
12	20cM	1.37	24cM	0.90
13	68cM	1.45	68cM	2.17
14	32cM	1.11	28cM	0.58
15	100cM	2.97	100cM	2.33
16	0cM	1.41	0cM	1.38
17	80cM	2.24	80cM	2.99
18	28cM	3.36	24cM	3.14
19	8cM	3.65	4cM	**4.15**
20	60cM	1.95	60cM	2.63
21	48cM	3.31	44cM	3.00
22	76cM	1.44	0cM	1.18
23	68cM	1.28	68cM	1.00
24	44cM	1.48	40cM	1.22
25	12cM	2.07	16cM	2.13
26	0cM	1.72	68cM	1.25
27	36cM	1.00	64cM	0.83
28	0cM	0.83	48cM	0.93
29	40cM	0.69	64cM	0.70

A = fixed effects: sire, breed, and cohort. B = fixed effects: sire, breed, cohort and myostatin genotype. Significance threshold F > 4

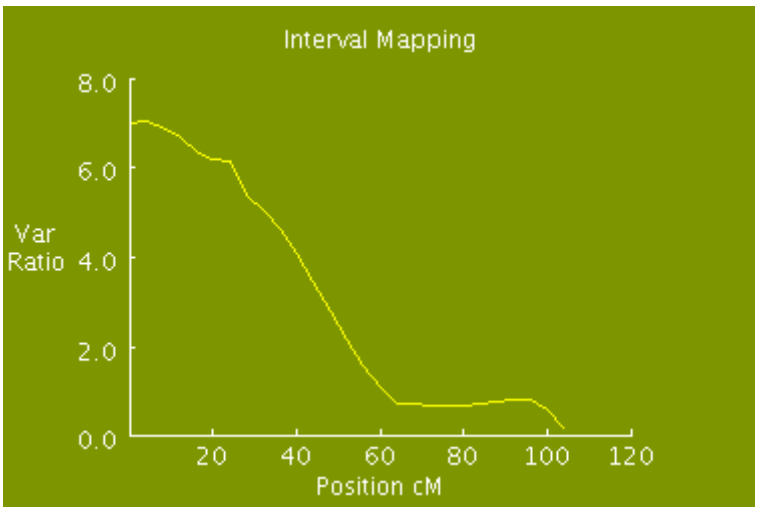


Figure 3.7: Intermuscular fat Quantitative Trait Locus on BTA 2. Myostatin genotype not included in the model.

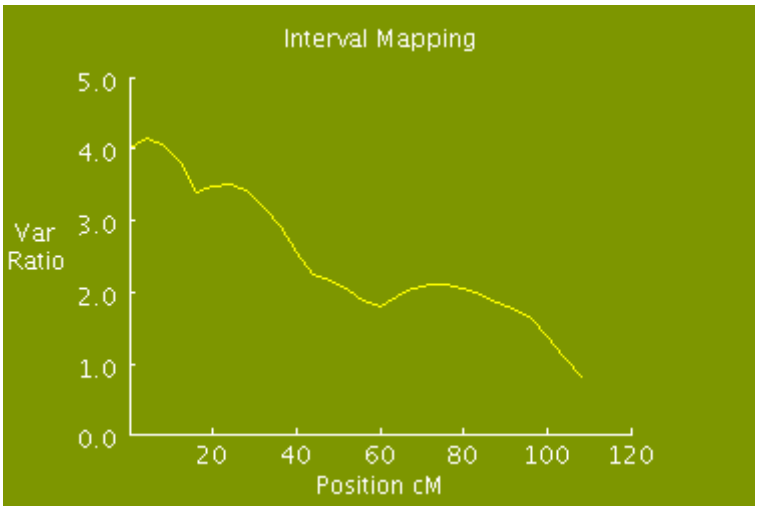


Figure 3.8: Intermuscular fat Quantitative Trait Locus on BTA 19. Myostatin genotype included in the model.

3.3.2 Intramuscular fat

3.3.2.1 Davies Gene mapping Herd

If image analysis is to be accurate, it must yield a similar result to the visually assessed marble score used at the abattoir, i.e. it must mimic the visual assessment as much as possible but be more repeatable. Visual assessment is likely to be influenced by the number and size of the fat flecks, as well as the concentration of those flecks. Therefore, four parameters were used to determine the correlation between the Matlab image analysis result and the visually assessed marble score; total fleck number, total

fleck area, fleck number as a proportion of muscle area and fleck area as a percentage of muscle area. Furthermore, different upper and lower thresholds were assessed as very small flecks may either not be detected by normal human vision or be the result of glare during the image capture, and very large flecks may be connective tissue rather than fat (Appendix B). As a result, it was determined that the most consistent parameters were fleck area as a percentage of muscle area (effectively intramuscular fat percent) and fleck number as a proportion of muscle area (Table 3.4), with lower and upper thresholds of 10 and 150mm^2, respectively.

There was considerable glare on some photographs from the Davies herd (Figure 3.9). In order to determine how much effect this glare had on the marble fleck results, the correlations between the visually assessed marble score and the Matlab generated data were repeated three times using 1) all of the images, 2) the 155 'better' images and 3) the 90 'best' images (little or no glare). Removing the glare affected images increased the correlations significantly, particularly in the 1998 steer cohort where the fleck area as a percentage of muscle area correlation with marble score improved from zero to 0.61 (Table 3.4). As expected, the correlations were generally higher when using fleck area as a percentage of muscle area rather than fleck number as a proportion of muscle area. This suggests that the effect of fleck area is greater than simply the number of flecks in visual assessment. However, the difference was not large, suggesting the additional information on fleck size only added marginally to the description of marbling.

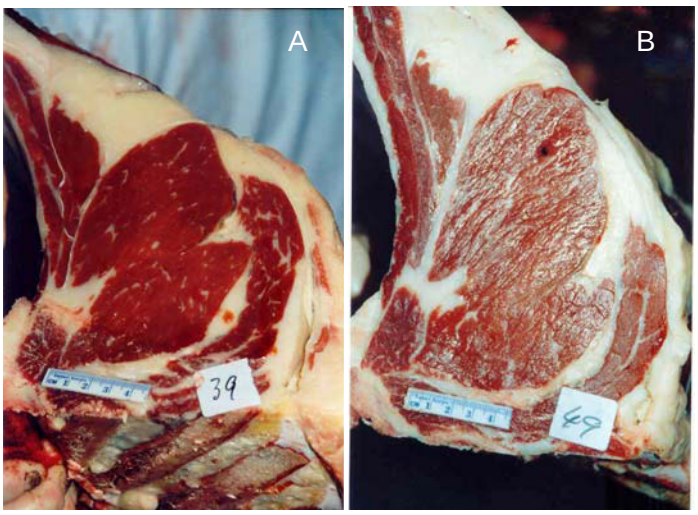


Figure 3.9: Images of steaks showing no glare (A) and glare (B).

Table 3.4: Correlations between image analysis and marble score or intramuscular fat %, Davies Gene mapping herd.

			FA/MA x 100		FN/MA x 100	
Cohort	**Photo quality**	**n**	**MBUSMS**	**IMF%**	**MBUSMS**	**IMF%**
1996	All	58	0.45	0.27	0.43	0.30
	Good	25	0.65	0.64	0.56	0.60
	Best	16	0.73	0.69	0.68	0.71
1997H	All	67	0.29	0.46	0.32	0.40
	Good	52	0.37	0.54	0.43	0.46
	Best	32	0.50	0.66	0.58	0.59
1997S	All	81	0.38	0.32	0.32	0.26
	Good	31	0.63	0.63	0.63	0.56
	Best	22	0.74	0.81	0.74	0.77
1998H	All	59	0.31	0.42	0.26	0.37
	Good	27	0.45	0.43	0.27	0.38
	Best	12	0.88	0.69	0.84	0.77
1998S	All	54	0.00	0.12	-0.07	0.00
	Good	20	0.39	0.27	0.10	0.07
	Best	8	0.61	0.66	0.11	0.21

Total fleck area as a percent of muscle area (FA/MA x 100), total number of flecks as a percent of muscle area (FN/MA x 100). Marble score (MBUSMS) and intramuscular fat % (imf). 1996 heifers and steers were slaughtered on the same day and therefore image quality did not vary between heifers and steers so were grouped together. Flecks smaller than 10mm and larger than 150mm were not included in the analysis.

In a further attempt to assess the effect of image glare, the Matlab data were used to generate marbling QTL using QTLExpress. These were compared to the QTL for marble score determined previously (Esmailizadeh, 2006) (Table 3.5). Using all of the images, no QTL were detected that matched the previously established marbling and intramuscular fat % QTL. This did not change when the most glare affected images were excluded. When only the best images were used, there were QTL detected on

two chromosomes that had marbling or intramuscular fat % QTL previously established, but these were not in the same location on the chromosome. Therefore, no new QTL matched the established QTL. This indicated that the quality of the images was below the standard required for accurate analysis and therefore, no other fleck analysis was conducted using this data set. However, as the correlations of the Matlab results with marbling increase as the lower quality images are removed (Table 3.4), it does suggest that the process would be successful if better quality photos with no glare were available.

Table 3.5: Comparison of QTL detected using different quality images with the established marbling and intramuscular fat % Quantitative Trait Loci.

	Quantitative trait loci: BTA and approximate position (cM)
***Established marbling and IMF% QTL**	1 (69), 3 (38), 4 (102), 7 (58), 9 (58, 61, 76, 78, 104), 10 (11), 12 (61), 16 (2), 24 (55), 28 (8)
QTL using all Davies images	2 (40), 18 (44)
QTL using better 278 images	22 (52)
QTL using best 180 images	1 (0), 4 (68), 11 (12)

Myostatin was not included as a fixed effect in the analysis.
*** QTL taken from Esmailizadeh, 2006.**

3.3.2.2 Trangie Residual Feed Intake (RFI) Selection Line

Due to the more controlled method of image capture used with the Trangie RFI selection line steaks, glare was not a major factor in the analysis. Although it was still important to exclude any small flecks that may actually have been glare or fat flecks smaller than would be easily visible, the correlations with marble score showed little difference whether there was no threshold or a lower threshold of 5mm (Table 3.7). An increase in the lower threshold was accompanied by a decrease in correlation with marble score, but this was expected as most of the marble flecks (85%) were less than 20mm (Table 3.6).

Table 3.6: Number and percentage of marble flecks in each fleck area (10mm^2) range

Range (mm^2)	Number of flecks	Percentage
<10	3719	56.22
10 to 20	1879	28.41
20 to 30	569	8.60
30 to 40	238	3.60
40 to 50	108	1.63
50 to 60	40	0.60
60 to 70	28	0.42
70 to 80	19	0.29
80 to 90	13	0.20
>90	2	0.03

Although the image quality was improved, the correlations in the Trangie images (Table 3.8) were generally lower than the correlations using the better Davies images (Table 3.4). This may have been due to the different position where the marbling was assessed and the images were taken in the herds, i.e. the 10th/11th rib (Davies) compared to the 5th/6th rib (Trangie). At the 5th/6th rib, there were three muscles of similar size, whereas at the 10th/11th rib the dominant *Longissimus dorsi* muscle is surrounded by much smaller muscles. The three larger muscles and the large amount of intermuscular (seam) fat may have influenced the visual assessment. Alternatively, the methodology used to differentiate intermuscular fat from intramuscular fat for the image analysis may have differed from that used in the visual assessment (Sections 3.3.1.1 and 3.3.1.2).

Table 3.7: Correlations of marble fleck area and number with marble score and intramuscular fat %.

	MSAMB correlation				IMF% correlation			
	Fleck area	**FA/MA**	**Fleck number**	**FN/MA**	**Fleck area**	**FA/MA**	**Fleck number**	**FN/MA**
All flecks	0.44	0.48	0.29	0.33	0.46	0.64	0.28	0.49
< 100mm	0.45	0.49	0.29	0.33	0.47	0.65	0.28	0.49
< 150mm	0.44	0.48	0.29	0.33	0.46	0.64	0.28	0.49
5 – 100mm	0.46	0.47	0.44	0.47	0.50	0.61	0.50	0.65
> 5mm	0.45	0.46	0.44	0.47	0.49	0.60	0.50	0.65
20 – 100mm	0.33	0.32	0.32	0.31	0.36	0.41	0.38	0.43
>20mm	0.31	0.30	0.31	0.30	0.34	0.38	0.37	0.43
>30mm	0.29	0.28	0.30	0.30	0.27	0.31	0.30	0.35
>40mm	0.24	0.22	0.28	0.27	0.20	0.24	0.25	0.31

FA/MA = fleck area as a percent of eye muscle area; FN/MA = fleck number as a proportion of eye muscle area.

Correlations between the image analysis results and marble score in distinct marble score ranges were investigated to determine if correlations were improved with higher or lower marbling (Table 3.8 and Appendix C). Only correlations with steaks in the 530 – 610 marble score range were higher than the overall correlations. However, the correlations with all other ranges were reduced significantly. Therefore, the accuracy of image analysis was not dependent on the amount of marbling present.

Table 3.8: Correlations using 5 – 100mm thresholds with selected marble score ranges, Trangie RFI Selection Line.

marble score range	n	fleck area	FA/MA	fleck number	FN/MA
		Marble score (MSAMB)			
350 - 390	36	0.09	0.14	0.12	0.15
400 - 480	74	-0.05	-0.01	0.00	0.07
490 - 520	32	0.13	0.10	0.25	0.24
530 - 610	32	0.51	0.50	0.52	0.49
620 - 830	33	-0.14	-0.16	0.10	0.08
		Intramuscular fat percent (IMF%)			
350 - 390	36	0.16	0.25	0.24	0.37
400 - 480	74	0.35	0.50	0.39	0.59
490 - 520	32	0.44	0.55	0.39	0.51
530 - 610	32	0.59	0.71	0.56	0.67
620 - 830	33	0.15	0.37	0.15	0.46

FA/MA = total fleck area / muscle area x 100. FN/MA = total fleck number / muscle area.

3.3.2.3 Fleck characteristics

Averages and standard deviations of fleck characteristics were calculated for each steer, and these were used to calculate the overall herd maximum, minimum, average and standard deviation of each characteristic (Table 3.9). When compared to the corresponding averages, the large standard deviations suggested there was considerable variation within each fat fleck characteristic.

Table 3.9: Fleck characteristics, Trangie RFI Selection Line.

Fleck characteristic	max	min	ave	ave SD
Area	19.7	7.0	12.8	9.8
Eccentricity	5.3	2.7	3.6	1.8
Ellipticity	10.5	2.1	5.7	6.8
Normalised ellipticity	0.46	0.26	0.37	0.16
Major axis length	4.7	2.6	3.5	1.5
Minor axis length	1.4	0.8	1.1	0.5
Orientation	15.8	-47.2	-18.8	48.4
x coordinate	12.3	-9.5	0.6	22.5
y coordinate	8.0	-7.2	0.0	19.2
Distance from centre	33.1	20.9	27.6	10.6

Max = maximum average of each steer, min = minimum average of each steer, ave = average of each steer average, ave SD = average standard deviation of each steer.
Area = average fleck area (mm), Eccentricity = average roundness of fleck, Ellipticity = average number of pixels outside of the ideal ellipse (mm^2), Normalised ellipticity = average percentage of pixels outside of the ideal ellipse, Orientation = average angle of each fleck ellipse (degrees), x coordinate (mm), y coordinate (mm), Distance from centre = average distance from arbitrary centre point of *M. longissimus dorsi* (mm).

Fleck eccentricity

Eccentricity measures the ratio of major to minor axes of the best fitting ellipse, giving an indication of the relative roundness of the flecks, where a result of 1 would mean the fleck was completely round (Figure 3.10). The smallest eccentricity in the complete data set had ratio of 1.014, and was, therefore, closest to round. However, only 7% of the flecks had a ratio of less than 1.5, almost 50% of the flecks had a ratio of major to minor axes of between 2 and 4, and 33% of the ratios were between 4 and 10 (Table 3.10, Figure 3.11). These results indicate the majority of flecks were elongated, most having a relatively oval shape, and the remainder being significantly elongated. This may indicate a tendency for intramuscular fat to extend either along blood capillaries or through the fascia between muscle fibre bundles.

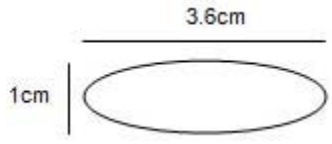


Figure 3.10: Diagrammatic representation of average eccentricity of marbling flecks, Trangie Residual Feed Intake line.

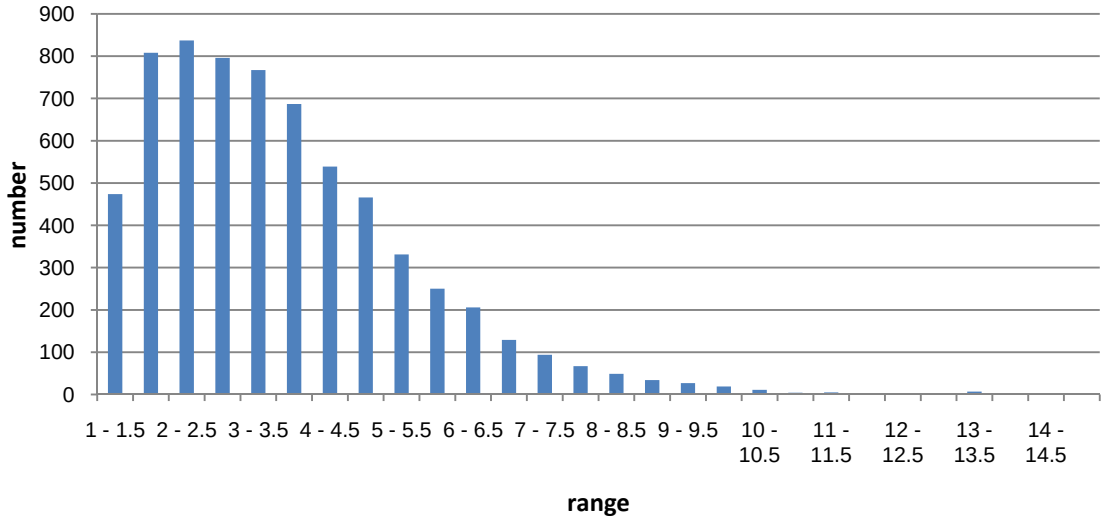


Figure 3.11: Number of fat flecks in each range of eccentricity, ratios from 1 – 15.

Table 3.10: Number of fat flecks in each range of eccentricity, ratios from 1 – 15.

Range	Number	Percent of total flecks
1 - 1.5	474	7.17
1.5 - 2	808	12.21
2 - 2.5	837	12.65
2.5 - 3	796	12.03
3 - 3.5	767	11.59
3.5 - 4	687	10.39
4 - 4.5	539	8.15
4.5 - 5	466	7.04
5 - 5.5	331	5.00
5.5 - 6	250	3.78
6 - 6.5	206	3.11
6.5 - 7	129	1.95
7 - 7.5	94	1.42
7.5 - 8	67	1.01
8 - 8.5	49	0.74
8.5 - 9	34	0.51
9 - 9.5	27	0.41
9.5 - 10	19	0.29
10 - 10.5	11	0.17
10.5 - 11	4	0.06
11 - 11.5	5	0.08
11.5 - 12	3	0.05
12 - 12.5	1	0.02
12.5 - 13	1	0.02
13 - 13.5	7	0.11
13.5 - 14	0	0.00
14 - 14.5	1	0.02
14.5 - 15	2	0.03

Fleck ellipticity

An ideal ellipse was assigned for each fleck, and the number of pixels outside of the ideal ellipse provided an indication of the relative branching of each fleck. This result was normalised for the area of the fleck to avoid variation due to size. The normalised ellipticity of all fat flecks ranged from 0.04 to 1.0 (average 0.37), with 60% of the flecks less than 0.5 (Table 3.11, Figure 3.12). On an individual animal basis, the average normalised ellipticity of all the flecks for each animal ranged from 0.26 – 0.46 (average 0.37). These results suggest there was a low level of branching within these marble flecks.

Table 3.11: Number of flecks in each normalised ellipticity range.

Range	Number	Percent of total flecks
<0.1	104	1.6
0.1 - 0.2	881	13.3
0.2 - 0.3	1533	23.2
0.3 - 0.4	1448	21.9
0.4 - 0.5	1168	17.7
0.5 - 0.6	817	12.4
0.6 - 0.7	456	6.9
0.7 - 0.8	172	2.6
0.8 - 0.9	29	0.4
0.9 - 1.0	7	0.1

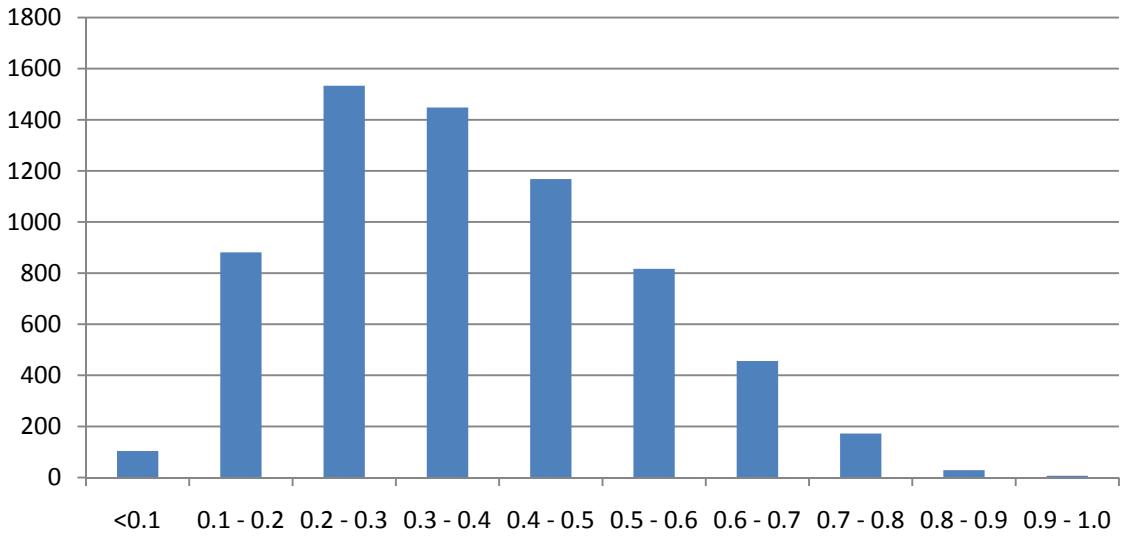


Figure 3.12: Number of flecks in each normalised ellipticity range.

Correlations

Fleck eccentricity and ellipticity correlations with marble score and intramuscular fat % were calculated (Table 3.12). There was a low negative correlation between eccentricity and both marble score (r = -0.26) and intramuscular fat % (r = 0.20), which suggests that as intramuscular fat increases, the flecks are more round. When the data were log-transformed because the distributions were not normal, the correlations between normalised ellipticity and marble score and intramuscular fat % did not improve. Normalised ellipticity was not correlated with marble score (r = -0.0002) nor intramuscular fat % (r = 0.03), and therefore, the amount of branching of the flecks did not vary with the amount of marbling or the intramuscular fat %. Similar to eccentricity, when the data were log-transformed due to the skewed distribution, the correlations between normalised ellipticity and marble score and intramuscular fat % did not improve.

Table 3.12: Eccentricity and ellipticity correlations with marble score and intramuscular fat %

	msamb	IMF%
average eccentricity of fleck	-0.26	-0.20
log of eccentricity	-0.24	-0.20
average normalised ellipticity	-0.0002	0.03
log of norm ellipticity	0.010	0.04

Heritability and genetic correlations

The heritability of fleck eccentricity, fleck normalised ellipticity, fleck area as a percent of muscle area (FA/MA) and fleck number as a proportion of muscle area (FN/MA) were calculated and compared to the heritability of marble score and intramuscular fat % (Table 3.13). The heritability of intramuscular fat % was higher but similar to those previously reported (Reverter *et al.*, 2003, Robinson and Oddy, 2004), whereas marble score was much lower (h^2 = 0.06) (Marshall, 1994, Bergen *et al.*, 2006). Fleck area as

a percent of muscle area is essentially a measure of intramuscular fat % and the heritability reflected this fact. Interestingly, eccentricity and ellipticity were not heritable and are therefore, likely to be the result of physiological factors.

Table 3.13: Heritabilities of fleck characteristics, Trangie RFI herd.

Eccentricity	Normalised ellipticity	FA/MA	FN/MA	IMF%	MSAMB
0.009	0*	0.36	0.27	0.46	0.06

FA/MA = fleck area as a percent of eye muscle area, FA/MA = fleck number as a proportion of muscle area, MSAMB = marble score, IMF% = intramuscular fat %.
*** value is outside the accepted boundary. Fixed effects of age and pen.**

As expected, the genetic correlation between fleck area (FA/MA) and fleck number (FN/MA) was very high, as were the correlations between these and intramuscular fat % and marble score (Table 3.14). The moderate, negative correlations between eccentricity and both marble score and intramuscular fat % were similar to the phenotypic correlations between these traits (Table 3.12). Although the negative correlations between normalised ellipticity and intramuscular fat % and marble score were higher than the phenotypic correlations (Table 3.12), these were still low and suggest that there is only a weak genetic relationship between these traits. Thus, the amount of branching in marble flecks is unlikely to be related to the amount of intramuscular fat.

Table 3.14: Genetic correlations of fleck characteristics, Trangie RFI herd.

	Normalised ellipticity	Eccentricity	FA/MA	FN/MA	IMF%	MSAMB
Normalised ellipticity	1					
Eccentricity	-0.33	1				
FA/MA	0.23	-0.60	1			
FN/MA	0.11	-0.65	0.88	1		
IMF%	-0.12	-0.37	0.54	0.57	1	
MSAMB	-0.15	-0.31	0.54	0.43	0.48	1

FA/MA = fleck area as a percent of eye muscle area, FA/MA = fleck number as a proportion of muscle area, MSAMB = marble score, IMF% = intramuscular fat %.

Fleck position on *Longissimus dorsi*

The Matlab program assigned a centre point of each *Longissimus dorsi* image, and the distance (mm) of each marble fleck from the centre point was calculated. The average distance from the centre point was 27.6mm (Table 3.9), indicating the flecks were not concentrated near the centre of the muscle. The standard deviation of 10.6mm indicated that there was general separation of the flecks. There were low or no correlation between these measures and either marble score or intramuscular fat % (Table 3.15). These results suggest that there was good spatial distribution of the marble flecks, and this was not related to the amount of intramuscular fat in the muscle. However, when each *Longissimus dorsi* image was separated into quarters (Figure 3.13) and each fleck allocated to a quarter, the results indicated that there were more flecks in two of these regions (B and C) compared to the other two regions (A and D) (Table 3.16).

Table 3.15: Fleck position correlations with marble score and intramuscular fat %

	average distance from centre	SD of average distance from centre
MSAMB	0.09	-0.02
IMF%	-0.05	-0.15

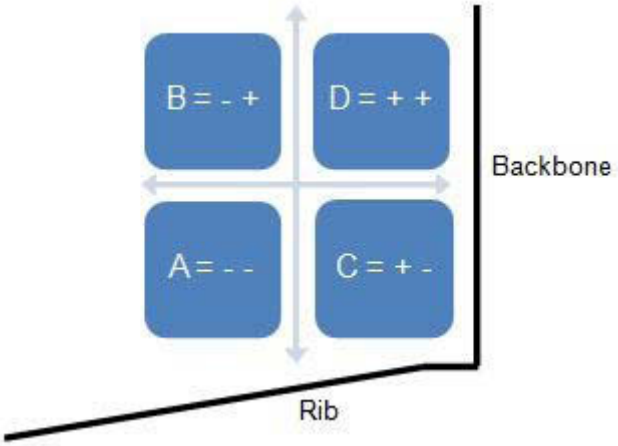


Figure 3.13: Schematic of quarters used to assess marble fleck placement.

Table 3.16: Average number of flecks in each quarter of *M. longissimus dorsi*, Trangie RFI Selection Line.

Quarter	max	min	ave	SD
A	16	0	6.57	2.64
B	20	1	9.40	3.61
C	20	1	9.41	3.18
D	15	0	6.39	2.68

Positions A, B, C and D are as indicated in Figure 3.13

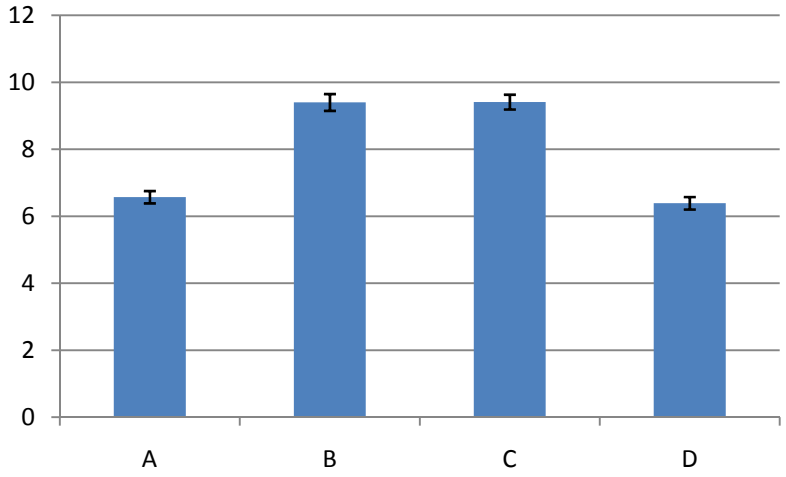


Figure 3.14: Average number of fat flecks in each quarter of the *M. longissimus dorsi.*
Positions A, B, C and D are as indicated in Figure 3.13.

3.4 Discussion

The analysis of images of the *Longissimus dorsi* taken from the Davies Gene Mapping herd and the Trangie RFI Selection line herd demonstrated the need to use high quality images. Although the images from the Davies Gene Mapping herd were not suitable for analysing marbling characteristics, these images were used to quantify the area of intermuscular fat, which was in turn used to identify a quantitative trait loci for seam fat in that herd. Furthermore, the preliminary investigations herein suggested there was no relationship between seam fat and carcass weight, eye muscle area, marble score or intramuscular fat % in either herd. However, this relationship was investigated further (Chapter 4).

Analysis of marble fleck characteristics indicated that there was no correlation between any of these parameters (Appendix D). Similarly, there was no correlation between the fleck ellipticity and marble score or intramuscular fat %. These results suggest that although there was large variation in all of the measures, the variation in fat flecks is largely independent of other the variation in other marbling factors. The variation is likely due to physiological factors yet to be determined. Although the positive correlations between fleck number and area (Table 3.7) are intuitive, there was a low, negative correlation between fleck eccentricity and both marble score and intramuscular fat % (Table 3.12). This suggests fat flecks become more round as intramuscular fat increases, and may indicate the increase is due more to hyperplasia (adipogenesis) rather than hypertrophy (lipid deposition). However, a larger number of better quality images are required to confirm this relationship.

Two aspects of the marbling flecks were quite interesting; the predominance of oval shaped and elongated fat flecks over round, and the variation in number of fat flecks in the four regions of the muscle. These may both be the result of fat developing either in

near proximity to blood vessels, or through the fascia between muscle fibre bundles where there would be less physical resistance. Further research involving a much larger data set would be required to firstly, confirm these results and secondly, to identify the cause of the shape and position of marble flecks.

Another interesting observation was the spatial distribution of the flecks in the steak, wherein more flecks were found in two quadrants (B and C). There is no obvious reason for this difference. It is likely that intramuscular fat develops in concert with blood vessels (Bornstein *et al.*, 2000), and if so, it is possible that there was a denser array of capillaries in regions where there was a greater number of fat flecks. However, this requires more investigation, initially with a much increased sample size to confirm this phenomenon. Although these images were positioned consistently, the irregularity of the shape of the *Longissimus dorsi* and the subsequent variation in the allocated centre point may have also affected the result. If the variation in fleck numbers in each region is confirmed, a thorough analysis of the vasculature in this muscle via standard histology or a three dimensional image, corrosion cast (Kondo, 1998, Minnich and Lametschwandtner, 2010) may address this question.

Chapter 4 Fat distribution traits

4.1 Introduction

Marker assisted selection (MAS) has the potential to be a powerful tool in animal breeding programs. However, for MAS to be used effectively to improve the body composition of beef cattle (e.g. increased marbling and decreased intermuscular fat), it is essential that the various fat depots do not have a strong genetic relationship or more preferably, no relationship at all. Despite its importance, the information available regarding the genetic relationships between fat depots is relatively scarce, and what information is available has generally compared the changes in specific fat depots to the total carcass fat (Bergen *et al.*, 2006) rather than to other specific fat depots. Although this is informative (Pethick *et al.*, 2004), comparing individual fat depots may prove to be equally, and quite possibly more, valuable. Another deficiency in the available information is that kidney and omental fat have often not been measured or included in the calculations (Robelin, 1986). For completeness of the analysis and understanding of the genetics and biology of fat deposition, all depots need to be considered. This may lead to further elucidation of the currently accepted description of fat depots.

Johnson *et al*. (1972) compared intermuscular, intramuscular, subcutaneous, channel and kidney fat to total carcass fat in a mixture of cattle breeds and ages. The authors found that as carcass weight increased, intermuscular fat remained relatively constant, subcutaneous fat increased, while kidney, channel and intramuscular fat declined as a percentage of total carcass fat. Kempster *et al*. (1976) examined the results from 643 steers of various breeds and found that across all breeds, subcutaneous fat increased and in most breeds, intermuscular fat decreased relative to total fat, as total fat increased. In a study of Friesian bulls, Robelin (1986) noted that as carcass weight increased, so did subcutaneous, omental and kidney fat, while intermuscular fat

decreased, all as a proportion of total body fat. Perry and Arthur (2000) found that, as a proportion of total carcass fat, subcutaneous, kidney and channel fat increased but omental and intermuscular fat decreased, as total fat accumulated in Angus steers.

As far back as 1965, it was noted that for the large amount of data generated for carcass traits to be useful to industry, it was crucial that the results were analysed carefully, and furthermore, the commonly used method of converting a trait measurement to percent of body weight was often not the best method (Dinkel *et al.*, 1965). However, adjusting for carcass weight will highlight any variation in carcass composition (Rios-Utrera *et al.*, 2005), which would enable a better evaluation of the variation between carcass composition traits. Therefore, the choice of endpoint in fat trait data analysis is important.

There are two fundamental questions that need to be answered in order to gain a better understanding of fat deposition in young beef cattle and use this knowledge to improve the selection and management of stock. First, are fat traits related genetically? Second, how do the individual fat depots develop in relation to other fat depots, as well as to the age and size of the animal?

4.2 Methods

4.2.1 Cattle

Data were obtained from the Davies gene mapping herd, the AgResearch gene mapping herd and the Trangie residual feed intake (RFI) selection line (NSW Department of Industry and Investment) commercial feedlot trial (as described in sections 2.1.1, 2.1.2 and 2.1.3, respectively).

4.2.2 Data analysis

The Davies gene mapping herd data were analysed using general linear regression with fixed effects of cohort (6 cohorts; steer or heifer in years 1 – 3), breed of dam (Jersey or Limousin), 3 F1 sires and the myostatin variant F94L (nested in breed of dam). To allow for the variation in weight between breeds, hot standard carcass weight (hscw) was used as a co-variate. The AgResearch gene mapping herd data were analysed with a similar model containing the effects of slaughter group (28 slaughter groups; 18 in year 1, 10 in year 2), breed of dam (Jersey or Limousin), 3 F1 sires (half-sibs to those in Australia) and the myostatin variant F94L (nested in breed of dam). Again, to allow for the variation in weight between breeds, hot standard carcass weight (hscw) was used as a co-variate. The Trangie RFI selection line data were analysed using general linear regression (GenStat 10.1) with fixed effects of age at slaughter, pen and sire.

Least squares means and correlations between residuals were calculated from the linear regression models described above. Regression coefficients of carcass weight were calculated, divided by the mean of each trait and multiplied by 10 to determine the percent change in each trait for every 10kg increase in carcass weight.

Based on the residual correlation matrix, a cluster analysis was undertaken to test how closely traits were related. This was done using a single link, hierarchical cluster analysis (GenStat 10.1). Also based on the residual correlation matrix, principal components were calculated. The output was a series of eigen values and vectors.

For the Trangie RFI selection line, the sire effects, heritability and genetic correlations could be determined because there were a sufficient number of sires. Sire effects were calculated using a mixed model with fixed effects of carcass weight, age at slaughter and pen. Sire was fitted as a random effect resulting in estimation of sire variances and best linear unbiased predictions (BLUP) for the sires. The variances were used to calculate heritabilities and the BLUPs were correlated to give an indication of genetic correlations between traits. The between-sire variance was assumed to be ¼ of the additive genetic variance from which the heritability was calculated.

4.3 Results

4.3.1 Summary statistics

Substantial variation was observed for all fat related traits within both the Jersey – Limousin mapping herds. The most variation occurred in the intermuscular, rump and rib fat in the Davies herd (Table 4.1), in the omental, kidney and pericardial fat in the AgResearch herd (Table 4.2), and in the rib and intermuscular fat in the Trangie herd (Table 4.3). The coefficient of variation for the fat deposition traits ranged from 31% to 52% in the Davies herd, 26% to 57% in the AgResearch herd, and 22% to 49% in the Trangie herd.

Table 4.1: Summary of trait data from the Davies gene mapping herd.

Trait	Abbreviation	N	Mean	Std Dev	Min	Max	CV (%)
hot standard carcass weight (kg)	hscw	356	335	61.7	168	480	18
marble score	mbms	356	1.73	0.68	0	3.4	39
eye muscle area (cm^2)	ema	355	81	17	26	166	21
rump fat at P8 (mm)	p8am	356	12.3	5.23	3	30	43
rib fat (mm)	rbft	356	9.7	3.64	3	24	38
kidney fat (kg)	kdft	356	12.5	3.86	4.7	22.8	31
omental fat (kg)	omental	266	12.0	4.14	3.3	24.5	34
intramuscular fat content (%)	imf%	355	5.2	1.71	1.4	11.1	33
melting point (°C)	meltpt	355	37.4	3.09	31	46	8
intermuscular fat (mm^2)	seamfat	320	308	160.1	25	1180	52
trimmable fat (%)	fat%	330	13.6	2.7	3.9	21.4	20

Table 4.2: Summary of trait data from the AgResearch gene mapping herd.

Trait	Abbreviation	N	Mean	Std Dev	Min	Max	CV (%)
hot standard carcass weight (kg)	hscw	415	228	43.8	136	369	19
eye muscle area (cm^2)	ema	328	59.0	13.6	37.3	112	23
rib fat depth (mm)	cfat	327	7.40	2.91	1	20	39
intramuscular fat in eye muscle (%)	marbpc	343	4.21	2.27	0.4	12.8	54
omental fat (kg)	omental	415	3.95	2.10	0.58	12.5	53
kidney fat (kg)	kidfat	415	7.08	3.24	1.46	19.5	46
pericardial fat (kg)	perifat	411	0.51	0.21	0.14	1.76	42
subcutaneous fat at flank (kg)	flankfat	402	2.94	0.97	0.8	6.74	33
subcutaneous fat at rump (kg)	rumpfat	402	0.47	0.15	0	1	32
subcutaneous fat at topside (kg)	topfat	402	0.29	0.17	0	0.95	57
subcutaneous fat at silverside (kg)	silvfat	402	0.54	0.14	0.17	1.02	26
subcutaneous fat at porterhouse (kg)	portfat	402	0.79	0.41	0	2.17	52
subcutaneous fat at forequarter (kg)	fqfat	402	4.43	1.26	1.25	11.6	29
subcutaneous fat at hindquarter (kg)	hqfat	402	5.03	1.52	1.51	11.2	30
total fat (%)	fatpc	402	8.97	2.41	3.48	17.3	27
melting point (°C)	mpt	405	37.2	2.61	28.5	44	7
unsaturated fatty acids (% of total triacylglycerides)	ufa	406	57.4	3.47	47.6	79.8	6

Table 4.3: Summary of trait data from the Trangie RFI steers.

Trait	Abbreviation	N	Mean	Std Dev	Min	Max	CV (%)
hot standard carcass weight (kg)	hscw	208	415	27.4	354	494	7
marble score	msamb	208	504	107	350	830	21
eye muscle area (cm^2)	ema	208	77	3.28	68	85	4
rib fat depth (mm)	rbft	208	17.9	5.62	6	34	31
intramuscular fat content (%)	imf%	207	14.5	3.12	8.29	22.7	22
melting point (°C)	melt pt	207	40.3	1.89	33	44	5
intermuscular fat (cm^2)	seamfat	207	24.2	6.21	9.67	44.2	26
rib fat gain, 440d to slaughter (mm)	fat gain	208	10.2	4.95	-2	24	49
dressed carcass (%)	dresspc	208	58.5	1.44	54.1	64.6	2

4.3.2 Significance effects

Each trait was regressed against cohort, breed of dam, sire and the myostatin genotype F94L (the DNA variant responsible for increased muscling in the Limousin breed) in both the Davies and AgResearch gene mapping herds. In the Davies herd, breed of dam was significant for all traits except for subcutaneous fat (P8 and rib fat) (Table 4.4). Sire was significant for all traits except subcutaneous and omental fat. The

F94L myostatin variant was significant for eye muscle area (ema), rump fat (P8) and seam fat and marginally significant for carcass weight (hscw) and intramuscular fat % (imf) (Table 4.4).

Table 4.4: Tests of significance (F-probabilities) for the Davies gene mapping herd.

	hscw	mbms	ema	p8am	rft	kdft	omental	imf	meltpt	seamft
cohort	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0003
breed	<0.0001	<0.0001	<0.0001	0.3427	0.1648	<0.0001	<0.0001	<0.0001	<0.0001	0.0036
sire	<0.0001	0.0456	<0.0001	0.9246	0.9547	0.0101	0.327	0.016	0.0011	<0.0001
MSTN	0.0347	0.0931	<0.0001	0.0009	0.1166	0.3081	0.7576	0.0212	0.3227	<0.0001

Cohort + breed of dam + sire + Myostatin (nested in breed of dam). Carcass weight (hscw) marble score (mbms), eye muscle area (ema), rump fat at P8 (p8am), rib fat (rft), kidney fat (kdft), omental fat (omental), intramuscular fat (imf%), melting point (meltpt), intermuscular fat (seamft). Significance defined as ≤0.05.

Hot standard carcass weight was added to the model to test the effect of animal size. Cohort and sire were unchanged, but breed of dam became significant for rib and rump fat as was the myostatin genotype for rib and kidney fat (Table 4.5).

Table 4.5: Tests of significance (F-probabilities) for the Davies gene mapping herd with carcass weight as a covariate.

	mbms	ema	p8am	rft	kdft	omental	imf	meltpt	seamfat
hscw	<0.0001	<0.0001	0.002	0.2591	0.1518	<0.0001	<0.0001	0.6211	0.0016
cohort	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0062
breed	<0.0001	<0.0001	0.0003	0.0016	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
sire	0.0586	0.007	0.5786	0.5893	0.0305	0.106	0.009	0.0025	<0.0001
MSTN	0.0943	<0.0001	<0.0001	0.0317	0.0549	0.8211	0.0114	0.3504	<0.0001

Hot standard carcass weight + cohort + breed of dam + sire + Myostatin (nested in breed of dam). Marble score (mbms), eye muscle area (ema), rump fat at P8 (p8am), rib fat (rft), kidney fat (kdft), omental fat (omentl), intramuscular fat (imf%), melting point (meltpt), intermuscular fat (seamft).Significance defined as ≤0.05.

Breed of dam was significant for all traits in the AgResearch herd, although subcutaneous fat at the topside region (topfat) was only marginally significant (Table 4.6). Sire was significant for carcass weight (hscw), eye muscle area (ema), the internal fat areas of kidney (kidfat) and pericardial (perifat), the subcutaneous fat depots at the flank (flankfat) and forequarter (fqfat) as well as melting point of fat (mpt). Sire was marginally significant for subcutaneous fat at the porterhouse region (portfat) and hindquarter (hqfat), and there was a trend for sire to affect rib fat depth (cfat) (p =

0.085). The F94L myostatin variant was significant for all traits except intramuscular fat % (marbpc), pericardial fat (perifat) and subcutaneous fat in the topside region (topfat) (Table 4.6).

When hot standard carcass weight was added to the model, carcass weight was significant for all traits except subcutaneous fat at the silverside region (silvfat) (Table 4.7). Cohort (slgrp) was unchanged as was breed of dam. Sire was more significant for omental and subcutaneous fat at the topside region (topfat) but not significant for subcutaneous fat at the flank (flankfat), porterhouse (portfat), forequarter (fqfat) and hindquarter (hqfat). Myostatin genotype became significant for intramuscular fat % (marbpc), pericardial fat (perifat) and subcutaneous fat at the topside region (topfat) (Table 4.7).

Table 4.6: Tests of significance (F-probabilities) for the AgResearch gene mapping herd.

	hscw	ema	cfat	marbpc	omental	kidfat	perifat	flankfat	rumpfat	topfat	silvfat	portfat	fqfat	hqfat	fatpc	mpt
slgrp	<0.0001	0.0003	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0066
breed	<0.0001	<0.0001	0.0178	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0009	0.0321	<0.0001	0.0002	0.0156	<0.0001	<0.0001	<0.0001
sire	<0.0001	<0.0001	0.0845	0.1541	0.1056	<0.0001	<0.0001	0.0082	0.814	0.4423	0.7882	0.0339	0.0002	0.0517	0.5349	0.0005
mstn	0.0008	<0.0001	0.0039	0.0952	0.024	0.0005	0.2364	<0.0001	<0.0001	0.3306	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0071

Slaughter group (slgrp) + breed of dam + sire + Myostatin (nested in breed of dam). Carcass weight (hscw), eye muscle area (ema), rib fat depth (cfat), intramuscular fat in eye muscle, (marbpc), omental fat (omental), kidney fat (kidfat), pericardial fat (perifat), subcutaneous fat at flank (flankfat), subcutaneous fat at rump (rumpfat), subcutaneous fat at topside (topfat), subcutaneous fat at silverside (silvfat), subcutaneous fat at porterhouse (portfat), subcutaneous fat at forequarter (fqfat), subcutaneous fat at hindquarter (hqfat), total fat (fatpc) and melting point (mpt). Significance defined as ≤0.05.

Table 4.7: Tests of significance (F-probabilities) for the AgResearch gene mapping herd with carcass weight as a covariate.

	ema	cfat	marbpc	omental	kidfat	perifat	flankfat	rumpfat	topfat	silvfat	portfat	fqfat	hqfat	mpt
hscw	<0.0001	0.0055	0.0018	<0.0001	0.0234	0.0002	<0.0001	<0.0001	<0.0001	0.121	<0.0001	<0.0001	<0.0001	0.0021
slgrp	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0016
breed	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
sire	<0.0001	0.1309	0.0889	0.0533	<0.0001	<0.0001	0.4175	0.1045	0.0134	0.1156	0.5441	0.1905	0.6802	0.0092
mstn	<0.0001	0.0002	0.0295	0.0003	<0.0001	0.0274	<0.0001	<0.0001	0.0433	<0.0001	<0.0001	<0.0001	<0.0001	0.0008

Hot standard carcass weight + slaughter group (slgrp)+ breed of dam + sire + Myostatin (nested in breed of dam). Eye muscle area (ema), rib fat depth (cfat), intramuscular fat in eye muscle, (marbpc), omental fat (omental), kidney fat (kidfat), pericardial fat (perifat), subcutaneous fat at flank (flankfat), subcutaneous fat at rump (rumpfat), subcutaneous fat at topside (topfat), subcutaneous fat at silverside (silvfat), subcutaneous fat at porterhouse (portfat), subcutaneous fat at forequarter (fqfat), subcutaneous fat at hindquarter (hqfat) and melting point (mpt). Significance defined as ≤0.05.

The Trangie Residual Feed Intake steers were allocated to pens according to their mid-parent RFI estimated breeding values, so that there were high, medium and low RFI pens. Pen was significant for most traits measured in the Trangie herd (Table 4.8). Intermuscular fat (seam fat) was marginally significant, and there was a trend for pen to affect carcass weight (p = 0.059), whereas age was only significant for about half of the traits. Adding carcass weight to the model had little effect (Table 4.9). Residual feed intake pen was less significant for intermuscular fat, and age was less or no longer significant for intramuscular fat %, rib fat and dressing percent.

Table 4.8: Tests of significance (F-probabilities) for the Trangie RFI steers.

	hscw	ema	imf%	msamb	seamfat	ribfat	fat gain	dresspc	meltpt
Final age	<0.001	0.389	0.045	0.001	0.536	0.056	0.219	0.004	0.523
Pen	0.059	<0.001	0.015	<0.001	0.034	<0.001	0.037	0.462	<0.001

Age at slaughter + pen + sire (random variable). Carcass weight (hscw), eye muscle area (ema), intramuscular fat % (imf), marble score (msamb), intermuscular fat (seamfat), rib fat (ribfat), rib fat depth increase since 400 day scan (fat gain), dressing percent (dresspc) and melting point (meltpt). Significance defined as ≤0.05.

Table 4.9: Tests of significance (F-probabilities) for the Trangie RFI steers with carcass weight as a covariate.

	ema	imf%	msamb	seamfat	rft	fat gain	dresspc	meltpt
hscw	0.001	0.197	0.014	0.154	0.005	0.027	<0.001	0.707
Final age	0.888	0.091	0.009	0.272	0.213	0.555	0.258	0.577
Pen	0.001	0.019	<0.001	0.053	<0.001	0.018	0.71	<0.001

Hot standard carcass weight + age at slaughter + pen + sire (random variable). Eye muscle area (ema), intramuscular fat % (imf), marble score (msamb), intermuscular fat (seamfat), rib fat (ribfat), rib fat depth increase since 400 day scan (fat gain), dressing percent (dresspc) and melting point (meltpt). Significance defined as ≤0.05.

4.3.3 Trait variation

The least squares means showed variation in traits within cohort, breed of dam, sire and myostatin genotype (Tables 4.10 and 4.11). The Limousin had greater carcass weight (hscw) and eye muscle area (ema) compared to the Jersey, but most fat traits were greater in the Jersey progeny. The sire effect was less overall, and the myostatin genotype had a large effect on all traits, with the variant genotype tending to have larger muscles and reduced fat. Deviation within cohort, breed of dam, sire and

myostatin genotype of both the Davies and AgResearch herds was evident (Figures 4.1 to 4.7).

Table 4.10: Least squares means of muscle and fat traits in the Davies gene mapping herd.

	hscw	mbms	ema	p8am	rft	kdft	omental	imf%	meltpt	seamfat	fat%
cohort 96H	348	1.52	82.9	16.7	11.5	15.1	.	5.97	35	303	15.6
cohort 96S	372	1.54	79.3	11.9	10.8	16.5	.	6.12	35.3	395	15.2
cohort 97H	302	1.99	70.1	11.3	10.0	12.5	17.1	4.79	37.6	399	13.2
cohort 97S	355	1.25	87.4	8.6	6.6	13.6	8.9	5.46	37.8	317	12.1
cohort 98H	299	1.83	75.8	15.4	10.0	11.0	10.8	4.95	39.5	245	14.0
cohort 98S	371	2.09	88.1	12.7	10.8	9.0	11.7	4.48	37.5	357	13.9
breed XJ	301	1.92	70.2	12.3	10.2	13.7	12.7	6.12	36.3	336	15.1
breed XL	382	1.49	90.9	13.2	9.7	12.2	11.6	4.47	38	303	12.9
sire 361	352	1.61	81.6	13.0	10.0	13.0	11.8	5.09	36.4	351	13.8
sire 368	341	1.75	82.5	12.7	9.9	13.5	12.1	5.24	37.3	350	14.2
sire 398	331	1.75	77.6	12.6	10.0	12.2	12.5	5.55	37.6	266	14.0
mstn(breed) AC XJ	302	1.92	71.9	12.3	9.9	13.3	12.7	6.02	36.4	316	14.9
mstn(breed) CC XJ	299	1.92	68.5	12.4	10.5	14.0	12.7	6.23	36.2	356	15.3
mstn(breed) AA XL	392	1.31	101.2	10.9	8.9	11.9	11.5	3.98	37.6	217	10.5
mstn(breed) AC XL	375	1.51	87.6	13.5	10.1	12.3	11.1	4.58	38.4	319	13.2
mstn(breed) CC XL	379	1.64	84.0	15.3	10.2	12.3	12.0	4.84	37.9	372	15.0
SEM – min	2.72	0.04	0.81	0.34	0.25	0.23	0.22	0.10	0.20	12.2	0.16
SEM - max	8.93	0.14	2.67	1.11	0.82	0.77	0.88	0.34	0.66	36.9	0.52

Carcass weight (hscw - kg) marble score (mbms), eye muscle area (ema – cm^2), rump fat at P8 (p8am - mm), rib fat depth (rft - mm), kidney fat (kdft - kg), omental fat (kg), intramuscular fat (imf%), melting point (meltpt – °C), intermuscular fat (seamfat – mm^2) and fat %. Omental fat was not measured in the 1996 cohorts.

Table 4.11: Least squares means of muscle and fat traits in the AgResearch gene mapping herd.

	hscw	ema	cfat	marbpc	omental	kidfat	perifat	flankfat	rumpfat
SG 1	201	57.8	7.34	4.31	2.03	4.91	0.43	2.21	0.46
SG 2	200	66.0	4.94	6.05	1.96	4.52	0.37	2.50	0.41
SG 3	199			4.27	1.87	5.04	0.49	2.20	0.40
SG 4	203				2.03	4.39	0.54	2.77	0.45
SG 5	207			4.14	2.19	5.08	0.34	2.69	0.51
SG 6	225	63.4	5.41	4.17	2.19	3.99	0.30	2.42	0.35
SG 7	211	61.6	7.81	4.79	1.02	5.55	0.49	2.71	0.46
SG 8	234	61.4	5.18		2.25	3.61	0.39	2.89	0.38
SG 9	212			4.41	3.19	6.46	0.51	2.92	0.44
SG 10	235			3.85	2.54	5.31	0.47	2.10	0.39
SG 11	215			6.49	3.94	7.85	0.57	3.46	0.56
SG 12	255	66.1	5.71	4.41	2.46	4.62	0.39	3.09	0.17
SG 13	217	57.8	7.56	5.67	3.29	7.59	0.29	1.75	0.48
SG 14	258	63.6	7.31	4.15	4.27	6.73	0.65	3.63	0.49
SG 15	256	62.6	6.18	5.47	4.46	6.94	0.67	2.67	0.48
SG 16	259	61.6	7.90	5.17	5.25	8.01	0.63	2.83	0.53
SG 17	276	68.1	7.98	6.16	4.78	8.03	0.70	3.31	0.60
SG 18	270	65.7	7.67		5.57	8.64	0.69	3.00	0.50
SG 19	211	58.9	9.80	2.87	3.33	7.25	0.51	2.25	0.47
SG 20	214	61.1	5.87	2.15	3.54	6.63	0.38	2.38	0.36
SG 21	217	58.0	8.44	2.48	3.66	6.86	0.39	2.55	0.45
SG 22	222	63.7	6.76	2.74	3.93	7.43	0.43	2.95	0.54
SG 23	227	63.0	6.16	2.33	3.70	7.62	0.56	3.08	0.55
SG 24	257	65.0	9.00	4.04	5.71	9.19	0.50	2.81	0.54
SG 25	261	63.2	8.44	3.68	5.39	8.68	0.81	2.86	0.51
SG 26	268	63.6	8.04	3.46	6.18	9.62	0.58	4.16	0.49
SG 27	272	65.7	8.36	3.17	6.14	9.41	0.58	4.34	0.50
SG 28	277	64.9	9.71	3.79	7.23	10.07	0.38	4.50	0.60
breed XJ	206	51.0	7.70	4.97	4.57	8.24	0.54	3.05	0.48
breed XL	263	74.7	6.99	3.37	2.86	5.33	0.46	2.74	0.45
394	227	60.3	7.33	4.40	3.79	5.56	0.51	2.73	0.46
402	241	66.6	7.81	4.24	3.85	7.12	0.44	3.01	0.47
417	234	61.6	6.89	3.87	3.52	7.68	0.55	2.94	0.47
mstn(breed) AC XJ	206	52.1	7.25	4.78	4.55	7.85	0.52	2.94	0.46
mstn(breed) CC XJ	205	49.9	8.14	5.16	4.60	8.63	0.56	3.16	0.51
mstn(breed) AA XL	273	79.4	5.96	2.82	2.40	4.51	0.45	2.36	0.39
mstn(breed) AC XL	259	68.8	7.33	3.52	2.96	5.64	0.48	2.98	0.47
mstn(breed) CC XL	257	76.0	7.68	3.75	3.23	5.84	0.47	2.89	0.49
SEM – min	1.32	0.46	0.18	0.13	0.07	0.13	0.01	0.04	0.01
SEM - max	7.61	2.75	1.09	0.74	0.41	0.78	0.06	0.25	0.04

Carcass weight (hscw - kg), eye muscle area (ema – cm^2), rib fat depth (cfat – mm), intramuscular fat in eye muscle, (marbpc – %), omental fat (omental – kg), kidney fat (kidfat – kg), pericardial fat (perifat – kg), subcutaneous fat at flank (flankfat – kg) and subcutaneous fat at rump (rumpfat – kg). SG = slaughter group.

Table 4.11 continued.

	topfat	silvfat	portfat	fqfat	hqfat	fatpc	mpt	ufa
SG 1	0.40	0.53	0.75	3.80	4.43	8.88	39.53	57.04
SG 2	0.23	0.47	0.83	4.36	4.46	9.45	36.83	56.90
SG 3	0.15	0.52	0.49	4.30	3.82	8.82	37.05	58.19
SG 4	0.24	0.50	0.55	4.78	4.51	9.59	37.15	57.27
SG 5	0.21	0.57	0.68	4.60	4.66	9.32	36.65	58.18
SG 6	0.15	0.49	0.60	3.84	4.01	7.22	37.50	58.96
SG 7	0.14	0.56	0.69	4.68	4.56	9.10	36.34	58.80
SG 8	0.20	0.48	0.55	3.87	4.50	7.43	38.74	55.49
SG 9	0.20	0.53	0.63	4.27	4.72	8.86	37.39	56.47
SG 10	0.16	0.50	0.50	4.25	3.67	6.89	36.53	57.36
SG 11	0.32	0.69	1.06	5.86	6.08	11.26	36.83	58.08
SG 12	0.21	0.57	0.87	4.45	4.91	7.54	38.80	55.40
SG 13	0.19	0.58	0.72	4.39	3.71	7.74	37.75	56.94
SG 14	0.26	0.56	0.70	4.84	5.66	8.46	36.82	56.90
SG 15	0.22	0.60	0.43	4.73	4.39	7.46	36.11	56.32
SG 16	0.22	0.59	0.48	4.83	4.64	7.63	36.26	55.86
SG 17	0.40	0.66	0.58	5.04	5.55	8.06	36.64	55.85
SG 18	0.27	0.62	0.60	4.65	4.99	7.48	37.31	56.68
SG 19	0.15	0.61	0.54	3.73	4.01	8.40	37.37	57.37
SG 20	0.27	0.41	0.76	3.81	4.18	8.47	37.82	56.88
SG 21	0.26	0.43	0.62	3.74	4.31	8.37	36.92	59.39
SG 22	0.19	0.63	0.87	4.00	5.19	9.29	36.20	59.01
SG 23	0.33	0.42	1.21	3.71	5.58	9.38	36.75	58.78
SG 24	0.32	0.54	1.28	5.44	5.48	9.72	38.08	57.83
SG 25	0.37	0.53	1.13	4.63	5.39	8.74	39.23	56.11
SG 26	0.53	0.49	1.19	5.03	6.86	10.24	37.91	56.97
SG 27	0.58	0.48	1.05	4.94	6.95	9.90	37.87	56.18
SG 28	0.61	0.53	1.37	4.96	7.61	10.33	36.98	56.78
breed XJ	0.30	0.56	0.83	4.56	5.22	10.05	36.49	58.44
breed XL	0.26	0.52	0.72	4.41	4.70	7.38	38.18	55.99
394	0.29	0.54	0.74	4.22	4.76	8.60	37.70	57.54
402	0.28	0.54	0.84	4.82	5.14	8.91	36.66	57.58
417	0.27	0.54	0.75	4.41	4.98	8.64	37.65	56.53
mstn(breed) AC XJ	0.29	0.55	0.76	4.37	4.99	9.63	36.86	58.11
mstn(breed) CC XJ	0.31	0.57	0.90	4.74	5.45	10.48	36.13	58.77
mstn(breed) AA XL	0.26	0.45	0.56	3.63	4.03	5.96	39.09	55.05
mstn(breed) AC XL	0.28	0.55	0.80	4.64	5.09	8.00	38.39	56.11
mstn(breed) CC XL	0.23	0.56	0.81	4.96	4.97	8.17	37.06	56.81
SEM – min	0.007	0.008	0.02	0.07	0.07	0.10	0.15	0.20
SEM - max	0.04	0.04	0.11	0.40	0.40	0.58	0.85	1.15

Subcutaneous fat at topside (topfat - kg), subcutaneous fat at silverside (silvfat – kg), subcutaneous fat at porterhouse (portfat – kg), subcutaneous fat at forequarter (fqfat – kg), subcutaneous fat at hindquarter (hqfat - kg), total fat (fatpc - %), melting point (mpt – °C) and unsaturated fatty acids (ufa – % of total triacylglycerides). SG = slaughter group.

The variation in the least square means of traits recorded for the Trangie herd showed that the steers in the medium RFI pen were generally larger than the other cohorts and had greater fat deposition, with the exception of rib fat and fat gain (Table 4.12).

Table 4.12: Least squares means of muscle and fat traits in the Trangie RFI steers.

	hscw	ema	imf%	msamb	seamfat	rft	melt pt	fat gain	dresspc
Low RFI	417	76.1	14.3	475.0	24.7	15.5	8.9	41.1	58.5
Medium RFI	423	78.6	15.7	573.1	25.6	17.8	10.4	40.2	58.9
High RFI	405	76.1	13.4	461.2	22.1	20.5	11.3	39.4	58.0
SEM – min	3.13	0.36	0.35	11.2	0.71	0.62	0.57	0.21	0.16
SEM - max	3.22	0.37	0.36	11.5	0.74	0.64	0.59	0.22	0.17

Carcass weight (hscw - kg), eye muscle area (ema – cm^2), intramuscular fat %, marble score (msamb), intermuscular fat (seam fat – cm^2), rib fat depth (rft - mm), melting point (°C), rib fat depth increase since 400 day scan (fat gain) and dressed percent (dresspc).

4.3.4 Effect of carcass weight

To estimate the extent to which the fat traits were related to the generally larger frame of the Limousin, regression coefficients were used to indicate the relationship between the fat traits and carcass weight. Increasing carcass weight by ten kilograms was associated with an alteration of fat deposition traits of between 0.1% and 3.5% in the Davies herd (Table 4.13), 3.2% and 7.7% in the AgResearch herd (Table 4.14), and 0.1% and 3.3% in the Trangie herd (Table 4.15).

Table 4.13: Regression coefficients of hot standard carcass weight with standard errors and percent changes of traits for the Davies gene mapping herd.

	Regression coefficient	Standard error	Percent change (%trait/10kg hscw)
ema	0.17	0.01	2.1
p8am	0.04	0.005	3.5
ribfat	0.02	0.004	1.8
kidfat	0.03	0.01	2.3
omental	0.01	0.23	1.1
seamfat	1.05	0.004	3.4
mbms	0.0001	0.01	0.1
imf%	0.003	0.0008	0.6

Eye muscle area (ema), rump fat at P8 (p8am), rib fat (rft), kidney fat (kdft), omental fat (omentl), intermuscular fat (seamft), marble score (mbms), intramuscular fat (imf%), melting point (meltpt).

Table 4.14: Regression coefficients of hot standard carcass weight with standard errors and percent changes of traits for the AgResearch gene mapping herd.

	Regression coefficient	Standard error	Percent change (%trait/10kg hscw)
ema	0.18	0.02	3.1
marbpc	0.01	0.01	3.2
omental	0.02	0.003	4.1
kidfat	0.04	0.005	5.8
perifat	0.002	0.0004	4.2
cfat	0.03	0.01	4.5
flankfat	0.02	0.001	6.5
rumpfat	0.002	0.0003	5.2
topfat	0.002	0.0003	6.0
silvfat	0.002	0.0003	3.5
portfat	0.01	0.001	7.7
fqfat	0.03	0.002	6.3
hqfat	0.03	0.002	6.2

Eye muscle area (ema), intramuscular fat in eye muscle, (marbpc), omental fat (omental), kidney fat (kidfat), pericardial fat (perifat), rib fat depth (cfat), subcutaneous fat at flank (flankfat), subcutaneous fat at rump (rumpfat), subcutaneous fat at topside (topfat), subcutaneous fat at silverside (silvfat), subcutaneous fat at porterhouse (portfat), subcutaneous fat at forequarter (fqfat), subcutaneous fat at hindquarter (hqfat) and melting point (mpt).

Table 4.15: Regression coefficients of hot standard carcass weight with standard errors and percent changes of traits for the Trangie RFI steers.

	Regression coefficient	Standard error	Percent change (%trait/10kg hscw)
ema	0.022	0.01	0.3
ribfat	0.050	0.01	2.8
seamfat	0.023	0.02	1.0
imf%	0.001	0.01	0.1
msamb	0.181	0.26	0.4
fat gain	0.034	0.01	3.3
dresspc	0.024	0.003	0.4

Eye muscle area (ema), intramuscular fat % (imf), marble score (msamb), intermuscular fat (seamfat), rib fat (ribfat) and rib fat depth increase since 400 day scan (fat gain).

4.3.5 Cohort effects

There was greater variation in fat deposition between cohorts in the AgResearch herd than in the Davies herd. The average difference between highest and lowest in the Davies herd was 1.9 standard deviations (Figure 4.1), whereas, in the AgResearch herd, it was 3.2 standard deviations (Figure 4.2). However, the average difference between the subcutaneous fat and internal fat depots was more consistent in the

AgResearch herd (subcutaneous, 3.2 – internal, 3.5 sd.) than in the Davies herd (subcutaneous, 1.9 – internal, 3 sd.). In both herds, the rump and omental fat depots had the greatest difference between the highest and lowest deposition, and in the Davies herd, seam fat showed the least variation between cohorts.

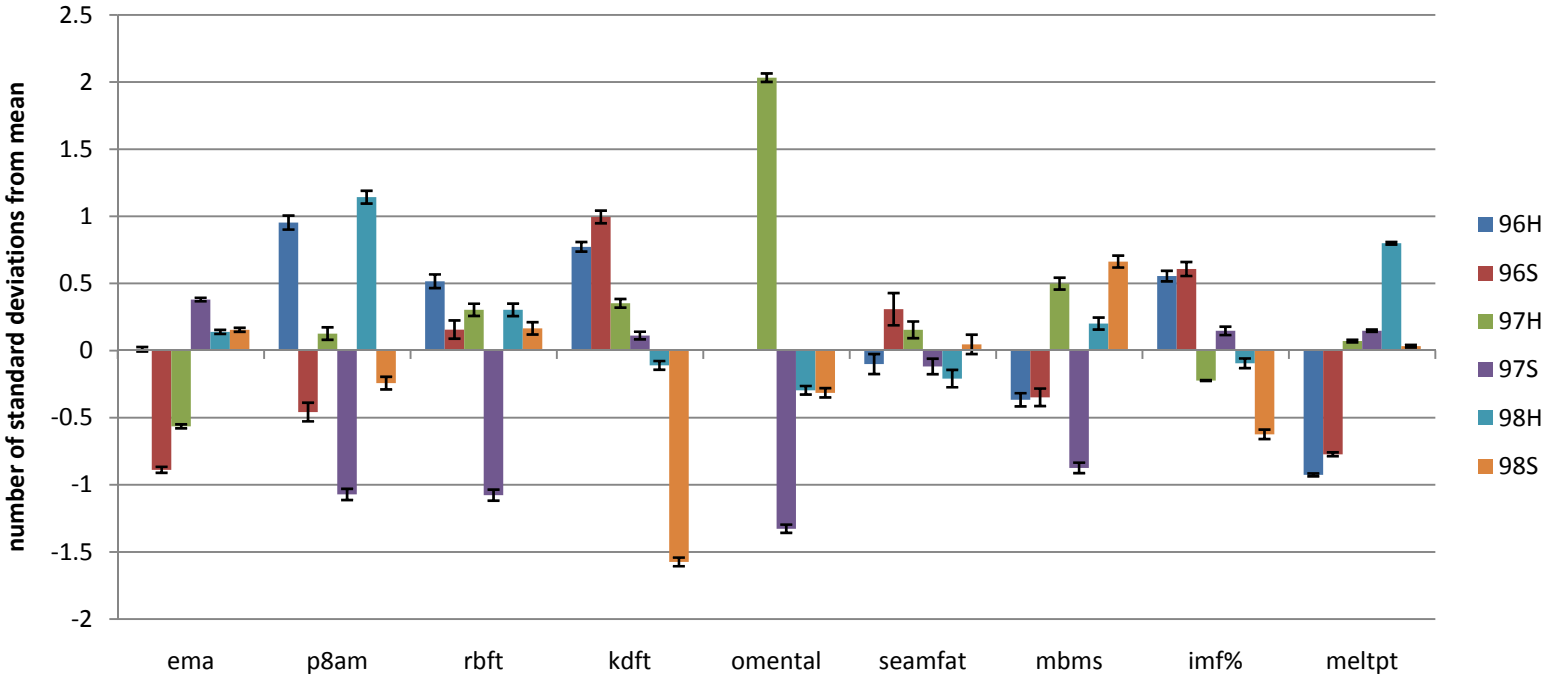


Figure 4.1: Davies gene mapping herd cohort effects.
Measured as the number of standard deviations from the overall mean, adjusted for carcass weight. Eye muscle area (ema), rump fat at P8 (p8am), rib fat (rbft), kidney fat (kdft), omental fat, intermuscular fat (seamfat), marble score (mbms), intramuscular fat (imf%) and melting point (meltpt). Omental fat was not measured in the 1996 cohorts.

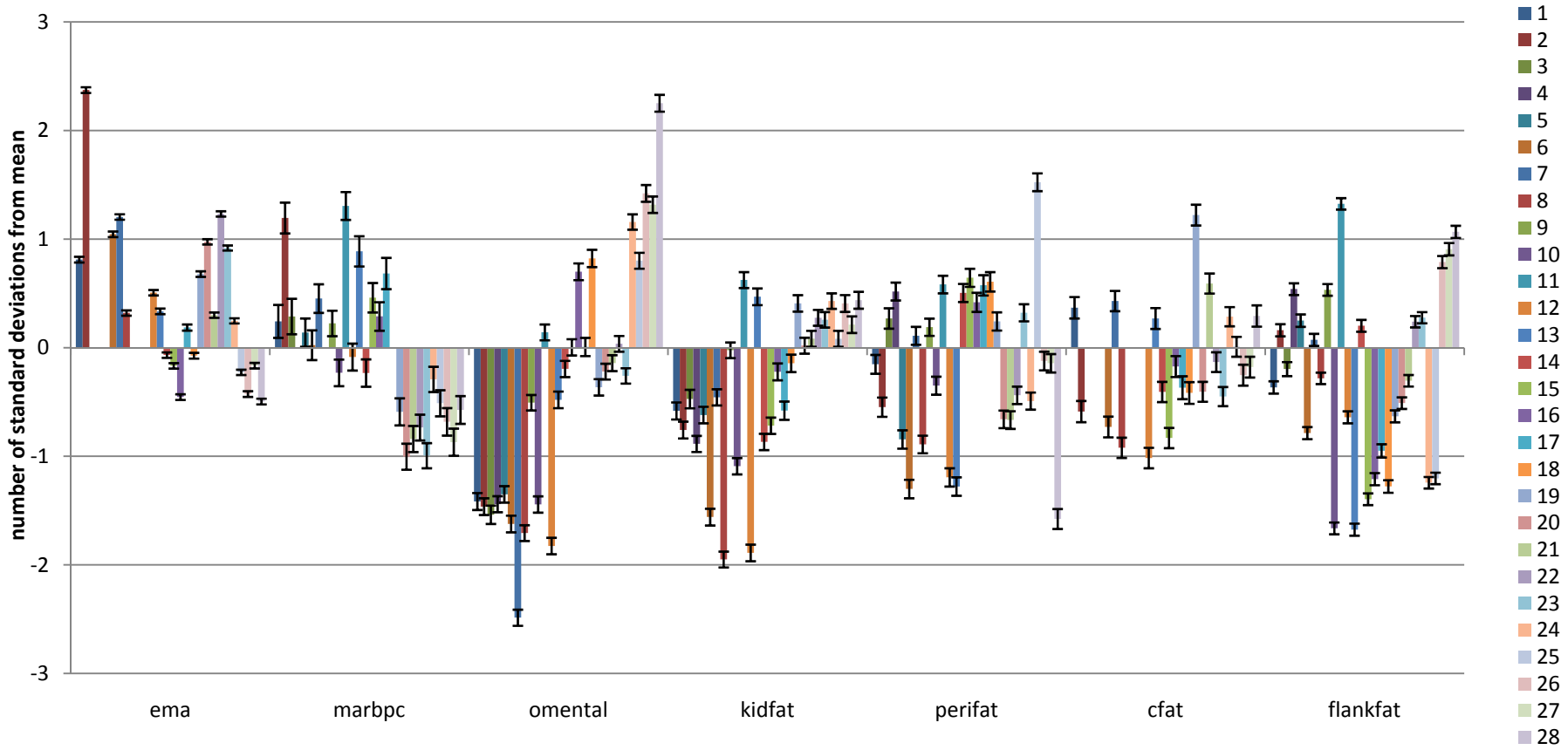


Figure 4.2: AgResearch gene mapping herd slaughter group effects.
Measured as the number of standard deviations from the overall mean, adjusted for carcass weight. Eye muscle area (ema), intramuscular fat in eye muscle, (marbpc), omental fat (omental), kidney fat (kidfat), pericardial fat (perifat), rib fat depth (cfat), and subcutaneous fat at flank (flankfat). Eye muscle area and ribfat depth (cfat) were not measured in slaughter groups 3, 4, 5, 9, 10, and 11, and intramuscular fat (marbpc) was not measured in slaughter groups 4, 8 and 18.

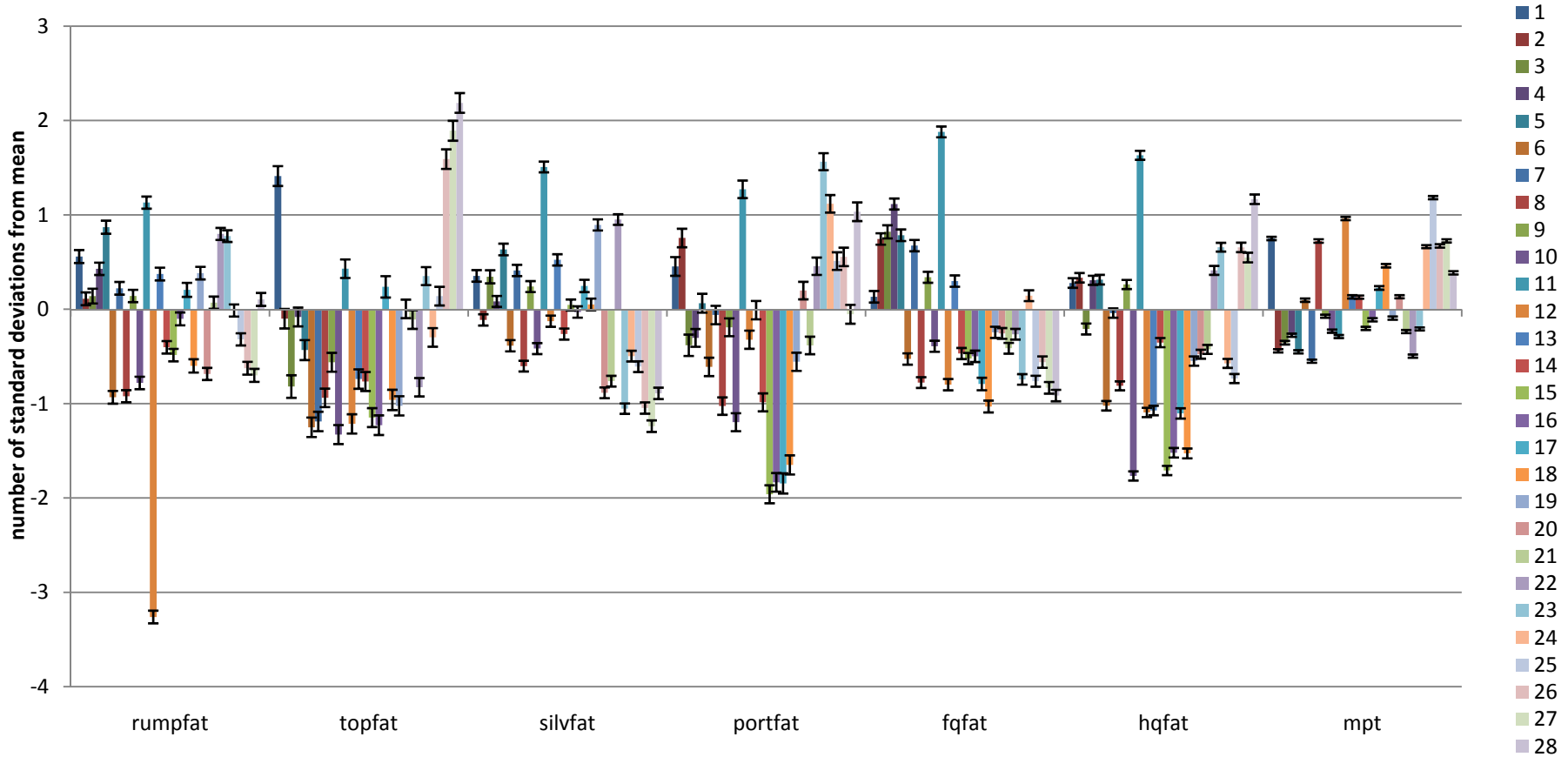


Figure 4.2 continued: AgResearch gene mapping herd slaughter group effects.
Measured as the number of standard deviations from the overall mean, adjusted for carcass weight. Subcutaneous fat at rump (rumpfat), subcutaneous fat at topside (topfat), subcutaneous fat at silverside (silvfat), subcutaneous fat at porterhouse (portfat), subcutaneous fat at forequarter (fqfat), subcutaneous fat at hindquarter (hqfat) and melting point (mpt).

The medium RFI Trangie group, which had the highest average daily feed intake, had the greater muscle weight and more fat than the other RFI groups in most depots. Rib fat was largest in the high RFI steers. The low and high RFI groups had similar eye muscle areas and marble scores but varied in all of the other measured traits (Figure 4.3).

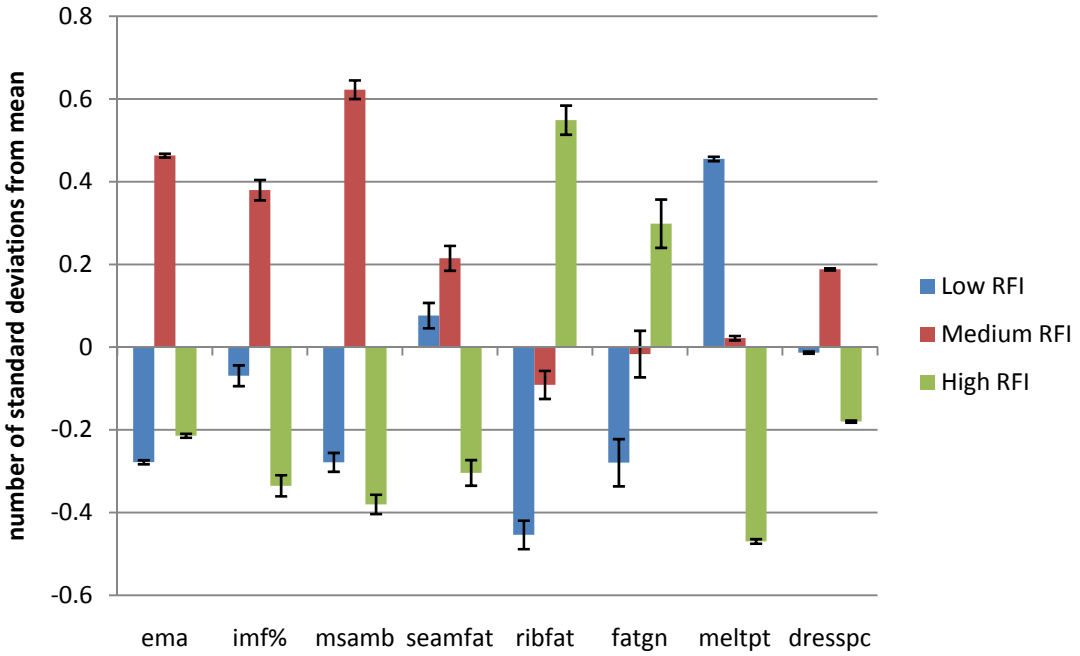


Figure 4.3: Pen effects on muscle and fat deposition traits in the Trangie RFI steers.
Measured as the number of standard deviations from the mean, adjusted for carcass weight. Eye muscle area (ema), intramuscular fat % (imf), marble score (msamb), intermuscular fat (seamfat), rib fat (ribfat), rib fat depth increase since 400 day scan (fatgn), melting point (meltpt) and dressing percent (dresspc).

4.3.6 Breed effects

Breed effects on fat deposition were greater for the AgResearch herd than the Davies herd, but in both herds, the Jersey animals were fatter in all depots than the Limousin. On average, the difference between the Jersey and Limousin was 0.9 standard deviations in the Davies herd (Figure 4.4) and 1.8 in the AgResearch herd (Figure 4.5). In both herds, there was more variation in the internal fat depots than in the subcutaneous fat.

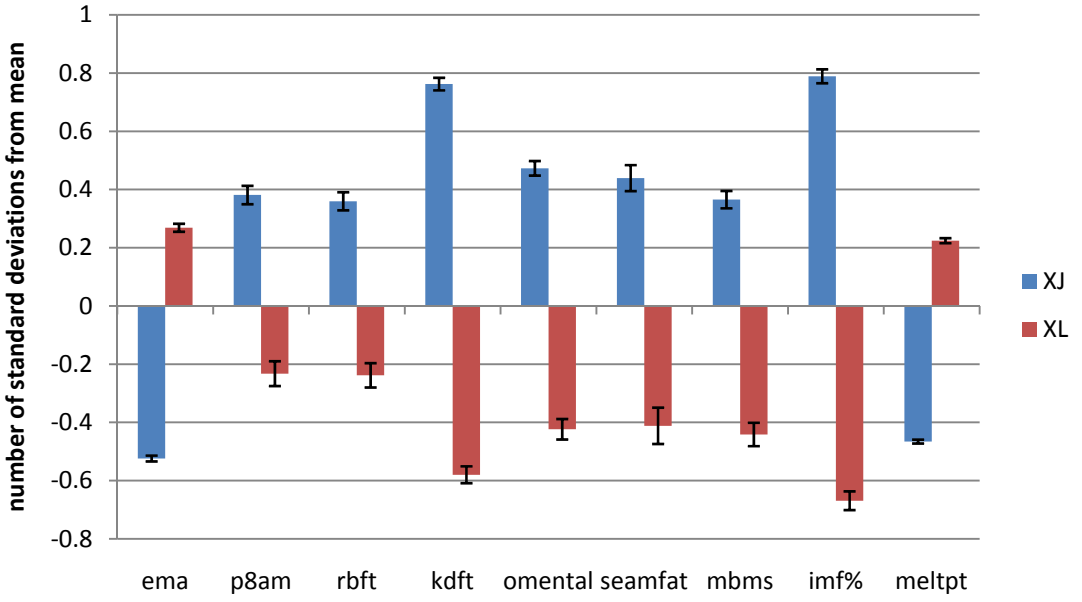


Figure 4.4: Breed of dam effects in the Davies gene mapping herd.
Measured as the number of standard deviations from the overall mean, adjusted for carcass weight. Eye muscle area (ema), rump fat at P8 (p8am), rib fat (rbft), kidney fat (kdft), omental fat, intermuscular fat (seamfat), marble score (mbms), intramuscular fat (imf%) and melting point (meltpt). Jersey (XJ) and Limousin (XL).

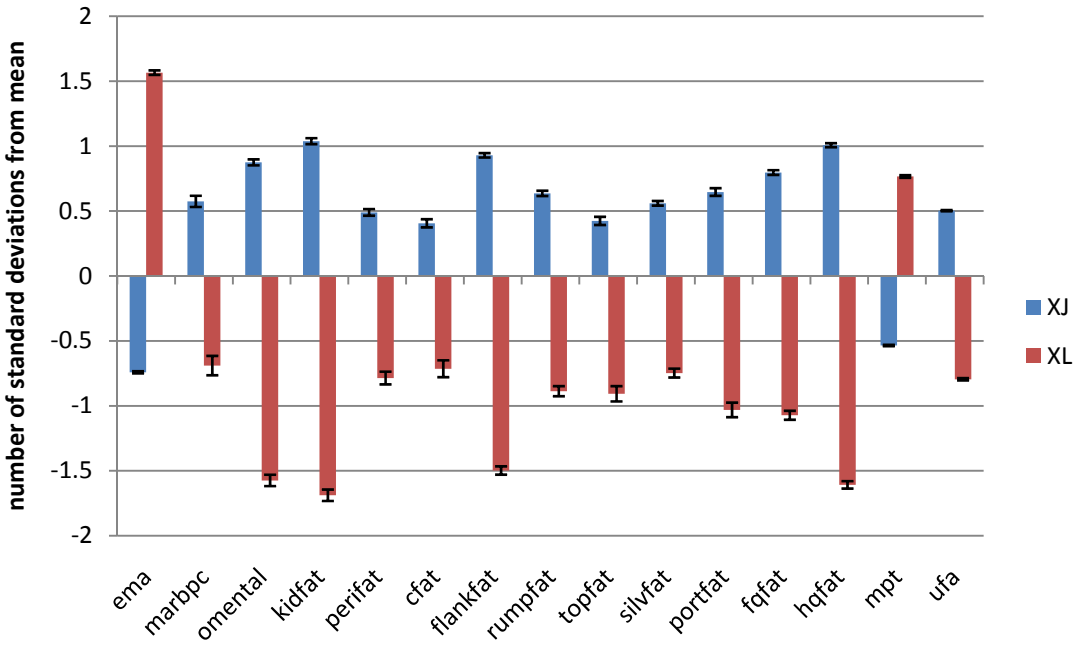


Figure 4.5: Breed of dam effects in the AgResearch gene mapping herd.
Measured as the number of standard deviations from the overall mean, adjusted for carcass weight. Eye muscle area (ema), intramuscular fat in eye muscle, (marbpc), omental fat (omental), kidney fat (kidfat), pericardial fat (perifat), rib fat depth (cfat), subcutaneous fat at flank (flankfat), subcutaneous fat at rump (rumpfat), subcutaneous fat at topside (topfat), subcutaneous fat at silverside (silvfat), subcutaneous fat at porterhouse (portfat), subcutaneous fat at forequarter (fqfat), subcutaneous fat at hindquarter (hqfat), melting point (mpt) and unsaturated fatty acids (ufa). Jersey (XJ) and Limousin (XL).

4.3.7 Sire effects

Sire effects on fat depots in the Davies herd were similar to the AgResearch herd; the average difference between the highest and lowest in each fat depot being 0.3 and 0.4 standard deviations, respectively (Figures 4.5 and 4.6). In both herds, there was more variation in the internal fat depots than in the subcutaneous fat depots. No sire was consistently highest or lowest for all depots in either herd, although in the Davies herd, the progeny of sire 361 tended to be leaner in more fat deposition traits, while the progeny of sire 398 were fatter in more of these traits. Also, the progeny of sire 398 had much more intramuscular fat and much less seam fat than the progeny of the other two bulls (361 or 368) in the Davies herd.

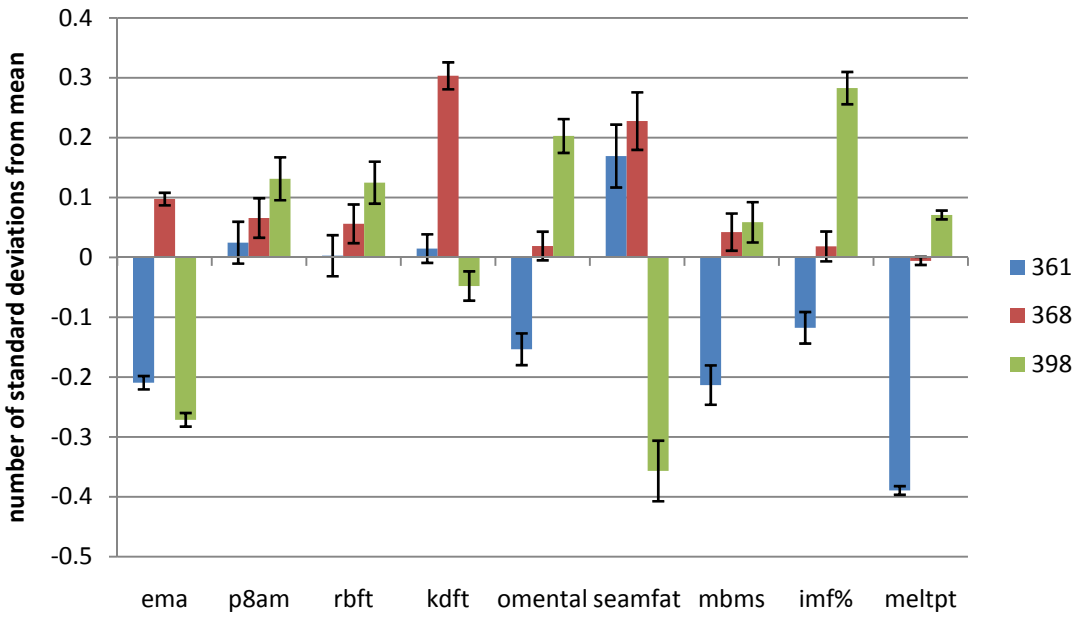


Figure 4.6: Sire effects in the Davies gene mapping herd.
Measured as the number of standard deviations from the overall mean, adjusted for carcass weight. Eye muscle area (ema), rump fat at P8 (p8am), rib fat (rbft), kidney fat (kdft), omental fat, intermuscular fat (seamfat), marble score (mbms), intramuscular fat (imf%) and melting point (meltpt).

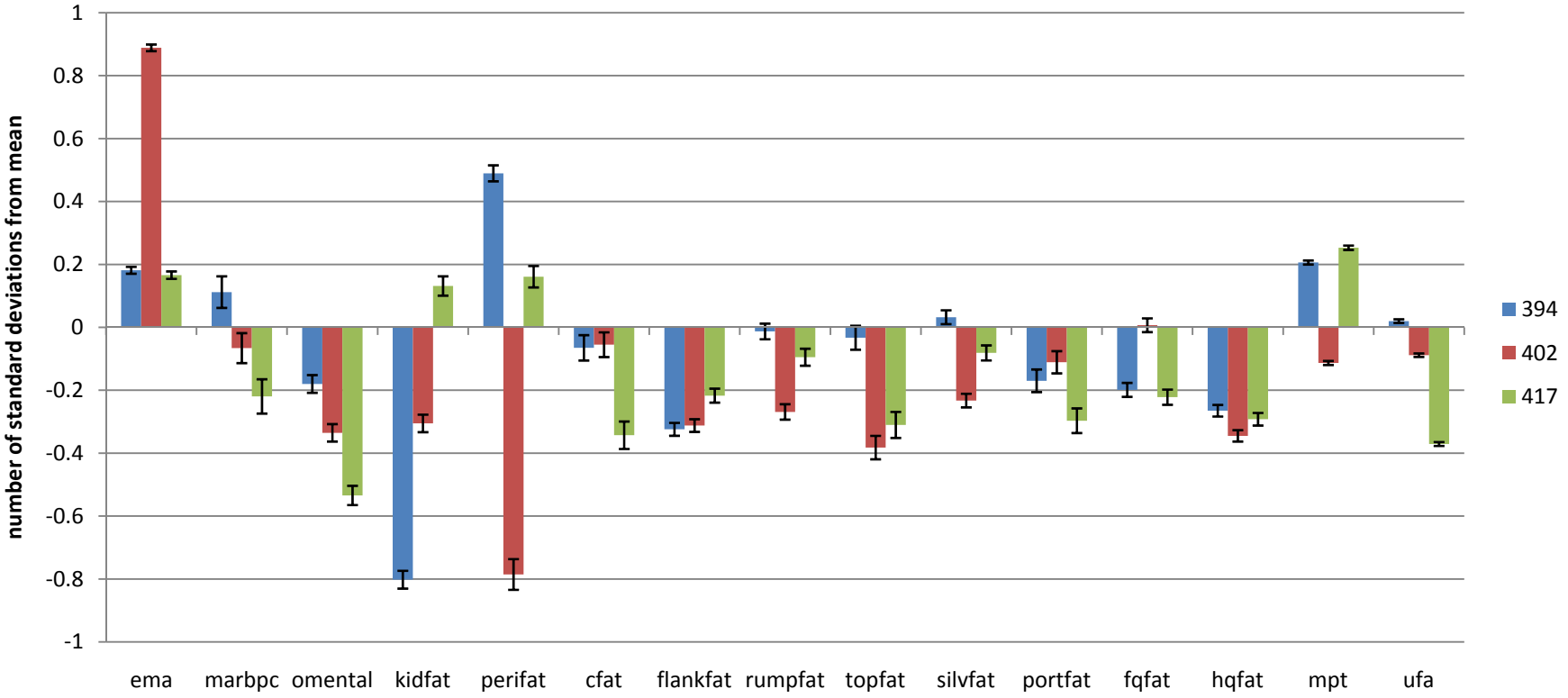


Figure 4.7: Sire effects in the AgResearch gene mapping herd.
Measured as the number of standard deviations from the overall mean, adjusted for carcass weight. Eye muscle area (ema), intramuscular fat in eye muscle, (marbpc), omental fat (omental), kidney fat (kidfat), pericardial fat (perifat), rib fat depth (cfat), subcutaneous fat at flank (flankfat), subcutaneous fat at rump (rumpfat), subcutaneous fat at topside (topfat), subcutaneous fat at silverside (silvfat), subcutaneous fat at porterhouse (portfat), subcutaneous fat at forequarter (fqfat), subcutaneous fat at hindquarter (hqfat), melting point (mpt) and unsaturated fatty acids (ufa).

Table 4.16: Trangie RFI herd sire effects

sire	ema	imf%	msamb	seamfat	ribfat	fatgain	dresspc	meltpt	progeny
NDAU005	76.5	14.8	504	24.1	18.3	10.5	58.1	39.6	16
NDAZ008	76.5	14.6	497	22.4	18.1	10.4	58.6	40.3	6
NDAZ010	78.4	14.2	506	24.1	18.1	10.5	59.2	40.2	4
NDAY015	77.6	14.0	503	25.3	17.9	10.3	58.6	40.3	8
NDAT024	76.3	14.6	497	24.9	17.8	10.0	57.5	40.6	21
NDAY025	76.7	14.5	503	25.1	17.8	10.0	58.7	40.5	6
NDAZ030	76.9	13.8	498	23.3	17.7	10.1	58.7	40.3	4
NDAZ031	77.0	15.4	507	23.4	17.9	10.2	58.4	40.6	4
NDAT034	77.5	13.9	504	22.4	18.0	10.2	58.7	39.9	2
NDAU040	77.2	13.2	505	22.9	17.8	10.1	58.5	40.0	4
NDAU051	77.3	14.0	504	23.0	17.9	10.2	58.4	40.4	1
NDAZ056	77.4	15.3	506	25.8	18.0	10.3	58.3	40.4	4
NDAZ068	77.1	13.9	502	22.6	17.7	10.1	58.3	40.2	4
NBBX73	77.3	14.8	500	25.3	17.9	10.1	58.0	40.6	11
NDAU077	77.1	15.3	506	23.8	18.0	10.2	59.1	40.6	13
NDAT095	77.5	14.7	504	22.9	18.2	10.5	58.9	40.6	7
NEPW102	77.5	14.8	512	23.7	17.8	10.1	58.7	39.8	11
NBBW118	76.0	14.3	500	26.9	17.9	9.8	59.1	40.2	14
NDAT119	76.3	13.0	494	23.0	17.6	10.0	58.2	40.2	16
NDAU124	76.8	15.2	508	25.4	17.8	10.1	58.4	40.5	8
NDAY182	77.2	14.5	504	23.5	17.9	10.2	58.3	40.2	1
NDAU227	76.9	13.9	502	22.4	18.1	10.5	58.5	40.6	5
NDAY265	77.5	15.5	507	24.3	17.9	10.2	59.1	40.2	11
NWPX392	76.9	14.6	514	23.9	18.2	10.4	58.4	40.0	16
NDAS472	77.5	13.4	502	22.3	18.0	10.3	58.5	40.1	8
NDAS537	77.3	14.0	505	23.4	18.1	10.4	58.9	40.5	3
Average	77.1	14.4	503.6	23.8	17.9	10.2	58.5	40.3	
SEM - min	0.58	0.62	12.54	1.22	0.61	0.60	0.27	0.33	
SEM - max	0.88	1.01	14.90	1.93	0.70	0.69	0.49	0.50	

Eye muscle area (ema), intramuscular fat % (imf), marble score (msamb), intermuscular fat (seamfat), rib fat (ribfat), rib fat depth increase since 400 day scan (fat gain), dressing percent (dresspc), melting point (meltpt) and number of progeny of each sire.

The sire effect was not always consistent across the fat deposition traits in the Trangie RFI herd (Table 4.16). When comparing intramuscular fat %, marble score, intermuscular fat and rib fat, progeny of sires NDAT119 and NDAS472 were average or lower in all traits, progeny of sires NBBW118 and NDAY15 were high in intermuscular fat but average or lower in the other depots, and progeny of sire NWPX392 were high in marble score and rib fat but average in the other traits (Table 4.16). The inconsistent

sire effects were also evident in the best linear unbiased prediction of sire effect (BLUP) (Figures 4.8, 4.9 and 4.10). There was a trend towards a linear increase in intramuscular fat percent and intermuscular (seam) fat area in the progeny, but this was not consistent for all sires (e.g. NDAY015 and NBBW118, Figure 4.8). However, there was no relationship between rib fat and either intramuscular fat percent (Figure 4.9) or intermuscular fat area (Figure 4.10).

Table 4.17: Heritabilities of fat traits and eye muscle area, Trangie RFI herd

	ema	imf%	msamb	seamfat	ribfat	dresspc
age+pen	0.25	0.46	0.06	0.44	0.10	0.76
hscw+age+pen	0.33	0.47	0.08	0.41	0.06	0.71

Eye muscle area (ema), intramuscular fat % (imf), marble score (msamb), intermuscular fat (seamfat), rib fat (ribfat) and dressing percent (dresspc).

Dressing percent had the highest heritability (0.71) but marble score and rib fat were very low, 0.08 and 0.06 respectively (Table 4.17). Genetic correlations were highest between intramuscular fat % and marble score (r = 0.48), intramuscular fat % and intermuscular fat (r = 0.45), and eye muscle area and dressing percent (r = 0.40) (Table 4.18).

Table 4.18: Genetic correlations of fat traits and eye muscle area, Trangie RFI herd

	ema	imf%	msamb	seam fat	rib fat	dresspc
ema	1					
imf%	0.05	1				
msamb	0.47	0.48	1			
seamfat	-0.20	0.45	0.08	1		
ribfat	0.20	0.26	0.34	-0.11	1	
dresspc	0.40	0.08	0.29	-0.01	0.17	1

Eye muscle area (ema), intramuscular fat % (imf), marble score (msamb), intermuscular fat (seamfat), rib fat (ribfat) and dressing percent (dresspc).

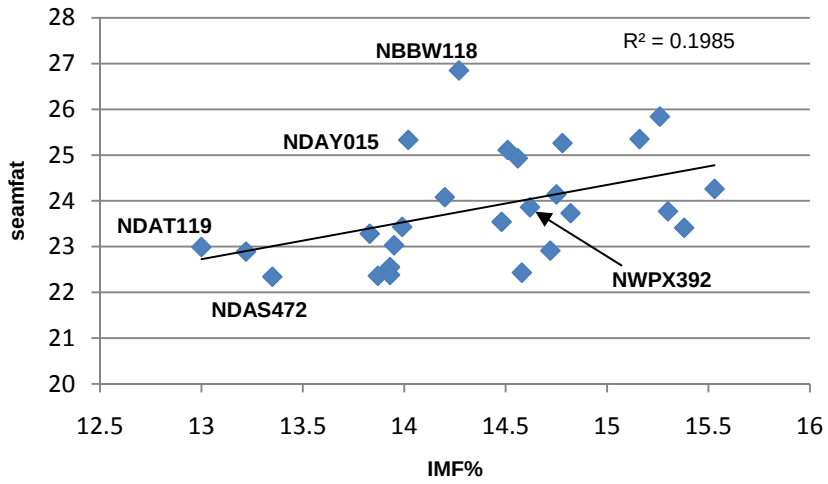


Figure 4.8: Best linear unbiased prediction of Trangie sire effects on intermuscular fat (seam fat) and intramuscular fat %.

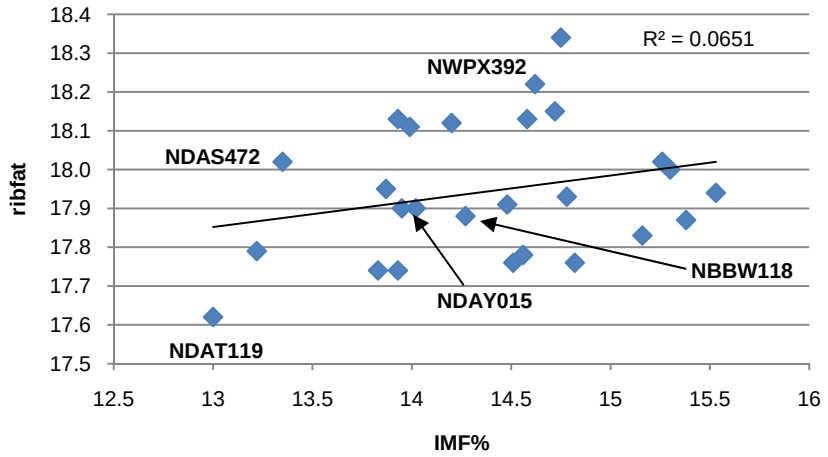


Figure 4.9: Best linear unbiased prediction of Trangie sire effects on rib fat and intramuscular fat %.

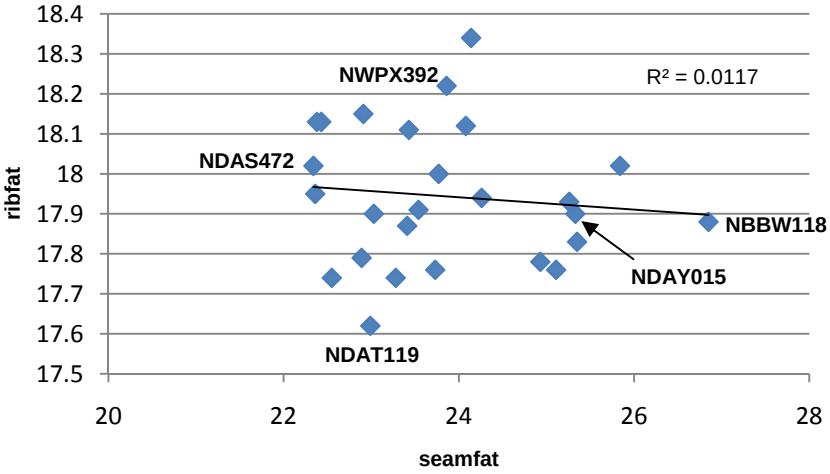


Figure 4.10: Best linear unbiased prediction of Trangie sire effects on rib fat and intermuscular fat (seam fat).

4.3.8 Myostatin genotype effects

The effects of the myostatin genotype demonstrate the increased muscle and decreased fat deposition of the 'A' variant in both the Davies and AgResearch herds (Figures 4.11 and 4.12). In the fat deposition traits, the AA genotype in the Limousin (AAXL) animals resulted in the least fat deposited and the CC genotype in the Jersey (CCXJ) had the most fat deposition, although this also reflects the breed effect, as the Jersey is a generally fatter breed. The A allele in the F94L variant is partially recessive to the C allele. Therefore, within each breed and herd, there was little difference in the effect of the CC and AC genotypes on the fat depots. As there was only a small number of CCXL animals in each herd, the most apporpriate comparison was the AAXL with ACXL. In this comparison, there was a large difference between the AAXL and ACXL in almost all of the traits. This effect was consistent across fat traits and therefore, the myostatin genotype appears to have little or no effect on fat distribution *per se*. Within each herd, the average difference from the overall mean was similar for the subcutaneous and internal fat depots.

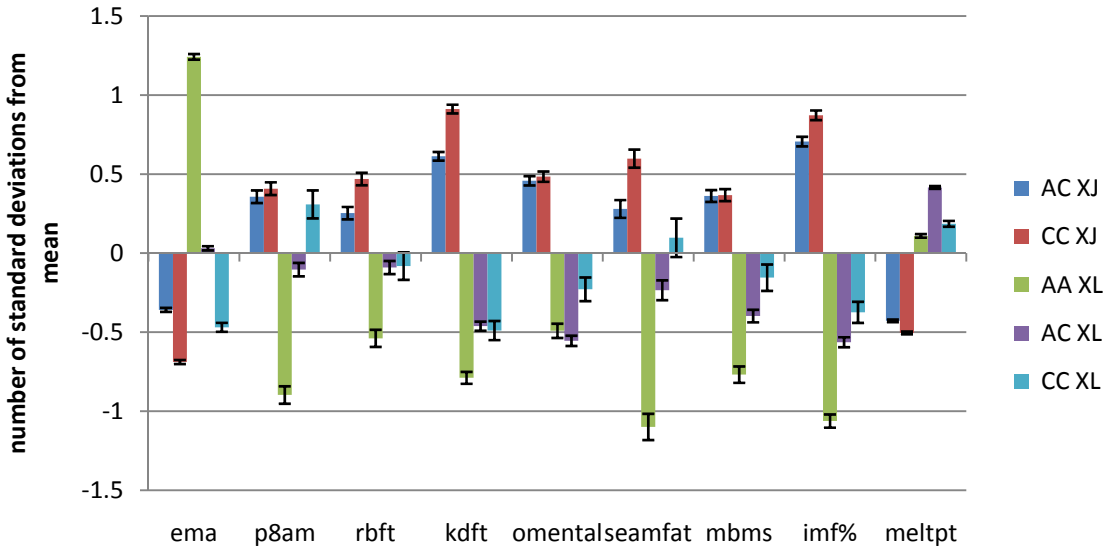


Figure 4.11: Myostatin genotype effects in the Davies gene mapping herd.
Measured as the number of standard deviations from the overall mean, adjusted for carcass weight. Eye muscle area (ema), rump fat at P8 (p8am), rib fat (rbft), kidney fat (kdft), omental fat, intermuscular fat (seamfat), marble score (mbms), intramuscular fat (imf%) and melting point (meltpt).

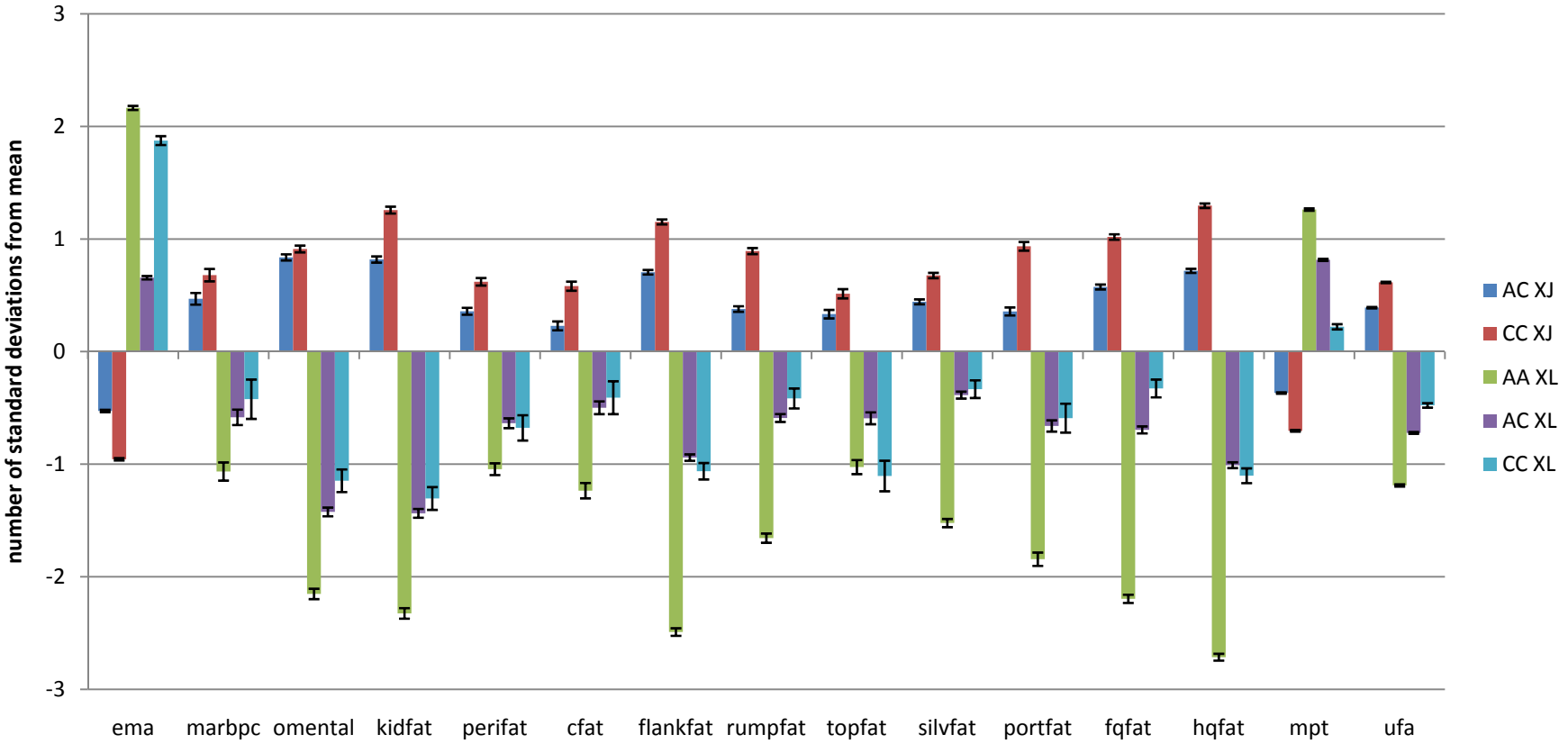


Figure 4.12: Myostatin genotype effects in the AgResearch gene mapping herd.
Measured as the number of standard deviations from the overall mean, adjusted for carcass weight. Eye muscle area (ema), intramuscular fat in eye muscle, (marbpc), omental fat (omental), kidney fat (kidfat), pericardial fat (perifat), rib fat depth (cfat), subcutaneous fat at flank (flankfat), subcutaneous fat at rump (rumpfat), subcutaneous fat at topside (topfat), subcutaneous fat at silverside (silvfat), subcutaneous fat at porterhouse (portfat), subcutaneous fat at forequarter (fqfat), subcutaneous fat at hindquarter (hqfat), melting point (mpt) and unsaturated fatty acids (ufa).

4.3.9 Trait Phenotypic Correlations

Phenotypic residual correlations were categorised as being high (greater than r = 0.6), moderate (r = 0.4 – 0.6) and low (r = 0.2 – 0.4). In the Davies herd, the correlation between residual feed intake and daily feed intake was high (r = 0.91), as was the correlation between fat% and fat to bone ratio (r = 0.94), while the fatty acid composition related measures had moderate to high correlations. Rib and rump fat (p8am) had a low correlation of 0.21. Fat percent and fat-to-bone ratio had low or moderate correlations with most of the fat deposition traits. There was no correlation between the internal fat depots, and omental fat was not correlated to any other fat trait (Table 4.19).

The correlations in the fat traits measured in the AgResearch herd (Table 4.20) were generally higher than those recorded for the Davies herd. There were low to moderate correlations within the internal fat depots; kidney fat and the omental fat had a correlation of 0.53, the kidney and pericardial fat correlation was 0.33, and omental and pericardial fat correlation was 0.24. Rib fat depth (cfat) had a moderate correlation with flank (flankfat), hindquarter (hqfat) and combined hindquarter and forequarter fat (fat), and low correlations with other subcutaneous fat depots. Rib fat depth also had low correlations with omental and kidney fat (r = 0.23). The highest correlations were observed between the individual subcutaneous fat depots and forequarter fat (fqfat), hindquarter fat (hqfat) and combined hindquarter and forequarter fat (fat). Eye muscle area had low negative correlations with most of the subcutaneous fat depots.

Correlations between traits in the Trangie herd (Table 4.21) were similar to the Davies herd and therefore, generally lower than those of the AgResearch herd. Rib fat and fat gain had a correlation of 0.95, and marble score had a moderate correlation with intramuscular fat % (0.40) and eye muscle area (0.33). Seam fat had a low correlation

with intramuscular fat % (0.26) but no correlation with eye muscle area. The melting point of intramuscular fat, dressing percent and ossification score were not correlated with any other trait (Table 4.21).

Table 4.19: Residual correlations between traits in the Davies gene mapping herd.

	ema	mbms	p8am	rft	kdfat	omental	imf%	mltpt	seamfat	dfi	rfi	adjdob	fatpct	fttobn	dent	desat	elong	mufa	ossms	sfa
ema	1																			
mbms	-0.04	1																		
p8am	-0.12	0.00	1																	
rft	-0.11	0.08	**0.21**	1																
kdfat	-0.14	0.12	0.02	0.02	1															
omental	-0.15	0.09	0.09	-0.07	0.10	1														
imf%	-0.13	**0.51**	0.07	0.15	0.15	0.08	1													
mltpt	0.02	0.02	0.09	-0.04	-0.07	-0.08	0.07	1												
seamfat	-0.09	0.13	0.11	0.09	0.11	-0.01	0.17	0.03	1											
dfi	-0.09	0.17	-0.02	0.06	0.13	0.18	0.12	-0.04	-0.01	1										
rfi	-0.04	0.12	-0.06	0.00	0.04	0.10	0.09	0.01	-0.07	**0.91**	1									
adjdob	0.00	-0.05	-0.08	-0.10	0.03	0.07	-0.09	-0.03	-0.07	0.02	0.09	1								
fatpct	**-0.41**	**0.22**	**0.41**	**0.21**	**0.22**	0.15	**0.35**	0.00	**0.27**	0.12	0.05	-0.04	1							
fttobn	**-0.28**	**0.20**	**0.40**	0.19	0.18	0.12	**0.31**	-0.01	**0.28**	0.08	0.04	-0.05	**0.94**	1						
dent	-0.02	0.06	0.02	0.00	0.01	-0.06	0.05	-0.05	0.01	-0.05	-0.06	**-0.22**	0.01	0.00	1					
desat	-0.02	0.02	-0.05	0.03	0.09	0.05	0.03	**-0.69**	0.02	0.00	-0.05	0.01	0.06	0.05	0.05	1				
elong	0.06	-0.01	-0.02	-0.08	-0.05	0.14	-0.05	0.03	0.05	0.02	-0.01	-0.03	0.01	0.03	0.06	-0.18	1			
mufa	0.02	-0.03	-0.08	-0.04	0.03	0.11	-0.03	**-0.62**	0.02	-0.02	-0.07	-0.02	0.05	0.05	0.06	**0.76**	**0.44**	1		
ossms	0.09	-0.02	0.06	0.06	-0.05	-0.03	-0.06	-0.01	0.12	-0.06	-0.09	-0.03	0.04	0.06	-0.09	0.07	0.04	0.10	1	
sfa	-0.03	-0.01	0.07	0.01	-0.05	-0.13	0.02	**0.67**	-0.01	0.00	0.06	0.00	-0.05	-0.05	-0.06	**-0.81**	**-0.37**	**-0.97**	-0.10	1

Eye muscle area (ema), marble score (mbms), rump fat depth at P8 (p8am), rib fat depth (rft), channel fat (kdft), omental fat (omental), intramuscular fat (imf%), melting point (mltpt), intermuscular fat (seamfat), daily feed intake (dfi), residual feed intake (rfi), adjusted date of birth (adjdob), total fat% (fat%), fat to bone ratio (fattobn), dentition (dent), desaturation index (desat), elongation index (elong), mono-unsaturated fatty acids – percent of triacylglyceride (mufa), ossification score (ossms), saturated fatty acids – percent of triacylglyceride (sfa).

Table 4.20: Residual correlations between traits in the AgResearch gene mapping herd.

	ema	marbpc	omenfat	perifat	kidfat	cfat	fqribfat	fqcutfat	flankfat	rumpfat	topfat	silvfat	portfat	fqfat	hqfat	fat	mpt	ufa
ema	1																	
marbpc	-0.06	1																
omenfat	-0.07	0.11	1															
perifat	-0.09	0.07	**0.24**	1														
kidfat	-0.08	0.13	**0.53**	**0.33**	1													
cfat	**-0.27**	0.00	**0.23**	0.13	**0.23**	1												
fqribfat	-0.17	**0.22**	**0.34**	**0.33**	**0.45**	**0.28**	1											
fqcutfat	**-0.24**	0.19	**0.34**	**0.36**	**0.41**	**0.26**	**0.62**	1										
flankfat	**-0.25**	0.17	**0.42**	**0.27**	**0.41**	**0.41**	**0.61**	**0.62**	1									
rumpfat	-0.18	**0.20**	**0.25**	**0.27**	**0.35**	**0.30**	**0.48**	**0.49**	**0.40**	1								
topfat	-0.03	0.13	0.09	0.09	0.13	0.10	0.21	0.17	**0.27**	**0.31**	1							
silvfat	**-0.23**	0.14	**0.23**	0.16	**0.23**	0.17	**0.40**	**0.44**	**0.41**	**0.34**	0.06	1						
portfat	-0.15	0.11	**0.26**	0.18	**0.35**	**0.28**	**0.56**	**0.41**	**0.43**	**0.33**	**0.24**	**0.34**	1					
fqfat	**-0.23**	**0.23**	**0.37**	**0.39**	**0.47**	**0.30**	**0.86**	**0.93**	**0.68**	**0.54**	**0.21**	**0.47**	**0.52**	**1**				
hqfat	**-0.26**	**0.20**	**0.43**	**0.29**	**0.46**	**0.43**	**0.70**	**0.67**	**0.92**	**0.57**	**0.42**	**0.55**	**0.70**	**0.76**	**1**			
fat	**-0.26**	**0.23**	**0.43**	**0.36**	**0.50**	**0.39**	**0.84**	**0.86**	**0.85**	**0.59**	**0.33**	**0.54**	**0.65**	**0.94**	**0.93**	1		
mpt	0.001	-0.17	**-0.20**	-0.13	**-0.36**	-0.16	**-0.25**	**-0.25**	**-0.23**	**-0.28**	0.01	-0.13	-0.16	**-0.28**	**-0.24**	**-0.28**	1	
ufa	0.05	0.12	**0.23**	0.15	**0.32**	0.13	**0.26**	0.19	**0.21**	**0.27**	0.01	0.09	0.14	**0.24**	**0.23**	**0.25**	**-0.73**	1

Eye muscle area (ema), marble percent (marbpc), omental fat (omental), pericardial fat (perifat), kidney fat (kidfat), rib fat depth (cfat), forequarter rib fat (fqribft), forequarter trimmed fat (fqcutfat), subcutaneous fat at flank (flankfat), subcutaneous fat at rump (rumpfat), subcutaneous fat at topside (topfat), subcutaneous fat at silverside (silvfat), subcutaneous fat at porterhouse (portfat), subcutaneous fat at forequarter (fqfat), subcutaneous fat at hindquarter (hqfat), forequarter and hindquarter fat (fat), melting point (mpt), unsaturated fatty acids (ufa).

Table 4.21: Residual correlations between traits and estimated breeding values (EBV) in the steers from the Trangie RFI herd.

	ema	ribfat	imf%	msamb	seamfat	fatgn	dresspc	RBY-EBV	meltpt	oss
ema	1									
ribfat	0.06	1								
imf%	0.04	-0.04	1							
msamb	**0.33**	0.003	**0.40**	1						
seamfat	0.09	0.004	**0.26**	0.05	1					
fatgn	0.12	**0.95**	-0.08	0.02	0.05	1				
dresspc	0.13	0.09	0.06	0.09	0.09	0.08	1			
RBY-EBV	**0.32**	-0.18	**-0.20**	0.07	-0.04	0.00	0.12	1		
meltpt	0.05	0.09	0.03	0.11	-0.08	0.13	-0.11	-0.04	1	
oss	0.08	0.14	0.18	-0.01	0.10	0.12	0.08	-0.08	-0.03	1

Eye muscle area (ema), rib fat depth (ribfat), intramuscular fat % (imf%), marble score (msamb), seam fat, fat gain since 400 day scan (fatgn), dressing percent (dresspc), retail beef yield% EBV (RBY-EBV), melting point of intramuscular fat (meltpt) and ossification score (oss).

4.3.10 Trait clusters

Cluster analysis of the residual values was used to determine how closely the fat traits varied together. Eye muscle area and eight fat traits were included from the Davies herd and sixteen fat traits were included from the AgResearch herd. These traits aligned into 4 clusters in the Davies herd and 5 in the AgResearch herd (Table 4.22).

Table 4.22: Clusters formed for fat traits in Davies and AgResearch gene mapping herds.

Davies gene mapping herd		AgResearch mapping herd	
Cluster	**Variable**	**Cluster**	**Variable**
1	eye muscle area	**1**	pericardial fat
	rump fat depth at P8		forequarter rib fat
	rib fat depth		forequarter trimmed fat
			subcutaneous fat at flank
			subcutaneous fat at rump
			subcutaneous fat at silverside
			subcutaneous fat at porterhouse
			subcutaneous fat at forequarter
			subcutaneous fat at hindquarter
			forequarter and hindquarter fat
2	marble score	**2**	melting point
	intramuscular fat %		unsaturated fatty acids
3	channel (kidney) fat	**3**	eye muscle area
	intermuscular fat		rib fat depth
4	omental fat	**4**	omental fat
	melting point		kidney fat
		5	intramuscular fat in eye muscle
			subcutaneous fat at topside

Similar traits from both herds were selected to construct cluster diagrams and for principal component analysis. Only fat deposition traits were included. Marble score was not included for the Davies herd as this is visually assessed rather than directly measured. Fat melting point was also excluded because it is related to fatty acid composition and not fat deposition *per se*. Although intramuscular fat (marbpc) was also assessed visually (via video image), it was included in the AgResearch correlation analysis as this was the only measure of marble score or intramuscular fat available for that herd.

The cluster diagrams indicated which traits varied together versus independently. In the Davies herd, the two measures of subcutaneous fat (ribfat and P8 fat) were grouped as were the two internal fat depots, channel (kidney) and omental fat as well as intramuscular fat % (imf) and intermuscular fat (seam fat) (Figure 4.13). This result was similar in the AgResearch herd, where kidney and omental fat were grouped as were forequarter and rump fat. Pericardial fat did not vary with the other internal fat depots (Figure 4.14). It should be noted that the Davies herd clusters were not well defined, whereas in the AgResearch herd, the clusters were tighter.

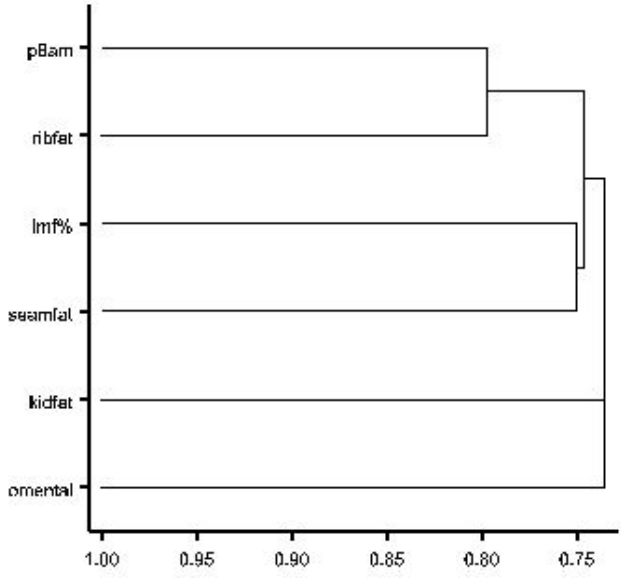


Figure 4.13: Cluster analysis diagram for the Davies gene mapping herd.
Rump fat at P8 (p8am), rib fat (ribfat), intramuscular fat % (imf), intermuscular fat (seamfat), channel fat (kidft) and omental fat (omental).

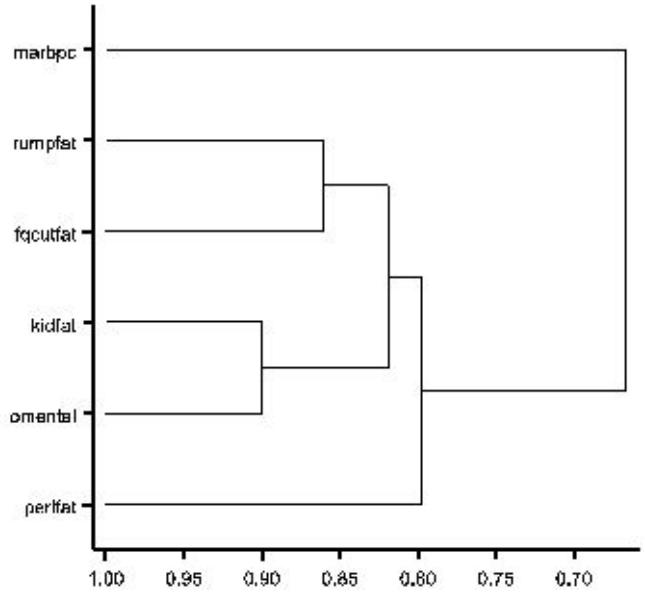


Figure 4.14: Cluster analysis diagram for the AgResearch gene mapping herd.
Intramuscular fat in eye muscle (marbpc), subcutaneous fat at rump (rumpfat), forequarter trimmed fat (fqcutfat), kidney fat (kidfat), omental fat (omental) and pericardial fat (perifat).

From the principal component analysis, there were two eigenvalues greater than the accepted level of significance of 1 and these accounted for 43% of the variation in fatness in the Davies herd. The third eigenvalue was 0.99 and could be considered significant. Adding this eigenvalue gave three principal components that together accounted for 60% of the variability in the fatness traits (Table 4.23).

Table 4.23: Eigenvalues and proportions of fat principal components in the Davies gene mapping herd.

	Davies Gene Mapping Herd					
	fatpc1	**fatpc2**	**fatpc3**	**fatpc4**	**fatpc5**	**fatpc6**
Eigenvalue	1.48	1.12	0.99	0.84	0.84	0.72
proportion	0.25	0.19	0.17	0.14	0.14	0.12
cumulative	0.25	0.43	0.60	0.74	0.88	1.00
Eigenvectors						
p8am	0.41	-0.25	0.61	-0.32	0.31	-0.44
ribfat	0.46	-0.46	0.02	0.48	0.25	0.54
kidfat	0.29	0.54	-0.37	-0.11	0.69	0.01
omental	0.11	0.63	0.63	0.13	-0.19	0.39
imf%	0.52	0.19	-0.19	0.48	-0.40	-0.51
seamfat	0.50	-0.04	-0.25	-0.64	-0.42	0.32

Rump fat at P8 (p8am), rib fat (ribfat), channel fat (kidfat), omental fat (omenfat), intramuscular fat % (imf%) and intermuscular fat (seamfat).

The six traits analysed in the AgResearch gene mapping herd resulted in only one eigenvalue greater than 1 (Table 4.24). The first eigenvalue of 3 accounted for 41% of the variability in fatness. The second was 0.99, and therefore, could be considered significant. Thus, in the AgResearch herd data, the first 2 principal components accounted for almost 60% of the variance (Table 4.24) in contrast to the three that were required for the Davies herd data (Table 4.23). These results reflect the lower residual correlations between these traits in the Davies herd (Table 4.19) than in the AgResearch herd (Table 4.20).

Table 4.24: Eigenvalues and proportions of fat principal components in the AgResearch gene mapping herd.

AgResearch Gene Mapping Herd						
	fatpc1	**fatpc2**	**fatpc3**	**fatpc4**	**fatpc5**	**fatpc6**
Eigenvalue	2.44	0.99	0.87	0.70	0.53	0.48
proportion	0.41	0.17	0.14	0.12	0.09	0.08
cumulative	0.41	0.57	0.72	0.83	0.92	1.00
Eigenvectors						
marbpc	0.21	0.86	0.25	0.39	0.05	-0.03
omental	0.41	-0.27	0.62	0.05	-0.20	0.58
perifat	0.37	-0.25	-0.53	0.69	0.13	0.16
kidfat	0.47	-0.25	0.33	-0.02	0.40	-0.67
fqcutfat	0.48	0.09	-0.26	-0.22	-0.76	-0.26
rumpfat	0.44	0.23	-0.32	-0.57	0.46	0.35

Subcutaneous fat at rump (rumpfat), forequarter trimmed fat (fqcutfat), marble percent (marbpc), kidney fat (kidfat), omental fat (omental) and pericardial fat (perifat).

When the principal component eigenvectors were plotted, the Davies principal components indicated that rump (p8am) and rib fat segregate together, as does channel (kidney) and omental fat, and intermuscular fat (seam fat) and intramuscular fat (IMF%) (Figure 4.15). However, when principal components 2 and 3 are plotted together, neither the internal fat depots (omental and channel fat) nor the subcutaneous fat depots (rump and rib fat) were as strongly linked (Figure 4.16).

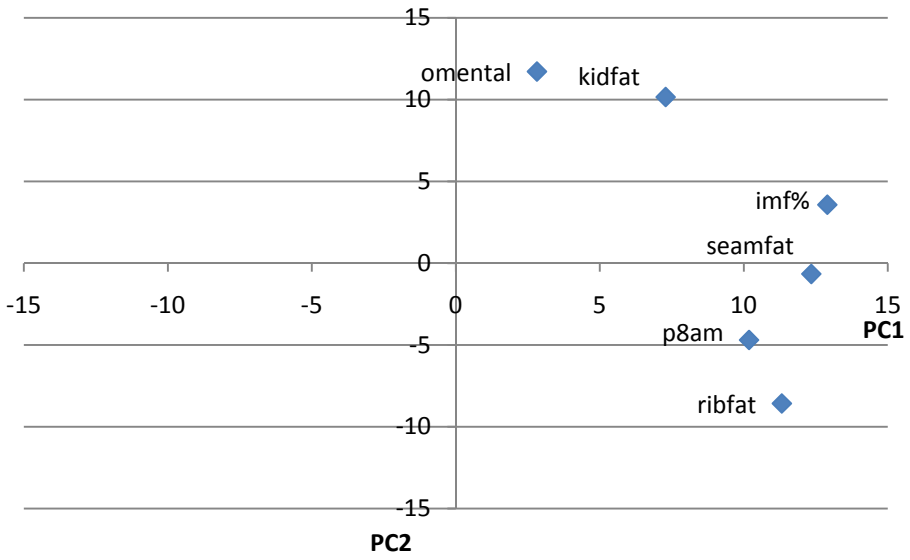


Figure 4.15: Davies gene mapping herd principal component 1 v principal component 2 (eigenvector x proportion x 100).
Intramuscular fat % (imf%), intermuscular fat (seam), channel fat (kidfat), rump fat at P8 (p8am), omental and rib fat (rbft).

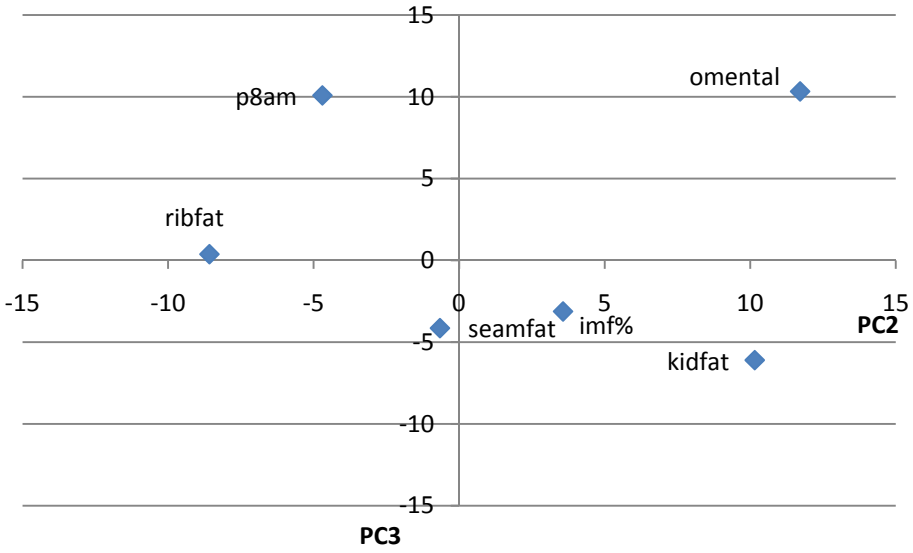


Figure 4.16: Davies gene mapping herd, principal component 2 v principal component 3 (eigenvector x proportion x 100).
Intramuscular fat % (imf%), intermuscular fat (seam), channel fat (kidfat), rump fat at P8 (p8am), omental and rib fat (rbft).

In the principal component analysis of the AgResearch herd data, principal components 1 and 2 segregated together except for intramuscular fat (marbpc) (Figure 4.17). Principal components 2 and 3 indicate that the internal and subcutaneous fat

depots were segregating separately, and pericardial fat was no longer segregating with the other traits (Figure 4.18).

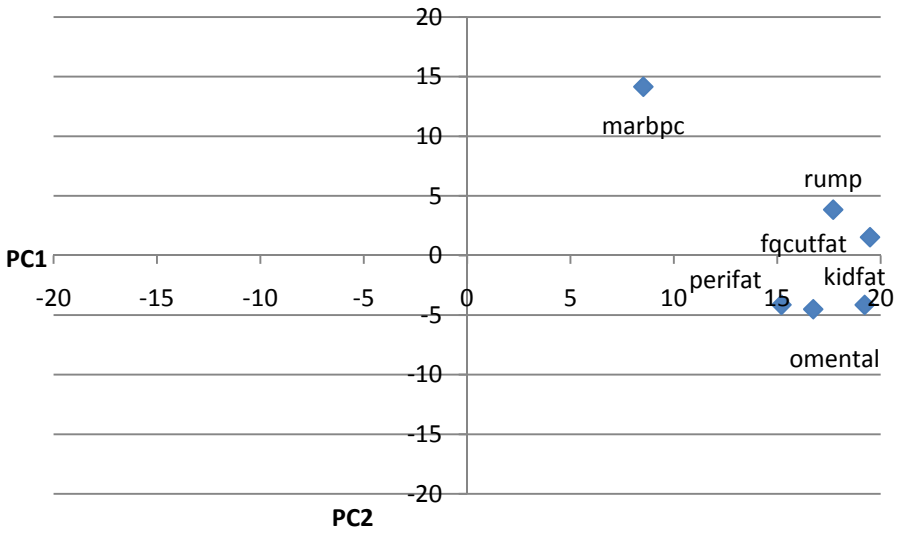


Figure 4.17: AgResearch gene mapping herd, principal component 1 v principal component 2 (eigenvector x proportion x 100).
Intramuscular fat (marbpc), omental fat (omental), pericardial fat (perifat), kidney fat (kidfat), forequarter trimmed fat (fqcutfat) and subcutaneous fat at rump (rumpfat).

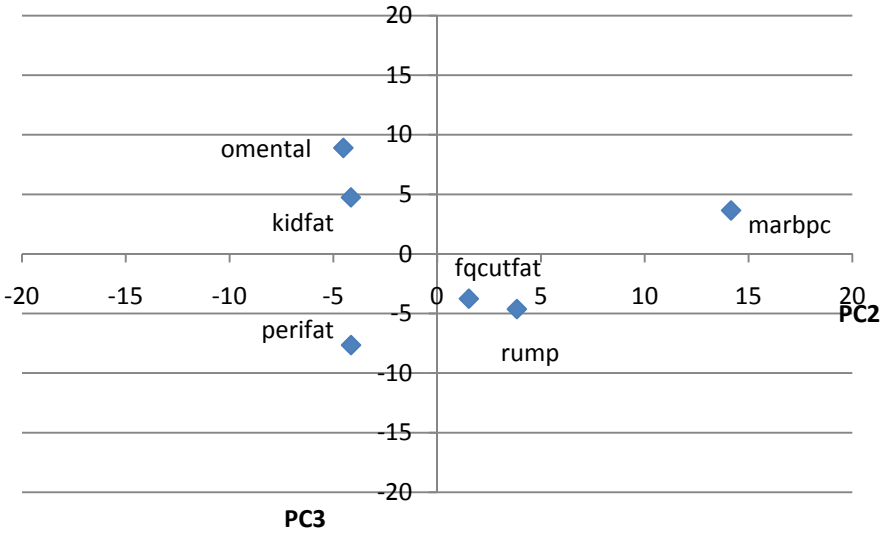


Figure 4.18: AgResearch gene mapping herd, principal component 2 v principal component 3 (eigenvector x proportion x 100).

4.4 Discussion

Three cattle herds with fat deposition trait data were used to evaluate the distribution and partitioning of fat in cattle. Two of these herds were Jersey – Limousin double backcrosses. Both of these herds utilised three sires and each sire was a paternal half brother to one sire in the other herd. The third herd consisted of Angus steers that were progeny of a herd divergently selected for residual feed intake. In addition to breed, the herds varied in location, feed management and age at slaughter.

As a result of the differing management regimes and locations of the herds, carcass measurement practise also varied and therefore, not all fat depots were measured in each herd. However, there was sufficient overlap of traits measured to enable a comprehensive evaluation of fat deposition. Although fat deposition traits are the principal traits included in this analysis, carcass weight and eye muscle area were included in order to determine if there was any influence on, or correlation with, any fat traits. While it is not a fat depot, melting point of the intramuscular fat was also included because it is a guide to the fatty acid composition. The variation in melting point led to inclusion of other measures of fatty acid composition such as saturated fatty acids and the desaturase and elongase indices, as these may affect marbling score.

There was large variation in all fat depots (coefficient of variation 20% to 50%), as well as carcass weight and eye muscle area. The amount of variation was not consistent across all depots, with intermuscular fat (seam fat) having the greatest variation of all traits (Tables 4.1, 4.2 and 4.3). Differences between the least squares means indicates the differences in the means of each trait and therefore, the influence of cohort, breed of dam, sire and myostatin genotype on each trait (Tables 4.10, 4.11 and 4.12).

4.4.1 Carcass weight and fat deposition

The regression coefficients on carcass weight indicate the measured change in each trait that was observed for every ten kilogram increase in carcass weight. There was little difference between measured fat traits in the Trangie herd, intramuscular fat % increased by 0.1%, seam fat increased 1.0% and rib fat increased 2.8%, for each 10kg increase in carcass weight (Table 4.15). This was most likely due to the more evenly sized Angus steers in the Trangie herd compared to the dissimilar breeds of the Davies and AgResearch herds. The carcass weight of the Trangie steers ranged from 354 to 494kg, whereas the AgResearch herd varied from 136 to 369kg and the Davies herd range was 168 to 480kg. Thus, using carcass weight as a covariate had a much bigger effect in the analyses of the Davies and AgResearch herds compared to the Trangie herd because of the greater variation in carcass weights within those herds. Carcass weight was associated with a larger change in the fat depots in the AgResearch herd (3.5% to 7.7% - Table 4.14) than the Davies herd (1.0% to 3.5% - Table 4.13). However, the size of the change was more consistent across fat depots in the AgResearch herd than the other herds, particularly the Davies herd, where the effect of increasing carcass weight varied greatly between similar depots. For the internal fat depots, the effect on kidney fat was double the effect on omental fat (2.3% and 1.1%), and the effect on rump fat (P8) was almost double the effect on rib fat (3.5% and 1.8%). More mature animals display more variation in fat accretion between depots (Hopkins *et al.*, 1993), and these results suggest that this increased variation also exists within fat depots.

4.4.2 Cohort, slaughter group and pen effects

In the Jersey – Limousin herds, cohort or slaughter group had the greatest effect on all fat depots with the exception of seam fat, which was only measured in the Davies gene

mapping herd. The cohort or slaughter group effect reflects the sex effect as well as the differences in management and environment that occurred between years. The age at entry to the feedlot, the length of time in the feedlot and the age at slaughter varied between cohorts and age at slaughter also varied between slaughter groups. Cohort and slaughter group was significant for all traits. The AgResearch herd slaughter groups were balanced for breed, sire and live weight prior to the first group being slaughtered (Gibbs *et al.*, 2009), but the groups were slaughtered sequentially, therefore the age of the groups increased as the slaughter progressed. Although each slaughter group in the AgResearch herd was single sex, and therefore, the sex was adjusted for in the model, the impact of sex bias in the cohort or slaughter groups cannot be ignored. Firstly, the combination of hormonal differences and the earlier maturation of heifers will lead to increased intramuscular fat at an earlier age (Malau-Aduli, 1998). Secondly, steers have less subcutaneous fat (Murphey *et al.*, 1985, Jones *et al.*, 1990, Choat *et al.*, 2006) and intermuscular fat (Jones *et al.*, 1990) than heifers. Furthermore, Murphey *et al.* (1985) noted that the subcutaneous fat depth variation between steers and heifers is not consistent across the subcutaneous fat depots. However, in the Davies herd, intramuscular fat % was higher in the 1996 and 1997 steers than the heifers, and the 1996 and 1998 steers had higher marble scores than the heifers (Figure 4.1).

There was greater variation in slaughter group effect in the AgResearch herd than the Davies herd cohort effect (average for all fat traits – 3.2 standard deviations compared to 1.9). However, in the AgResearch herd, there was a tendency for a slaughter group that was low in one depot to be low in most others (Figure 4.2), whereas this was not the case in the Davies herd. The Davies herd also had greater variation between the internal and subcutaneous fat depots than did the AgResearch herd. This may have been influenced in part by the age differences between these trials. Hopkins *et al.*

(1993) noted more consistent fat deposition across depots in younger animals than in older animals. Although their study compared subcutaneous fat depth at the rump and rib, it is reasonable to assume that this would also be the case when comparing different depots (e.g. subcutaneous, internal and intramuscular fat). Seam fat was only minimally affected by cohort, suggesting that the primary influence in the variation of this depot is genetic.

The significance of cohort or slaughter group on all traits reflects firstly, the differences in feed that occurred due primarily to variation in environment, and secondly, the effect of sex. However, as the effect was not constant for cohorts/slaughter groups of the same sex, the feed component was likely the more important factor. The results indicate the impact feed had on the general fatness of the cattle, but also suggest that this effect is not equal in all fat depots. Channel fat (kdft) had a similar pattern of fat deposition to intramuscular fat % across cohorts, although the magnitude of variation differed (Figure 4.1). However, channel fat was not similar to omental fat, rump fat (P8) or rib fat. The similarity between channel fat and intramuscular fat was not demonstrated in the cluster diagram (Figure 4.13) and the residual correlation between these depots was just 0.15 (Table 4.19).

The Trangie herd steers were progeny of 26 sires. The number of progeny per sire ranged from 1 to 21 with some sires represented across pens. Sire was added as a random effect and the residual feed intake pen effect was still significant. Consequently, pen was the only fixed effect evaluated in this herd. The Trangie steers were separated into three pens based on residual feed intake (RFI) estimated breeding values (EBV). Each pen was supplied feed *ad libitum* (adjusted for under- or over-feeding) and consequently, the feed intake differed between pens. The pen that consumed the most feed had the greatest carcass weight (hscw), intermuscular fat (seam fat), intramuscular fat % (imf%) and to a lesser extent, marble score (msamb).

Eye muscle area (ema) was also largest in the group which consumed the most feed, but did not vary between the other groups. Rib fat did not follow the trend of the other depots. For cattle at the same age and fatness, the fat depots most closely related were intermuscular fat (seam fat) and intramuscular fat % (r = 0.24). The obvious implication of this is that increasing daily feed consumption increases size and fatness, but it also indicates that the fat depots are not affected by the amount of feed consumed in the same manner. However, as the steers were allocated to pens based on their residual feed intake estimated breeding values, residual feed intake may have caused some of the variation in fat depots (Egarr *et al.*, 2009) (RFI data was not presented in this thesis). Nevertheless, due to the lack of pen replication in this trial, these residual feed intake pen effects should be treated with caution.

4.4.3 Breed effect

Breed was significant for all traits in the AgResearch herd and most traits in the Davies herd. When carcass weight (hscw) was added to the model, breed was significant in all traits. As expected, the Limousin backcross had a greater carcass weight and eye muscle area and were generally less fat than the Jersey backcross. When the results were adjusted for carcass weight, the greater fatness of the Jersey breed was more evident (Figures 4.4 and 4.5). The Jerseys were fatter across all fat depots by an average of 0.9 standard deviations in the Davies herd and 1.8 in the AgResearch herd. The difference between herds was similar when the fat depots were separated into subcutaneous and internal fat groups. When compared in this manner, the average range of subcutaneous fat was 0.6 and 1.7 standard deviations, and internal fat 1.1 and 2.2 standard deviations in the Davies and AgResearch herds, respectively. In the Davies herd, breed was not significant for subcutaneous fat (P8 and rib fat), but when carcass weight was included as a covariate, breed became significant for these depots (Table 4.5). Dairy breeds deposit more internal than subcutaneous fat (Kempster *et al.*,

1976), and the breed difference was greater for the internal fat depots and intramuscular fat % than subcutaneous fat (Figures 4.4 and 4.5). However, the carcass weight adjustment was more pronounced for the subcutaneous fat depots than the internal fat (Appendix E). These results indicate that subcutaneous fat depth was related to carcass weight (Tables 4.13 and 4.14). The Jersey backcross had a similar subcutaneous fat depth to the Limousin backcross but had a much lower carcass weight. Therefore, when adjusted to a constant carcass weight, the Jersey was proportionately a fatter breed. However, it is clear from this work that even with two very different breeds, the breed effects on fat distribution were negligible.

4.4.4 Sire effect

The sire effect was greater in the AgResearch gene mapping herd than the Davies herd. The difference between sires was greater for internal fat than subcutaneous fat in both herds, with highest to lowest fat differing by 0.35 and 0.1 standard deviations for internal and subcutaneous fat in the Davies herd and 0.85 and 0.2 standard deviations in the AgResearch herd (Figures 4.6 and 4.7). However, no sire was consistently higher or lower for all fat traits in either herd. Sire was significant for about half of the traits in both herds but this was not consistent for similar depots (Tables 4.4 – 4.7). In the Davies herd, sire differences were significant for kidney fat but not omental fat, both of which are measures of internal fat. In the AgResearch herd, the sire effect was also significant for some but not all subcutaneous and internal fat depots. In the Davies herd, sire 398 was lower than the other bulls for kidney fat, much lower for seam fat and also considerably higher for intramuscular fat % and to a lesser extent, marble score.

The results of the Trangie RFI herd also suggest there is a sire effect on individual fat deposition traits. The progeny of these sires were not always high, average or low in all

fat depots (Table 4.16). The progeny of NBBW118 were high in intermuscular fat (seam fat) but average or lower in the other fat depots, and the progeny of NWPX392 were high in marble score and rib fat but average in the other traits. Although there was a general trend of a similar increase in intramuscular fat % and intermuscular fat (seam fat), this was not the case for all sires, as highlighted by sires NDAY15 and NBBW118 (Figure 4.8). Furthermore, there was no such relationship between rib fat and intramuscular fat %, or rib fat and intermuscular fat (Figures 4.9 and 4.10).

The heritabilities of intramuscular fat %, rump fat and rib fat have been reported as 0.38, 0.36 and 0.27 (Reverter *et al.*, 2003) and 0.33, 0.43 and 0.42 (Robinson and Oddy, 2004). Bergen (2006), reported heritabilities for marble score, subcutaneous, intermuscular and internal fat of feedlot finished steers, calculated at different endpoints (carcass weight, slaughter age, subcutaneous fat depth and marble score). These heritabilities were, on average for all end points, for subcutaneous fat, 0.4; intermuscular fat, 0.4; internal fat, 0.24; and marble score, 0.42. In a review of literature at that time, Marshall (1994) reported heritabilities for subcutaneous fat depth ranged between 0.24 and 0.68, and marble score ranged between 0.23 and 0.47. Schenkel (2004) reported the heritability of backfat as 0.36, and intramuscular fat % as 0.14, while Pitchford *et al.* (2002) reported heritabilities of 0.26 and 0.18 for rump (P8) and intramuscular fat %, respectively. When heritability was calculated in the Trangie herd (Table 4.17), intramuscular fat % (0.47) and seam fat (0.41) were moderately heritable, but marble score (0.08) and rib fat (0.06) were lowly heritable. The heritabilities of marble score and rib fat in the Trangie herd were much lower than the published data, and may be less reliable due to the much smaller population used in the Trangie herd calculations (Trangie, 208; Reverter >3,500; Robinson and Oddy, >1400; Bergen, >1000; Marshall, 377 to >10,700). However, the results overall suggest that these fat

traits are heritable but the effect of environment is greater on some fat depots than others.

The genetic correlations from the sire best linear unbiased predictions (BLUPs) could only be calculated for the Trangie RFI herd (Table 4.18) as there were only three sires for each of the Davies and AgResearch herds. Rib fat had correlations of 0.34 with marble score and 0.26 with intramuscular fat %. This was similar to previous reports of a correlation of 0.35 between back fat and marble score (Koots *et al.*, 1994), 0.36 between rump (P8) and intramuscular fat % (Pitchford *et al.*, 2002), and 0.34 between rump (P8) and intramuscular fat %, 0.22 rump and marble score, 0.21 rib fat and intramuscular fat %, and 0.12 between rib fat and marble score (Reverter *et al.*, 2003). However, Robinson and Oddy (2004) reported higher genetic correlations between intramuscular fat % and rump (P8) (r_g = 0.48), and intramuscular fat % and rib fat of (r_g = 0.45). There was also a correlation of -0.11 between rib fat and intermuscular fat (seam fat) in the Trangie herd. The low to moderate correlations between these traits suggest that there is only a weak to moderate genetic relationship between the traits. Therefore, there is an opportunity for selection to alter fat distribution, not just overall fatness.

4.4.5 Myostatin genotype effect

Myostatin genotype had a large effect on all traits, with the variant AA genotype tending to have more muscle and reduced fat. The average variation was 1.4 standard deviations in the Davies herd and 2.7 in the AgResearch herd, with the effect on subcutaneous and internal fat depots being very similar within each herd (Figures 4.11 and 4.12). The AA variant is responsible for increased muscling and is not found in the Jersey breed (Sellick *et al.*, 2007). In all fat deposition traits, the AA (Limousin only) was associated with the lowest fat deposition, while the CC (wild type) Jerseys were

the fattest. However, breed was still a factor as the CC Limousins were leaner than average for all fat deposition traits. The AC Limousins were, in most cases, of similar variation from average fatness to the CC Limousins rather than the AA Limousins, which is in agreement with previous studies (Martinez *et al.*, 2010). The myostatin genotype was significant for most fat traits in the Davies herd, and all traits in the AgResearch herd. As expected, the myostatin genotype was significant for carcass weight and eye muscle area. Myostatin is a negative regulator of muscle growth, being expressed from embryogenesis through to maturity (Lee and McPherron, 2001). Mutations in the *myostatin* gene result in a large increase in muscle mass via both hyperplasia and hypertrophy (McPherron *et al.*, 1997). It is clear that animals with myostatin mutations have less fat mass than earlier maturing animals (Rodgers and Garikipati, 2008, Martinez *et al.*, 2010) such as the Jersey breed. The role of myostatin in adipogenesis is unclear, although it appears to work through Wnt/β-catenin pathway to inhibit the transcription factors C/EBPa and PPARg, which are key regulators of adipogenesis (Guo *et al.*, 2008). Results from cell culture are conflicting, with increased levels of myostatin both promoting and inhibiting differentiation of preadipocytes (Rosen and MacDougald, 2006, Rodgers and Garikipati, 2008). The *in vivo* effect may be due to regulation of adipogenesis by myostatin as fat and muscle cells both originate from the multipotent mesenchymal stem cells (Lin *et al.*, 2002). However, the relatively lower level of fat in the AA genotype may be due to the increased energy expenditure associated with the increased muscle mass (Rodgers and Garikipati, 2008).

4.4.6 Phenotypic correlations

Phenotypic residual correlations were generally higher in the AgResearch herd than the Davies and Trangie herds. In the Davies gene mapping herd, the high correlation (r = 0.94) between fat% and fat-to-bone ratio is a logical result as fat-to-bone ratio is

calculated from the fat% (Table 4.19). The moderate correlations observed between these and most other fat deposition traits were also expected. In the Trangie herd, subcutaneous fat (ribfat) was not correlated to marble score, intermuscular (seam) fat or carcass dressing percent (dresspc) (Table 4.21). There was a high correlation between rib fat and fat gain, but fat gain is a measure of the change in rib fat depth from approximately 400 days of age until slaughter. Jones *et al.* (1990) reported that seam fat and marble score were positively correlated (r = 0.67). There was no correlation between these depots in the Davies or Trangie herds, although there was a low correlation between seam fat and intramuscular fat % in the Trangie herd (r = 0.26).

The residual correlation between rump (P8) and rib fat in the Davies herd was only low (r = 0.21). As these are both measures of subcutaneous fat, it was expected that the correlation between these depots would be higher. Sex, breed, age, carcass weight and diet all affect the distribution of subcutaneous fat, and therefore, the correlation between the depots (Hopkins *et al.*, 1993). Of these, sex, breed and carcass weight would have been factors in the Davies herd, and were accounted for in the model used to generate the residual correlations. However, the raw correlation was only moderate (r = 0.40) between rump and rib fat. Subcutaneous fat is used as a measure of overall fatness and carcass composition (Kempster *et al.*, 1976, Priyanto *et al.*, 1993, Bergen *et al.*, 2006). Ramsey *et.al.* (1962) reported that subcutaneous fat depth was a good predictor of carcass fat. However, the accuracy of this measure will be low if all of the fat depots are not significantly correlated, and the work reported herein demonstrates a low correlation between fat depots, particularly in the more mature Davies herd. Furthermore, subcutaneous fat depth increases as a proportion of total fat as the animal matures (Kempster *et al.*, 1976, Robelin, 1986, Perry and Arthur, 2000), and

therefore, this fat depot would not be a useful tool for estimating total body fat unless breed and maturity are taken into account.

The only measures of maturity available in this study were adjusted date of birth (adjdob), ossification score (ossms) and dentition (dent). However, as the age of the animals within cohort only varied by a maximum of 87 days, these measures of maturity are not likely to be very informative herein, and this was reflected in the correlations between these traits (Table 4.19). Dentition was negatively correlated to adjusted date of birth ($r = -0.22$) but there was no correlation with ossification score. Although ossification score was related to seam fat ($P = 0.013$) and date of birth was borderline significant for P8 fat ($P = 0.064$) and intramuscular fat % ($P = 0.052$), neither these traits nor dentition were correlated to any of the fat deposition traits. There was also no correlation between adjusted date of birth, dentition or ossification score and any fatty acid composition trait. As cattle get older, the fatty acids become less saturated (Wood, 1984) and the proportion of C18 to C16 and C14 fatty acids increases (Malau-Aduli, 1998) so a correlation was expected.

The melting point of fat is related to the length of the carbon chain and the saturation of the carbon bonds (Wood, 1984) and this was reflected in the strong correlations between melting point and saturated and monounsaturated fatty acids, and the elongase and desaturase indices. Desaturase is the enzyme responsible for inserting a double bond in the carbon chain, while elongase adds two carbon units to fatty acid chains of C16 or greater. There was no correlation between melting point and marble score which is contrary to an earlier report that melting point had high negative correlations with marble score and intramuscular fat (Malau-Aduli, 1998). This contradiction may be the result of different cattle breeds used in the respective studies. Also, as the slaughters were conducted in commercial abattoirs, there may have been different conditions and chilling times employed which could alter the visible

intramuscular fat and therefore, affect the marble score. Smith *et al.* (2009a) noted that the concentration of oleic acid (C18:1) increased with an increase in intramuscular fat. There was some evidence of this in a low correlation between monounsaturated fatty acids and intramuscular fat % (r = 0.24), although there was no correlation with marble score (r = -0.12).

If the genetics that underpin the partitioning of fat to the individual depots were strongly related to each other, it would be reasonable to expect a strong correlation between these traits. However, that was not always the case herein (Tables 4.19 and 4.20). The Davies gene mapping herd had residual correlations between fat depots of 0.21 or lower, whereas the AgResearch herd had correlations as high as 0.76. The greater correlation may be due to many similar traits being measured in this herd. Of the 16 traits measured in the AgResearch herd, 10 were measures of subcutaneous fat. However, in the Davies herd, the correlation between similar traits was low (P8 and rib fat, r = 0.21). The conflicting correlations observed in the Davies and AgResearch herds suggest that other factors, such as environmental differences between herds, influenced the results. The two major differences were in management and slaughter age; the AgResearch herd was maintained on pasture feed throughout life and slaughtered at approximately two years of age, whereas the Davies herd was feedlot finished on a hay/grain ration for at least 6 months and slaughtered at approximately three years of age.

The difference in feeding systems is quite likely to have influenced these results. The AgResearch herd was slaughtered in early spring to mid summer. Although the winter feed is often restricted, these animals were supplemented with hay and silage and hence, it would be expected that the animals were fully fed. The Davies herd, being grain fed, would also have been fully fed. Therefore, any difference resulting from the finishing of each herd should be due to the inherent differences of a grain vs. pasture

diet. Grain fed cattle grow faster and deposit fat more rapidly than cattle fed hay, although the amount of stored fat is similar once the hay fed animals reach a comparable weight (Leat, 1977). However, the increased energy content of grain results in more variation in fat deposition (Camfield *et al.*, 1999). Grain fed cattle have more subcutaneous fat (Kempster *et al.*, 1976, Bidner *et al.*, 1981) and more marbling (Bidner *et al.*, 1981, Gibbs *et al.*, 2009). Bennett *et al.*, (1995) reported that subcutaneous fat and marble score increased, but internal fat remained constant in cattle fed a grain diet. Kerth *et al.,* (2007) found that marble score, rib and internal fat increased with grain feeding, although the increase in marbling was not statistically significant. McCurdy *et al*., (2010b) found that internal, rib fat and marble score were all higher in grain fed cattle than in their pasture fed counterparts, although none of these were significant. However, in that study, the diets of pasture and grain fed animals were balanced so that the energy intake and body weight gain were similar across the trial. Therefore, the variation in fat deposition between the treatment groups in that trial was due to diet composition not energy intake, which may explain the smaller variation between treatments.

It is likely that the lower correlations in the Davies herd are also a result of the age at slaughter. At two years of age, cattle are still growing and therefore, maturing, whereas a three year old has reached maturity and is close to, if not fully grown. In cattle, individual fat depots vary as a percentage of total fat depending on the age and maturity (Johnson *et al.*, 1972, Kempster *et al.*, 1976, Robelin, 1986, Perry and Arthur, 2000). Fat partitioning is more consistent across depots in younger cattle (Hopkins *et al.*, 1993) and marbling develops at a younger age in grain fed compared to grass fed cattle (Camfield *et al.*, 1999). As the AgResearch herd were approximately two years old at slaughter and the Davies herd were approximately three years old, it is reasonable to suggest that the variation in fat depots had become more obvious in the

more mature animals of the Davies herd. However, the steers of the Trangie herd were also slaughtered at approximately two years of age, yet the correlations between fat traits were low, similar to the correlations in the older Davies herd. Therefore, it is likely that the increased maturity combined with the increased rate of fat accretion resulting from the grain diet would account for the different results between these herds. The extent to which each of these factors contributed to the variation is yet to be determined.

4.4.7 Cluster analysis

Cluster analysis demonstrated which traits varied together (Table 4.22). The cluster analysis of the Davies herd placed rump (P8) and rib fat in their own cluster (Figure 4.13). This was not surprising as these are both measures of subcutaneous fat. Intramuscular fat % and marble score were also in their own cluster, and again this was expected. However, it is interesting to note that although they are classed as the same depot, channel (kidney) and omental fat were in separate clusters. The Davies herd clusters were weak but the AgResearch results were much stronger and similar traits clustered to those in the Davies herd. The analysis of the AgResearch herd also placed omental and channel (kidney) fat in a separate cluster to pericardial fat even though these are all internal fat depots (Figure 4.14). All but two of the various measures of subcutaneous fat were in a single cluster. Not only are these results further evidence that the fat depots act independently, they suggest that the partitioning of fat within each depot also varies.

4.4.8 Principal Component Analysis

Principal component analysis based on residual correlations between traits in the Davies gene mapping herd showed that the first principal component accounted for

25% of the variation in fat traits, the second accounted for 19% and the third 17% (Table 4.23). When the first two principal components of the Davies herd were plotted together, the results suggested that the fat traits of the same depots group together, i.e. intramuscular fat % (IMF%) and intermuscular (seam) fat, omental and channel (kidney) fat, rump (P8) and rib fat (Figure 4.15). Furthermore, intramuscular fat %, intermuscular fat, rump and rib fat segregated together. However, when PC2 and PC3 were plotted, omental and channel (kidney) fat separated as did rump (P8) and rib fat, while intramuscular fat % (IMF%) and seam fat remained together (Figure 4.16). This result is not surprising as the residual correlations between fat traits were not strong (Table 4.19). Rib and P8 fat had a correlation of 0.21, and what appears to be the most closely related traits, intramuscular fat % and intermuscular fat had a correlation of only 0.17. The principal component results reflect the low residual correlations between these traits and therefore, there was no strong overall fatness principal component in the Davies herd, which is in contrast to the AgResearch herd results.

Although there was large variation in fat deposition in the AgResearch gene mapping herd, the principal component analysis indicated that the fat traits were more closely related to each other than for the Davies herd. The first principal component, which would be considered to be overall fatness, accounted for 41% of the variation in fatness and the first eigenvalue was high (Table 4.24) while the second principal component accounted for 17%. The second principal component was mainly related to intramuscular fat (marbpc) as this was the only trait separated from the others. It is important to note from these results, that 57% of the variation in the fat deposition traits in the AgResearch herd was accounted for by two principal components, whereas it required three principal components to account for the fat traits in Davies herd. This reflects the higher residual correlations in the AgResearch herd (Table 4.20) compared to the Davies herd (Table 4.19).

In the AgResearch herd, the third principal component was low (0.87) and therefore, would not normally be included. Although this effect is low, the eigenvectors for omental and pericardial fat were higher than the other traits, which suggests that pericardial and omental fat are separate to the other traits, and therefore, this principal component may be of interest. It would be expected that the traits from the same fat depots would separate in a similar manner. However, the results from the Davies herd demonstrated that this is not always the case. Adipose develops later than the other carcass tissues, and the rate at which the adipose tissue increases varies between depots (Perry and Arthur, 2000). Therefore, the pericardial and omental fat may be an indication that the fat depots are acting independently, but that this has been masked in the younger animals in the AgResearch herd and that maturity would cause a more significant difference in the traits. Fat reserves also fluctuate with energy intake and the result here may have been influenced by the 'hard' winter on pasture compared to the Davies herd which was fully fed on grain for the 180 days preceding slaughter. The results of the Trangie herd lend support to this possibility, as the Trangie steers were fully fed on grain for the 250 days immediately preceding slaughter. Although they were a similar age to the AgResearch animals, the Trangie steers were heavier and fatter. Some of the extra fatness would be explained by breed differences between the Angus and Jersey x Limousin herds. Nevertheless, it is reasonable to infer that a proportion of the extra fat depth was due to better feeding regimen leading up to slaughter.

While principal component analysis does not necessarily identify directions that can be used to separate traits from each other (Ringner, 2008), it does indicate how much traits are related to each other, and therefore, the likelihood that the traits vary either together or independently. In this case, if the fat traits varied together, it would suggest a general fatness trait, in which the level of fatness in all depots would increase or decrease at the same, or very similar, rate. Although the AgResearch herd does

suggest general fatness to be the case, the results from the older, grain fed cattle of the Davies herd are evidence that the fat depots do vary independently. The similarity of P8 and rib fat was not unexpected, as these are both measures of subcutaneous fat. The similarity between kidney fat and intramuscular fat percent, (Figure 4.16) was a little surprising as these are considered to be different fat depots, although Johnson *et al.,* (1972) did note that these two depots followed a similar pattern of reduction as a percentage of total fat.

4.5 Conclusion

This study indicates that selection for or against a particular fat depot will not have a direct effect on other depots. In a recent review Hocquette *et al.* (2010) noted a similar independence between intramuscular fat and total body fat in both chickens and trout. Kempster (1981) commented that if the relationship between these traits was weak, selecting for reduced fat in one depot will not reduce overall fatness, implying that this would be unfavourable. However, with an increasing demand for highly marbled meat with less seam fat, and the knowledge that reducing the overall fatness of the animal is not necessarily advantageous for fertility, the ability to select for and against individual fat depots, while leaving other depots largely unaffected, would be a great advantage to the beef industry.

The results are particularly important for seam fat (intermuscular fat) as this depot appears to vary independently from the other fat depots. Excessive seam fat can result in downgrading of carcasses due to the difficulty in removing it from the prime cuts (Kempster, 1981, Christensen *et al.*, 1991, Bergen *et al.*, 2006). It is an ideal candidate, therefore, to target through selective breeding, specifically marker assisted selection, provided a suitable marker can be identified. There has been some disagreement as to the partitioning of intermuscular fat in relation to other fat depots. As the animal grows, it has been reported as staying relatively constant (Johnson *et al.*, 1972) or decreasing (Kempster *et al.*, 1976, Robelin, 1986, Perry and Arthur, 2000). If the amount of seam fat does reduce as a proportion of total fat, it may be feasible to grow the animal to a sufficient level of maturity such that the seam fat is of little consequence, particularly as marble score increases as the animal matures (Pethick *et al.*, 2004). However, this would not be an option if the amount of seam fat does not reduce as a proportion of the eye muscle area. In this case, seam fat would continue to cause a reduction in carcass

quality, particularly of the prime cuts. Another consideration here is that seam fat may act as a separate depot to the other intermuscular fat depots. It has already been demonstrated in this work that subcutaneous fat is not strongly correlated between rib and rump fat, and internal fat differs between the pericardial, kidney and omental regions. Regardless of whether intermuscular fat generally stays constant or reduces as a proportion of total fat as the animal grows, the specific seam fat depot may not behave in the same manner as the other intermuscular fat depots. Therefore, further research is required to determine the growth pattern of the seam fat depot.

The results of this investigation indicate that in cattle, the partitioning of fat to and within individual fat depots varies independently of the other fat depots. This variation becomes more evident as the animal reaches maturity and is accentuated by the use of high energy, grain based diets. There is no strong evidence that cohort, breed of dam and myostatin genotype affect fat distribution in these populations. However, there was a strong sire effect on fat distribution, and therefore, it can be assumed that there is a large genetic component in fat deposition, an aspect that may be exploited in the future.

Chapter 5 Candidate genes

5.1 Introduction

Phenotypic traits fall into two categories, qualitative (e.g. eye colour), and quantitative (e.g. height). Quantitative, or continuous, traits are under the control multiple genes, usually of small effect (Risch, 2000). As phenotypic variation is a function of the interaction of genetic and environmental variation (Brookes, 1999), identifying the genes contributing to a quantitative trait is one of the most difficult areas of genetics (Risch, 2000, Darvasi and Pisante-Shalom, 2002). However, qualitative, or discreet, traits are generally single gene traits and although there is often an environmental component to the phenotype, the gene effect is large and therefore, the gene responsible is usually easier to identify.

Candidate gene association studies have been reasonably successful in identifying the genes controlling qualitative traits (Zhu and Zhao, 2007). In this approach, candidate genes are selected based on their perceived involvement in a trait, either directly or indirectly, due to the biological or physiological function (Loos, 2009). This requires extensive knowledge of, or the ability to predict, gene function (Tabor *et al.*, 2002). However, when considering quantitative traits, the involvement of multiple genes and their variants, and the influence of environment, selection of candidate genes can be difficult.

The positional candidate gene method uses linkage analysis of phenotypes with polymorphic DNA markers, often microsatellites, spread relatively evenly throughout the genome to establish quantitative trait loci (QTL). Quantitative trait loci are chromosomal regions that are associated with a particular trait, and therefore, a gene or genes affecting that trait are likely to be located within that region. Because a QTL region can be large, up to 50cM (centimorgans) (Darvasi and Pisante-Shalom, 2002), depending on the density of DNA markers and number of informative meioses, there

are usually many genes located within the QTL. Therefore, candidate genes within the region are selected based on their function. Although the nature of quantitative traits (multiple genes combined with environmental effects) means that the identification of candidate genes is still uncertain, it is the most appropriate method available.

Following the selection of candidate genes, the genes are sequenced to identify variants (Andersson and Georges, 2004), such as variable number tandem repeats (VNTRs; mini- or micro-satellites), one or more base insertions or deletions (in/dels) and single nucleotide polymorphisms (SNPs) (Chakravarti, 1999). Single nucleotide polymorphisms occur approximately every 0.7kb in the bovine genome (Lee and Kim, 2009), while in the human genome they occur approximately every 1kb (Taillon-Miller *et al.*, 1998, Miller and Kwok, 2001) and possibly as frequently as every 0.1kb (Wang *et al.*, 2006b). Less than 2% of these SNPs are located in exons (Lee and Kim, 2009), while the remainder are in non-coding regions; the 5' untranslated region (5'UTR), 3' untranslated region (3'UTR), introns and regions between genes (Wang *et al.*, 2006a). The lower SNP density in exons compared to introns may be because SNPs in exons are more likely to be deleterious and therefore, will be automatically selected against (Cargill *et al.*, 1999, Zhao *et al.*, 2003). Due to their abundance in the genome and the relatively cheap methods of genotyping SNPs (Collins *et al.*, 1998, Brookes, 1999, Chakravarti, 1999, Miller *et al.*, 2001), these are the preferred variant for this type of research.

5.2 Methods

Candidate genes were selected and then sequenced to locate DNA variants, principally single nucleotide polymorphisms. Two main criteria were used to select candidate genes. The first criterion was the proximity of the gene to established quantitative trait loci (QTL) for fat deposition traits (Esmailizadeh, 2006) and the new QTL for intermuscular (seam) fat (Table 3.3, Section 3.3.1). The second criterion was the biological or physiological function of the gene and therefore, the perceived role in fat deposition.

5.2.1 Cattle

DNA was obtained from the Davies gene mapping herd (section 2.1.1) using phenol:chloroform extraction as described by Sellick (2002). The three mapping sires (361, 368, 398) were genotyped for sequence variants and the parents of these sires then sequenced for verification of the variants.

5.2.2 Sequencing

5.2.2.1 Polymerase chain reaction

All aspects of the standard polymerase chain reactions are detailed in section 2.2 (General Methods) and Appendix A.

5.2.2.2 Preparation of DNA product for sequencing

Two 25µl PCR reactions for each animal to be sequenced were pooled and confirmation of a single, correct sized product was determined via gel electrophoresis. The remaining product was purified of all excess primers, salts, enzyme and dNTPs using an Ultraclean™ PCR Clean Up column (*Mo Bio Laboratories*). The standard

protocol was varied in two ways. Firstly, all centrifuge times were doubled and secondly, in order to achieve a higher DNA concentration in the elutant, only 30µl of elution buffer was added and samples were allowed to stand for 60 seconds prior to the final centrifugation. Samples were then further concentrated by leaving the open 1.5ml tube (*Axygen Inc.*) in a 65°C oven for 20 – 30 minutes.

In those PCR where a single product was unable to be achieved, four 25µl reactions were resolved in a 2% agarose gel and stained as detailed above (2.2.2.4). The gel was then examined on a Transilluminator 2020E (Stratagene) UV visualiser and the target DNA bands cut out of the gel using a 'LabGadget' (*Geneworks*). DNA was extracted from the resulting gel piece using an Ultraclean™ Gel Spin column (*Mo Bio Laboratories*) with the same alterations to the standard spin column protocol as detailed above.

2µl of the elution was used to determine the concentration of DNA using a NanoDrop® ND100 spectrophotometer (*Thermo Scientific*) and the remainder stored at 4°C.

5.2.3 Sequencing reaction

Sequencing reactions consisted of 70 – 100ng DNA, 3.7µM primer (final concentration), 1µl glycogen, 2µl Big Dye® Terminator (*Applied Biosystems)* and H_2O to a final volume of 10µl. Primer concentration was reduced to 2.5µM for lower DNA concentrations (<70ng) or shorter DNA products (< 500bp). Reactions were carried out in 200µl, thin walled tubes (*Axygen Scientific*) using a Palmcycler (*Corbett Life Science Research*) heated lid thermal cycler. The sequencing program was 25 cycles of 95°C for 30 sec, 50°C for 15 sec, 60°C for 4 min, followed by an indefinite hold at 4°C.

Sequencing reactions were precipitated using 75% isopropanol. The completed reactions were transferred to a clean tube and 80µl isopropanol added. This was briefly

vortexed and allowed to stand at room temperature for 20 minutes. The mix was then centrifuged for 20 minutes at 13,000g in a Centra-M2 (*International Equipment Company*) bench centrifuge. The supernatant was decanted and the tube inverted to air dry for 10 minutes. 250µl of 75% isopropanol was added and the mix was vortexed and centrifuged again at 13,000g for 20 minutes. Tubes were placed in the centrifuge in the same orientation as the previous centrifugation. The supernatant was decanted and the tube inverted to air dry for a minimum 1 hour prior to sequencing on an ABI3730 (*Applied Biosystems*) automated sequencer at the Institute of Medical and Veterinary Science, Adelaide. The sequencing results were analysed using Sequencher® 4.8 software (*Gene Codes Corporation*).

5.3 Results and Discussion

5.3.1 Candidate genes

Nine candidate genes were selected based on both function and proximity to the fat deposition quantitative trait loci (QTL) (Table 5.1). Genes involved in adipogenesis or fatty acid synthesis were the obvious choice. However, there are other biological functions and pathways that may, or have already been shown to, affect adipose tissue formation.

Table 5.1: Fat deposition candidate genes sequenced for polymorphisms.

BTA	Region (Mb)	Gene symbol	Gene name	Function
1	82.2	**AdipoQ** APM1 Adipo_Bovin,	Adiponectin	Involved in the control of fat metabolism and insulin sensitivity. May play a role in angiogenesis.
3	15.9	**LMNA** Q3SZI2_Bovin	Lamin A/C	Lamin A inhibits adipogenesis. Defects in lamins implicated in muscular dystrophy and lipodystrophy.
3	44.9	**EDG1** S1PR1_Bovin	Sphingosine 1-phosphate receptor 1	Postulated to regulate the differentiation of endothelial cells.
8	17.5	**TEK1** TIE2_Bovin, TEK,	Tyrosine kinase, endothelial,	Regulates endothelial cell development, growth and branching of blood vessels.
9	26.4	**NCOA7** A6QNZ3_Bovin ERAP140	Nuclear receptor coactivator 7	Involved in the coactivation of different nuclear receptors, including ESR1, PPARG and RARA.
9	92.2	**ESR1** ESR1_Bovin, ESR alpha	Estrogen receptor	Estrogen is involved in lipid metabolism and implicated in increased adipose tissue.
19	12.8	**ACACA** ACACA_Bovin,	Acetyl-CoA carboxylase 1	Involved in fatty acid synthesis.
19	26.8	**ENO3** ENOB_Bovin	Beta Enolase	Appears to have a function in striated muscle development and regeneration.
22	58.3	**PPARG** PPARG_Bovin PPARgamma	Peroxisome proliferator-activated receptor gamma	Regulator of adipocyte differentiation.

Gene symbols used commonly in this thesis are listed first and in bold. Functions are adapted from GeneCards® version 3 (www.genecards.org).

5.3.1.1 Adipogenesis and lipogenesis

Adipogenesis is highly regulated (Gregoire, 2001). It involves an array of transcription factors and cofactors, as well as extracellular stimuli, that act through both positive and negative regulation (Rosen and MacDougald, 2006). There are several major pathways involved in adipogenesis. For instance, adipogenesis is inhibited by extracellular

agents through the Wnt signalling pathway and DLK1/PREF1 signalling, while the MAPK pathway both enhances and inhibits adipogenesis (Rosen and MacDougald, 2006) as does vitamin A (Bonet *et al.*, 2003). Other pathways which have primary roles in different tissues can also affect adipogenesis. For example, the myostatin pathway, which controls muscle development, also affects adipogenesis. Polymorphisms in *myostatin* result in increased muscle mass and decreased adipose tissue (Section 4.4.5), although the biology controlling this is unclear (McPherron and Lee, 2002).

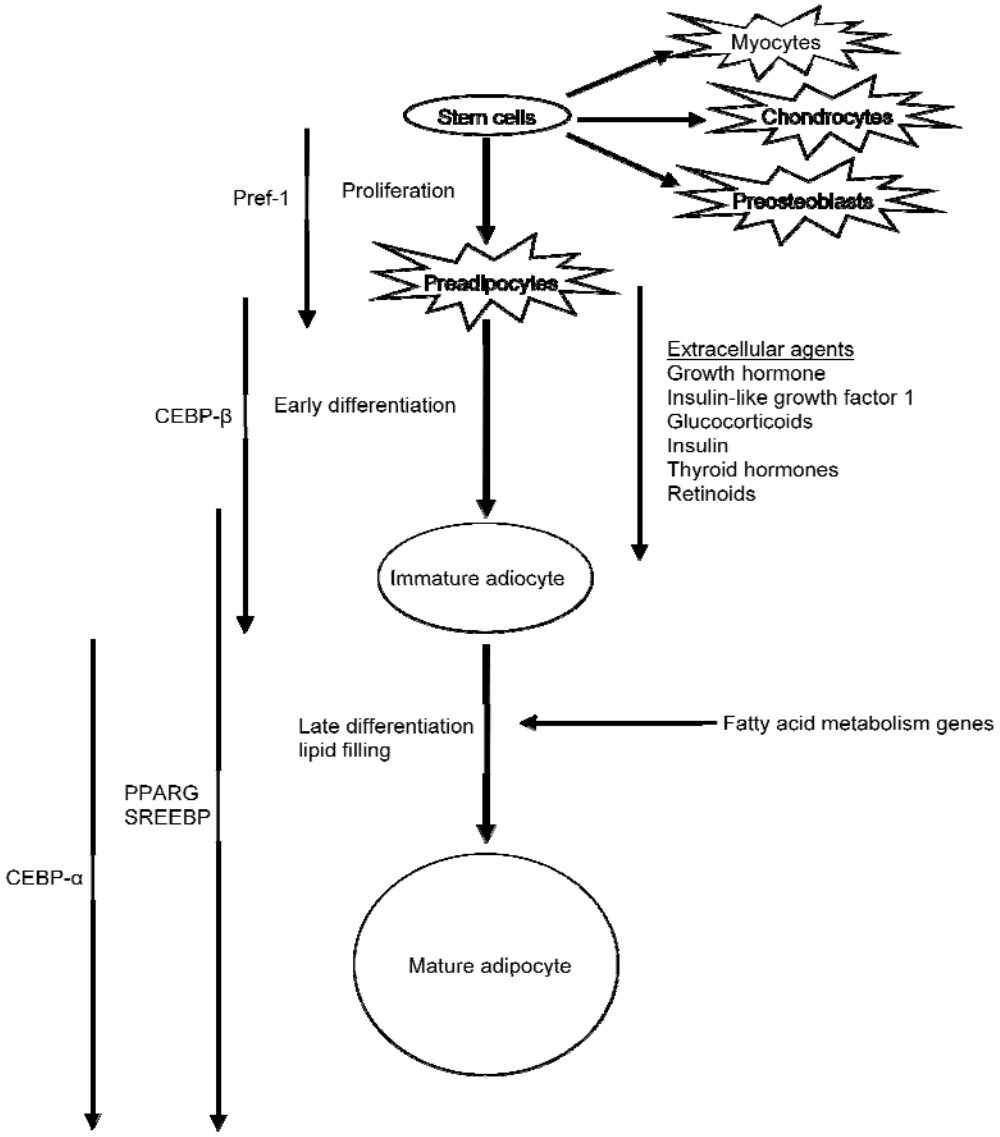


Figure 5.1: Schematic of adipogenesis
Adapted from Boone *et al.* (2000) Hausman *et al.* (2001) Agarwal and Garg (2006) and Roh *et al.* (2006).

As a consequence of the number of pathways and extracellular agents that affect adipogenesis, there are a large number of candidate genes that represent many biological functions. Five of these genes that directly affect adipogenesis and lipogenesis, or interact with genes that do, were selected for more detailed investigation.

Acetyl-CoA carboxylase alpha – ACACA

Acetyl-CoA carboxylase alpha (*ACACA*) catalyses the formation of malonyl-CoA (Ponce-Castañeda *et al.*, 1991, Abu-Elheiga *et al.*, 1995), in the production of long chain and unsaturated fatty acids (Zhang *et al.*, 2010b), and therefore, the expression of *ACACA* is correlated to fatty acid synthesis (Ponce-Castañeda *et al.*, 1991, Zhang *et al.*, 2010b). *ACACA* is expressed principally in adipose tissue, mammary gland and liver (Ponce-Castañeda *et al.*, 1991, Abu-Elheiga *et al.*, 1995, Zhang *et al.*, 2010b). Zhang *et al.* (2010b) identified eight single nucleotide polymorphisms (SNPs) in the promoter region of *ACACA*, which were associated with marble score, (adjusted) subcutaneous fat thickness, fatty acid composition and fatty acid concentration in cattle.

Adiponectin – AdipoQ

Adiponectin (*AdipoQ*) is an adipocyte derived cytokine (Sato *et al.*, 2001, Morsci *et al.*, 2006), involved in energy homeostasis (Scherer *et al.*, 1995, Sato *et al.*, 2001) through the regulation of lipid and glucose metabolism (Fu *et al.*, 2005, Morsci *et al.*, 2006) and has direct anti-diabetic, anti-atherogenic and anti-inflammatory activities (Fu *et al.*, 2005, Tilg and Moschen, 2006, Ouchi *et al.*, 2011). Over-expression of adiponectin in cell culture increases the rate of adipocyte differentiation and fibroblast proliferation (Fu *et al.*, 2005), which suggests that adiponectin may have a role in cell differentiation or growth. Adiponectin is negatively correlated with obesity in mice and humans (Hu *et al.*,

1996, Arita *et al.*, 1999, Sato *et al.*, 2001, Fu *et al.*, 2005, Morsci *et al.*, 2006), increases lipid oxidation and glucose utilisation in muscle cells (Fu *et al.*, 2005), and inhibits lipogenesis (Morsci *et al.*, 2006). Therefore, variants in this gene may be associated with variation in fat trait phenotypes, in particular, intramuscular fat % and marble score (Morsci *et al.*, 2006).

Nuclear receptor coactivator 7 – NCOA7

Nuclear receptor coactivator 7 (*NCOA7*) is a transcription factor that enhances the function of estrogen receptor alpha and peroxisome proliferator-activated receptor gamma (Shao *et al.*, 2002), two nuclear receptors involved in adipogenesis (discussed below). The *NCOA7* gene product appears to also function in the protection of DNA from oxidative damage (Durand *et al.*, 2007).

Estrogen receptor alpha – ESR1

Estrogen is involved in the positive and negative regulation of various physiological processes and tissues, including the reproductive system, brain, cardiovascular system, lipid metabolism and plasma lipid concentration, skeletal system and osteoporosis, as well as breast and endometrial cancers (Ohlsson *et al.*, 2000, Sato *et al.*, 2001, Demissie *et al.*, 2006). Reduced levels of estrogen result in increased adipose tissue (Cooke and Naaz, 2004) and atherosclerosis (Ohlsson *et al.*, 2000). Estrogen affects adipose tissue mass through inhibiting lipogenesis, regulating adipogenesis, reducing energy intake and increasing energy expenditure (Cooke and Naaz, 2004). The gender difference in fat distribution in humans is caused by different levels of estrogen (Cooke and Naaz, 2004). Two nuclear receptors, estrogen receptors α and β, mediate the effect of estrogen (Ohlsson *et al.*, 2000, Sato *et al.*, 2001, Cooke and Naaz, 2004, Demissie *et al.*, 2006). Mice that have ESRα inactivated became obese, whereas there is little effect in ESRβ knockout mice, demonstrating that ESRα

(*ESR1*) has the larger effect (Ohlsson *et al.*, 2000, Cooke and Naaz, 2004), and Okura *et al.* (2003) reported a polymorphism in *ESR1* was associated with an increase in abdominal fat in women, but had no effect on men.

Peroxisome proliferator-activated receptor gamma – PPARG

Peroxisome proliferator-activated receptor gamma (*PPARG*) is a member of the PPAR subfamily of nuclear receptors (Meirhaeghe and Amouyel, 2004, Anghel and Wahli, 2007). In order to be activated, *PPARG* forms a heterodimer with the retinoid X receptor (RXR), another nuclear hormone receptor (Rosen *et al.*, 1999, Way *et al.*, 2001, Meirhaeghe and Amouyel, 2004, Anghel and Wahli, 2007). *PPARG* is highly involved in the formation and regulation of adipose tissue (Blumberg, 2011). Firstly, *PPARG* is instrumental in adipocyte differentiation of both preadipocytes and their progenitor cells (Crossno *et al.*, 2006, Farmer, 2006, Anghel and Wahli, 2007), and secondly, in the regulation of the synthesis, transport, release and uptake of free fatty acids as well as the esterification of these to triacylglycerides and subsequent storage (Way *et al.*, 2001, Yong *et al.*, 2008).

The importance of *PPARG* in relation to fat deposition is indicated by the association between a mutation in this gene resulting in an amino acid change (Arg425Cys) and one form of familial partial lipodystrophy (Agarwal and Garg, 2002). Furthermore, another amino acid change (Pro12Ala) lowers insulin and free fatty acid concentrations (Tschritter *et al.*, 2003). However, an earlier review of *PPARG* reported conflicting results from multiple studies, which indicated that the alanine genotype in the alanine/proline mutation was responsible for a higher, lower, or no change in body mass index, and concluded that the effect of the amino acid substitution was altered firstly by environment, and secondly, by interactions with other genes, specifically a mutation in adiponectin (*AdipoQ*) (Meirhaeghe and Amouyel, 2004).

Just as adipocytes are essential for energy balance, and therefore, life, *PPARG* is essential to the development and function of adipose tissue. *PPARG* knockout mice are embryonic lethal, and when *PPARG* was ablated in mature wild type mice, the mice died within a few days (Anghel and Wahli, 2007). Conversely, gain of function leads to obesity, and it has been demonstrated in both humans and mice, that the level of *PPARG* expression is correlated to adiposity (Anghel and Wahli, 2007). While *PPARG* mutations cause severe alterations to fat deposition or death, minor alterations to the regulation of this gene do induce smaller changes to the amount of fat deposited or its distribution (Tsai and Maeda, 2005).

5.3.1.2 Angiogenesis and vascularisation

Adipose tissue is highly vascularised (Crandall *et al.*, 1997, Stacker *et al.*, 2000) and the formation of adipose tissue is usually preceded by, and dependent on, angiogenesis (Rosen and Spiegelman, 2000, Rupnick *et al.*, 2002, Dallabrida *et al.*, 2003, Fukumura *et al.*, 2003). Fukumura *et al.* (2003) demonstrated the interconnectedness of blood vessel formation and remodelling with adipocyte cell differentiation and adipose tissue formation, a relationship previously suggested by Rosen and Spiegelman (2000). Blood flow is associated with adipocyte growth patterns and metabolism (Crandall *et al.*, 1997, Hausman *et al.*, 2001), and it is likely that intramuscular fat is promoted by increased intramuscular vascularisation (Yamada *et al.*, 2009a).

Tyrosine kinase, endothelial – TEK1

Tyrosine endothelial kinase (*TEK1*) is involved in the development of endothelial cells (Davis *et al.*, 1996, Stacker *et al.*, 2000) and the growth and branching of blood vessels during angiogenesis (Liu *et al.*, 2000, Stacker *et al.*, 2000, Otrock *et al.*, 2007). *TEK1* interacts with its ligand angiopoietin (*ang1*) (Stacker *et al.*, 2000, Otrock *et al.*, 2007),

and this interaction facilitates blood vessel remodelling (Fukumura *et al.*, 2003). Furthermore, Stacker *et al.* (2000) demonstrated that angiopoietin was upregulated in cell lines during adipocyte differentiation.

Sphingosine 1-phosphate receptor 1 – EDG1

The sphingosine 1-phosphate receptor 1 (*EDG1*), originally known as the endothelial differentiation gene (Hla and Maciag, 1990), is a G protein coupled receptor that binds to sphingosine 1-phosphate with high specificity and affinity (Pyne, 2000). It has been suggested that *EDG1* is involved in the regulation of endothelial cell differentiation (Hla and Maciag, 1990, Pyne, 2000) and formation of blood vessels (Liu *et al.*, 2000).

EDG1 has previously been associated with marbling in a trial comparing two Holstein steers and two steers cloned from a Japanese Black bull with a high estimated breeding value for marbling (Sasaki *et al.*, 2006). Three single nucleotide polymorphisms (SNPs), two in the 5'UTR and one in the 3'UTR, were associated with marbling in Japanese Black cattle (Yamada *et al.*, 2009a, Yamada *et al.*, 2009b). Furthermore, the frequency of the alleles associated with higher marbling was high in Japanese Black, but very low or nonexistent in Japanese Brown, Japanese Shorthorn, Holstein and Brown Swiss, breeds not traditionally heavily selected for marbling (Watanabe *et al.*, 2010).

5.3.1.3 Muscle development and structure

Fat and muscle cells are mesenchymal in origin. As increases in fat appear to occur at the expense of muscle (Chapter 4), genes or gene pathways affecting muscle growth or stability, such as *myostatin*, have effects on fatness, particularly intramuscular and intermuscular fat. Similarly, transdifferentiation of myocytes and adipocytes has been

demonstrated (Section 1.6), and in particular, high levels of glucose induce adipogenesis in muscle satellite cells (Yue *et al.*, 2010).

Enolase 3 – ENO3

Enolase 3 (β Enolase, *ENO3*) is one of three enolase isoenzymes involved in the glycolytic pathway (Fougerousse *et al.*, 2001, Wu *et al.*, 2008). *ENO3* is involved in skeletal muscle cell development and regeneration, being expressed during myogenesis and in proliferating adult myoblasts (Fougerousse *et al.*, 2001, Wu *et al.*, 2008), and is implicated in glycolytic pathway dysfunction specifically in skeletal muscle (Comi *et al.*, 2001). Wu *et al.* (2008) identified a single nucleotide polymorphism in *ENO3* that was associated with intramuscular fat %, marble score, rib fat thickness and carcass fat percentage in pigs.

Lamin A/C (LMNA)

Lamins are grouped into two classes, A type (lamin A and C) and B type (lamin B1 and B2). Lamin proteins are involved in nuclear stability, with mutations in lamin A/C (*LMNA*) causing Emery-Dreifuss muscular dystrophy (EDMD) (Sullivan *et al.*, 1999) and Dunnigan-type familial partial lipodystrophy (FPLD) (Murase *et al.*, 2002, Boguslavsky *et al.*, 2006). FPLD patients exhibit a loss of subcutaneous fat from the arms and legs, gain excess fat around the face and neck (Boguslavsky *et al.*, 2006) while maintaining intermuscular fat (Garg *et al.*, 1999, Flier, 2000). Because of the link between FPLD and *LMNA* mutations, it has been suggested that polymorphisms in *LMNA* affect adipocyte size rather than number (Weyer *et al.*, 2001). A SNP in *LMNA* has been associated with adiposity in a population of indigenous Canadians (Hegele *et al.*, 2000) and over-expression of lamin A in cell culture. *LMNA* knockout mice have confirmed that lamin A inhibits adipogenesis (Boguslavsky *et al.*, 2006). Furthermore, a

SNP in exon 10 of LMNA is associated with insulin resistance in humans (Murase *et al.*, 2002).

5.3.2 Other candidate genes

The work reported herein forms part of a much larger research program, involving other fat traits, muscle traits and residual feed intake of cattle. Gene variants in all of the candidate genes are routinely tested for association with all traits investigated within the research program, using an ASREML mixed model. Four of these genes, peroxisome proliferator-activated receptor alpha (*PPARGA*), ß, ß-carotene 15, 15'-monooxygenase (*BCMO1*), aldehyde dehydrogenase 8 family, member A1 (*ALDH8A1*) and ATPase Ca^{++} transporting, plasma membrane 4 (*ATP2B4*), were selected for a more directed association analysis with fat traits in the mapping progeny as they indicated association with fat distribution traits using the mixed model (chapter 6). *BCMO1* and *ALDH8A1* are involved in vitamin A metabolism. However, the connection between *ATP2B4* and fat deposition is not immediately obvious.

5.3.2.1 Vitamin A pathway

Adipogenesis and adipose tissue development are inhibited by high levels, and promoted by low levels of vitamin A in the diet (Ribot *et al.*, 2001, Bonet *et al.*, 2003). Siebert *et. al.* (2006) found that Angus steers fed a diet with reduced vitamin A had increased intramuscular fat %. There are two forms of dietary vitamin A; pre-formed vitamin A and provitamin A (β,β-carotene), of which β,β-carotene has the most effect on adiposity in mammals (Lobo *et al.*, 2010). β,β-carotene is metabolised to three forms of vitamin A or retinoids; retinol, retinaldehyde and retinoic acid (Bonet *et al.*, 2003). Retinoic acid is the most biologically active of the retinoids (Villarroya *et al.*, 1999, Ribot *et al.*, 2001, Bonet *et al.*, 2003) and in high concentrations is an inhibitor of adipocyte differentiation, while at low levels, retinoic acid increases differentiation

(Safonova *et al.*, 1994, Bonet *et al.*, 2003, Lobo *et al.*, 2010). Retinoic acid is a strong inhibitor of peroxisome proliferator-activated receptor gamma (*PPARG*) and CAAT/enhancer binding protein alpha (*C/EBPα*), two transcription factors that are key elements in adipogenesis (Villarroya *et al.*, 1999, Ribot *et al.*, 2001, Lobo *et al.*, 2010). Retinoic acid also possibly contributes to a reduction in adipocyte storage capacity (Lobo *et al.*, 2010). Two genes involved in the conversion of ß, ß-carotene to retinoic acid were selected as candidate genes for other fat traits and included herein for further study based on their association with fatness. ß, ß-carotene 15, 15'-monooxygenase (*BCMO1*) catalyses the conversion of ß, ß-carotene to retinaldehyde (Lietz *et al.*, 2010, Lobo *et al.*, 2010) and aldehyde dehydrogenase 8 family, member A1 (*ALDH8A1*) catalyses the conversion of retinaldehyde to retinoic acid (Stacker *et al.*, 2000, Grapes and Rothschild, 2006). As well as their roles in vitamin A metabolism, *ALDH8A1* is associated with increased fatness in pigs (Grapes and Rothschild, 2006) and it has been suggested that *BCMO1* may be involved in lipid metabolism (Hessel *et al.*, 2007, Lietz *et al.*, 2010).

5.3.2.2 Lipid metabolism

Like *PPARG* (Section 5.3.1.1), peroxisome proliferator-activated receptor alpha (*PPARA*) is a member of the PPAR subfamily of nuclear receptors (Meirhaeghe and Amouyel, 2004). While *PPARG* is mainly expressed in adipose tissue, *PPARA* is expressed in brown adipose tissue and the liver (Kersten *et al.*, 1999). *PPARA* is involved in energy balance during fasting (Kersten *et al.*, 1999) by controlling genes involved in lipid metabolism (Aoyama *et al.*, 1998, Meirhaeghe and Amouyel, 2004), and has been reported to affect abdominal fat in chickens (Zhang *et al.*, 2010a). *PPARA* knockout mice tend to develop obesity in later life (Lewitt and Brismar, 2002) with greater effects in females than males (Costet *et al.*, 1998). Although *PPARA* knockout mice have increased internal fat (Costet *et al.*, 1998) and intracellular lipid in

the heart and liver (Leone *et al.*, 1999), it is likely that the loss of *PPARA* can be mediated by the abundance of *PPAR delta* (*PPARD*) in skeletal muscle (Muoio *et al.*, 2002).

5.3.2.3 Unknown function

The remaining candidate gene, ATPase Ca^{++} transporting, plasma membrane 4 (*ATP2B4*), is associated with fatty acid composition, having a large effect on fatty acid chain elongation and desaturation (Pitchford unpublished data). Therefore, because of the relationship between fatty acid composition and marble score (Malau-Aduli, 1998), *ATP2B4* was also selected as a candidate gene. However, the link between *ATP2B4* and any fat trait is unclear. ATP2B4 catalyses the conversion of ATP to ADP and also plays a role in calcium homeostasis in the cell by transporting calcium bivalent ions out of the cell (Brandt *et al.*, 1992, Di Leva *et al.*, 2008). Ca^{++} concentration is important in regulating of cellular processes that are involved in the differentiation and proliferation of mesenchymal stem cells (Kawano *et al.*, 2006), the precursors of fat and muscle cells.

5.3.3 Sequencing

In general, most exons and the adjacent intron regions of the candidate genes were sequenced in the three gene mapping sires of the Davies Jersey – Limousin backcross herd (Table 5.2). However, in some genes, there were regions that were unable to be amplified for sequencing due to technical difficulties with those particular DNA regions. Multiple alterations to the standard PCR were used for any region that would not amplify at the first attempt, including different annealing temperatures, the use of alternate polymerases and polymerase concentrations, inclusion of enhancers such as betaine to improve strand separation in GC rich regions, and new primer design. In the case of *ACACA* which had 52 exons, only the 5' region, exon 3, which included the

translation initiation site, exon 42, which was a larger exon than most, and exon 56, the last exon, were sequenced. There was some doubt to the accuracy of the published *ESR1* sequence. In the second assembly of the bovine genome, when *ESR1* was initially chosen as a candidate gene and sequenced, the gene sequence included 20 exons, 7 of which varied between 4 and 21bp. Also, there were large regions where the sequence had not been confirmed, including the region immediately after exon 1. Comparing the bovine and human *ESR1* sequences confirmed that the human exons 1, 2, 3, 4, 7, and 8 aligned with the bovine exons 1, 4, 5, 6 and 7 (combined), 19 and 20 respectively. Although the human exons 5 and 6 were not aligned with the bovine *ESR1*, it was decided to sequence the equivalent regions of the bovine genome, along with bovine exons 1, 4, 5, 6, 7, 19 and 20. Exon 3 was also included because it was located close to exon 4 and could be sequenced in the same reaction. When the third bovine genome assembly was released, the number of exons had been reduced to 12 including a new exon 2, (near exon 1), but there was still an unknown region preceding exon 2. Moreover, some of the exons from assembly 2 were not included in the third assembly. Using comparative analysis of the human and bovine *ESR1* DNA sequences and the bovine mRNA sequence (National Centre for Biotechnology Information NM_001001443.1), primers were designed for all of the bovine mRNA and flanking regions. This resulted in the exons in assembly 2 that were not included in assembly 3, and the regions homologous to human exons 5 and 6 being sequenced, as was the previously unknown region between exon 1 and the new exon 2.

5.3.4 Candidate gene variants

There were 109 variants identified in the candidate genes (Tables 5.2 and 5.3). Most of these were single nucleotide polymorphisms (SNPs). However, 18 were insertion/deletions (in/dels); either single base pair (10), 2 base pair (3), 4 base pair (1), 5 base pair (2), or 9 base pair (1). In one in/del, a four base sequence was replaced

with an alternate, 2 base sequence (*TEK1* SNP34). One SNP (*ENO3*, SNP9[‡]) and one 2 base pair in/del (*PPARG* SNP2[‡]) were identified only in a dam of one of the bulls. Fourteen SNPs were located in either the 5' or 3' untranslated (UTR) or flanking regions, five were located in exons (*ENO3*, 1; *ESR1*, 1; *TEK1*, 3), and the remainder were intronic. All exonic SNPs were synonymous, i.e., the SNP did not cause a substitution of the amino acid. The frequency of polymorphisms varied between genes; *LMNA* had the lowest number (1:8,470bp) while *ACACA* had the highest number (1:300bp) (Table 5.2).

Table 5.2: Candidate genes and regions sequenced.

Gene symbol	Full length (bp)	Transcript length (bp)	Total exons	Number of exons sequenced	Bases sequenced	Variants	Bases/variant	GC%
ACACA	335,865	7,254	57^	4 (5', 3, 42, 56)	2,100	7	300	50.0
ADIPOQ	13,160	2,483	3*	3	3,050	2	1,525	49.9
EDG1	4,655	3,030	2*	2	2,770	2	1,385	52.5
ENO3	5,158	1,439	12^	12	6,050	12	504	54.8
ESR1	137,928	1,098	20*	7 (1, 3, 5, 6, 19, 20, 3' and remainder of transcribed region)	9,050	14	646	48.4
LMNA	18,230	2,204	12*	8 (5', 2, 3, 4, 6, 7, 8, 10, 12)	8,470	1	8,470	59.6
NCOA7	11,784	2,792	13*	11 (not 1 or 11)	9,050	22	431	40.4
PPARG	72,704	1,827	7[§]	7	5,150	6	858	44.9
TEK1	102,979	4,612	23*	22 (not 12)	14,300	43	376	44.8

*** = Ensembl Btau 2, [§] = Ensembl Btau 3.1, ^ = Ensembl Btau 4**

Table 5.3: Variants identified in the candidate genes.

SNP No	Variant	Region [Φ]	Sequence context
			ACACA
1	TC	5'	ggactactaaacccttgtgc[t/c]acaactagagaaagcccaca
2	AC	Intron 2	taacttgtggctttcctcag[a/c]ttattatttcctcaggttat
3	TC	Intron 3	gaagctgaacctttttctta[t/c]tgagc[t]tgtcctattggttt
4	TC	Intron 3	gaacctttttctta[t]tgagc[t/c]tgtcctattggtttcactga
5	TC	Intron 41	ataatcttagaaaagtatgc[t/c]agaatatttgctctagttt
6	AG	Intron 41	aagcttgtattgatttgtct[a/g]tctgtttatgcctttgcaga
7	GT	Intron 42	gatccttacaaggtacagat[g/t]aagagaagataatacttccg
			ADIPOQ
1	TC	Intron 2	acagaaaagtcccctatgta[t/c]gactggctgagcctgcacat
2	AC	5'	tgtcttctgtattggcataa[a/c]gtggttctataccacttgta
			EDG1
1	T in/del	3' UTR	accaagagagttttttttt[t/a]aattagtttatctaacaata
2	GC	3'	ttctataatctcagtatagt[g/c]ttcaattattattatttttt
			ENO3
1	G in/del	Intron 1	agtgcagctttgtgggagat[g/-]gggttcagcaagtggtggag
2	AG	Intron 2	gtgatcttccaattcctccc[a/g]tcccaggccgattccg[a]gca
3	AG	Exon 3	ccc[g]tcccaggccgattccg[a/g]gcagctgtgcccagtggcgc
4	CG	Intron 6	tggagacaagctgctaggtt[c/g]gaatcctggtcttgtctctt
5	AG	Intron 6	ggtaagcaggtgatatcccc[a/g]ttttatgaaaatgagggcag
6	G in/del	Intron 6	ccatgttgacctcacccccc[g/-]ccccccactccagtcctttc
7	CT	Intron 8	gcagctgcacagcacaccgg[c/t]ctcggcccctcctctgccac
8	CG	Intron 9	ctacccttccagctaccct[c/g]ttgtccactaactccaggtc
9	GT[‡]	Intron 9	taactccaggtctgactctc[g/t]cttggctcctgacccacccc
10	AG	Intron 9	ctaactcctgacctgacccc[a/g]gaacttttgtcattgtcac[c]
11	CG	Intron 9	[g]gaacttttgtcattgtcac[c/g]ctgcctcaatccatccccac
12	AG	Intron 10	cccagtgtctgagttttctt[a/g]gggttcctggccccctgccc
			ESR1
1	T in/del	Intron 3	gattcactgggtttttttttt[t/-]gttttgttttgttttttctc
2	GC	Intron 5	acctgccatgatgtccttgt[g/c]catcacccactgctggctgt
3	CT	Intron 18	acccctttatccgctttgagt[c/t]tctctct[gt]ctttctctgcac
4	GT in/del	Intron 18	tccgctttgagt[c]tctctct[gt/-]ctttctctgcacattcagga
5	G in/del	Intron 19	tgagcctgaggctgatctga[g/-]cggtcctgtctgtgtctccc
6	CT	Exon 20	ctgcacgccccagccaacttt[c/t]gggagcgcacctccagagga
7	GA	5'	gggagggctggggccagcaa[g/a]gcatctgatccaagtggatc
8	AG	5'	ttctaatatattatatatac[a/g]tatataatgtacttgagcag
9	CA	Intron 1	ctacacccgcctccgcagcc[c/a]ctctcgcccttcctgcaccc
10	CA	Intron 1	ccgcgccccgtcggggtggt[c/a]gccgcgcgccgggcggcggg
11	GA	Intron 1	cccgcaggccgcggggctgg[g/a]cgcccggccgcagccgcagc
12	TA	Intron 1*^	aatttttttttaatttttaat[t/a]tttttccttctccaccgccc
13	AG	Intron 1	ttttagttggggaggggcc[a/g]ccccagagccaatacacgta
14	AG	3'	gttcatgctccatgaagaga[a/g]tgggctgtggtttttttgtga

Table 5.3 continued.

SNP No	Variant	Region [Φ]	Sequence context
			LMNA
1	AC	Intron 2	gactagagtcagccaccaag[a/c]ctctgtccagggctctcttc
			NCOA7
1	GA	Intron 3	ttaagatattttctaaagac[g/a]tgatggggcatttttata[g]g
2	GA	Intron 3	c[g]tgatggggcatttttata[g/a]gttcatatatatatgcttta
3	GT	Intron 3	ggtgattatttttgaaggat[g/t][g]aagacatttacctttttataa
4	GT	Intron 3	ggtgattatttttgaaggat[g][g/t]aagacatttacctttttataa
5	T in/del	Intron 3	ataaataaataatatttttt[t/-]agtgtttctatttgtggaaa
6	AG	Intron 6	gaaatatttcagtatcacca[a/g]tattattaataatggagttt
7	AA in/del	Intron 8	aagatgaagaaggggggaaa[aa/--]aaaaacgcacaacaatgagg
8	AT	Intron 8	ctctgcgctctgccactcat[a/t]tg[g]tttgaaacgctttctaa
9	GT	Intron 8	tgcgctctgccactcat[a]tg[g/t]tttgaaacgctttctaa
10	CT	Intron 9	tgggttctccctggggatga[c/t]ggtaggggccttccagacct
11	CT	Intron 9	tattactactaggaaaaact[c/t]ccctttctcttagttttgtt
12	CG	Intron 9	ttcagtatcatccctgagag[c/g]gaatacatgctctggggagc
13	CT	Intron 9	aatacatgctctggggagca[c/t]ccctgccaaggtaaacagtg
14	GT	Intron 9	gatggttttgccttctcagt[g/t]tatagtggcaa[g]tatttgct
15	G insert	Intron 9	ttctcagt[g]tatagtggcaa[g/-]tatttgctcgagtaaatggg
16	AG	Exon 10	tggagactcgcgtacagcac[a/g]ctagagcatgggaccagctt
17	CT	Exon 10	gtactattggtcatcaaaga[c/t]atggataatcaggtgaggct
18	AG	Intron 10	tggataatcaggtgaggctt[a/g]ttcctcttatagagaaacat
19	CT	Intron 10	tgcgacaggagagagacgga[c/t]acaatgaact[c]gatcagcca
20	CT	Intron 10	agagacgga[c]acaatgaact[c/t]gatcagccagaccgcgcagt
21	AG	Intron 12	gacagtgtggtctaacacat[a/g]acactaagaatcgaagataa
22	AT	3'	ggcagacgatgaagaaagaa[a/t]ttgaagttcggatcgttgaa
			PPARG
1	CT	5'	agctattgctccacaactga[c/t]gatggaacttttgctaatct
2	AT insert[‡]	5'	tcgatggaccagatatatat[at/--]gtattctgctcggggaatat
3	CG	Intron 2	gaattggactaagctctcgt[c/g]cacagtagggtaaatgctcc
4	9bp in/del	Intron 2	ggtatactataacgacatgc[cctgctgcc]tgtttagcattgtttttcct
5	AG	Intron 4	cgagggccgtggctgaaaac[a/g]tgttcagttttcccacagat
6	AG	Intron 5	ggtgttgaccgtgaatgaga[a/g]atttccaactcagccacttt
			TEK1
1	C in/del	5'	acaggcagatcatggcagac[c/-]tgggattagtacccagaatc
2	AT	Intron 1	aggtttggctttattattatt[a/t]ttttttttttttaagttttttt
3	GT	Intron 4	actgatttaaaggctgacaa[g/t]caagttccagaaaatatctt
4	T in/del	Intron 8	tctctctttttttttttttt[t/-]ggtagaacaaattatagctt
5	CT	Intron 8	gactgatcttcttaatctct[c/t]ctagaaaacttcttatttaa
6	GT	Intron 9	gccttttggatgttctccag[g/t]atactaagccttaagcaaaa
7	AG	Exon 10	gtcatcaacatcagctctga[a/g]ccttactttggggatggacc
8	AG	Intron 10	tggcagggaaatgctccagc[a/g]agagatgggacaccaggaaa
9	CG	Intron 10	tccccaaatgtagaaatcct[c/g]tc[t]ctcaatctcaaccctct
10	CT	Intron 10	ccaaatgtagaaatcct[c]tc[c/t]ctcaatctcaaccctctacc

Table 5.3 continued.

SNP No	Variant	Region [Φ]	Sequence context
			***TEK1* continued**
11	AG	Intron 10	gtgggtctacctttctgaaa[a/g]tcatggtgtaaaatggggca
12	CT	Intron 10	caatgcttcctgtccaggtt[c/t]ctctggatctccagtcccat
13	GT	Intron 10	tggatctccagtcccatttt[g/t]actctgtcttgcttggtcat
14	4bp in/del	Intron 10	atgtatgaatggcacagaga[tact]tacatgaaaaagccttgttt
15	CT	Intron 13	tgcaaatggtcgtggcctat[c/t]tccccatctagactaacctg
16	AG	Intron 14	aaaatttacttgtaaaggaa[a/g]tatttcccttaggcaagttt
17	CT	Intron 14	ctaccaggctcctccgtcca[c/t]gggatacctattagatgaac
18	5bp in/del	Intron 15	ctagggtgggggacagttt[gactt]cctgttgaatctttcctcta
19	A in/del	Intron 15	aaccacaagaagcattggcc[a/-]aaaatgtggacagccaagcc
20	AG	Intron 15	gctaacgttttccagaccca[a/g]tgacagctgagtgtacttga
21	AG	Exon 16	agggactttgctggggaact[a/g]gaggttctttgtaaacttgg
22	AG	Intron 16	agataaaatggaattttata[a/g]gcagtccccgaggaccaaag
23	CT	Intron 17	atcagtttagatttttttt[c/t]cccagttcac[indel]ttccttaa
24	5bp in/del	Intron 17	attttttttt[t]cccagttcac[ctctc]ttccttaattccttctcaaa
25	GT	Intron 17	atgctgaaaatatatgactt[t/g]ttttttttttt[t]aatttcag
26	AT	Intron 17	tatgactt[t]ttttttttttt[t/a]aatttcagtttattcacagg
27	CT	Exon 18	tacgtagccaagatagccga[c/t]tttggattatcccgaggtca
28	GT	Intron 18	gcccctccctgaccatttct[g/t]ccatttcataaggcctcagc
29	AG	Intron 18	acgtggtgaagttgttgaac[a/g]tgcaagcct[c]gtgctggagg
30	CT	Intron 18	gttgttgaac[g]tgcaagcct[c/t]gtgctggaggggctggggtc
31	AG	Intron 18	agacccccaagtctggtctc[a/g]ttcagcatcttctggggagc
32	T in/del	Intron 18	gacacacacatacacacaca[t/-]cat[t]atttttctttacatga
33	CT	Intron 18	acacacatacacacaca[t]cat[c/t]atttttctttacatgaaac
34	2-4bp in/del	Intron 18	gtgggacatgcaaaaatatc[tc/ccct]gactctgccatgccttctgt
35	T in/del	Intron 19	ggatttttttttttttttt[ttt]ggacccagaaaaatatccat
36	AG	Intron 19	atttgacaaaggttttgaag[a/g]gaagcaggaatattgcatcc
37	CG	Intron 19	agtcagttgccccagctttg[c/g]gggcagaacgctgactggcc
38	AG	Intron 19	gaacgctgactggccaacgc[a/g]tgcgctgatt[a]ctgttttct
39	AG	Intron 19	ggccaacgc[g]tgcgctgatt[a/g]ctgttttctctttcatccct
40	CG	Intron 20	cacaaacggagtgggcccccc[c/g]cc[c]cccgtgctccttcacca
41	AC	Intron 20	aaacggagtgggcccccc[c]cc[a/c]cccgtgctccttcaccatct
42	C in/del	Intron 20	cacaaacggagtgggcccccc[c]cc[c]cccgtgctccttcaccatct
43	CG	Intron 22	gttcttttgaaccaacctga[c/g]gtgctacagggccgtaaaag

Φ = based on Ensembl build (Btau) listed in table 5.2. [‡] = SNP only in grandparent, *^ = intron 1 in Btau 2, exon 2 in Btau 4

5.3.5 Inferred genotypes

Any sequence after a heterozygous in/del is difficult to interpret. To address this problem, all regions were sequenced in both the forward and reverse directions which,

in most cases, allowed the in/del and the sequence on either side of the in/del to be confirmed. In the case of TEK1 exon 19, there were three in/dels and two SNPs (SNPs 32 – 36, Table 5.3). Mapping sires 368 and 398 were each homozygous for all of these polymorphisms except one in/del (SNP 35), therefore, the sequences of both these sires were confirmed (Table 5.4). However, sire 361 was heterozygous for two of the in/dels (variants 32 and 35), and therefore, the sequence between these variants was unconfirmed. Nevertheless, by comparing the chromatogram with the published sequence and the sequences of the other two mapping sires, it was possible to infer the sequence in that region (Figure 5.2). Therefore, it was assumed that the genotypes of polymorphisms 33 and 34 were the same in sire 361 as sire 398.

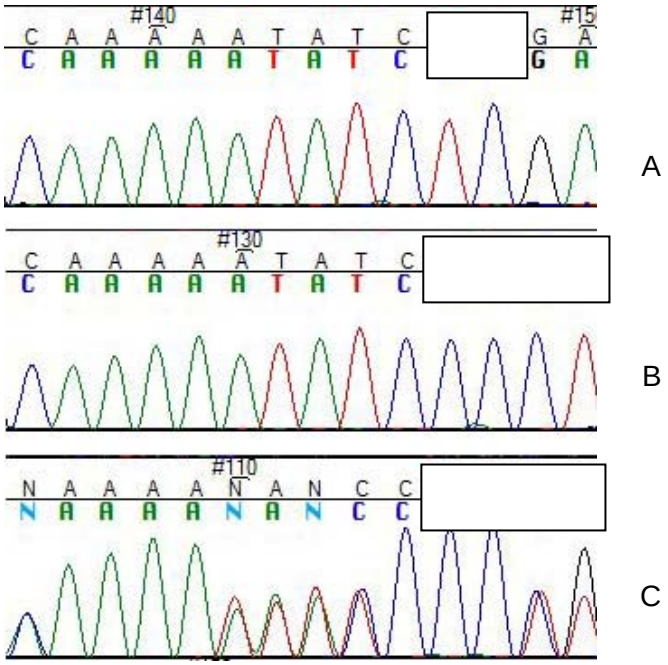


Figure 5.2: Chromatograms showing TEK1 polymorphism 34 in mapping sires.
Sire 368 (A) with polymorphism TC instead of the published sequence CCCT seen in sire 398 (B). Sire 361 (C) shows the effect of an upstream in/del where each base has moved upstream (left) one position, resulting in the sequence showing a stutter. It is clear that this sequence is not TC as is 368 (A), but more likely CCCT as is 398 (B), particularly when the single base shift is considered.

5.3.6 Mononucleotide DNA regions

There is often an in/del at the end of a mononucleotide repeat. Whether these are true in/dels is uncertain because mononucleotide repeats can cause the 'polymerase' to slip

during extension *in vivo* as well as *in vitro* (either during PCR or the sequencing reaction), resulting in a 'stutter' in the sequence. This occurs most frequently with mononucleotide repeats of 8bp or greater (Shinde *et al.*, 2003, Fazekas *et al.*, 2010). The *ENO3* variant 6 demonstrates this phenomenon, in that there is a single G with a 6bp 'C' repeat in both the 5' and 3' directions (Figure 5.3). Sire 368 was homozygous for this sequence, had no in/del and was sequenced clearly in both directions. However, sire 361 had a heterozygous G – C substitution (or, less likely, a G deletion), which caused the sequence beyond the polymorphism to overlap. Furthermore, sire 398 had a homozygous G substitution or deletion, but the sequence after this polymorphism was unclear, similar, but not identical, to sire 361. This demonstrates that the 6bp repeat did not affect the fidelity of the PCR in contrast to the longer repeat. A heterozygous deletion of G in sire 361 would explain the poor sequence quality after the deletion. However, if sire 398 had a homozygous deletion, the sequence pattern would have been obvious unless the repeat sequence caused a slippage during the replication. Although these in/dels are unconfirmed, they have been included but should not be assumed to be verified.

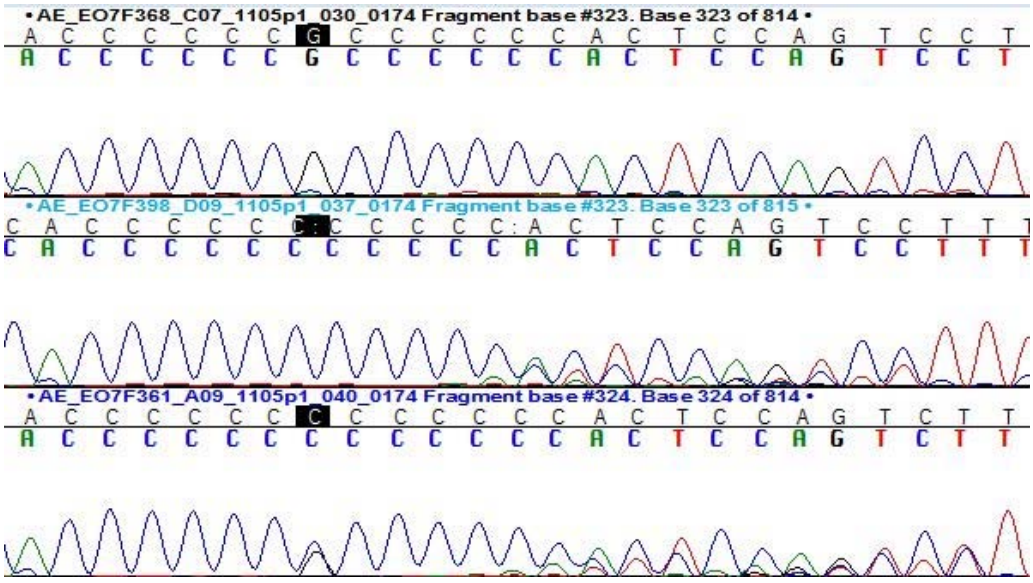


Figure 5.3: Chromatograms showing effects of mononucleotide repeats.
Sire 368 (A) wild type mononucleotide repeat no greater than 6 resulting in easily interpreted DNA sequence. Sire 398 (B) G deletion or G/C substitution resulting in difficult to interpret DNA sequence. Sire 361 (C) heterozygous for wild type and G deletion/substitution resulting in difficult to interpret DNA sequence slightly different to Sire 398.

Table 5.4: Mapping sire genotypes

SNP	Variant	Region [Φ]	Bull			Genotyped
			361	368	398	
			ACACA			
1	TC	5'	T/T	T/T	T/C	HRM FAILED
2	AC	Intron 2	A/A	A/A	A/C	
3	TC	Intron 3	T/T	T/T	T/C	
4	TC	Intron 3	T/T	T/T	T/C	
5	TC	Intron 41	T/T	T/T	T/C	
6	AG	Intron 41	A/A	A/A	A/G	HRM
7	GT	Intron 42	G/G	G/G	G/T	
			ADIPOQ			
1	TC	Intron 2	T/C	T/T	T/C	ILLUMINA
2	AC	5'	A/C	A/A	A/C	HRM FAILED
			EDG1			
1	TA	3'UTR	T/A	T/T	T/A	
2	GC	3'	G/C	G/G	G/C	HRM / ASP
			ENO3			
1	G in/del	Intron 1	G/G	G/G	-/-	
2	AG	Intron 2	A/G	G/G	A/A	
3	AG	Exon 3	A/G	A/A	G/G	
4	CG	Intron 6	C/G	G/G	C/C	
5	AG	Intron 6	A/G	G/G	A/A	HRM
6	G in/del	Intron 6	G/-	G/G	-/-	
7	CT	Intron 8	C/T	C/C	C/C	
8	CG	Intron 9	C/G	C/C	C/C	
9[‡]	GT[‡]	Intron 9	G/G	G/G	G/G	
10	AG	Intron 9	A/G	A/G	G/G	
11	CG	Intron 9	C/C	C/G	C/C	HRM / ASP
12	AG	Intron 10	A/G	G/G	G/G	
			ESR1			
1	T in/del	Intron 3	T/-	T/-	-/-	
2	GC	Intron 5	C/G	C/G	C/G	HRM / ASP
3	CT	Intron 18	C/T	C/C	C/T	
4	GT in/del	Intron 18	GT/-	-/-	GT/-	
5	G in/del	Intron 19	G/-	G/-	-/-	
6	CT	Exon 20	T/T	T/T	C/C	
7	GA	5'	G/A	G/G	G/G	
8	AG	5'	A/G	A/A	A/A	
9	CA	Intron 1	C/C	C/C	C/A	
10	CA	Intron 1	C/C	C/C	C/A	
11	GA	Intron 1	G/G	G/G	G/A	
12	TA	Intron 1*^	A/T	A/A	A/T	
13	AG	Intron 1	A/A	A/A	A/G	
14	AG	3'	A/A	G/A	A/A	HRM

Table 5.4 continued.

SNP	Variant	Region [Φ]	Bull			Genotyped
			361	368	398	
			LMNA			
1	A/C	Intron 2	C/C	A/C	C/C	HRM / ASP
			NCOA7			
1	GA	Intron 3	G/G	G/A	G/G	HRM
2	GA	Intron 3	G/G	G/A	G/G	HRM
3	GT	Intron 3	G/G	G/T	G/G	
4	GT	Intron 3	G/G	G/T	G/G	
5	T in/del	Intron 3	-/-	T/-	-/-	
6	AG	Intron 6	G/G	G/A	G/G	
7	AA in/del	Intron 8	A7/A7	A9/A9	A7/A9	
8	AT	Intron 8	A/T	A/A	A/T	
9	GT	Intron 8	T/T	G/G	G/T	
10	CT	Intron 9	T/T	C/T	T/T	
11	CT	Intron 9	C/T	T/T	T/T	
12	CG	Intron 9	G/G	C/C	G/C	
13	CT	Intron 9	C/C	T/T	C/T	
14	GT	Intron 9	T/T	G/G	T/G	
15	G insert	Intron 9	-/-	G/G	-/G	
16	AG	Exon 10	A/A	G/G	A/G	
17	CT	Exon 10	C/C	T/T	C/T	
18	AG	Intron 10	A/A	G/G	A/G	ILLUMINA
19	CT	Intron 10	T/T	C/C	T/C	
20	CT	Intron 10	T/T	C/C	T/C	
21	AG	Intron 12	G/A	A/A	A/A	ILLUMINA
22	AT	3'	T/A	A/A	A/A	ILLUMINA
			PPARG			
1	CT	5'UTR	C/T	C/T	T/T	HRM
2[‡]	AT insert[‡]	5'UTR	WT	WT	WT	
3	CG	Intron 2	C/G	C/G	C/G	HRM / ASP
4	9bp in/del	Intron 2	CCTGCTGCCT/----------	WT	WT	
5	AG	Intron 4	G/G	A/A	A/A	
6	AG	Intron 5	A/A	G/G	G/G	
			TEK1			
1	C in/del	5'	C/-	-/-	C/-	
2	AT	Intron 1	AA	AA	AT	
3	GT	Intron 4	GG	GG	GT	
4	T in/del	Intron 8	TG	TG	TG	
5	CT	Intron 8	TC	CC	TC	HRM
6	GT	Intron 9	GG	GT	GG	HRM
7	AG	Exon 10	AG	GG	GG	HRM
8	AG	Intron 10	GG	AA	GA	
9	CG	Intron 10	GG	CC	CG	
10	CT	Intron 10	TC	TT	TT	

Table 5.4 continued.

SNP	Variant	Region [Φ]	Bull			Genotyped
			361	368	398	
			***TEK1* continued**			
11	AG	Intron 10	AA	GG	AG	
12	CT	Intron 10	TT	CC	CT	
13	GT	Intron 10	TT	GG	GT	
14	4bp in/del	Intron 10	-/-	TACT	TACT/-	
15	CT	Intron 13	CT	CC	CC	
16	AG	Intron 14	GA	GG	GG	
17	CT	Intron 14	CC	TT	CC	
18	5bp in/del	Intron 15	GACTT ins	WT	WT	
19	A in/del	Intron 15	A/-	AA	AA	
20	AG	Intron 15	AA	GG	AA	
21	AG	Exon 16	GG	AA	GG	
22	AG	Intron 16	GG	AA	GG	
23	CT	Intron 17	TT	CC	TT	
24	5bp in/del	Intron 17	WT	CTCTC/CTCTC	WT	
25	GT	Intron 17	TT	GG	TT	
26	AT	Intron 17	AA	TT	AA	
27	CT	Exon 18	TT	CC	TT	
28	GT	Intron 18	GG	TT	GG	
29	AG	Intron 18	GG	AA	GG	
30	CT	Intron 18	CC	TT	CC	
31	AG	Intron 18	AA	GG	AA	
32	T in/del	Intron 18	T/-	DEL/DEL	TT	
33	CT	Intron 18	TT[x]	CC	TT	
34	2bp/4bp in/del	Intron 18	ATC[CCCT]GAC ATC[CCCT]GAC[x]	ATC[TC]GAC ATC[TC]GAC	ATC[CCCT]GAC ATC[CCCT]GAC	
35	T in/del	Intron 19	16T/17T	20T/21T	16T/17T	
36	AG	Intron 19	GG	AA	GG	
37	CG	Intron 19	GG	CC	GG	
38	AG	Intron 19	GG	AA	GG	
39	AG	Intron 19	AA	GG	AA	
40	CG	Intron 20	CG	CC	CC	
41	AC	Intron 20	CC	CC	AC	
42	C in/del	Intron 20	10C/10C	8C/8C	11C/10C	
43	CG	Intron 22	CC	GG	CG	ILLUMINA

Φ = based on Ensembl build listed in table 5.2. HRM = High Resolution Melt, ASP = allele specific PCR, [‡] = SNP only in grandparent, *^ = intron 1 in Btau 2, exon 2 in Btau 4, WT = sequence is the same as published, [x] = inferred genotype.

5.3.7 Density of Single Nucleotide Polymorphisms

The average number of bases between SNPs (SNP density) varied between the candidate genes, and was particularly evident in *LMNA,* where there was only one SNP

identified in more than 8,000bp sequenced (Table 5.2). Chromosomal regions with a high guanine – cytosine (GC) content have an increased SNP density (Brookes, 1999, Miller and Kwok, 2001, Zhao *et al.*, 2003). This is most likely because of the frequency of deamination of methylated cytosine (C) to thymine (T) at methylated CpG dinucleotides, and the observation that most SNPs are C – T transitions (Brookes, 1999, Miller and Kwok, 2001). However, Miller *et al.* (2001) found low SNP density in regions of high and low GC content. In the work reported herein, the number of SNPs in each gene was not correlated to the GC content (Table 5.2). The gene with the highest GC content (*LMNA;* 60%) only had one SNP in the 8,470 bases sequenced, whereas the gene with the lowest GC content (*NCOA7*; 40%) had a higher SNP density than the expected average of 1 per 700 – 1000bp (Section 5.1). This would suggest that *LMNA*, although it has a very high GC content, is not highly methylated.

A recent 'selective sweep', where a particularly advantageous allele is fixed in the population, or a population bottleneck may explain the lack of heterozygosity in *LMNA*. However, a bottleneck would reduce the variation in all regions of the genome and therefore, a selective sweep is the more logical explanation. The single SNP located in bovine *LMNA* is in stark contrast to the human version, in which at least 56 SNPs have been identified (Urbanek *et al.*, 2009). This may reflect the importance of *LMNA* in animals. Mutations in *LMNA* cause Emery-Dreifuss muscular dystrophy (EDMD) in humans (Sullivan *et al.*, 1999). A similar effect in domestic cattle would be heavily selected against. Alternatively, a SNP in *LMNA* has been associated with obesity in one human population that was not affected by muscular dystrophy (Hegele *et al.*, 2000). If one *LMNA* variant was associated with a particularly favourable fat trait in cattle and therefore, heavily selected for, a selective sweep may have occurred. However, it must be remembered that the results reported herein are from only three bulls resulting from a cross between two dissimilar cattle breeds. As noted previously

(Section 4.4.3), the Limousin is a more muscular breed with less overall fat than the Jersey which has been selected for dairy production and is generally fatter. Therefore, it is unlikely that there is any mutually favourable fat trait in these breeds that would have caused a selective sweep, unless it is associated with general fatness, similar to the results noted by Hegele *et al.* (2000).

5.3.8 Single Nucleotide Polymorphisms

Single nucleotide polymorphisms (SNPs) occur in exons, introns and the intergenic regions of genomic DNA. SNPs in exons can either be non-synonymous, in which there is an amino acid substitution, or synonymous, sometimes referred to as silent mutations, where the amino acid is not altered (Liao and Lee, 2010). Non-synonymous SNPs can introduce premature stop codons, producing a truncated protein, or alter the amino acid sequence and possibly change the protein structure. Although synonymous SNPs may not be obvious in the protein sequence, these and SNPs in non-coding regions, can affect gene function (Wang *et al.*, 2006a) and expression (Chorley *et al.*, 2008). Synonymous SNPs can cause altered gene expression, protein structure (Chamary *et al.*, 2006, Kimchi-Sarfaty *et al.*, 2007, Sauna *et al.*, 2007) and stability (Liao and Lee, 2010). SNPs in the 5'UTR (Rieder *et al.*, 2005) and 3'UTR (Subramaniam *et al.*, 2004, Wang *et al.*, 2006a, Liao and Lee, 2010) have altered gene regulation, as well as promoter activity and mRNA stability (Yamada *et al.*, 2009a). A SNP in the *leptin* promoter region has been associated with subcutaneous fat thickness (Chung *et al.*, 2008), while a SNP in an enhancer for the transcription factor *PU1*, is associated with an increase in acute myeloid leukaemia (Steidl *et al.*, 2007). An alteration to gene function has been associated with an intronic polymorphism (Fu *et al.*, 2004), although it has been suggested that this may be due to the SNP being in linkage disequilibrium with a causal mutation, rather than being causal in itself (Wang *et al.*, 2006a). The extensive research in this area indicates that variation in gene

function occurs not just via SNPs causing amino acid substitution, but also SNPs in regulatory elements.

Another form of regulatory element is microRNA (miRNA). These short lengths of single stranded RNA, approximately 20 – 25 nucleotides (Lin *et al.*, 2006, Georges *et al.*, 2007, Wilfred *et al.*, 2007), occur in introns, exons and intergenic regions (Lin *et al.*, 2006, Wilfred *et al.*, 2007, Liu *et al.*, 2010). miRNAs bind to complementary RNA sequences (target sites) which can cause protein degradation (Lin *et al.*, 2006) or alteration of expression of genes both by down– and up–regulation (Liu *et al.*, 2010). Most of the miRNA target sites are located in the 3'UTR (Georges *et al.*, 2007, Liu *et al.*, 2010). Many biological processes are affected by miRNAs (Liu *et al.*, 2010), including adipogenesis and the regulation of adipose cell function (Wilfred *et al.*, 2007). SNPs within the miRNA or target sites can prevent recognition or potentially introduce another target site (Liu *et al.*, 2010). These SNPs are another cause of phenotypic variation (Georges *et al.*, 2007, Liu *et al.*, 2010). For example, a SNP in the 3'UTR region of the Texel *myostatin* gene creates a miRNA target site that causes down regulation of *myostatin* and hence, an increase in muscle hypertrophy in these sheep (Clop *et al.*, 2006).

Further complicating the search for causal polymorphisms is the possibility of interactions within the gene. For example, a polymorphic alteration to the structure that affects the binding efficiency, can be either accentuated or negated by a regulatory polymorphism altering the concentration of the protein (Dimas *et al.*, 2008).

Thus, although none of the SNPs or in/dels identified herein caused amino acid changes, affected intron splice sites or appeared to otherwise alter gene function, this does not preclude any of the polymorphisms from having an effect on a trait. Therefore, any of these SNPs shown to have association to fat traits would warrant further

investigation into the mechanism causing the effect by these ‘silent’ polymorphisms. Regardless, these SNPs can be used to increase DNA marker density in the Davies gene mapping herd and for association studies in other cattle populations.

Chapter 6 Genotyping and association analysis

6.1 Introduction

Genotyping single nucleotide polymorphisms (SNPs) is a common procedure that can be achieved using a variety of methods. These methods include direct sequencing, restriction fragment length polymorphism (RFLP), allele specific polymerase chain reaction (ASP), high resolution melt analysis (HRM), and multiplex systems, such as SNPlex® (Applied Biosystems™) and BeadXpress® (Illumina®). The suitability of each system varies depending on the type and position of the particular SNP. Furthermore, these genotyping methods vary in cost, speed and accuracy. The relative importance of each of these factors in a given study determines which system is chosen in a particular situation.

High resolution melt (HRM) is a fast and relatively cost effective method of genotyping single nucleotide polymorphisms (SNPs) in large populations (Liew *et al.*, 2004, Reed *et al.*, 2007, Croxford *et al.*, 2008). This two-step process involves PCR amplification of a short section of DNA surrounding the polymorphism, with the inclusion of a dye that fluoresces in double stranded DNA. Once amplification is completed, it is immediately followed by a gradual increase in temperature until the DNA strands separate. As the strands separate, there is a corresponding decrease in the measured fluorescence. The temperature at which the DNA strands separate is related to the length, the percentage of guanine and cytosine nucleotides (GC content), and the sequence (Reed *et al.*, 2007). As the DNA amplified by PCR for a particular polymorphism is the same length for each sample and the GC content is the same, any difference in the melt temperature is the result of the sequence change at the polymorphism.

Heterozygotes are almost always easily distinguished from homozygotes due to the altered shape of the melt curve caused by the different melting temperature of the alternate alleles of the heterozygote. However, because homozygotes have two copies

of the same allele, the shapes of the melt curves are similar, only differing in the melting temperature. The variation in melting temperature is principally due to differences in energy required to break the triple hydrogen bonds of C:G base pairing compared to the double hydrogen bonds of A:T base pairing. More than 80% of mammalian SNPs are an A/G or C/T substitution, and due to the alteration in number of hydrogen bonds in this type of SNP, there is a difference in melting temperature of approximately 1°C (Liew *et al.*, 2004, Reed *et al.*, 2007). The remaining SNPs (A to T or G to C) are neutral because there is no change in the number of hydrogen bonds. Therefore, the differences in melting temperature are the result of the interaction between the SNP and the adjacent bases. Consequently, the differences in melting temperature are less than 0.4°C (Liew *et al.*, 2004) and accurate genotyping of these neutral SNPs can be problematic compared to other SNPs. In particular, if there is symmetry in the adjacent bases (Figure 6.1), there will be no difference in the melting temperatures of the homozygotes (Liew *et al.*, 2004, Reed *et al.*, 2007).

Figure 6.1: Nearest-neighbour symmetry at a G/C SNP.

Genotyping the mapping herd enabled analysis to identify any association between gene sequence polymorphisms and fat trait variation, and how much, if any, of that variation is explained by the polymorphism. The low correlations between individual fat deposition traits (Chapter 4) suggest that it is unlikely that any of the candidate genes will be associated with variation in all, or even many, of the fat traits. Furthermore, the complex nature of the quantitative traits investigated herein suggests that the effects of each DNA variant are likely to be small and the overall phenotype will be the cumulative effect of multiple gene variations. However, a small effect does not preclude it from being biologically relevant (Hegele *et al.*, 2000).

6.2 Methods

6.2.1 Genotyping

All candidate genes (listed in chapter 5) were genotyped. Where there were more than two single nucleotide polymorphisms (SNPs) in a gene, the SNPs were selected based on independent haplotypes and suitability for genotyping.

Fourteen SNPs were genotyped via high resolution melt (HRM) analysis using the Rotorgene 6000 (*Corbett Life Science*), while the remainder were either outsourced to the Department of Primary Industries, Victoria, for genotyping on the Illumina® BeadXpress® platform, or data were supplied by other members of the research group. In some cases, when only the heterozygous and one homozygous genotype were available or the second homozygote was not clearly identified by HRM, the SNPs were genotyped using allele specific polymerase chain reaction (ASP).

6.2.1.1 Genotyping reaction mix

All PCR reactions were performed as per Section 2.2, with the exception of the addition of an intercalating dye (Appendix A.1). The enzyme selected for a particular SNP genotyping assay was decided upon during optimisation of the primers. Both KapaTaq (*Kapa Biosystems*) and Sensimix™ (*Quantace Ltd*) were trialled and the enzyme and dye combination that provided the better HRM pattern differentiation for that SNP was used for genotyping.

6.2.1.2 High Resolution Melt

The high resolution melt was completed immediately after amplification (Appendix A.2), with the temperature increased by 0.2°C per step with a 2 second wait at each step. The temperature range for the melt step was derived during the optimisation, starting at

10°C below to 10°C above the melt temperature of the PCR product for a particular SNP. If the melt temperature was within 8°C of the final extension (72°C), another temperature hold step of 60°C for 10 minutes was added between the final extension and the melt step to allow complete formation of the DNA duplex.

6.2.1.3 Allele Specific Polymerase Chain Reaction

Primers were designed such that the 3' base was specific to one allele of the SNP. Therefore, the reaction would only proceed if the DNA sample was either homozygous or heterozygous for that allele. The genotype was determined either by agarose gel separation or using real time PCR, as the homozygous and heterozygous patterns were easily distinguishable. Inconclusive results were confirmed by repeating or using the alternative allele primers. One SNP (PPARG-2) was genotyped using multiplex PCR, where two primers, specific to alternate alleles, were designed to amplify products of significantly different lengths. The reactions were resolved by agarose gel separation to determine genotypes. All PCR reactions were standard with the exception that the number of cycles was reduced to a maximum of 30 to minimise the possibility of amplification of false products.

6.2.2 Data analysis

In addition to the 19 SNPs genotyped, as outlined in this chapter, there were 14 other SNPs analysed for association to fat traits. These were chosen based on other analyses indicating an effect on fat traits (Section 5.3.2).

Single nucleotide polymorphism (SNP) and fat trait association was determined using general linear regression (GenStat 10.1) with fixed factors in two models: 1) cohort, breed of dam, and sire, and 2) cohort, breed of dam, sire, and myostatin variant F94L genotype (nested within breed of dam). Carcass weight was added as a co-variate to

model 1 for subcutaneous fat, internal fat and marble scores, but this did not alter the results.

The interaction between SNPs within a gene was tested for association with each trait using the fixed factors of cohort, breed of dam, sire, myostatin variant F94L genotype (nested within breed of dam) and SNPxSNP. This same model was also used to test interactions between SNPs in different genes for epistatic interactions.

The additive and dominance effects were calculated for SNPs that were significant for any fat trait using general linear regression (GenStat 10.1) with fixed effects of cohort, breed of dam, sire and myostatin variant F94L genotype (nested within breed of dam). Correlations between the additive effects of each SNP on each trait were calculated as an indication of the genetic correlations between the fat depots.

The SNP variance as a percentage of total variance was calculated using a linear mixed model, with fixed effects of cohort, breed of dam, sire and myostatin variant F94L genotype (nested within breed of dam). The variance (residual) was used to calculate the SNP effect as the number of standard deviations from the mean.

Least squares means were calculated using an unbalanced ANOVA with fixed effects of cohort, breed of dam, sire and myostatin variant F94L genotype. These were used to estimate the allele substitution effect as a percentage of the lowest of the least squares means for genotype.

6.3 Results

6.3.1 High Resolution Melt Analysis

The difference in melt patterns between homozygotes is sometimes ambiguous, depending on the type of SNP. Neutral transversion SNPs (C to G or A to T) have little difference in melting temperature and therefore, the melt patterns are very similar (Figures 6.2 and 6.3) compared to transition polymorphisms (A/G or C/T) (Figures 6.4 and 6.5).

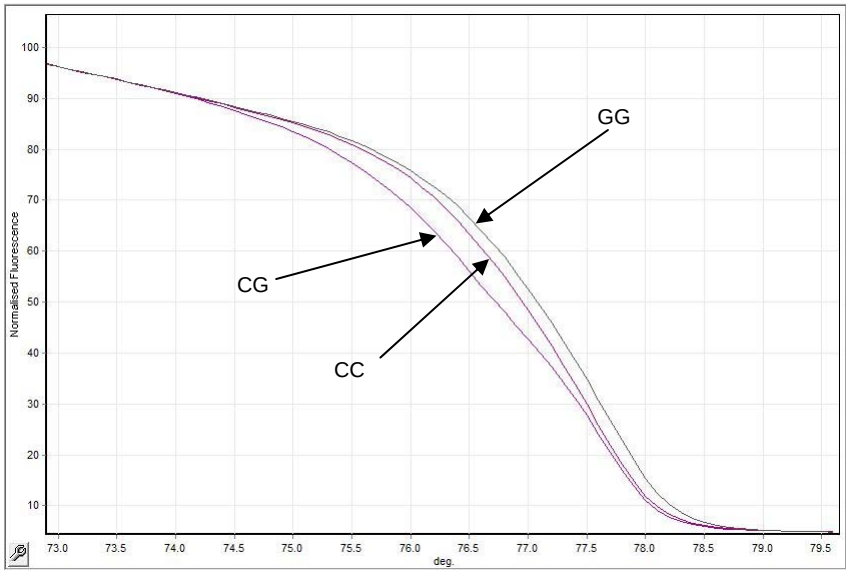


Figure 6.2: Melt curve of SNP PPARG-2.

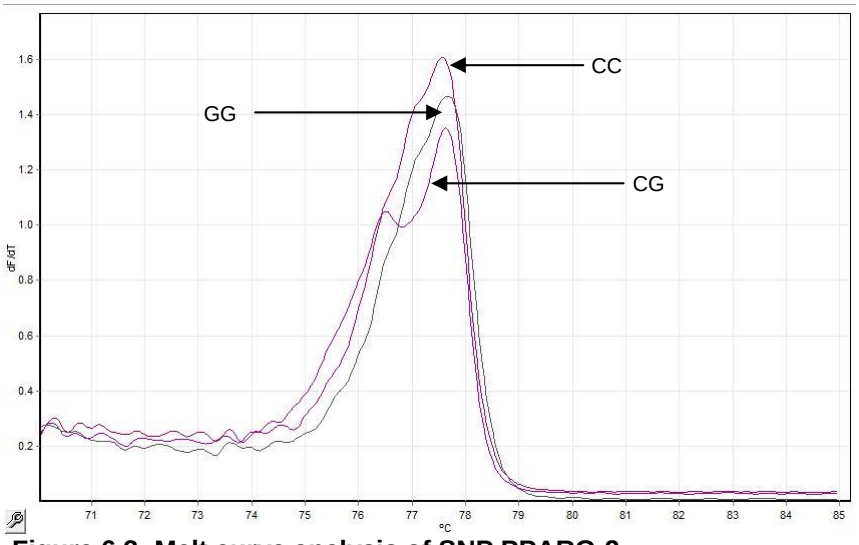


Figure 6.3: Melt curve analysis of SNP PPARG-2.

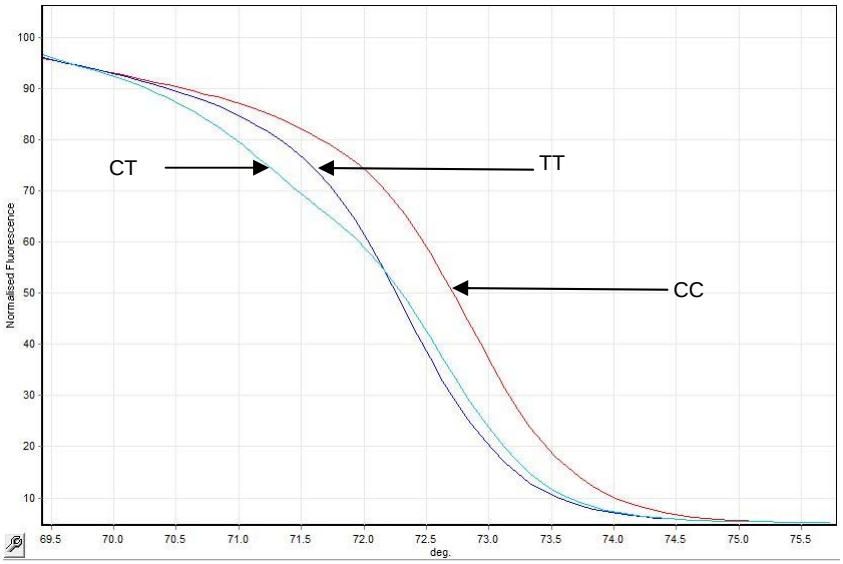


Figure 6.4: Melt curve of SNP TEK1-4

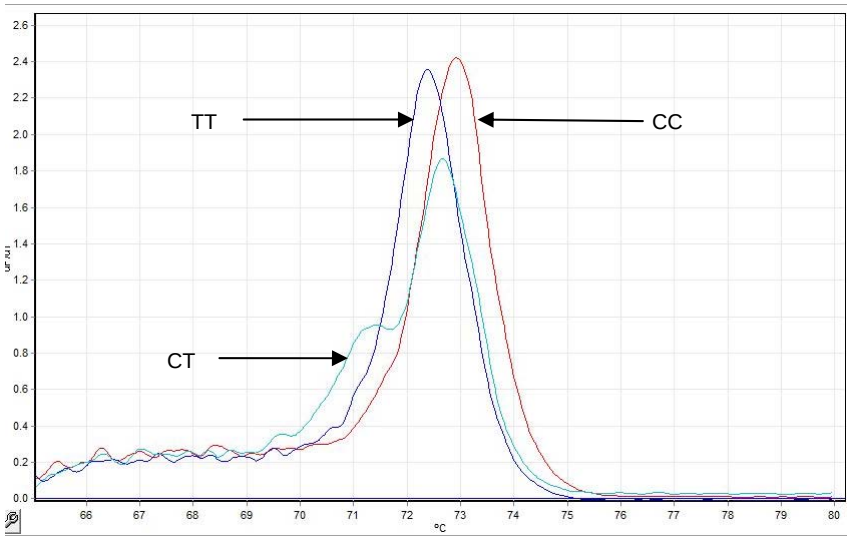


Figure 6.5: Melt curve analysis of SNP TEK1-4

All progeny genotypes were checked against the sire genotype to identify obvious genotyping errors. There were instances of false genotyping results, mainly when there was only one of the homozygous controls available for HRM optimisation. Provided the amplification of each sample was equivalent (Figure 6.6), the HRM analyses should be reliable. However, there were cases where there was a distinct difference in the melt curve, the difference graph and the melt curve analysis, but sequencing revealed another genotype. For example, from the HRM analyses, progeny 917 was assumed to

be GG at the ENO3-11 locus (Figures 6.7 – 6.9). However, sequencing results for that animal confirmed that it was actually CC (Figure 6.10).

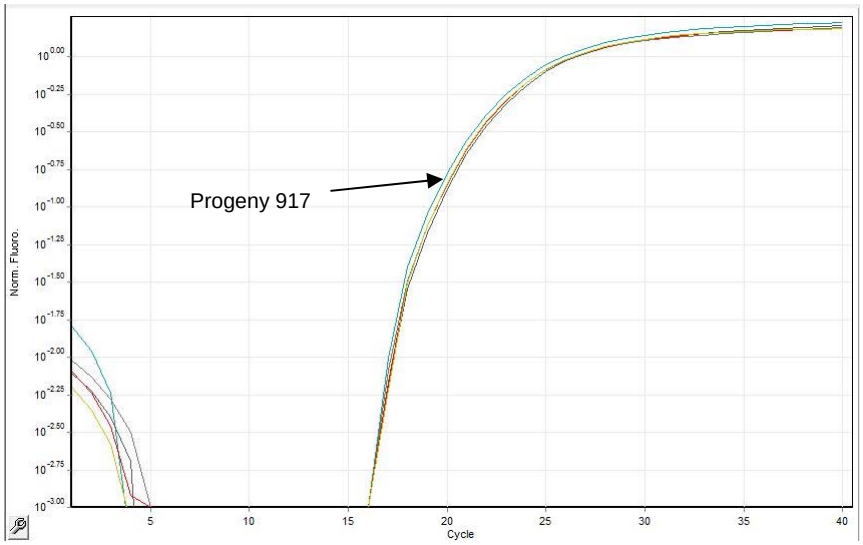


Figure 6.6: ENO3-11 quantitation curve for PCR amplification prior to HRM.

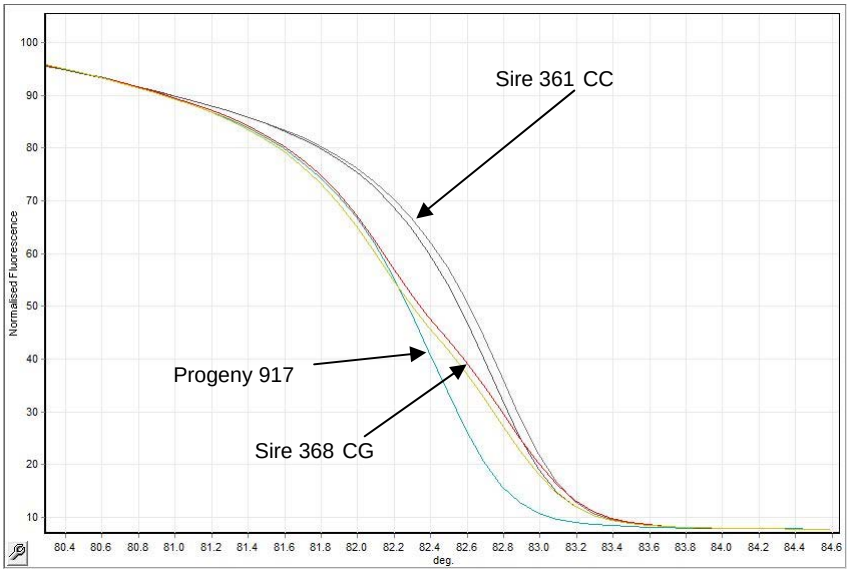


Figure 6.7: ENO3-11 HRM melt curve.

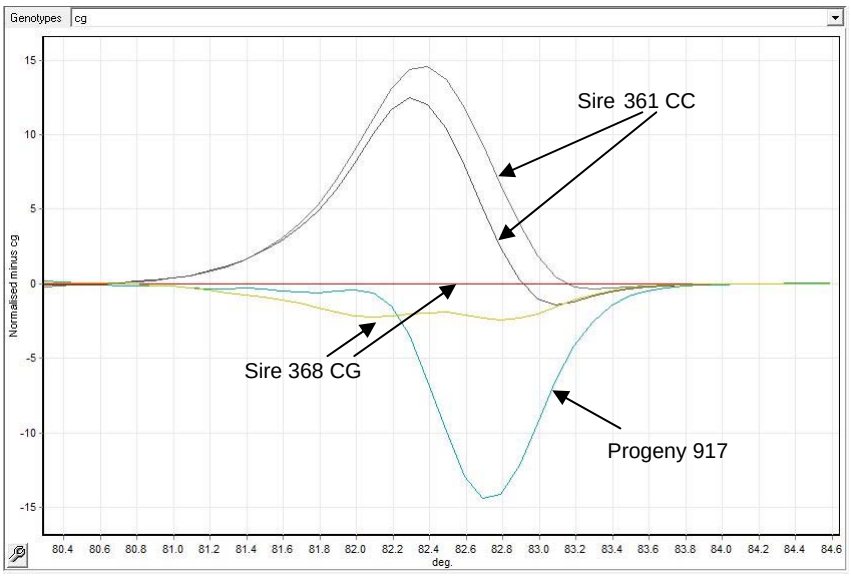


Figure 6.8: ENO3-11 HRM difference graph.

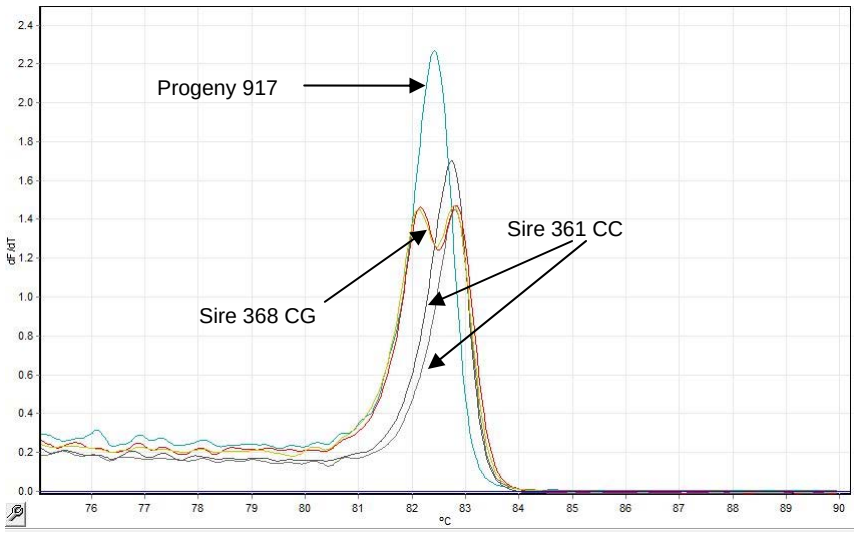


Figure 6.9: ENO3-11 HRM melt curve analysis.

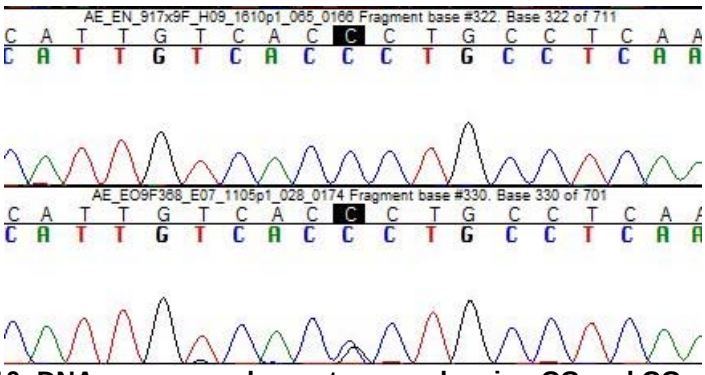


Figure 6.10: DNA sequence chromatagram showing CC and CG genotypes (marked – black highlight). Progeny 917 (top) CC and Sire 368 (bottom) CG.

6.3.2 Allele Specific Polymerase Chain Reaction

Therefore, allele specific PCR (ASP) was used to confirm the genotype results of some SNPs. The heterozygous samples usually amplify with the same or similar efficiency as the homozygotes targeted by the allele specific primer. The heterozygotes had previously been determined by the HRM analysis, and therefore, the two homozygotes were easily distinguished using ASP (Figure 6.11).

Two allele specific primer pairs were designed for SNP PPARG-2. The forward primer of one pair was specific for the G allele, while the reverse primer of the other pair was specific for the C allele. These were used in the same PCR and resolved on an agarose gel, where the three genotypes were easily identifiable (Figure 6.12).

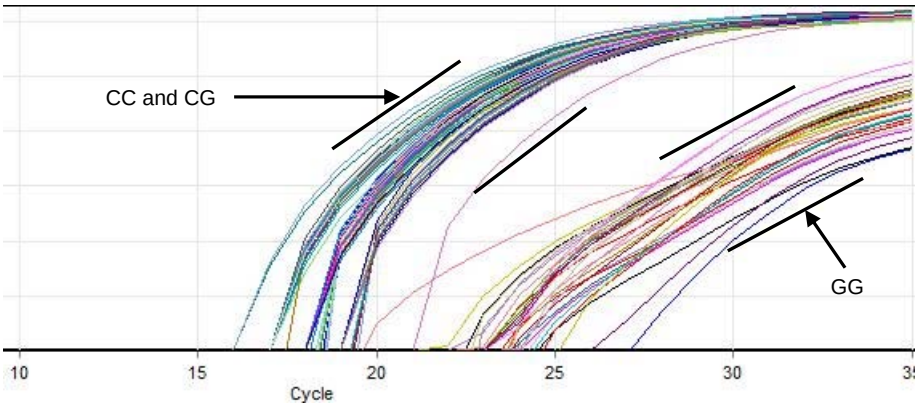


Figure 6.11: ESR1-2 allele specific PCR quantitation curve.
The similar amplification efficiency of the heterozygote and one homozygote is shown by the CG and CC genotypes compared to the GG genotype.

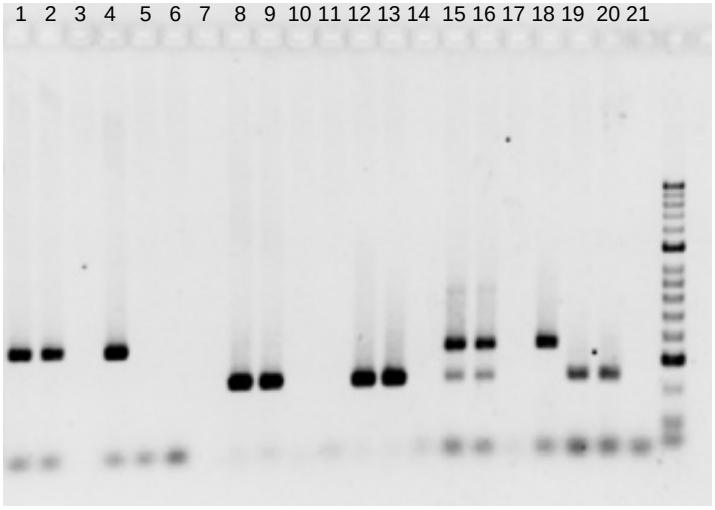


Figure 6.12: Image of agarose gel showing result of multiplexed allele specific PCR trial of known genotypes.
Lanes 1 – 7 G allele primers, 8 – 14 C allele primers, 9 – 21 G and C allele primers. Lanes 1, 2, 8, 9, 15 and 16 were GC. Lanes 3 (failed), 4, 10, 11, 17 (failed) and 18 were GG. Lanes 5, 6, 12, 13, 19 and 20 were CC. Lanes 7, 14 and 21 were no template control.

6.3.3 HRM genotyping of two SNPs within one PCR fragment

HRM genotyping can also be used for two variants within a single amplicon. Herein, HRM was used to genotype amplicons that included two SNPs 18bp apart, and indicated the presence of five of the nine possible genotypes (Figures 6.13 to 6.19). Direct sequencing was used to confirm these as GG/GG, GA/GA, AA/AA, GA/AA and GG/GA (Figure 6.20).

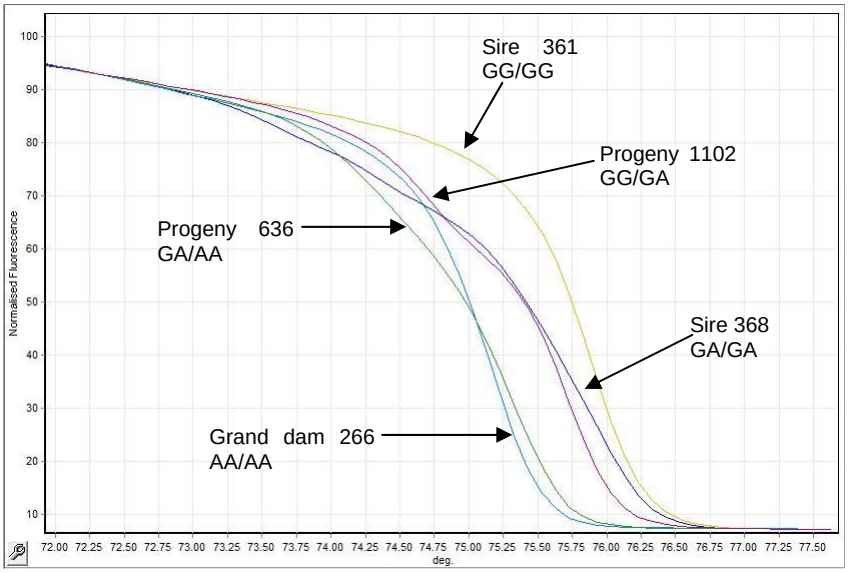


Figure 6.13: NCOA7 SNP 1 and 2 HRM melt curve.

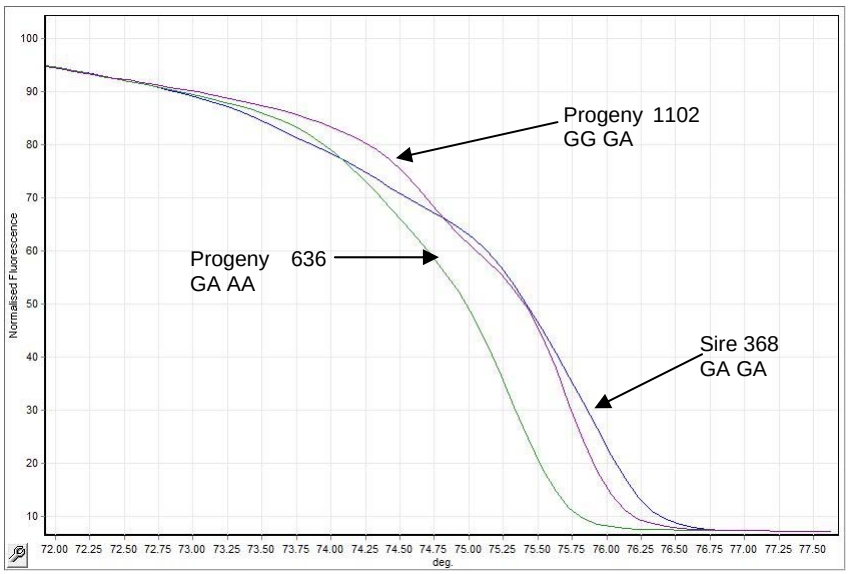


Figure 6.14: NCOA7 SNP 1 and 2 HRM melt curve without homozygous controls.

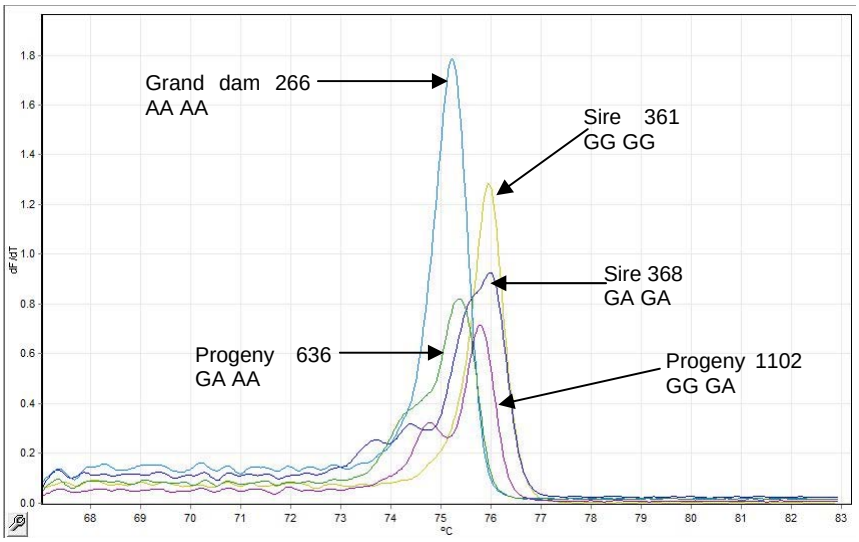


Figure 6.15: NCOA7 SNP 1 and 2 HRM melt curve analysis.

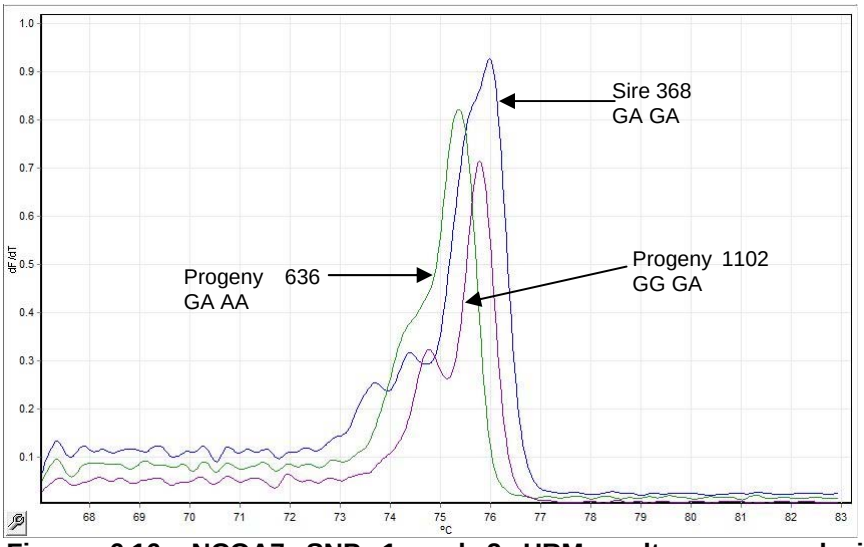


Figure 6.16: NCOA7 SNP 1 and 2 HRM melt curve analysis without homozygous controls.

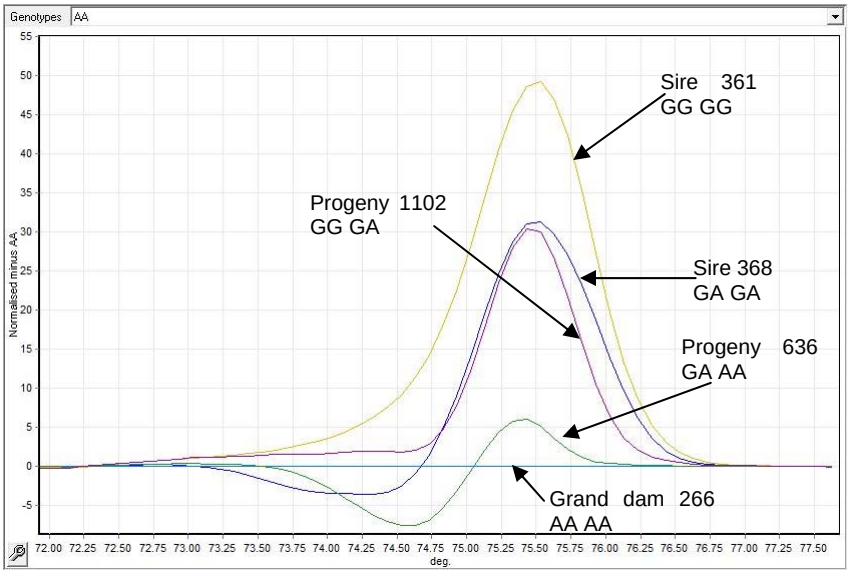


Figure 6.17: NCOA7 SNP1 and 2 HRM difference graph. Sample compared to AA genotype.

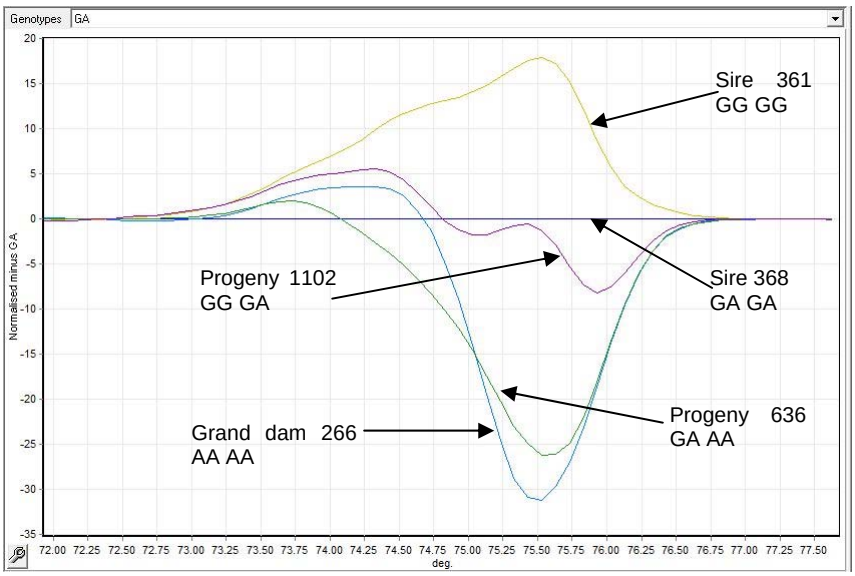


Figure 6.18: NCOA7 SNP1 and 2 HRM difference graph. Sample compared to GA genotype.

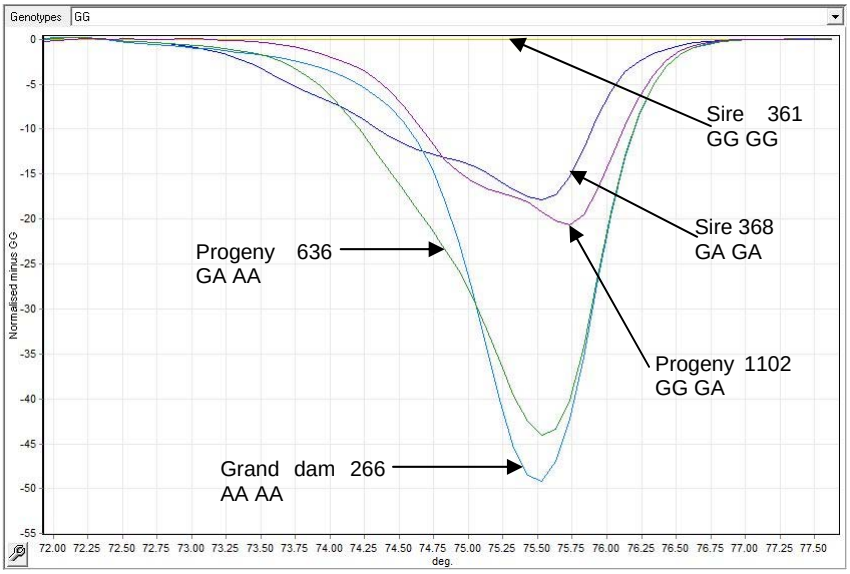


Figure 6.19: NCOA7 SNP1 and 2 HRM difference graph. Samples compared to GG genotype.

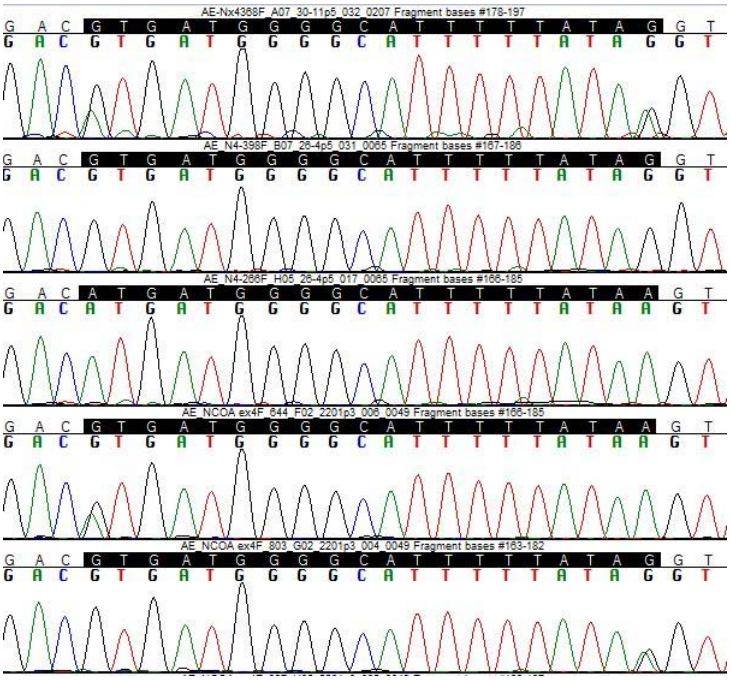


Figure 6.20: Sequence chromatograms of five genotypes at NCOA7 -1 and 2 SNPs.
SNPs are at both ends of the highlighted sequence. In order from top, genotypes are; GA GA, GG GG, AA AA, GA AA, GG GA.

6.3.4 Genotype frequencies

Although all of the 366 steers and heifers from the Davies Gene Mapping Herd were genotyped, the genotypes of some animals were not confirmed due to repeated reaction failure or inconclusive results. There were less than 10 animals not genotyped for any one SNP, except for NCOA7-21 (10) and TEK1-7 (23). The minor allele genotype frequencies of the 19 single nucleotide polymorphisms (SNPs) genotyped in this study and the 14 other SNPs included for the association studies varied from 0.09 to 0.48 (Table 6.1). Two SNPs (PPARa-4 and TEK1-5) had a minor allele frequency less than 10%, while the highest was 48% (EDG1-UTR, ESR1-2 and PPARG-2). Only one of the homozygote genotypes was represented in the TEK1-5 polymorphism, and in 7 other polymorphisms, the alternate homozygote occurred in less than 10 individuals (Table 6.1).

Table 6.1: Genotyped single nucleotide polymorphisms and allele frequencies.

SNP	genotype	11	12	22	Missing genotypes	p	q*
ACACA-7	GA	21	155	189	1	0.73	0.27
ALDH8A1-10	AG	151	187	27	1	0.67	0.33
ALDH8A1-15	CT	242	114	9	1	0.82	0.18
ALDH8A1-16	AG	16	190	158	2	0.70	0.30
ADIPOQ	GA	20	140	201	5	0.75	0.25
ATP2B4-32	AG	21	131	209	5	0.76	0.24
ATP2B4-33	AC	221	115	22	8	0.78	0.22
BCMO1-4	AC	7	79	279	1	0.87	0.13
BCMO1-7	CT	11	142	212	1	0.78	0.22
BCMO1-8	CG	221	132	12	1	0.79	0.21
BCMO1-13	CT	55	171	139	1	0.62	0.38
EDG1-UTR	GC	77	226	62	1	0.52	0.48
ENO3-5	GA	61	175	129	1	0.59	0.41
ENO3-11	GC	1	78	286	1	0.89	0.11
ESR1-2	GC	85	176	102	3	0.52	0.48
ESR1-14	GA	9	97	259	1	0.84	0.16
LAMIN-1	CA	270	85	10	1	0.86	0.14
NCOA7-1/2#	genotype GA GA	1111 1112 173 51	1212 113	1222 2222 8 19	2	0.74	0.26
NCOA7-1	GA	224	121	19	2	0.78	0.22
NCOA7-2	GA	173	164	27	2	0.70	0.30
NCOA7-18	GA	117	171	72	6	0.56	0.44
NCOA7-21	GA	18	113	225	10	0.79	0.21
NCOA7-22	AT	17	110	236	3	0.80	0.20
PPARa-4	GA	330	30	1	5	0.96	0.04
PPARG-2	GC	96	190	78	2	0.52	0.48
PPARG-6	CT	172	156	37	1	0.68	0.32
TEK1-1	AG	5	268	87	6	0.61	0.39
TEK1-2	AT	59	185	115	7	0.58	0.42
TEK1-3	GC	59	189	112	6	0.57	0.43
TEK1-4	CT	67	171	126	2	0.58	0.42
TEK1-5	GT	0	67	297	2	0.91	0.09
TEK1-6	GA	206	150	8	2	0.77	0.23
TEK1-7	CT	118	174	51	23	0.60	0.40

 # NCOA7-1AB is a combination of two SNPs genotyped in one reaction, which resulted in five genotypes.
*** q = minor allele frequency**

6.3.5 Association studies

Linear regression analysis was used to identify any association between each SNP and the measured traits (Table 6.2). The initial model of cohort, breed of dam, sire and SNP

(SNP effect), showed all traits, with the exception of rib fat, were associated with at least one SNP. As the fat deposition traits (i.e. not fatty acid composition traits) are likely to be affected by the size of the animal, carcass weight (hscw) was included as a covariate. The most noticeable effect from this was with the TEK1-5 polymorphism, which was more significant for channel fat (P = 0.095 to 0.041). Also, the association was more significant between ACACA-7 and seam fat (P = 0.06 to 0.028) and ALDH8A1-16 and marble score (marbam, P = 0.06 to 0.007).

The myostatin F94L variant genotype has a large impact on muscle size, and therefore, this was included in the model, as was the interaction between the F94L variant and each SNP. Including the myostatin variant had little impact on the results. Most SNPs that were associated with a fat trait in the first model were also associated with that trait when myostatin was included in the model. The exceptions to this were ACACA-7 (marble score), EDG1-UTR (marble score), and ESR1-2 (eye muscle area). Conversely, the associations of BCMO1-13, TEK1-1 and TEK1-5 with IMF%, EMA and seam fat respectively, only became significant when the effect of the myostatin variant was included.

Overall, *BCMO1* and *TEK1* appeared to have the most widespread influence on fat and muscle traits. Both of these genes were associated with variation in omental, channel and seam fat, with *BCMO1* also associated with P8 fat depth and carcass weight (HSCW), and *TEK1* associated with eye muscle area. Only these two genes were associated with both internal fat depots (omental and channel fat) and no gene was associated with both subcutaneous fat depots (P8 and rib fat). No SNP was directly associated with variation in rib fat, with or without the myostatin variant included in the model. However, there was evidence of an interaction between the myostatin F94L variant and polymorphisms in both *LMNA* and *NCOA7* being associated with variation in rib fat.

In total, 16 SNPs from eight genes had a significant interaction with the myostatin F94L variant on fat and muscle traits, without the SNP directly affecting that trait (Table 6.2). Of these genes, individually *ENO3* had no effect on any trait except for a marginal effect (ENO3-5; F = 0.059) on melting point, and *ATP2B4* was only associated with variation in the desaturase index. With the exception of P8 fat depth, all fat deposition traits (as well as carcass weight (HSCW) and eye muscle area) were associated with an interaction between *myostatin* and at least one gene.

Table 6.2: Traits affected by SNPs (F probability)

SNP	SNP effect	SNP effect with hcsw	SNP effect with MSTN	MSTNxSNP interaction
ACACA-7	mbusms (0.018)	mbusms (0.019)		
ACACA-7	seamfat (0.060)	seamfat (0.028)		
ALDH8A1-10	marbam (0.004)	marbam (0.004)	marbam (0.003)	
ALDH8A1-10			mbms (0.051)	
ALDH8A1-15	mbusms (0.059)		mbusms (0.052)	
ALDH8A1-16	marbam (0.007)	marbam (0.007)	marbam (0.006)	
ADIPOQ-1	mbms (0.048)	mbms (0.044)	mbms (0.035)	
ATP2B4-32				hscw (0.048)
ATP2B4-32				elong (0.023)
ATP2B4-33	desat (0.008)		desat (0.003)	
ATP2B4-33				hscw (0.048)
ATP2B4-33				mbms (0.013)
ATP2B4-33				mbusms (0.017)
ATP2B4-33				omental (0.029)
BCMO1-13	hscw (0.006)		hscw (0.012)	
BCMO1-13			IMF% (0.044)	
BCMO1-4	channel (0.033)	channel (0.034)	channel (0.036)	
BCMO1-4	omental (0.041)	omental (0.040)	omental (0.042)	
BCMO1-4	P8 (0.003)	P8 (0.004)	P8 (0.004)	
BCMO1-7	hscw (0.031)		hscw (0.026)	
BCMO1-8	channel (0.009)	channel (0.009)	channel (0.012)	
BCMO1-8	P8 (0.021)	P8 (0.021)	P8 (0.022)	
BCMO1-8	seamfat (0.006)	seamfat (0.006)	seamfat (0.012)	
EDG1-UTR	mbusms (0.048)	mbusms (0.049)		mbusms (0.031)
EDG1-UTR	seamfat (0.006)	seamfat (0.003)	seamfat (0.011)	
EDG1-UTR				IMF% (0.018)
ENO3-11				EMA (0.031)
ENO3-11				marbam (0.046)
ENO3-11				seamfat (0.026)
ENO3-5	meltpt (0.059)	meltpt (0.059)		meltpt (0.038)

Table 6.2 continued.

SNP	SNP effect	SNP effect with hcsw	SNP effect with MSTN	MSTNxSNP interaction
ESR1 - 2	EMA (0.017)			EMA (0.008)
ESR1 - 2	omental (0.015)	omental (0.014)	omental (0.017)	
ESR1 - 2				hscw (<0.001)
ESR1 - 2				seamfat (0.038)
ESR1-14	marbam (0.042)	marbam (0.043)	marbam (0.019)	
ESR1-14			mbusms (0.052)	
LAMIN-1	fatpc (0.006)		fatpc (0.033)	
LAMIN-1	fattobn (0.013)		fattobn (0.038)	
LAMIN-1	meltpt (0.045)		meltpt (0.039)	
LAMIN-1				channel (0.002)
LAMIN-1				ribfat (0.002)
NCOA7-2				ribfat (0.051)
NCOA7-2				meltpt (0.059)
NCOA7-18	mbusms (0.045)	mbusms (0.044)	mbusms (0.025)	
NCOA7-21				fattobn (0.037)
NCOA7-21				omental (0.027)
NCOA7-21				ribfat (0.041)
NCOA7-22				omental (0.030)
NCOA7-22				ribfat (0.050)
PPARa-4	seamfat (0.003)	seamfat (0.006)	seamfat (0.019)	
PPARG-2	desat (0.026)		desat (0.030)	
PPARG-2	mufa (0.023)		mufa (0.025)	
PPARG-2	sfa (0.017)		sfa (0.018)	
PPARG-2				EMA (0.015)
PPARG-2				hscw (0.041)
PPARG-6				omental (0.018)
TEK1-1	channel (0.015)	channel (0.045)	channel (0.021)	
TEK1-1	desat (0.043)		desat (0.027)	
TEK1-1			EMA (0.050)	
TEK1-1			P8 (0.056)	
TEK1-2				EMA (0.035)
TEK1-4				omental (0.012)
TEK1-5		channel (0.041)		channel (0.037)
TEK1-5	omental (0.002)	omental (0.001)	omental (0.003)	
TEK1-5			seamfat (0.041)	
TEK1-7				EMA (0.027)

hscw = hot standard carcass weight, MSTN = myostatin F94L genotype fitted as a co-variate.

6.3.6 Single nucleotide polymorphism effects

The single nucleotide polymorphisms (SNPs) that had a significant effect on any fat or muscle trait (Table 6.2) were analysed for additive, dominance, and allele substitution effects, and the variance due to each SNP (SNP effect) (Table 6.3). The SNP effects on fat traits ranged from 0.8% to 27.5%, with nearly half (17) being 5% or greater. Two SNPs in *TEK1* had large effects on channel fat (27.5%, TEK1-1) and omental fat (14.1%, TEK1-5) as well as a smaller, but still significant, effect on seam fat (5.5%, TEK1-5). However, the effects on rump fat thickness (P8) and marble score (marbam) were lower, 3% and 1%, respectively. Two of the *BCMO1* SNPs affected fat deposition traits, BCMO1-4 (P8, channel and omental fat) and BCMO1-8 (P8, channel and seam fat). Although the BCMO1-4 SNP effect on omental and channel fat was similar (5.5% and 5.4%), as was the BCMO1-8 SNP effect on channel fat (4.1%), the effect of these SNPs on rump fat (P8) was very different (19.7% and 3.3%). The C allele substitution in BCMO1-4 indicates that this SNP increased P8 fat but decreased both omental and channel fats (Figures 6.21 – 6.23).

The allele substitution effect indicated the additive effect of each allele (Table 6.3). The effects ranged from 0.3% to 30.3% of the minimum least squares means value of that SNP, but most of the effects were less than 10%. Only one of the mapping herd progeny was homozygous for the alternate PPARa-4 allele, and there were no progeny homozygous for the alternate TEK1-5 allele (Table 6.1), and therefore, there were no allele substitution effects available for these SNPs. When the allele substitution effect was expressed as the number of standard deviations from the mean, the range was 0.01 (BCMO1-8, P8) to 1.05 (PPARa-4, seam fat) with an average of 0.3 standard deviations.

Table 6.3: Single nucleotide polymorphism effects, including additive and dominance.

SNP	trait	SNP effect (var. %)	Trait average	Allele effect (% mean)	Allele effect (St. Dev)	slope (se)		t pr	
						additive	dominance	additive	dominance
ACACA-7	mbusms	2.4	381	4.3	0.24	15.9 (8.76)	1.22 (9.67)	0.071	0.900
ALDH8A1-10	marbam	6.4	1.53	15.8	0.31	0.20 (0.07)	0.01 (0.08)	0.005	0.941
ALDH8A1-10	mbms	3.9	1.73	10.0	0.27	0.15 (0.06)	0.11 (0.07)	0.015	0.114
ALDH8A1-15	mbusms	9.1	381	7.5	0.43	-28.3 (12.4)	-32.5 (13.7)	0.022	0.019
ALDH8A1-16	marbam	4.0	1.53	10.7	0.23	-0.15 (0.09)	-0.07 (0.10)	0.099	0.518
ADIPOQ	mbms	5.0	1.73	10.3	0.31	0.17 (0.07)	-0.07 (0.08)	0.014	0.399
ATP2B4-33	desat	12.8	79.1	2.1	0.42	1.60 (0.47)	0.98 (0.56)	<0.001	0.083
BCMO1-13	hscw	5.6	334	2.8	0.26	-9.33 (3.38)	7.19 (4.03)	0.006	0.075
BCMO1-13	imf%	2.7	5.2	4.3	0.16	-0.21 (0.13)	0.35 (0.15)	0.099	0.024
BCMO1-4	p8am	19.7	12.3	16.5	0.47	1.99 (0.93)	-3.38 (1.04)	0.032	0.001
BCMO1-4	channel	5.4	12.5	13.2	0.44	-1.30 (0.65)	0.42 (0.73)	0.045	0.565
BCMO1-4	omental	5.5	12.0	9.6	0.37	-1.03 (0.70)	0.02 (0.78)	0.143	0.981
BCMO1-7	hscw	3.2	334	1.0	0.10	-3.27 (6.05)	13.5 (6.53)	0.589	0.039
BCMO1-8	p8am	3.3	12.3	0.3	0.01	-0.01 (0.72)	-1.47 (0.82)	0.986	0.074
BCMO1-8	channel	4.1	12.5	0.9	0.04	-0.09 (0.5)	-1.18 (0.57)	0.858	0.040
BCMO1-8	seamfat	6.7	308	26.5	0.39	56.3 (25.3)	12 (28.8)	0.027	0.677
EDG1-UTR	seamfat	4.1	308	5.3	0.12	-17 (14.6)	-50.3 (17.4)	0.245	0.004
EDG1-UTR	mbusms	2.5	381	3.9	0.21	14.2 (6.37)	-4.73 (7.68)	0.026	0.539
ENO3-5	meltpt	1.8	37.4	0.9	0.14	-0.35 (0.21)	-0.49 (0.30)	0.165	0.105
ESR1 - 2	omental	3.7	12.0	2.8	0.12	-0.30 (0.24)	0.88 (0.33)	0.213	0.009
ESR1 - 2	EMA	1.4	80.7	0.3	0.02	0.23 (0.81)	-2.61 (1.16)	0.781	0.025
ESR1-14	marbam	9.1	1.53	30.3	0.47	-0.29 (0.125)	0.13 (0.13)	0.014	0.326
ESR1-14	mbusms	3.3	381	4.7	0.25	-16.5 (12.5)	-4.6 (14)	0.188	0.744
LAMIN-1	fatpc	12.7	13.6	7.2	0.44	0.88 (0.35)	0.93 (0.41)	0.013	0.023
LAMIN-1	fattobn	11.8	0.78	10.0	0.45	0.07 (0.03)	0.063 (0.03)	0.012	0.038
LAMIN-1	meltpt	9.2	37.4	2.7	0.37	0.96 (0.44)	1.29 (0.52)	0.031	0.013
NCOA7-18	mbusms	3.4	381	3.5	0.19	12.3 (6.6)	15.49 (7.49)	0.062	0.040
PPARa-4	seamfat	10.6	308		1.05	-77.5 (32.7)		0.019	
PPARG-2	mufa	3.0	49.2	1.3	0.18	0.67 (0.28)	0.60 (0.39)	0.019	0.120
PPARG-2	sfa	3.4	47.0	1.5	0.19	-0.72 (0.29)	-0.63 (0.40)	0.013	0.110
PPARG-2	desat	2.9	79.1	0.9	0.19	0.77 (0.31)	0.48 (0.42)	0.013	0.249
TEK1-1	EMA	0.8	80.7	8.1	0.53	-5.58 (2.77)	6.93 (2.84)	0.045	0.015
TEK1-1	channel	27.5	12.5	27.4	0.74	-2.24 (0.80)	1.96 (0.82)	0.006	0.018
TEK1-1	desat	4.0	79.1	1.0	0.21	-0.76 (1.00)	-0.57 (1.03)	0.447	0.582
TEK1-1	p8am	3.0	12.3	15.0	0.35	-1.57 (1.15)	0.31 (1.18)	0.174	0.796
TEK1-5	omental	14.1	12.0			1.61 (0.53)		0.003	
TEK1-5	seamfat	5.5	308			-56.3 (27.4)		0.041	

Effect: SNP = SNP variance as a percentage of total variance, Allele % = change in each trait per allele as a percentage of the lower least squares mean, Allele St. Dev = number of residual standard deviations from the trait mean, per allele.
mbusms: USDA marble score, marbam: AUS-MEAT marble score, mbms: MSA marble score, channel: channel fat, desat: desaturation index, hscw: hot standard carcass weight, p8am: rump fat depth at P8, omental: omental fat, seamfat: intermuscular fat area, meltpt: melting point, fat%: total fat%, fattobn: fat to bone ratio, imf%: intramuscular fat %, elong: elongation index, mufa: mono-unsaturated fatty acids – percent of triacylglyceride, sfa: saturated fatty acids – percent of triacylglyceride, EMA: eye muscle area.

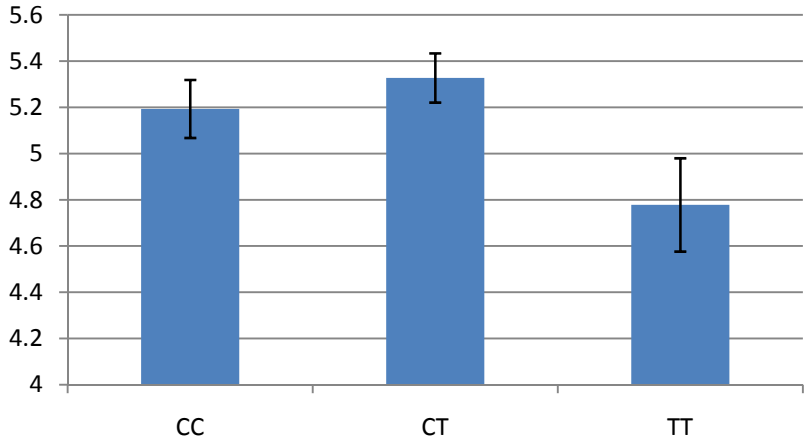


Figure 6.21: Least squares means of BCMO1-4 SNP effect on subcutaneous (P8) fat.

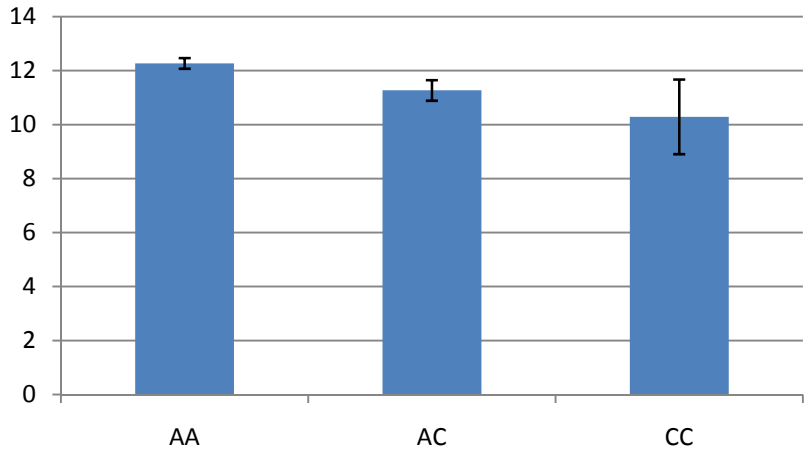


Figure 6.22: Least squares means of BCMO1-4 SNP effect on omental fat.

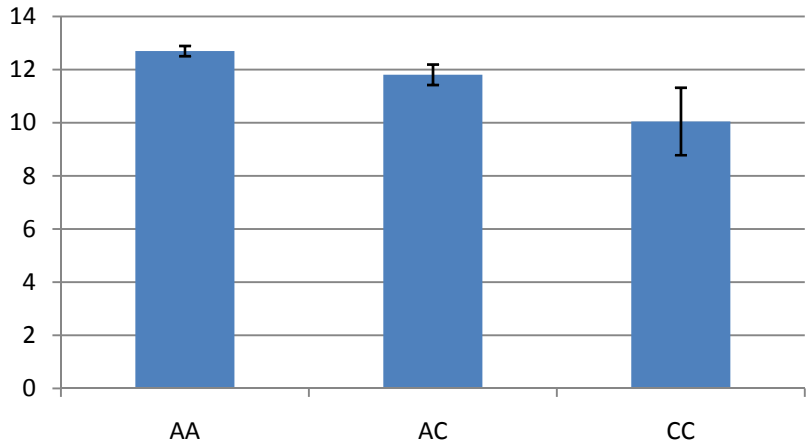


Figure 6.23: Least squares means of BCMO1-4 SNP effect on channel fat.

6.3.7 Correlations

Genetic correlations were categorised as being high (greater than r_G = 0.6), moderate (r_G = 0.4 – 0.6), low (r_G = 0.2 – 0.4), and nonexistent (r_G = 0 – 0.2) (Table 6.4). The correlations were highest between intramuscular fat % and melting point of intramuscular fat (r_G = 0.94), intramuscular fat % and marble score (marbam) (r_G = 0.82), and melting point and marble score (r_G = 0.81). Of the 120 correlations tested, 24 were greater than 0.4, while 41 were less than 0.1, including no correlation between eye muscle area and intermuscular (seam) fat or intramuscular fat % and subcutaneous (P8) fat.

Table 6.4: Genetic correlations calculated from SNPs for fat and muscle traits.

	channel	desat	elong	ema	hscw	imf%	marbam	mbms	mbusms	meltpt	mufa	omental	p8	ribfat	seam fat	sfa
channel	1	**0.47**	0.16	**0.40**	**0.40**	0.13	0.28	0.01	-0.14	0.07	**0.46**	**0.53**	0.05	0.28	0.15	**-0.53**
desat		1	-0.22	0.01	0.02	0.16	0.35	0.28	0.19	0.07	0.57	-0.01	-0.20	0.15	0.28	**-0.63**
elong			1	**0.49**	0.02	0.24	0.06	-0.11	-0.32	0.16	**0.52**	**0.48**	0.12	-0.05	-0.30	**-0.55**
ema				1	0.70	0.11	0.29	0.07	-0.24	0.09	0.21	0.14	**0.45**	0.14	0.00	-0.35
hscw					1	0.04	0.22	-0.09	-0.35	0.03	-0.15	-0.11	**0.62**	0.01	-0.02	0.05
imf%						1	**0.82**	**0.40**	0.05	**0.94**	-0.03	0.27	0.00	0.09	0.08	-0.27
marbam							1	**0.64**	0.25	0.81	-0.03	0.29	0.02	0.24	0.14	-0.31
mbms								1	**0.85**	0.38	0.01	0.01	-0.10	0.18	0.27	-0.16
mbusms									1	0.06	-0.02	-0.08	-0.27	0.22	**0.44**	0.02
meltpt										1	-0.19	0.30	-0.04	0.02	0.12	-0.20
mufa											1	0.35	-0.11	0.08	0.02	**-0.88**
omental												1	-0.26	0.04	-0.15	**-0.49**
p8													1	-0.15	0.06	0.17
ribfat														1	**0.47**	-0.01
seam fat															1	-0.06
sfa																1

channel: channel fat, desat: desaturation index, elong: elongation index, ema: eye muscle area, hscw: hot standard carcass weight, imf%: intramuscular fat %, marbam: AUS-MEAT marble score, mbms: MSA marble score, mbusms: USDA marble score, meltpt: melting point, mufa: mono-unsaturated fatty acids – percent of triacylglyceride, omental: omental fat, p8am: rump fat depth at P8, ribfat: rib fat, seamfat: intermuscular fat area, sfa: saturated fatty acids – percent of triacylglyceride.

6.3.8 Cluster analysis

A cluster diagram was generated from the genetic correlations to indicate which fat deposition and muscle traits have similar genetic influences (Figure 6.24). All of the traits tested with this method were highly correlated, and therefore, it was anticipated there would be a strong clustering effect. As expected, the internal fat depots (omental and channel fat) were clustered together, as were the marble scores and intramuscular fat % (mbusms, mbms, marbam and IMF%). However, the subcutaneous depots were not clustered; P8 fat was associated with eye muscle area and carcass weight, whereas rib fat was associated with intermuscular (seam) fat.

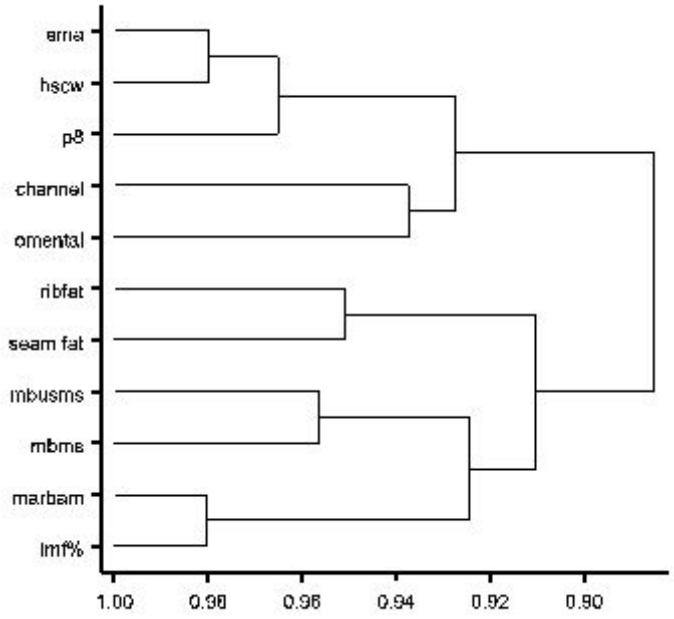


Figure 6.24: Cluster analysis of genetic correlations, Davies Gene Mapping herd.
ema: eye muscle area, hscw: hot standard carcass weight, p8: rump fat depth at P8, channel: channel fat, omental: omental fat, ribfat: rib fat, seamfat: intermuscular fat area, mbusms: USDA marble score, mbms: MSA marble score, marbam: AUS-MEAT marble score, imf%: intramuscular fat %.

6.3.9 Within gene SNP interactions

To determine if haplotype effects might be present, the interaction between SNPs within a particular gene was tested using general linear regression (Table 6.5). In general, there was little similarity between individual SNP association and interactions between those SNPs. Although all of the *ALDH8A1* SNPs individually were associated with variation in marble score (Table 6.2), the only interaction between these SNPs was associated with seam fat. This was also observed with SNPs in *NCOA7* and *TEK1*. In *BCMO1,* although there was some similarity between individual SNP effects and interactions between these SNPs, there were other traits where the variation was only associated with SNP interactions. There was no significant association between SNPs in *ESR1* or *PPARG* and variation in fat traits.

Table 6.5: Within gene SNP interactions (significance).

SNPs	trait (Fpr)			
ALDH8A1				
10 x 15				
10 x 16				
15 x 16	seam fat (0.014)			
ATP2B4				
32 x 33	p8 (0.037)			
BCMO1				
113 x 18	fatpc (<0.001)	fattobn (0.001)	hscw (0.034)	p8 (0.019)
14 x 18	fattobn (0.024)	marbam(0.040)	p8 (0.025)	rbft (0.009)
14 x 113				
17 x 18	imf% (0.005)			
ENO3				
5 x 11	EMA (0.010)			
ESR1				
2 x 4				
NCOA7				
1 x 2	meltpt (0.041)	omental (0.033)		
1 x 18				
1 x 21	mbms (0.042)			
1 x 22				
2 x 18				
2 x 21	mbms (0.003)			
2 x 22	fattobn (0.048)			
18 x 21				
18 x 22	mufa (0.053)	sfa (0.027)		
21 x 22	meltpt (0.007)	desat (0.009)	sfa (0.035)	
PPARG				
2 x 6				
TEK1				
7 x 4	EMA (0.041)			
7 x 6	marbam (0.009)			
7 x 1	meltpt (0.045)			
7 x 2				
4 x 6	p8 (0.028)	rbft (0.015)		
4 x 3	mufa (0.018)	sfa (0.026)		
4 x 1				
5 x 6				
5 x 2	fattobn (0.011)	hscw (0.024)	p8 (0.001)	
3 x 1	hscw (0.044)			
3 x 2				
1 x 2				
5 x 3	marbam (0.027)	mbms (0.031)		
6 x 3	marbam (0.010)	mbms (0.003)	mbusms (0.018)	
6 x 1	marbam (0.015)	mbms (0.038)		

6.3.10 Between gene SNP interactions

To determine if there may be epistatic effects, the association of fat and muscle trait variation with the interactions of SNPs in different genes was assessed using general linear regression. Where there was more than one SNP in a gene, the results of all SNPs within the gene were combined, and gene by gene interactions were examined (Table 6.6). *BCMO1* (91)*, NCOA7* (86)*, ENO3* (70) and *ALDH8A1* (67) were involved in the most interactions associated with fat trait variation. The interaction between *BCMO1* and *NCOA7* were associated with variation in the most traits (13), followed by *NCOA7* and *ENO3* (12)*, BCMO1* and *TEK1* (11)*, ENO3* and *ESR1* (10) and *BCMO1* and *ENO3* (10)*.* Variation in seam fat was associated with the highest number of between gene interactions (36), while rib fat variation was associated with the least number (13).

Table 6.6: Interactions between genes. All SNPs within each gene are included.

Genes	EMA	fat%	fattobn	hscw	imf%	channel	marbam	mbms	mbusms	omental	p8am	ribfat	seam fat
ACACA*ADIPOQ						X							
ACACA*ALDH8A1		X	X		X					X			
ACACA*ATP2B4													X
ACACA*BCMO1	X			X		X				X	X		
ACACA*EDG1													
ACACA*ENO3				X	X							X	
ACACA*LAMIN													
ACACA*NCOA7	X	X				X			X				X
ACACA*PPARA	X									X			
ADIPOQ*ATP2B4													X
ADIPOQ*BCMO1	X			X				X		X		X	X
ADIPOQ*ENO3	X							X	X				
ADIPOQ*LAMIN													
ADIPOQ*NCOA7	X	X		X	X		X				X		
ADIPOQ*PPARA													
ALDH8A1*ADIPOQ	X		X						X				
ALDH8A1*ATP2B4	X	X		X			X				X		
ALDH8A1*BCMO1		X	X	X		X	X			X	X	X	X
ALDH8A1*ENO3		X	X	X		X	X	X	X	X	X		
ALDH8A1*LAMIN		X	X			X							X
ALDH8A1*NCOA7					X	X	X		X	X	X		X
ALDH8A1*PPARA												X	X
ATP2B4*BCMO1		X	X	X	X						X		X
ATP2B4*ENO3		X	X			X							X
ATP2B4*LAMIN		X	X										
ATP2B4*NCOA7				X	X	X	X	X	X				

Table 6.6 continued.

Genes	EMA	fat%	fattobn	hscw	imf%	channel	marbam	mbms	mbusms	omental	p8am	ribfat	seam fat
ATP2B4*PPARA													X
BCMO1*ENO3	X	X	X	X		X	X	X		X	X		X
BCMO1*LAMIN		X	X				X				X		X
BCMO1*NCOA7	X	X	X	X	X	X	X	X	X	X	X	X	X
BCMO1*PPARA	X						X	X	X				X
EDG1*ADIPOQ		X									X		
EDG1*ALDH8A1		X	X										
EDG1*ATP2B4													X
EDG1*BCMO1	X	X		X				X	X	X			X
EDG1*ENO3	X	X						X		X			
EDG1*LAMIN													
EDG1*PPARA													X
ENO3*LAMIN	X							X					
ENO3*NCOA7	X	X	X	X	X	X	X	X	X	X	X		X
ENO3*PPARA													X
ESR1*ACACA							X						X
ESR1*ADIPOQ													
ESR1*ALDH8A1	X	X	X		X			X		X	X		X
ESR1*ATP2B4								X	X		X	X	X
ESR1*BCMO1		X	X	X			X			X	X	X	X
ESR1*EDG1					X								
ESR1*ENO3	X	X	X		X		X	X	X		X	X	X
ESR1*Lamin1		X											
ESR1*NCOA7	X	X			X			X	X	X	X	X	X
ESR1*PPARA	X						X	X	X				X
LAMIN*NCOA7		X			X	X	X	X			X		X

Table 6.6 continued.

Genes	EMA	fat%	fattobn	hscw	imf%	channel	marbam	mbms	mbusms	omental	p8am	ribfat	seam fat
LAMIN*PPARA													
PPARA*NCOA7	X			X					X	X	X		X
PPARG*ACACA							X						
PPARG*ADIPOQ1				X	X	X		X	X				
PPARG*ALDH8A1	X	X	X	X		X	X		X		X		X
PPARG*ATP2B4		X	X						X				X
PPARG*BCMO1			X	X	X					X	X		X
PPARG*ENO3			X	X	X					X		X	X
PPARG*ESR1	X	X			X	X							
PPARG*LAMIN1	X	X	X			X			X				
PPARG*NCOA7	X	X	X	X		X		X	X	X		X	
PPARG*PPARA	X							X	X				X
PPARG*EDG1						X		X	X				
TEK1*ACACA	X					X	X		X	X			
TEK1*ADIPOQ1	X	X			X							X	
TEK1*ALDH8A1	X	X	X								X	X	
TEK1*ATP2B4		X	X	X				X			X		X
TEK1*BCMO1	X	X	X	X	X	X	X	X	X	X	X		
TEK1*EDG1					X								
TEK1*ENO3		X	X					X	X		X		X
TEK1*ESR1	X			X		X							
TEK1*LAMIN													
TEK1*NCOA7	X			X		X	X		X		X		
TEK1*PPARA													
TEK1*PPARG				X									

A full list including P values is included in Appendix K

6.4 Discussion

6.4.1 Single nucleotide polymorphisms for association studies

As none of the polymorphisms herein were obviously functional (i.e. altered amino acid sequence, Section 5.3.8), the polymorphisms were selected for genotyping the mapping herd progeny based on four criteria. Firstly, because the in/dels at mononucleotide sites were unconfirmed, these were excluded from consideration. Secondly, only polymorphisms suited to the two methods to be used to genotype the mapping herd (Section 6.2.1) were considered. Thirdly, SNPs where the three genotypes (both homozygotes and the heterozygote) were represented in the mapping sires were preferentially selected, but if this was not possible, at least one sire needed to be heterozygous. Lastly, haplotypes were formed to select SNPs that would be informative with respect to testing each haplotype likely to be inherited.

Generally, at least two SNPs in each gene were genotyped. However, there was only one SNP in *LMNA*, and one of the two variants in *EDG1* was an in/del and therefore, not genotyped. All of the SNPs in *ACACA* were in linkage disequilibrium and therefore, each SNP represented all of the other SNPs. Both of the *ADIPOQ* SNPs were tested but one was not suitable for high resolution melt genotyping (HRM). Although the aim was to genotype SNPs in which all three genotypes were represented in the mapping sires, in practice, this was not possible. For example, *TEK1* had a high SNP density which resulted in most of the SNPs being situated close to another SNP. This prevented the design of PCR primers that would anneal to sequence between the SNPs or that would produce an amplicon of an appropriate length for HRM genotyping, and therefore, these SNPs were not suitable for HRM genotyping. This included all of the five SNPs that had three genotypes. Therefore, the most appropriate of the available SNPs were genotyped. Also, there were only two genotypes for all of the

SNPs in *ESR1.* The *NCOA7* SNPs were genotyped using the Illumina system, and only those SNPs appropriate for this system were used. However, another region of *NCOA7* was later genotyped using HRM. Some of the *ENO3* SNPs were in linkage disequilibrium so one of these was selected and a second SNP some distance from the first was chosen, and there were only two SNPs in *PPARG* that were appropriate for genotyping.

Twenty two SNPs from the nine candidate genes initially selected for sequencing were genotyped in the mapping herd: *ACACA* (1), *ADIPOQ* (1), *EDG1* (1), *ENO3* (2), *ESR1* (2), *LMNA* (1), *NCOA7* (5), *PPARG* (2) and *TEK1* (7) (Table 5.4). In addition to these, there were data from ten SNPs from the four other candidate genes, *BCMO1*(4), *ALDH8A1* (3), *ATP2B4* (2) and *PPARA* (1), analysed for association with fat deposition.

6.4.2 High resolution melt analysis (HRM)

Eighteen SNPs were genotyped using HRM, either alone or jointly with allele specific polymerase chain reaction (ASP), in the work reported herein. Although HRM is a rapid and economical method of genotyping, it was not always successful for accurate genotyping, and hence, the need to combine HRM with ASP (Section 6.3.2). ASP was also used when both homozygous genotypes, and therefore all positive controls, were not immediately available. The combination of these methods enabled the genotyping of all 18 SNPs with a high degree of accuracy.

HRM separates PCR amplicons based on the variation in the temperature of DNA strand separation caused by the sequence variation of a SNP. The minor difference in melting temperature resulting from an A/T or G/C transversion can make the accurate detection of the temperature difference, and therefore accurate genotyping, quite

problematic. It is essential to have efficient amplification, and therefore, variation in DNA template concentration and quality must be avoided as far as practicable.

As well as a wide variation in melt temperatures (Section 6.3.1), accurate HRM genotyping is dependent on the availability of both homozygous and heterozygous controls. Although the third genotype can be putatively located by HRM and then confirmed by direct sequencing, this is not always practical when one allele is present at a low frequency. This was illustrated in the ENO3-11 polymorphism. The minor allele frequency was 11%, and the GG homozygous genotype was eventually only identified in one individual in the mapping herd. Without both homozygote controls, the HRM analysis indicated the presence of a homozygote GG animal (Section 6.3.1, Figures 6.6 – 6.9). However, sequencing results for that animal confirmed that it was actually CC (Figure 6.10). To overcome this problem in the ENO3-11 SNP, and also in four other SNPs where only two genotypes were available, allele specific PCR was used (Section 6.3.2).

Allele specific PCR (ASP) usually involves the use of PCR primers specific to an allele, in two separate assays, with the products of each assay resolved on an agarose gel to confirm the genotype of the DNA sample. The allele specific base at the 3' end binds preferentially to the matching allele, resulting in enhanced amplification of that allele. Although the specificity of the primer should prevent amplification of the alternate allele, there is often some amplification, albeit at a reduced rate (Germer *et al.*, 2000), and if the number of cycles is limited (20 – 25 cycles), the product of this false priming is not obvious on an agarose gel. ASP has also been used with real-time PCR to calculate allele frequencies in pooled DNA samples (Germer *et al.*, 2000, Liew *et al.*, 2004) and with HRM for individual genotyping (Germer and Higuchi, 1999).

An altered ASP protocol was used in conjunction with the standard HRM analysis to genotype five problematic polymorphisms (EDG1-UTR, ENO3-11, ESR1-2, LAMIN-1 and PPARG-2). A standard HRM analysis was completed and the heterozygous individuals identified. Allele specific primers were then designed for the minor alleles of EDG1-UTR, ENO3-11, ESR1-2 and LAMIN-1. These primers were used in a standard real-time PCR, and the homozygous samples were easily separated due to the obvious difference in amplification efficiency (Figure 6.11). Initially, the results were confirmed using gel electrophoresis, until the accuracy of the assay was evident.

For the remaining polymorphism (PPARG-2), allele specific primers were designed for both alleles. These primers were designed such that they could be multiplexed, amplified in a standard PCR and resolved via agarose gel electrophoresis. This approach was used in order to remove the need for separate reactions and therefore, reduce both the time and cost involved. Two primer pairs were designed, with the forward primer of one pair specific to one allele, and the reverse primer of the other pair specific to the other allele. The PCR products of both pairs were of a sufficiently different length (242bp and 363bp) to enable clear identification of each genotype (Figure 6.12).

As HRM genotyping relies on alteration to melt curves due to subtle differences in melting temperatures, usually only one SNP is genotyped in each amplicon. However, multiple genotypes from two SNPs in one amplicon have been successfully distinguished previously (Wittwer *et al.*, 2003). Therefore, it was decided to investigate how effectively this could be repeated for two SNPs 18bp apart in *NCOA7*.

Direct sequencing of the sires and their parents revealed that the genotype at SNP1 reflected the genotype at SNP2, indicating a high level of linkage disequilibrium, as was expected due to the close proximity of the SNPs. Sires 361 and 398, and their parents,

were homozygous for the G allele at both SNPs, sire 368 and his sire were heterozygous at both SNPs, while his dam was homozygous for the A allele at both SNPs. Primers were designed for a 142bp amplicon that included both SNPs, with NCOA7-1 69bp from the 5' end and NCOA7-2 54bp from the 3'end. Although only three genotypes (GG/GG, GA/GA, AA/AA) were present in the sires and their parents, HRM analysis of the mapping herd suggested the existence of two other genotypes (Figures 6.13 to 6.19). Direct sequencing confirmed these as GG/GA and GA/AA (Figure 6.20). However, although it was possible to identify each genotype, great care must be taken to ensure the accuracy of the results, including pedigree checks.

6.4.3 Association studies

Thirty two single nucleotide polymorphisms (SNPs) from 13 genes were genotyped in the Davies Gene Mapping herd, and then tested for association with fat and muscle traits recorded in that herd. The traits of main interest were related to fat deposition: subcutaneous fat (rib and P8 fat), internal fat (omental and channel fat), intermuscular fat (seam fat) and intramuscular fat (IMF% and marble score). Fatty acid composition traits (elongation index, desaturation index, mono-unsaturated fatty acid % and saturated fatty acid %), were included because of the link between fatty acid composition and marble score (Gilbert *et al.*, 2003, Smith *et al.*, 2009), and increased value of beef associated with higher monounsaturated fatty acid content (Siebert *et al.*, 2006). Eye muscle area and carcass weight were included because of the shared cell lineage and therefore, the possibility of transdifferentiation between adipocytes and myocytes (Sections 1.6 and 5.3.1.3).

6.4.3.1 Individual SNPs

Twelve SNPs were not associated with variation in any traits, and no single SNP was associated with variation in all of the measured traits. When the associations were

considered for each gene, *BCMO1* and *TEK1* were both associated with variation in four fat deposition traits, channel, omental, P8 and seam fat (Table 6.2). This could be attributed to the number of SNPs genotyped in these genes, four and seven respectively. However, only two of the *TEK1* SNPs were associated with variation in any traits. Furthermore, *NCOA7* had five SNPs genotyped but only one was associated with variation and in only one trait.

Although it is often assumed that increasing size is associated with increasing fatness, including carcass weight as a covariate had little effect on these results. Although with this model, TEK1-5 was more significantly associated with channel fat, with similar results for ACACA-7 (seam fat) and ALDH8A1-16 (marble score), these were single occurrences for each of these traits, and therefore, carcass weight was removed from all subsequent models for association analyses.

The *myostatin* genotype has a large impact on muscle mass (McPherron *et al.*, 1997) and fat deposition (Rodgers and Garikipati, 2008, Martinez *et al.*, 2010). Herein, the *myostatin* F94L variant of this gene was associated with eye muscle area, carcass weight, subcutaneous (P8) and seam fat, fat% and fat to bone ratio, intramuscular fat % (IMF%) and one of the marble scores (marbam) but not the other marble scores (mbms or mbusms) in the Davies Gene Mapping herd (Appendix L). Therefore, the *myostatin* F94L variant genotype was included both as a fixed effect and to test for interactions with each SNP. Including *myostatin* as a fixed effect had little effect other than on marble score, IMF%, intermuscular fat (seam fat) and eye muscle area. Since *Myostatin* mutations increase muscle mass and decrease fat deposition (Martinez *et al.*, 2010), the effect of the F94L genotype on eye muscle area was expected, as was the effect on IMF%, marble score and intermuscular (seam) fat, particularly as these fat depots are located within or between the muscles. An increase in muscle mass would likely reduce the amount of fat present due to increased energy requirement of the

muscle. Furthermore, due to the shared lineage of adipocytes and myocytes (Lin *et al.*, 2002), increased muscle hyperplasia would correspond to a decrease in adipose cell hyperplasia.

There was a significant interaction between SNPs from most genes and the *myostatin* F94L variant. *ATP2B4, ENO3* and *PPARG* were all only associated with variation in fatty acid synthesis (desaturation index, mono-unsaturated fatty acids, saturated fatty acids and melting point). However, SNPs in these genes interacted with *myostatin* to affect carcass weight, marble score, seam fat, omental fat and eye muscle area. There was only one trait (P8 fat) not associated with the interaction of *myostatin* and a candidate gene SNP, demonstrating the large effect *myostatin* has on fat and muscle development.

Although there is strong evidence of interactions, the biology behind these interactions is unclear and should be further investigated. For example, the *myostatin* F94L variant causes an increase in muscle mass, most likely at the expense of adipose tissue. Therefore, the interaction between the *myostatin* F94L variant and genes involved in adipogenesis (e.g. *ESR1* and *PPARG*) was not unexpected, particularly *PPARG*, as the protein from this gene has been shown to be reduced in *myostatin* knockout mice (Lin *et al.*, 2002). Similarly, the interactions between the *myostatin* variant and genes involved in muscle development (*ENO3*) and myocyte and adipocyte differentiation (*ATP2B4*) were expected. However, many of the interactions were associated with variation in internal fat deposition, which is not an intuitive result, although there is evidence of reduced internal organ size in *myostatin* knockout mice and Belgian Blue cattle, a breed that carries a *myostatin* deletion mutation (Lin *et al.*, 2002). Myostatin seems to work in concert with PPARG to increase marble score (Shibata *et al.*, 2006), but this did not appear to be the case herein. While there was an interaction between the *myostatin* F94L variant and *PPARG*, the effect was related to eye muscle area,

carcass weight and omental fat, not intramuscular fat percent or marble score. Furthermore, there was an interaction between the *myostatin* F94L variant and *TEK1*, but not with *EDG1*, although both of these genes are involved in endothelial call development and blood vessel growth and branching. Therefore, the interactions noted herein are not consistent with respect to fat traits or gene functions, and as such, further studies involving larger numbers and different breeds of cattle may serve to elucidate the underlying biology.

All of the candidate genes were selected for analysis because of the known function and proximity to quantitative trait loci established in the Davies Gene Mapping herd, or an association with fat deposition traits either in the Davies herd or available literature. Some of these genes demonstrated the expected association with a fat trait or traits, but others did not.

Acetyl-CoA carboxylase alpha (*ACACA*) is involved in fatty acid synthesis and has been associated with marble score and subcutaneous fat previously (Zhang *et al.*, 2010b). *ACACA* was associated with marble score in this study, as well as intermuscular fat but not subcutaneous fat. Adiponectin (*AdipoQ*) was associated with marble score, but not IMF% as might be expected (Morsci *et al.*, 2006). Estrogen has a large effect on levels of internal fat in humans (Cooke and Naaz, 2004) and defects in the estrogen receptor lead to general obesity in mice (Ohlsson *et al.*, 2000, Cooke and Naaz, 2004). Estrogen receptor alpha (*ESR1*) was associated with omental (internal) fat. However, it was not associated with variation in channel fat, the other internal fat depot, or any other fat depots. Although peroxisome proliferator-activated receptor gamma (*PPARG*) plays such a critical role in adipogenesis, the *PPARG* SNP was only associated with variation in fatty acid synthesis. This may be a reflection of the importance of this gene, as gross mutations result in death (Anghel and Wahli, 2007). Therefore, only minor alterations to this gene may be tolerated and these may result in

only minimal variation in fat deposition. Alternatively, there may be variants in *PPARG* that affect or are associated with variation in fat deposition traits but these do not exist within the Davies Gene Mapping herd.

Peroxisome proliferator-activated receptor alpha (*PPARA*) is a key regulator in fatty acid metabolism, and *PPARA* knockout mice have greatly increased internal fat (Costet *et al.*, 1998). However, the SNP in *PPARA* was only associated with intermuscular (seam) fat variation. Muoio *et al.* (2002) suggested that the high level of *PPARD* in skeletal muscle may compensate for the lack of *PPARA* in knockout mice. If the role of *PPARA* in skeletal muscle was being assumed by *PPARD*, little if any, change in intermuscular fat area would be expected. In addition, there was no association between the *PPARA* SNP and variation in internal fat. However, the *PPARA* knockout mice exhibited late onset obesity (Lewitt and Brismar, 2002) and therefore, the cattle may not have aged sufficiently for the expected changes in internal fat to become obvious.

Tyrosine kinase, endothelial (*TEK1*) and sphingosine 1-phosphate receptor 1 (*EDG1*) are both involved in angiogenesis, which may be important due to the highly vascularised nature of adipose tissue (Stacker *et al.*, 2000). SNPs in *EDG1* have previously been reported to be associated with marble score (Sasaki *et al.*, 2006, Yamada *et al.*, 2009a, Yamada *et al.*, 2009b, Watanabe *et al.*, 2010). EDG1-UTR was associated with marble score but the effect was less than the effect reported by Yamada *et al.* (2009b). The SNPs in the previous reports were in the 5' and 3' untranslated regions. Only one of these SNPs, -312A>G (Yamada *et al.*, 2009a), was in a region that was sequenced herein but only the GG genotype was present in the mapping sires. Watanabe *et al.* (2010) reported that the frequency of alleles associated with high marbling was higher in breeds of cattle heavily selected for marbling and lower in other breeds. Jersey cattle have more marbling than Limousin, although

neither breed has been heavily selected for increased marbling. However, the G allele of the -312A>G SNP, which was the allele associated with increased marble score in Japanese Black cattle, was the only allele in the Davies Jersey – Limousin mapping sires. Therefore, it is likely that the -312A>G SNP is not in strong linkage disequilibrium with the causative DNA variants reported in the previous work *(Yamada et al., 2009a, Yamada et al., 2009b, Watanabe et al., 2010)*.

Although *EDG1* was associated with marble score variation, *TEK1* was not associated with either intramuscular fat % or marble score. SNPs from both genes were associated with intermuscular (seam) fat, and *TEK1* was also associated with variation in channel and omental fat. Because adipose tissue is highly vascularised, it was anticipated that if genes involved in angiogenesis, such as *EDG1* and *TEK1,* are associated with variation in fat deposition, then the effect would be more global, and not restricted to just a few fat depots. However, the effect of vascularisation may be more important to some fat depots than others. These SNPs in *EDG1* and *TEK1* were associated with variation in intramuscular, intermuscular and internal fat, depots that are within or between muscles or surrounding internal organs. It may be that these depots fluctuate rapidly with changes in energy balance, whereas subcutaneous fat is less vascularised (Hausman and Thomas, 1986, Bornstein *et al.*, 2000) and may be more stable.

A SNP in *ENO3* has been associated with variation in subcutaneous fat, marble score and IMF% and body fat percentage in pigs (Wu *et al.*, 2008). However, neither of the *ENO3* SNPs reported herein were associated with any fat deposition traits. Also, the only SNP that was associated with fat% and fat to bone ratio was in *LAMIN A/C*, a gene that has been associated with lipodystrophy and muscular dystrophy, not general body fat.

ß, ß-carotene 15, 15'-monooxygenase (*BCMO1*) and aldehyde dehydrogenase 8 family, member A1 (*ALDH8A1*) are both involved in the vitamin A biosynthetic pathway Altering the level of dietary vitamin A affects adipose tissue development (Ribot *et al.*, 2001, Bonet *et al.*, 2003) and lowering vitamin A also increases IMF% (Siebert *et al.*, 2006, Kruk *et al.*, 2008). Herein, *ALDH8A1* was only associated with marble score, while *BCMO1* was associated with channel, omental, intermuscular, rump (P8) fat and IMF%. It is interesting that two genes in the same pathway were associated with very different fat deposition traits. Furthermore, *BCMO1* was associated with variation in rump fat but not rib fat, another measure of subcutaneous fat. Siebert *et al*. (2006) suggested that altering the vitamin A intake did not alter the amount of fat deposited, but rather, where it was deposited. There is some evidence of this occurring with the BCMO1-4 SNP, where the AA genotype resulted in increased internal fat (channel and omental) but decreased subcutaneous fat (P8), compared to the CC genotype (Figures 6.21 – 6.23). This also occurred to a lesser extent with the BCMO1-8 SNP (figures not shown).

Eight of the candidate genes had multiple SNPs genotyped. Of these genes, the SNPs in *ALDH8A1* were associated with variation in one or more of the measures of marble score (marbam, mbms, mbusms). The SNPs in *BCMO1*, *ESR1* and *TEK1* affected multiple traits and in the remaining genes, *ATP2B4*, *ENO3*, *NCOA7* and *PPARG*, only one SNP in each gene was associated with variation in the measured traits (Table 6.2). This disparity in the effects of SNPs within a gene is likely to be the result of each SNP having a small effect on a particular trait, or being in less linkage disequilibrium with a DNA variant that is causing the variation, and therefore, there is no overt effect.

6.4.3.2 Interactions between SNPs

There were relatively widespread effects of *BCMO1* SNPs on fat and muscle traits, whereas most other genes were only associated with variation in one or possibly similar traits (e.g. *PPARG*, which was associated with desaturation index, mono-unsaturated fatty acids and saturated fatty acids). The interaction between SNPs within each gene was tested for association with variation in all of the traits (Table 6.5) and in general, these effects did not reflect the effects of each individual SNP (Table 6.2). For example, the *ALDH8A1* SNPs were all associated with variation in marble score but the interaction between two of these SNPs was associated with intermuscular (seam) fat. Furthermore, only two of the seven *TEK1* SNPs were individually associated with fat trait variation, yet the interactions between pairs of the other five SNPs were associated with multiple fat deposition traits. This 'gain of function' through the interaction of two SNPs that have no effect singly, has occurred with two SNPs in diacylglycerol O-acyltransferase (*DGAT1*). Kong *et al.* (2007) reported that although the two SNPs in (*DGAT1*) had no significant individual effects on the beef carcass traits analysed, the interaction between these SNPs was associated with variation in marble score.

A similar result was observed when testing the interaction of SNPs in different genes, in that the interaction between genes was significantly associated with traits that were not associated with either gene alone. This phenomenon has been previously reported in human disease. For example, interactions between SNPs in different genes have been shown to increase breast cancer risk even though individually, these SNPs were not associated with an increased risk of breast cancer (Onay *et al.*, 2006). The multi-gene nature of diseases, such as breast cancer, is not dissimilar to the multi-gene quantitative traits. Therefore, the putative synergistic relationship between SNPs within and between genes reported here deserves more investigation. However, the number

of animals available within this study was insufficient for any further analysis. Increasing the number of SNPs and traits analysed within a fixed data set reduces the statistical power of the analysis and may increase the number of false positive and negative results (Lee and Kim, 2009). Therefore, a much larger data set of animals is required to increase the power of the analyses and enable more specific and reliable results, particularly as it has been estimated that approximately 5% of associations are likely to have a P value less than 0.05 (as used herein) without a true association (Newton-Cheh and Hirschhorn, 2005).

6.4.3.3 Correlations

While different from the traditional approach, 'genetic' correlations between traits were calculated using the significant additive effects of the genotyped single nucleotide polymorphisms (SNPs) on fat and muscle traits (Table 6.4). Some of the high correlations were expected, (for example, eye muscle area and carcass weight, intramuscular fat % and marble score, channel and omental fat, and the different measures of fatty acid composition). However, a low, negative 'genetic' correlation (r_G = -0.15) between P8 and rib fat was unexpected, as these are both measures of subcutaneous fat and it is logical that they would be under the same genetic influence. There are two points to be considered here. Firstly, P8 and rib fat were measured with potential for variation caused by hide removal and by different carcass assessors at separate times, P8 on the hot carcass and rib 24 hours later at <4°C. This may explain the modest phenotypic correlation of 0.21 between the P8 and rib fat depots (Table 4.19), although these depots were sufficiently related for them group together in both the cluster analysis and principle component analysis. Secondly, the SNPs were used to generate the 'genetic' correlations for the Davies herd. However, no SNPs tested herein were significantly associated with variation in rib fat. Therefore, it is probable

that the lack of a ‘genetic’ correlation with rib fat is a reflection of this rather than true differences in the genetic control of individual subcutaneous fat sites.

There was also variation in the correlations between marble scores, mbms and mbusms 0.85, mbms and marbam 0.64, marbam and mbusms 0.25. Like subcutaneous fat, these traits are measured separately, although on the same day and mbms and mbusms are measured by the same person. The MSA grading (mbms) is likely to be more accurate than AUS-MEAT (marbam) due to the greater level of training invested in the MSA graders. Also, marbam is measured in whole units whereas mbms is measured in increments of 0.1, and has proven to be more accurate indication of intramuscular fat % (Bindon, 2004). In practice, mbms and mbusms have similar scales, although different orders of magnitude (0 to 3.4 compared with 160 to 620, in this herd), which may explain the higher correlation between these two finer measures than correlations with marbam which is scored more grossly. Furthermore, there is potential for the level of intermuscular (seam) fat to influence marble score in that delineating seam fat from marbling can be difficult, in some instances.

There was little similarity between the genetic correlations reported herein and genetic correlations reported previously (Table 6.7). Davies herd correlations between eye muscle area (EMA) with intramuscular fat % (IMF%), subcutaneous fat at the rump (P8) and rib (Ribfat) were positive whereas those reported by Pitchford *et al.* (2006) and Reverter *et al.* (2003) were negative. Gregory *et al*. (1995) reported a positive correlation between EMA and IMF% but a negative correlation between EMA and subcutaneous fat. However, the strength of the genetic correlations between EMA and IMF%, and EMA and rib fat were the same herein with that of Reverter *et al.* (2003). Although correlations between carcass weight (hscw) and the fat traits were also different to those of Pitchford *et al.* (2006) and Reverter *et al.* (2003), there was a strong correlation between carcass weight and P8 fat but no correlation with rib fat.

There was also no similarity in the IMF% – rib fat – P8 correlations. The large difference between the Davies Gene Mapping herd correlations and the others may be due to the method of calculating the correlations. The correlations reported herein were calculated from the additive effects of each SNP and therefore, suggest the actual genetic effect rather than the parental effect. While these correlations were based on a limited number of SNPs, the near zero correlation between IMF% with both rib fat and P8 fat suggests there is an even greater opportunity to select for increased intramuscular fat without affecting subcutaneous fat depth than with selection based on the whole genome. The genetic correlations calculated using the Trangie RFI selection line sires were also different to those from previous literature, although the correlation between IMF% and rib fat was similar to those reported by Pitchford *et al.* (2006), Reverter *et al.* 2003 and Gregory *et al.* (1995). The discrepancies may be a result of the low number of progeny in the Trangie herd.

Table 6.7: Comparison of genetic correlations between carcass traits from the Davies Gene Mapping herd with previously published genetic correlations.

	Davies Gene Mapping herd	Trangie RFI Selection line	Pitchford et al.*	Reverter et al.*	Gregory et al.*
hscw – ema	0.70		0.63	0.45	0.66
hscw - p8am	0.62		0.22	-0.39	0.13
hscw - ribfat	0.01			-0.39	
hscw - imf%	0.04		-0.19	-0.12	0.26
ema - p8am	0.45		-0.21	-0.29	-0.06
ema – ribfat	0.14	0.20		-0.13	
ema - imf%	0.11	0.05	-0.37	-0.12	0.20
p8am - ribfat	-0.15			0.82	
imf% - p8am	-0.004		0.28	0.34	0.33
imf% - ribfat	0.09	0.26		0.21	

***Adapted from Gregory *et al.,* 1995, Pitchford *et al.,* 2006 and Reverter *et al.,* 2003.**
Eye muscle area (ema), rump fat depth at P8 (p8am), rib fat depth (ribfat), intramuscular fat (imf%), carcass weight (hscw).

Another unexpected result was that there was no correlation between eye muscle area and intermuscular (seam) fat. The theory was that seam fat would develop at the expense of muscle area, and therefore, a genetic influence to reduce eye muscle area would be associated with increased seam fat area, particularly if this relationship was

due to transdifferentiation within myocytes and adipocytes. However, these results suggest that this is not the case and that seam fat and eye muscle area are under independent genetic control, at least for the SNPs examined herein. Other genetic correlation estimates between eye muscle area and seam fat have not been reported.

There may be a link between the melting temperature of intramuscular fat and marble score, in that a higher melting temperature may make the intramuscular fat more visually obvious and hence, increase the visually assessed marble score, especially when assessed just 12 hours after slaughter and the carcass may still be cooling. There was a strong genetic correlation between the melting point of intramuscular fat and intramuscular fat % (IMF%) (r_G = 0.94, Table 6.4), which is much higher than that reported by Pitchford *et al.* (2002) (r_G = 0.06). There was also a strong correlation herein, between melting point and marbam (r_G = 0.81), a moderate correlation between melting point and mbms (r_G = 0.38) and no correlation between melting point and mbusms (r_G = 0.06). There were similar correlations between IMF% and marbam (r_G = 0.82), IMF% and mbms (r_G = 0.40) and IMF% and mbusms (r_G = 0.05). The differences in correlations between melting point and the three marble scores, and also IMF% and the three marble scores, is likely to be a result of the differences in marble score measurements mentioned previously. However, these correlations contradict the earlier report that mbms was a better indicator of intramuscular fat % than was marbam (Bindon, 2004), although it must be noted that these are genetic correlations and the previous report involved phenotypic relationships. Nevertheless, a more accurate phenotypic measure should correspond to a more accurate genotypic measure. However, the strong genetic correlation between intramuscular fat % and the melting point of intramuscular fat, and to a lesser extent marble score, suggests that these are under the same, or similar, genetic control.

The melting point of fat is influenced by the fatty acid composition, i.e. the length of the carbon chains and their level of saturation (Wood, 1984). Therefore, it was expected that the strong correlations between melting point of intramuscular fat and both marble score and intramuscular fat % would be accompanied by a high genetic correlation between these and the various measures of fatty acid composition (desaturation index, elongation index, mono-unsaturated fatty acids as a percent of total triacylglycerides and saturated fatty acids as a percent of total triacylglycerides). However, these genetic correlations were generally low to very low. The highest correlation with melting point was saturated fatty acids as a percent of total triacylglycerides (r_G = -0.20), while the highest of all these correlations were between the AUS-MEAT marble score (marbam) and desaturation index (r_G =0.35) and saturated fatty acids as a percent of total triacylglycerides (r_G = -0.31). These correlations were very different to those reported by Pitchford *et al.* (2002), where the most similar genetic correlation was between mono-unsaturated fatty acids as a percent of total triacylglycerides and elongation index (Table 6.8).

Table 6.8: Comparison of fatty acid composition genetic correlations with previously published genetic correlations.

	Davies	Pitchford et al.*
imf% - meltpt	0.94	0.06
imf% - mufa	-0.03	-0.27
imf% - desat	0.16	0.04
imf% - elong	0.24	-0.61
meltpt - mufa	-0.19	-0.42
meltpt - desat	0.07	-0.46
meltpt - elong	0.16	-0.05
mufa - desat	0.57	0.87
mufa - elong	0.52	0.69
desat - elong	-0.22	0.33

***Adapted from Pitchford *et al*., 2002.**
Intramuscular fat (imf%), melting point (meltpt), desaturation index (desat), elongation index (elong), mono-unsaturated fatty acids – percent of triacylglyceride (mufa).

6.4.3.4 Cluster analysis

The cluster analysis is a visual representation of the correlation estimates discussed above that show which traits are under the most common genetic control (Figure 6.24). The analysis placed the marble scores and intramuscular fat % in two closely grouped clusters. Also the two internal fat depots (omental and channel fat) clustered together, as did eye muscle area and carcass weight. Although rib and P8 fat are both measures of subcutaneous fat, these did not cluster together, but this is likely due to measurement variation or the SNP selection, as discussed previously (Section 6.4.3.3). However, it is interesting to note in the two overarching clusters, one included carcass weight, eye muscle area and P8, channel and omental fat, while the other cluster included the three marble scores, intramuscular fat %, rib and intermuscular (seam) fat. The alignment of the internal fat depots with eye muscle area and carcass weight rather than the other fat depots suggests that internal fat varies more closely with animal size than do the other fat depots. This may be a function of the breeds used in this trial, as both the Jersey and Limousin breeds generally have less subcutaneous fat compared to other breeds (Pitchford *et al.*, 2002). Nevertheless, it is evidence that the genetic control of the partitioning of fat to the individual depots is not strongly linked.

The AgResearch phenotypic correlations were stronger than those of the Davies herd, possibly because of differences in age and feeding regimes (Section 4.4.2) or more accurate phenotypic measurements (subcutaneous fat depth versus fat weight). Given the better correlations between fat depots, genotyping the AgResearch herd using the same SNPs used for the Davies herd may provide genetic correlations and hence, genetic clusters that that better resemble what was expected.

6.4.3.5 Single nucleotide polymorphism effects

The variation explained by the SNPs was calculated for all of the significant SNP associations (Table 6.3). The variation explained by an individual SNP ranged from 0.8% to 27.5%. The three SNPs affecting channel fat combined, explained 38% of the phenotypic variation, and similar results were found for intermuscular (seam – 26%), subcutaneous (P8 – 26%) and internal fat (omental – 23%). However, although the linear mixed model was considered to be the most appropriate method to calculate the effects, it must be noted that this model is likely to inflate the estimates. Also, the mapping herd is closed and although the sire has been accounted for in the model, these results should be validated in a larger, open population. Nevertheless, these results indicate that the DNA variants of these genes are associated with a significant variation in fat deposition, as usually a QTL explains less than 10% of the phenotypic variation (Darvasi and Pisante-Shalom, 2002). Whether any of the SNPs are causing a regulatory effect or are simply in linkage disequilibrium with a causative SNP is unclear.

SNP effects can be either additive or non-additive. Non-additive effects include dominance (interaction of different alleles at the same locus), and epitasis (interaction of genes at different loci) (Vanraden and Hoeschele, 1991). The additive effects of these DNA variants are particularly important for the selection of breeding stock, as they indicate the amount of genetic improvement that would be associated with each inherited allele. These are used in the calculation of estimated breeding values (EBV) and hence, the relative worth of any breeding animal whose genotype is known. Where the genotype is known, the additive and dominance effects can be used to predict the phenotype of that animal, although maternal (Montaldo and Kinghorn, 2003) and environmental effects (Gallardo *et al.*, 2010) will have a significant influence on the actual phenotype. Predicting the phenotype would be of benefit for identifying cattle suited for a particular purpose or market. Another non-additive effect is over

dominance, where the heterozygote phenotype is greater than both the homozygous phenotypes. Although there was evidence to suggest this occurred in six of the SNP/trait associations, a T-test indicated that over dominance was only a factor in the effect of EDG1-UTR on intermuscular (seam) fat and ESR1-2 on eye muscle area ($P < 0.05$).

The additive, or allele substitution, effect of the SNPs ranged from 0.3 to 30.3% of the trait for each allele inherited. In general, when comparing the effects of different SNPs on the same trait, the size of effect of each SNP was different. Similarly, when comparing the effect of a single SNP on multiple traits, the size of effect varied. This was also true when the allele substitution effect was expressed as the number of standard deviations from the mean. This was expected because of the complex regulation of fat deposition, involving many genes with multiple DNA variants. It is unlikely that any one DNA variant would have a consistent size of effect on multiple traits, nor would any trait be affected to the same extent by multiple DNA variants.

6.5 Conclusion

As none of the SNPs identified in the candidate genes were obviously functional, i.e., cause an amino acid change or alter an intron splice site, the SNPs selected for genotyping in the Davies Gene Mapping herd were chosen, as far as possible, to represent all possible haplotypes within each gene. This was not always possible or practical due to either the large number of SNPs within a gene (e.g. *TEK1*) or the suitability of the SNP for the genotyping methods employed herein. However, 32 SNPs were genotyped and the subsequent association analyses produced results worth noting.

Firstly, where there were multiple SNPs within a gene, not all of the SNPs were associated with the same trait. Secondly, where the SNPs within the same gene were associated with the same trait, the size of the SNP effect was not consistent for all SNPs. Thirdly, there were interactions between SNPs both within the same gene and between different genes, and these were often not associated with the same traits as the single SNP effects.

It could be argued that the different size of effect indicates that one SNP is responsible for the variation and the other shows an effect due to the proximity to the causal SNP. This would suggest that the size of effect was related to the level of linkage disequilibrium of each SNP with the causal polymorphism. However, this does not preclude these SNPs from being involved in the trait variation, because the complexity of the quantitative traits may be so great that a strong association with a polymorphism may not be obvious (Brookes, 1999).

The SNPs that were associated with variation in fat traits have the potential to be used as markers for genomic selection, provided they are the causative variant or remain in strong linkage disequilibrium with the causative variant. However, they must first be

confirmed in other breeds and validated in a much larger population to better quantify the size of effect.

Chapter 7 General discussion

7.1 Background

Rather than just a passive store of energy, adipose tissue has a much greater role in human health, including blood pressure regulation and immunity (Vigouroux *et al.*, 2011), and as an endocrine organ facilitating endocrine interaction with the central nervous system (Kershaw and Flier, 2004, Tilg and Moschen, 2006) and in the breeding efficiency of cattle. In humans, there is large variation in the deposition and distribution of fat between individuals, with evidence of a genetic basis to the variation (Heid *et al.*, 2010), similar to that identified in the Jersey-Limousin cattle herein. Although there is also variation in fat deposition and distribution in cattle, this variation is generally viewed as breed specific. However, there is variation within breeds, and it is possible that this variation may be exploited in order to improve individual animal composition and consequently, improve the end product. To achieve this improvement, it is necessary to understand the cause of the variation, whether that be environmental, genetic or a combination of these.

Although investigating environment effects in relation to fat distribution may provide a simple cause and effect hypothesis, this is unlikely to fully explain the variation observed. Understanding the genetics underlying fat deposition and distribution will help the biochemical effects to be elucidated. This, in turn, will increase the understanding of the environmental effects, which may provide methods for the manipulation of fat deposition and distribution, such as altering the quality or quantity of the diet. However, the complex nature of quantitative traits, such as fat deposition, means that identifying genes that have a true effect on these traits is difficult. The variation in quantitative traits is usually the result of many genes each with a small effect, and this small effect may not be obvious.

Traditional methods for genetic improvement involve the use of estimated breeding values. Although these can be accurate, generating accurate estimated breeding values involves the use of historical data from a large number of progeny, and takes a long time. Furthermore, some traits can only be measured accurately at slaughter (e.g. intramuscular fat percent, marble score and intermuscular fat). The use of molecular markers that are linked to a particular trait will reduce the time and cost required to identify animals that are genetically superior and improve the accuracy of selection (Schaeffer, 2006, Seidel, 2010). Achieving this in multiple breeds would require the identification of markers that are informative in each breed, as SNPs are not always common across breeds (e.g. *MSTN*, Section 4.4.5 and *EDG1*, Section 6.4.3.1). However, this will require a large number of cattle from multiple breeds being genotyped and measured for fat deposition traits, and this is not likely to occur in the immediate future. The preliminary step to this is to identify molecular markers that are associated with traits in a crossbred herd (such as the Davies Gene Mapping herd) and confirm the association in a similar herd (e.g. the AgResearch Gene Mapping herd). These molecular markers can then be tested in a random sampling of other breeds to determine if these or similar polymorphisms are present in all or some breeds, indicating whether a larger study is warranted.

If marker assisted selection is expanded to whole genome selection, where potentially hundreds of thousands of molecular markers can be used, the speed and accuracy of selection can be greatly improved (Schaeffer, 2006, Hayes *et al.*, 2009), as can the magnitude of phenotypic improvement. It has been estimated that to overcome the problem of markers that are linked to variation in one breed, but not other breeds, would require the use of approximately 300,000 SNPs (De Roos *et al.*, 2008). This technology is now available (for example, the illumina® BovineHD Genotyping Bead Chip incorporates 770,000 SNPs, (Illumina, 2011)), but this requires that a large

number of animals are phenotyped and genotyped for this to be exploited. Nevertheless, the results of this technology reported to date (e.g. Hayes *et al.*, 2009) are promising in situations where phenotypes are available (e.g. milk traits in dairy cattle). However, measurements for traits such as those related to fat distribution are often difficult to obtain. Thus, examining SNPs within genes that may be used for selection or elucidating traits is still of interest.

7.2 Fat deposition QTL

There have been many quantitative trait loci for fat deposition identified in cattle. However, these QTL have usually been the result of genome scans for QTL or analyses of candidate genes within an established QTL in which the number of fat traits have been limited. For example, Gutierrez-Gil *et al*. (2008) conducted a genome wide scan for QTL affecting sensory, chemical and physical properties of the *Longissimus dorsi* muscle but, only intramuscular fat % was included. As another example, Morsci *et al.* (2006) analysed polymorphisms in *adiponectin* and *somatostatin* for association with 19 traits, but the only fat depot measurements included were marble score, subcutaneous fat depth and intramuscular fat percent via ultrasound measurement.

Although there are QTL for marble score and subcutaneous fat on most chromosomes, there are only QTL for intramuscular fat percent on eight chromosomes, and there are no internal fat QTL currently (Table 7.1). Furthermore, no chromosome has a QTL for all of these traits. Although Esmailizadeh (2006) identified internal fat as well as intramuscular and subcutaneous fat QTL in the Davies gene mapping herd, again there was no chromosome with QTL for all fat traits (Table 7.2). However, prior to the work herein, no QTL for seam or intermuscular fat had been identified. The most objective and time efficient method of quantifying seam fat required the development of an image

analysis methodology, which can also be utilised for an accurate measurement of marble fleck characteristics, such as size and shape.

Table 7.1: Fat deposition quantitative trait loci from the National Animal Genome Research Program database, accessed 2011.

	Chromosome (BTA)																												
Trait	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**	**29**
IMF%	x	x	x											x					x	x		x						x	
marble	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
fatth	x	x	x	x	x	x	x	x	x		x	x	x	x	x	x	x	x	x	x	x	x	x	x	x		x	x	x
subfat	x	x			x	x	x			x	x	x	x	x	x				x					x				x	
sirfd					x									x															
ribf		x																x		x						x			
fatp	x	x				x				x							x					x			x			
fatcov		x		x											x			x						x					

Adapted from cattleQTLdb http://www.animalgenome.org/cgi-bin/QTLdb/BT/viewmap, (NAGRP, 2011).
IMF% – ether extractable intramuscular fat; marble – marble score; fatth – fat cover at 12th rib; subfat – subcutaneous fat; sirfd – thickness of fat on the sirloin; ribf – rib fat; fatp – weight of fat expressed as a proportion of weight of carcass or portion of carcass; fatcov – amount of fat on the outside of the carcass and inside the thoracic cavity.

Table 7.2: Fat deposition quantitative trait loci from the Davies Gene Mapping herd.

	Chromosome (BTA)																												
Trait	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**	**29**
IMF %							x		x			x												x					
Marble	x		x						x							x												x	
ribfat			x				x																					x	
P8	x																x								x				
channel	x														x			x		x									
fat%		x	x	x															x						x				

Adapted from Esmailizadeh, 2006.
IMF% – Intramuscular fat %; marble – marble score; ribfat – subcutaneous fat depth (mm); P8 – subcutaneous fat at P8; channel – channel fat; fat% - carcass fat%

7.3 Image analysis

Two of the principal fat depots to be considered are marbling and intermuscular (seam) fat. A large area of seam fat can have a negative impact on carcass value, whereas increased marbling improves the value. Furthermore, an accurate assessment of the number of marble flecks, and their shape, size and location on the muscle may help to provide a better understanding of the biology controlling marbling.

Image analysis was used to quantify seam fat and examine the various characteristics of marbling. Although seam fat is the fat between the muscles, the delineation of seam fat from subcutaneous fat is often unclear, as is the difference between seam fat and large marble flecks located adjacent to muscle boundaries. The method of delineation used herein was developed to enable a consistent area to be examined within each herd. However, the method differed between the Trangie and Davies herds due to the different body sites used to obtain the images (i.e. the $5^{th}/6^{th}$ rib compared to the $10^{th}/11^{th}$ rib, respectively as described in Sections 3.3.1.1 and 3.3.1.2). Measuring seam fat area in the Davies Gene mapping herd enabled the identification of a new quantitative trait loci for this trait on BTA19, and from this, two candidate genes were chosen.

The low quality of images from the Davies herd prevented the generation of any meaningful data for the marbling flecks. Nevertheless, the concept of measuring these marbling fleck characteristics was validated using the better quality images from the Trangie RFI selection line progeny. The results from the Trangie herd did identify some preliminary information that should be further investigated in a larger data set of better quality images. Firstly, there was low to no phenotypic correlation between the fleck characteristics (e.g. ellipticity, orientation, x and y coordinates) (Appendix D). Secondly, although the majority of marbling flecks were elongated, there was a negative correlation between fleck eccentricity (roundness) and both marble score and intramuscular fat percent, suggesting the flecks become rounder as intramuscular fat increases. However, there was no correlation between the amount of branching of the flecks (ellipticity) and either measure of intramuscular fat. Thirdly, the number of marbling flecks was greater in two quadrants (B and C, Figure 3.13) than the other two quadrants, which may be related to the blood vessel distribution in that muscle.

As there is currently no available genetic marker genotype data for this herd, no further analysis was possible. However, if better quality images were available in a gene mapping herd, such as the Davies or AgResearch herds, more information regarding the fleck characteristics may lead to the identification of genes involved in intramuscular fat deposition and therefore, increase understanding of the biology controlling the marbling phenotype and provide genetic markers for more favourable marbling features.

As the visually assessed marble score is most likely a combination of both the number and area of the marble flecks, a method to combine these measures may improve the correlation between the image analysis results and the visually assessed score. Also, a greater number of flecks of a small size rather than fewer large flecks is preferred by consumers (Albrecht *et al.*, 1996). Therefore, this is probably the most important feature of marbling to analyse, and use to identify molecular markers and candidate genes.

7.4 Fat distribution

The analysis of the seam fat area and the other fat deposition traits previously measured in the Davies Gene Mapping herd demonstrated that the fat depots are lowly phenotypically correlated (e.g. omental:channel, r = 0.01; omental:P8, r = 0.09; ribfat:P8, r = 0.21, Table 4.19). Similar fat deposition traits measured in the AgResearch Gene Mapping herd had moderate correlations, that were generally higher than the correlations in the Davies herd (e.g. omental:channel, r = 0.53; omental:perirenal, r = 0.24; omental:ribfat r= 0.23, Table 4.20).

Cluster analysis indicated which traits varied together (Table 4.22). As expected, the cluster analysis of the Davies herd placed rump (P8) and rib fat close in their own cluster, and also intramuscular fat % and marble score in their own cluster. However,

the internal fat depots (channel and omental) were in separate clusters (Figure 4.13). These results reflect the low residual correlations between fat deposition traits in the Davies herd, and suggest these traits are effectively uncorrelated in comparison to the AgResearch herd. The analysis of the AgResearch herd also placed omental and channel fats in a separate cluster to pericardial fat even though these are all internal fat depots. All but one measure of subcutaneous fat were in a single cluster in the AgResearch herd (Figure 4.14). Not only are these results further evidence that the fat depots act independently, they also suggest that the partitioning of fat within each body site varies.

Principal component analysis indicated how much traits were related to each other and therefore, the likelihood that the depots comprise a general fatness trait. The AgResearch herd results suggested general fatness could be the case (Table 4.24, Figures 4.17 and 4.18). However, in the older, grain fed cattle of the Davies herd, the fat depots were more distant and therefore, the Davies herd data provided more evidence that fat depots vary independently (Table 4.23, Figures 4.15 and 4.16). This segregation of fat depots may be more noticeable in the Davies herd due to the higher energy feed or the more mature animals, or possibly a combination of both factors.

The fixed effects of cohort (steer or heifer, years 1 – 3; Davies herd) or slaughter group (steer or heifer, 28 groups, 18 in year 1, 10 in year 2; AgResearch herd), breed of dam (Jersey or Limousin), sire (3 F1 sires) and *myostatin* F94L variant genotype (nested in breed of dam) were analysed for effects on fat distribution. There was variation in fat deposition associated with all of these fixed effects. Cohort and slaughter group included sex and environmental effects. Although the Davies herd was grain finished and therefore, fully fed for the final 180 days, the environmental effects on the backgrounding of each cohort cannot be ignored. Similarly, the AgResearch herd had supplementary feeding to complement the available pasture. However, as the

AgResearch herd groups were slaughtered sequentially, the environmental effects on the early groups slaughtered in late winter compared to the later groups slaughtered in early summer, were greater. Each slaughter group was also single sex, and as heifers are generally fatter than steers (Murphey *et al.*, 1985, Robelin, 1986), this combined with the environmental effects of the cohort or slaughter group to alter fat deposition.

The Limousin breed is generally larger and leaner than the Jersey, so as expected, breed of dam had an effect on general fat deposition. When adjusted for carcass weight, the Jersey was fatter than the Limousin in all depots (Figures 4.4 and 4.5). However, as breed of dam was significant for all fat depots, breed had no noticeable effect on fat distribution at the individual sites (Tables 4.5 and 4.7).

The *myostatin* F94L variant was included in the analyses as myostatin is a key regulator of muscle growth, and mutations in *myostatin* cause a significant increase in muscle mass and a corresponding decrease in fat deposition (Lee and McPherron, 2001, Rodgers and Garikipati, 2008, Martinez *et al.*, 2010). This effect on both muscle and fat deposition suggests that myostatin may be involved in regulating both processes (Lin *et al.*, 2002), and *in vitro* results indicate that myostatin can inhibit the differentiation of adipocytes (Kim *et al.*, 2001, Zimmers *et al.*, 2002, Rebbapragada *et al.*, 2003, Guo *et al.*, 2008). However, it is equally likely that the lower fat deposition is the result of the increased energy expenditure related to increased muscle mass (Rodgers and Garikipati, 2008). The results herein indicate that *myostatin* genotype does influence fat deposition. Animals with the variant AA *myostatin* genotype, unique to the Limousin (Sellick *et al.*, 2007), were leaner than the CC (wild type), although there was a large breed component involved, as the Limousin CC and AC variants were consistently leaner than their Jersey counterparts (Figures 4.11 and 4.12). However, in the Davies herd, the *myostatin* F94L variant was not significant for all fat depots (Tables 4.4 and 4.5), suggesting that the fat depots were not equally affected. It

is interesting to note that marble score was not significant (P = 0.9) but intramuscular fat % was significant (P = 0.01). Subcutaneous fat depots were significantly affected by the *myostatin* variant (P8, P <0.001; rib fat, P = 0.03), but neither internal fat depot was significant (channel/kidney fat, P = 0.055; omental fat, P = 0.8). These results indicate the effect of *myostatin* F94L genotype on fat deposition was not identical across fat depots.

Cohort and breed affected fat deposition generally but not fat distribution at the individual depots. However, sire did have some effect on fat distribution. No single sire in either the Davies or AgResearch herds had progeny that were consistently higher or lower for all fat traits, which suggests there is a genetic component to fat distribution as well as general fatness. The results from the Trangie RFI selection line were similar in that the progeny of no one sire were consistently high, average or low across all fat depots. Heritability and genetic correlations were only calculated in the Trangie herd, as there were only three sires each in the Davies and AgResearch herds. Both the heritability and genetic correlations (eye muscle area, intramuscular fat %, marble score, seam fat area, ribfat depth; h^2 = 0.06 – 0.46, r_g = 0.01 – 0.48, Tables 4.17 and 18) were low to moderate. This suggests there is only a weak to moderate genetic link between the fat traits, and this can be exploited in selection for or against specific depots without adversely affecting other fat depots.

7.5 Genetic associated lipodystrophies in humans

Lipodystrophy is a loss of fat, either from the entire body or selected regions (Vigouroux *et al.*, 2011), and with most lipodystrophies, there is an inverse relationship between fat and muscle development (Hu *et al.*, 1995). There is a genetic basis for a number of lipodystrophies in humans. For example, mutations in 1-acylglycerol-3-phosphate *O*-acyltransferase 2 (*AGPAT2*), seipin (or Berardinelli-Seip congenital

lipodystrophy 2, *BSCL2*) and zinc metalloproteinase (*ZMPSTE24*) result in general lipodystrophy, while lamin A/C (*LMNA*) and peroxisome proliferator-activated receptor gamma (*PPARG*) mutations result in partial lipodystrophy (Agarwal and Garg, 2006). The phenotypes are either evident at birth (*AGPAT2* and *BSCL2*) or tend to develop with the onset of puberty (*LMNA, ZMPSTE24, PPARG*) (Hegele, 2003, Garg, 2004). There are also genetic abnormalities causing general obesity in humans, such as Alstrom syndrome and Bardet–Biedl syndrome caused by mutations in the Alstrom syndrome 1 (*ALMS1)* and Bardet–Biedl syndrome (*BBS2)* genes, respectively, and Prader–Willi and Angelman syndromes which are caused by deletions on chromosome 15 (Hegele, 2003, Weinstein *et al.*, 2010). The variation of the genetically induced lipodystrophies on fat depots is more evidence of the multi-gene nature of fat deposition.

As these mutations and syndromes are accompanied by physiological problems, such as insulin resistance and other metabolic disorders (Chen and Garg, 1999, Tsai and Maeda, 2005) as well as the abnormal fat distribution, they are unlikely to be common in cattle. The nature of animal production is that animals that fail to thrive will be culled, and those that are unsuitable for production due to gross abnormalities in body conformation will not be used for breeding and are also likely to be culled early. For example, the mutations in *LMNA* associated with lipodystrophy mentioned above have not been identified in cattle as yet. Gross mutations in *LMNA* are unlikely to become fixed in production animals, as the disruption of nuclear function is likely to cause more serious physiological effects that would not be compatible to animal production systems. This may be reflected in the lack of polymorphisms located in *LMNA* herein, where only one polymorphism was identified in the almost 8,500bp sequenced in the mapping sires (Table 5.2), much less than the estimated average rate of one SNP per 700bp in the bovine genome (Gibbs *et al.*, 2009). However, if a mutation caused a

desirable phenotype, such as increased marbling, these animals would be selected for breeding and the mutation could become fixed. This seems to be occurring in Japanese Black cattle as there was a high frequency of *EDG1* alleles associated with higher marbling in Japanese Black cattle, whereas these alleles were either at a low frequency or non-existent in breeds that had not been heavily selected for increased marbling (Watanabe *et al.*, 2010). Interestingly, although marbling in beef production is valuable, intramuscular fat in humans is associated with disease (e.g. type 2 diabetes, insulin resistance) (Schaffer, 2003).

These lipodystrophic genes and gene pathways may provide alternative candidate genes for fat distribution in cattle. For example, ADAM metallopeptidase domain 12 (*ADAM12*) is involved in the regulation of myogenesis, adipogenesis, and obesity (Kurisaki *et al.*, 2003, Masaki *et al.*, 2005), and transgenic mice overexpressing *ADAM12* have been shown accumulate intramuscular fat (Kawaguchi *et al.*, 2002). Furthermore, investigating the gene function and pathways may help to increase the knowledge of the biology underlying variation in fat deposition and distribution. Although mutations in these genes result in a gross alteration in fat distribution in human diseases that would not be compatible with production animals, a minor mutation that resulted in altered gene expression may cause a more subtle shift in fat distribution. For example, mutations in *LMNA* and *PPARG* cause large alterations in fat distribution in humans (Agarwal and Garg, 2002, Garg, 2004), whereas the polymorphisms in *LMNA* and *PPARG* reported herein were only associated with minor variation in carcass fat percent and fatty acid composition, respectively.

7.6 Independence of fat depots

Why is there variation in fat distribution? The reasons for this are unclear. It would be easy to assume that, although adipose tissue is a dynamic rather than passive organ,

the various depots would be essentially the same and function in a similar manner. However, this is not the case. Just as there are different types of muscle (smooth, striated or cardiac) and epithelial tissue (stratified, cuboidal, columnar, squamous and combinations of these) depending on function, there are biological differences between adipose tissue depots (Lafontan and Berlan, 2003, Hausman *et al.*, 2009, Yamashita *et al.*, 2010) including preadipocyte proliferation and differentiation capacity (Tchkonia *et al.*, 2004, Grant *et al.*, 2008, Wan *et al.*, 2009). It is likely that these biological differences relate, at least in part, to the function of each depot.

There is extensive vascularisation in adipose tissue (Yamada *et al.*, 2010) with a clear spatial and temporal correlation between angiogenesis and adipogenesis (Hausman *et al.*, 1993, Nishimura *et al.*, 2007). However, the individual adipose depots vary in the structure of blood vessels and the density of the capillary network (Crandall *et al.*, 1997, Yamada *et al.*, 2010). Hausman and Thomas (1986) reported a higher capillary density per arteriole in perirenal fat compared to subcutaneous fat in pigs. A larger concentration of endothelial cells has been identified in human internal fat cell culture explants compared to subcutaneous fat explants (Bornstein *et al.*, 2000). Furthermore, the number of endothelial cells in explant cell cultures from human femoral and abdominal subcutaneous fat also varies (van Harmelen *et al.*, 2004), indicating differences also exist between adipose tissues considered to be the same depot. This may be a contributing factor in the unexpected, weak correlation between rump and rib fat herein.

Vasculature plays an important role in lipid metabolism and adipogenesis as blood flow is critical for both the deposition and utilisation of lipids (Crandall *et al.*, 1997, Hausman *et al.*, 2001) and provides a source of stem cells (Harper and Pethick, 2004). Blood flow to adipose tissue increases in response to fasting but the response is depot specific (Crandall *et al.*, 1997). In rats, the blood flow to mesenteric fat is greater than

the flow to retroperitoneal, epididymal (Mayerle and Havel, 1969) and subcutaneous fat (Crandall *et al.*, 1984). Also, Engfeldt and Linde (1992) reported increased blood flow to abdominal subcutaneous fat compared to femoral subcutaneous fat in fasting women.

There is a strong correlation between angiogenesis and adipogenesis (Bornstein *et al.*, 2000, Nishimura *et al.*, 2007). Adipocytes and their differentiation are intricately involved with angiogenesis and angiogenic growth factors (Rupnick *et al.*, 2002, Cho *et al.*, 2007, Nishimura *et al.*, 2007). Hutley *et al.* (2001) reported paracrine interactions between microvascular endothelial cells and preadipocytes. Varzaneh *et al.* (1994) concluded that extracellular matrix components were the signalling molecules, and Aoki *et al.* (2003) found that adhesion cell molecules that mediate cell-cell interaction seem to be critical in the differentiation and/or dedifferentiation of adipocytes. Using both *in vivo* and *in vitro* studies of mice, Fukumura *et al.* (2003) reported that inhibiting angiogenesis also inhibited preadipocyte differentiation, and while angiogenesis is required for adipogenesis, new vasculature does not develop in the absence of adipogenesis.

However, the order of development of blood vessels and adipose tissue appears to be specific for a given fat depot. For example, blood vessels develop prior to perirenal fat but after subcutaneous fat in prenatal pigs (Crandall *et al.*, 1997). It is clear that there is an integrated and complicated relationship between vasculature and adipose tissue, and the variation in vasculature in different fat depots is likely to induce differences between these depots.

There is also variation in the sympathetic nervous system activity in adipose tissue depots (Bartness and Song, 2007). These nerves tend to develop mainly around arteries and arterioles but rarely with capillaries (Crandall *et al.*, 1997). Sympathetic

nervous system innervations of adipose tissue appear to be associated with hormone synthesis, insulin sensitivity, lipid mobilisation and accumulation, and may affect adipocyte proliferation and differentiation (Bartness and Bamshad, 1998, Flier *et al.*, 2003). There are physiological differences between the neuroanatomy of internal and subcutaneous fat depots, which may cause variation in the partitioning of fat to these depots (Flier *et al.*, 2003). Denervated adipose tissue studies have demonstrated suppressed lipolysis compared to untreated regions (Bartness and Song, 2007).

The size of adipose cells also appears to vary between depots. There have been reports that internal fat cells are larger than subcutaneous fat cells in mice and pigs (Meade and Ashwell, 1980, Hausman and Thomas, 1986), and cattle subcutaneous and intramuscular fat cells were smaller than intermuscular and internal (mesenteric and renal) fat cells (Yamada *et al.*, 2010). Fat cells in different regions decrease in size differently in response to reduced energy intake (Bjorntorp *et al.*, 1975, Bartness and Song, 2007). The limit of oxygen diffusion can cause hypoxia in larger adipose cells, which causes an increase in angiogenesis (Yamada *et al.*, 2010). Therefore, the larger size of adipocytes in internal fat depots may explain the increased vasculature, although the hypoxia is most likely to occur in cells that are hypertrophic and the blood vessel pattern in adipose tissue is established very early in development (Crandall *et al.*, 1997).

There is some evidence of age related differences both in adipocyte size (Bjorntorp *et al.*, 1975) and differentiation (Crandall *et al.*, 1997). There is a negative correlation between age and subcutaneous fat proliferation but no correlation with omental fat, which is consistent with the increase in omental fat associated with ageing in humans (van Harmelen *et al.*, 2004).

There are also fat depot specific biochemical and endocrine responses (Lafontan and Berlan, 2003, Gesta *et al.*, 2007, Yamashita *et al.*, 2010). For example, there is greater secretion of leptin from subcutaneous fat than internal fat (Bornstein *et al.*, 2000), while the reverse is true for adiponectin (Lafontan and Berlan, 2003). Similarly, internal fat is more sensitive to catecholamine induced stimulation of lipolysis than is subcutaneous fat, and more resistant to the antilipolytic effect of insulin (Arner, 1995, Lafontan and Berlan, 2003). Moreover, Garden *et al.* (Gardan *et al.*, 2006) reported less lipogenesis and lower expression of leptin and adiponectin in intramuscular fat than subcutaneous and internal fat in pigs. Furthermore, Anderson and Kauffman (1973) found a difference in accumulation of fat between the three subcutaneous layers as piglets grew, and suggested this may be due to differences in enzyme activities.

In conclusion, fat deposition is a multifaceted process that appears to be centralised around the vascular network in adipose tissue. Firstly, adipogenesis and angiogenesis are positively correlated. Secondly, blood vessels facilitate the endocrine and biochemical effects on adipose tissue (e.g. lipid accumulation and mobilisation) and also the effects caused by adipose tissue hormones (e.g. leptin and adiponectin). Thirdly, there is a correlation between vasculature and the sympathetic nervous system. The sympathetic nervous system is involved in lipolysis, lipid accumulation, adipogenesis and hormone responses within the adipose tissue.

With the knowledge that the amount of vasculature varies between depots, it is not surprising that the partitioning of fat to the different adipose depots varies. Furthermore, it is likely that these fat depots have different functions in the animal. The variation in biochemical and hormone responses between fat depots point to a variation in physiological roles as well. Subcutaneous fat provides insulation for the body (Eckert *et al.*, 1988), internal fat provides protection for the internal organs (Bone, 1988) and intramuscular fat can be utilised as a ready supply of energy for the surrounding

muscles (Tume, 2004). Therefore, with this knowledge that fat deposition is intricately controlled by multiple factors, it is clear that variation in the expression of genes that affect adipogenesis, lipogenesis, lipolysis, angiogenesis, vasculature or innervation may affect fat distribution. Any of these genes can be considered candidates for variation in fat distribution.

7.7 Genes affecting fat distribution in cattle

There are many obvious candidate genes for fat deposition, due to their biological function (for example, *growth hormone*, *growth hormone receptor*, *insulin-like growth factor*, *insulin-like growth factor receptor*, *somatostatin* and *obese*). However, these genes had already been tested for association with variation in fat depots in the Davies Gene Mapping herd (Pitchford, unpublished data) and therefore, were not included herein.

Some of the candidate genes herein have now been tested by others for associations, but only for specific fat depots. For example, *acetyl-CoA carboxylase alpha* has been tested for association with fatty acid composition, marble score and subcutaneous fat thickness (Zhang *et al.*, 2010b); *adiponectin* has been examined for intramuscular fat %, marble score and subcutaneous fat depth (Morsci *et al.*, 2006); *enolase 3* has been examined for subcutaneous fat, intramuscular fat % and marble score (Wu *et al.*, 2008); and *sphingosine 1-phosphate receptor 1* only marble score (Yamada *et al.*, 2009a, Yamada *et al.*, 2009b, Watanabe *et al.*, 2010). As the results herein suggested a weak to moderate genetic link between fat depots (Section 7.4), one purpose behind the selection of these candidate genes was to test polymorphisms within the genes for association with variation in all fat depots.

In all, eleven candidate genes for fat deposition were selected and sequenced for association studies. The selection was based on the proximity to known quantitative

trait loci for fat depots or from the literature describing likely associations with fat deposition in other breeds of cattle or different species. The candidate genes were sequenced to identify polymorphisms that either caused an alteration in function or could be used for association analyses. Although none of the SNPs altered amino acid sequence, there were associations between these genes and various fat depots. Although it was planned to test these polymorphisms in the Trangie Residual Feed Intake herd, time constraints prevented this. Ideally, the Trangie herd will be genotyped in the near future.

The association studies support other results reported herein. For instance, the sire effect varied between fat depots and therefore, there was a large genetic component in fat distribution, whereas the cohort, breed and myostatin genotype effects were on general fat deposition principally (Chapter 4). The association studies showed that no SNP was associated with variation in all fat traits, which indicates a lack of global effect by these SNPs. That is, there were no genes that affected all fat depots. This is the observation also from the literature. For example, the obese gene (*OB* or *LEP*) encodes leptin, which is mainly produced in adipose tissue (Bornstein *et al.*, 2000). Leptin is involved in the regulation of energy expenditure and appetite to control body fat reserves (Delavaud *et al.*, 2002) (Section 1.7.2.8). Although the effect of mutations in the *obese* gene were initially reported as affecting general fatness (Alonso and Maren, 1980, Halaas *et al.*, 1995, Pelleymounter *et al.*, 1995), it has since been shown that polymorphisms in the promoter region do not alter fat deposition consistently. Chung *et al*. (2008) reported that SNPs in the promoter region were significantly associated with variation in subcutaneous fat but not marble score in cattle. Similarly, Nkrumah *et al*. (2005) reported *obese* SNPs associated with variation in both backfat and marble score, but the variation in subcutaneous fat was greater than that in marble score. Another example is *myostatin* genotype which has a large impact on muscle

mass and fat deposition (McPherron *et al.*, 1997, Rodgers and Garikipati, 2008, Martinez *et al.*, 2010). Although the *myostatin* F94L genotype did have a large impact on fat deposition herein, the effect was not equal across all fat depots (Figures 4.11 and 4.12, Appendix L). These examples further indicate the complexity of the genetics underlying fat distribution.

The association studies also showed that the size of effect was small for most of the SNPs tested. This suggests that the genetic cause of variation in fat traits is the result of many genes, each of relatively small effect. These small effects do not preclude the SNPs from having a real and significant effect on fat deposition, because the nature of quantitative traits is that these are very likely to be affected by multiple genes, and therefore, some of the associations may not be easily identified (Brookes, 1999, Hegele *et al.*, 2000). Moreover, when the effects of all SNPs in all genes affecting a single trait were combined, the overall effect was considerable in some cases (e.g. the effect on channel fat was 38%, Table 6.3 and Section 6.4.3.5). There were also a few SNPs (e.g. BCMO1-4 and TEK1-1) with very large effects, even rivalling the size of the *myostatin* genotype effects.

In genes with multiple SNPs, the SNPs were not always associated with the same trait or, if they were, the size of effect varied between SNPs. Both of these phenomena are likely to be the result of different levels of linkage disequilibrium between the SNP and the polymorphism causing the phenotype. However, testing these polymorphisms in other cattle populations would provide more information regarding their effect and validity, and also would be required for these to be used in the beef industry.

Interactions between SNPs both within and between genes were tested for association with variation in fat deposition. In most cases, these interactions were associated with a different fat depot than the one affected by the SNP individually. This inconsistency

with the single SNP effect has been reported previously (Onay *et al.*, 2006, Kong *et al.*, 2007) (Section 6.4.3.2) and is probably indicative of the multi-gene nature of quantitative traits.

7.7.1 Gene associations

Single nucleotide polymorphisms (SNPs) in four genes were associated with large variation in fat traits, ß, ß-carotene 15, 15'-monooxygenase (*BCMO1*), aldehyde dehydrogenase 8 family, member A1 (*ALDH8A1*), estrogen receptor alpha (*ESR1)* and tyrosine kinase, endothelial (*TEK1)* (*BCMO1*, P8 = 19.7%; *ALDH8A1*, mbusms = 9.1%; *ESR1,* marbam = 9.1%; *TEK1*, channel = 27.5%, Table 6.3 and Section 6.4.3.5). *BCMO1* is suggested to be involved in lipid metabolism (Hessel *et al.*, 2007, Lietz *et al.*, 2010), presumably through its role in the synthesis of vitamin A. Vitamin A inhibits peroxisome proliferator-activated receptor gamma (*PPARG*) and CAAT/enhancer binding protein alpha (*C/EBPα*) (Villarroya *et al.*, 1999, Ribot *et al.*, 2001, Lobo *et al.*, 2010) and subsequently, affects adipocyte differentiation (Safonova *et al.*, 1994, Bonet *et al.*, 2003, Lobo *et al.*, 2010) (Section 5.3.2.1). *ALDH8A1* is also involved in the vitamin A pathway and is associated with increased fatness in pigs (Grapes and Rothschild, 2006). Low dietary levels of vitamin A cause an increase in both adipocyte differentiation (hyperplasia) and lipid filling (hypertrophy) (Siebert *et al.*, 2011). SNPs in *BCMO1* were associated with variation in subcutaneous fat (BCMO1-4) and seam fat (BCMO1-8), while SNPs in *ALDH8A1* were associated with marble score herein. If seam fat is utilised as a ready supply of energy, due to its location between muscles, an alteration to the regulation of a gene associated with lipid metabolism is likely to affect the level of fat accretion in this depot. However, subcutaneous fat is less sensitive than internal fat to lipolysis (Arner, 1995), so the specific role of *BCMO1* in subcutaneous fat deposition is less clear. High levels of vitamin A inhibit, and low levels promote, adipogenesis as well as lipolysis. Although this suggests that variation in

vitamin A related genes would affect all fat depots equally, this was not found herein. The fact that variation in seam and subcutaneous fat, but no other fat depots, was associated with polymorphisms in *BCMO1* and only marble score in *ALDH8A1* suggests there are also physiological and/or endocrinal influences involved.

ESR1 has been implicated in general fatness (Section 5.3.1.1) and therefore, it was anticipated that an alteration to *ESR1* function would affect most, if not all, fat depots. However, one SNP (ESR1-14) only had a large effect on marble score, with another SNP (ESR1-2) having a very small effect on omental fat. Estrogen affects adipose tissue by regulating adipogenesis, inhibiting lipogenesis, reducing energy intake and increasing energy expenditure (Section 5.3.1.1). As intramuscular fat is situated within the muscles, it is likely to be utilised as a ready supply of energy (Tume, 2004), similar to seam fat. An alteration to the expression of *ESR1* may, therefore, alter the effect of estrogen on energy expenditure with the most obvious effect on the amount of marbling in the muscle.

Although it could be reasonably expected that, due to their roles in lipogenesis and adipogenesis, alterations to the functions of *ESR1* and *BCMO1* would have global effects on fat deposition, this was not the case. However, there are three points to consider. Firstly, none of the SNPs caused an amino acid change, altered intron splice sites or caused any other obvious alteration to gene function. Therefore, any effect caused by these SNPs is likely to be regulatory, and as such, may not have a large effect on protein function. Secondly, all of these fat deposition traits are quantitative and variation in these traits is due to many genes, exerting usually small effects. Therefore, these SNPs may be causing variation in more, or all, traits, but these small effects are being hidden by other factors, such as the environment or possibly other, yet to be identified, SNPs. Lastly, these SNPs may be in linkage disequilibrium with the

actual polymorphisms causing the variation in fat deposition and therefore, would only reflect some of the overall variation.

TEK1 is involved in angiogenesis and vascularisation (Section 5.3.1.2), which is intricately involved in adipogenesis and lipolysis. One SNP in *TEK1*, TEK1-1, was associated with a very large effect on channel (internal) fat and a smaller effect on P8 (subcutaneous) fat. The role *TEK1* plays in angiogenesis, and therefore adipogenesis, implies that alterations to gene function would also have a global effect on fat deposition. However, the results herein are consistent with the aforementioned variation in vasculature between internal fat and subcutaneous fat. Furthermore, although the SNP effect was not large, there was a highly significant effect on omental fat, another internal fat depot (P = 0.002, Table 6.2). *TEK1* is highly polymorphic in cattle, with 38 sequence variants identified at an average of 376 bases per variant herein, although the SNPs were often clustered in small areas. The effect of the TEK1-1 SNP on fat deposition warrants further investigation of this gene. Firstly, the remaining exon should be sequenced to identify polymorphisms that may be causing a more overt change in gene function. The exon (exon 12) was not sequenced due to its resistance to PCR amplification, and may require sub-cloning into a vector for amplification and then sequencing. Secondly, larger populations of cattle, including different breeds should be genotyped for the TEK1-1 SNP. If the TEK1-1 polymorphism remains in strong linkage disequilibrium with variation in internal fat in larger populations and different cattle breeds, this SNP would be a good candidate for marker assisted selection. Efficient reproduction is dependent, at least in part, on maintaining a level of overall fatness, rather than fat in a specific depot. Therefore, being able to select for increased intramuscular fat, a high value depot, while selecting against a less valuable depot, such as internal or intermuscluar fat, would be very advantageous.

The large effect of these SNPs in *BCMO1*, *ALDH8A1, ESR1* and *TEK1* on fat deposition suggests they are responsible for the variation. However, other SNPs within these genes were associated with variation in other fat traits. This does not imply a global effect, as no gene had SNPs associated with all fat traits. It is possible that this reflects the fact that different adipose depots vary in their sensitivity to hormones (e.g. estrogen) or metabolites (e.g. vitamin A). All the evidence herein suggests that the fat depots are genetically distinct. Therefore, the SNPs with effects herein may only affect one or two fat depots and account for the genetic distinction between the depots.

In summary, analysis of the relationship between the fat depots showed that cohort, breed and *myostatin* F94L genotype altered the amount of fat deposited in an animal, but sire influenced where that fat was deposited. Heritability and genetic correlations estimated in the Trangie RFI Selection Line supported this supposition (Table 4.17 and 4.18), and together these results suggest there is only a weak to moderate genetic relationship between the fat depots. The analysis of SNP association with fat deposition traits showed that no SNP was associated with variation in all fat depots. When these results are viewed together, they are a strong indication that there is only a weak genetic relationship between the fat depots, which is consistent with previous literature (Section 4.4.4). Consequently, there is scope for selecting for and against individual fat traits. As the variation resulting from individual SNPs is likely to be relatively small compared to the environmental effects, marker assisted selection will be the most efficient method of improving cattle breeding for fat distribution traits.

7.8 Project limitations

There are limitations inherent in all scientific investigations involving large, long gestational animals. For cattle, the costs involved in establishing and maintaining a resource herd of sufficient number, and the logistics of sampling this resource can be

prohibitive. Consequently, the number of cattle must be restricted or commercial partnerships utilised.

In the case of this research, the Davies Gene Mapping herd was the principal resource for examining fat distribution, gene association analysis and image analysis. The AgResearch Gene Mapping Project was incorporated to increase the number of cattle measured using the same breed. Using these herds enabled a comparison of fat distribution at different ages and under differing feed regimes while keeping the breed constant. The Trangie herd was included in the analysis because although the breed was different, this herd was slaughtered at a similar age to the AgResearch herd but was grain finished similar to the Davies herd. Furthermore, the Trangie herd provided a good source of highly marbled cattle for use in the image analysis.

The use of three different herds imposed some unavoidable limitations. The Davies herd was slaughtered in five separate cohorts over three years, the AgResearch herd in 28 groups over two years, and the Trangie herd were all slaughtered the same day, eight to ten years after the Davies and AgResearch herds. Each herd was slaughtered at a different abattoir and for different markets. The Trangie herd was prepared for export and therefore, was quartered at the $10^{th}/11^{th}$ rib, whereas the Davies herd were quartered at the $5^{th}/6^{th}$ rib. Furthermore, the multiple slaughters introduced different graders assessing marble score, an already partly subjective measurement. In addition to this, the different quartering sites may have altered the marbling results. More research is required to evaluate marbling along the length of the *Longissimus dorsi* as this may vary, as does the area of this muscle (Rutley *et al.*, 2002). As there were different graders involved in assessing marble scores, and these scores are to an extent, subjective, some caution should be used with these results particularly in regard to the association analysis (Chapter 6).

The measurement of subcutaneous fat also differed between herds. The Davies and Trangie herds subcutaneous fat was measured as depth whereas the AgResearch herd subcutaneous fat was predominantly measured as weight. While this was not ideal, the trait measurements were consistent within each herd and the amount of variation within each herd was comparable between the herds.

It could be argued the differences between the three herds (i.e. breed, environment, age and methods of measurement) used in this research could make interpreting the results problematic at best and unreliable at worst. However, without dismissing this argument, the incorporation of the three herds served to highlight variation in fat distribution between these herds which would have been unnoticed had the herds been evaluated in isolation. While the method of measurement, and therefore the accuracy, may have varied, it has raised the questions of firstly, is the variation real or a result of comparing different measurement practices? Secondly, if the variation is real, is it the result of age, diet, a combination of these or some other factor?

Less emphasis has been placed on the Trangie herd with regard to fat distribution. This herd introduces breed differences but more importantly, there were limited measurements of this herd and therefore, less direct comparisons can be made. It would have been preferable for measures of rump fat and internal fat to have been included to provide a better comparison to the Davies and AgResearch herds. This highlights a common problem in animal science. The Davies and AgResearch herds were bred and controlled by the respective research organisations and therefore, there was more control over what samples and measurements were taken, the restrictions inherent in slaughtering animals in a commercial abattoir notwithstanding. In comparison, the Trangie herd were the steers bred from the NSW Department of Industry and Investment, Trangie RFI selection line. These steers had been sold to a

commercial feedlot and therefore, there was little control of the sampling and measurements.

As cattle slaughters must be completed in a licensed abattoir, the time and space available for sampling will generally be limited. For this reason, image analysis of marbling would be an advantage. This would require either a section of the *Longissimus dorsi* being taken for analysis later, or an improved facility to photograph the cut site if a sample cannot be taken. The importance of a controlled setting for photographing the steaks was demonstrated in this research. The low quality images taken at the Davies herd slaughter were not suitable for marbling analysis, which prevented any QTL and association analysis of marbling characteristics. Image analysis of marbling would negate the subjective assessment used currently, remove the discrepancies between the AUS-MEAT and MSA grading (Section 6.4.3.3) and standardise the classification for all slaughters. Furthermore, image analysis will provide more information

Subcutaneous fat measures could also be standardised and likely made more accurate by measuring the fat weight rather than fat depth. Due to the method of hide removal, fat can be removed with the hide intermittently, therefore, reducing the accuracy of fat depth as a measure of total subcutaneous fat. Although fat weight would also be affected by the hide removal, the impact would be much less. However, this may be impractical in a commercial abattoir as disruption to the operation must be minimised.

The high cost of cattle research and the requirement to slaughter the animals in a licensed abattoir will usually require a commercial partnership or agreement. This will restrict the amount and consistency of data obtained from a given project. However, if more control of the resource can be retained by maintaining ownership and recognising

there is a cost to this research, there will be a corresponding increase in quality of data obtained.

7.9 Future directions

7.9.1 Validate SNPs

Although there were SNPs identified as being associated with variation in fat deposition, these need to be validated in larger populations and multiple breeds. The Davies Gene Mapping herd was a herd of 366 progeny from just three sires. To validate the SNPs, many more sires and their progeny are required to better calculate the heritability, genetic correlations between the SNPs and fat depots, and the size of effect. Also, multiple breeds are required to ensure the effects of these SNPs are present in all breeds. For example, the SNP in the sphingosine 1-phosphate receptor 1 (*EDG1*) gene that is associated with increased marbling in Japanese Black cattle (Yamada *et al.*, 2009a) was not present in the three sires of the Davies Gene Mapping herd. Another example is the GeneSTAR® marker for increased marbling which was not associated with marbling or intramuscular fat percent in Simmental steers (Rincker *et al.*, 2006). It cannot be assumed that a SNP associated with variation in a particular trait in one breed will be associated with that trait in other breeds. Where this occurs, other SNPs will be required to use for association studies.

There were interactions between SNPs within genes and in different genes, which were associated with variation in fat traits. However, the number of animals in the Davies herd was not sufficient to confirm the interactions. Therefore, increasing the number of animals tested for these associations would also allow confirmation of the interactions and better estimations of the size of effect.

7.9.2 Gene expression

Where an association between a gene and variation in a fat trait has been identified, it is important to determine whether that gene is causing the variation or the SNP is in linkage disequilibrium with the causative mutation. To do this, one approach is to determine the level of expression of that gene in the depot compared to reference genes and other fat depots. RNA extracted from adipose biopsies taken from different adipose depots, and also different regions within those depots, should be used for real time PCR quantification of gene expression. All genes identified as being associated with fat deposition should be assayed in this manner, not just those that have been shown to have a large effect. It is important to identify any difference in expression in the depots that were affected by these SNPs but also in the depots without the effect to ensure all genes affecting fat distribution are identified, and also to exclude genes that are only in linkage disequilibrium with the causative mutation.

7.9.3 Epigenetics

Epigenetics is variation in gene function that occurs without an alteration to the DNA sequence (Herrera *et al.*, 2011). This alteration to gene function is mediated by DNA methylation or histone modifications as a result of parental imprinting (Stoger, 2008, Herrera *et al.*, 2011), microRNAs which interfere with the regulation of gene transcription (Gluckman and Hanson, 2008, Campbell *et al.*, 2011) or environmental effects, principally nutrition (Waterland and Jirtle, 2004). Imprinting is most likely the result of competing needs of the parents. The sire breeds to produce bigger and stronger offspring from a single mating to ensure the continuation of the genetic line, whereas the dam's needs are to maintain a constant breeding efficiency, which can be compromised by carrying, and then feeding, an overly large offspring (Stoger, 2008). The epigenetic effect on energy balance, and hence obesity, in humans is greater than

that of DNA polymorphisms and it is expected that the epigenetic modification will affect a large range of genes (Stoger, 2008). Investigating epigenetics as it relates to fat deposition and distribution will be important, particularly as *PPARG*, investigated herein due to its critical role in adipogenesis, interacts with histone acetyltransferase (Fu *et al.*, 2004), and epigenetics is implicated in increased body mass index and obesity in humans (Herrera *et al.*, 2011). If there is a strong epigenetic effect on fat deposition and distribution, this will have important implications on the accuracy of genomic selection.

7.9.4 Vascularisation

Most of the research indicating the different level of vascularisation between fat depots has involved cell culture. Although cell culture closely mimics *in vivo* adipogenesis (Rosen and Spiegelman, 2000), it does have inherent limitations, as authentic replication of *in vivo* processes and fat depot specific aspects of adipose tissue is not guaranteed (Djian *et al.*, 1985, Rosen *et al.*, 2000, Selvarajan *et al.*, 2001) (Section 1.5). Histological studies of fat depots from cattle, similar to those used by Hausman and Thomas (1986) in their study of pigs, would help to identify the relationship between the capillary network and adipose cell development in cattle. This would also confirm the vasculature patterning across species. Other histological procedures such as corrosion casting (Kondo, 1998, Minnich and Lametschwandtner, 2010) has been used for tracing blood vessels in muscles and organs, and may be useful for studying vasculature in adipose tissue, although whether this is applicable for adipose and capillaries is unknown at this stage.

7.10 Conclusions

There were four important points from the results herein. Firstly, angiogenesis may be more important in the control of adipogenesis and fat distribution than first realised. As well as the role in adipogenesis, differences in vascularisation between fat depots is likely to impact the deposition and mobilisation of lipids, and also the hormone action both within and secreted from the adipose tissue.

Secondly, a QTL for seam fat area was identified and neither this nor any of the other QTL identified in the Davies Gene mapping herd previously (Esmailizadeh, 2006) were associated with all of the fat deposition traits, suggesting a level of genetic independence between these traits. In a recent review, Hausman *et al.* (2009) also noted that the separate QTL for subcutaneous fat and marble score indicates these traits have different genetic controls.

Thirdly, there were only low to moderate correlations between all fat traits in all the herds. The correlations between fat traits were higher in the AgResearch herd than the Davies herd. This may be due to the higher energy diet of the lot fed Davies herd, as increased energy diets produce faster growth, increased fat deposition and more variation in that fat deposition (Kempster *et al.*, 1976, Leat, 1977, Bidner *et al.*, 1981, Camfield *et al.*, 1999, Kerth *et al.*, 2007, Smith *et al.*, 2009, McCurdy *et al.*, 2010) (Section 4.4.6). Alternatively, the difference in fat depots may increase as the animal ages and therefore, the difference was more noticeable in the three year old Davies animals than in the two year old AgResearch animals. Nevertheless, the low correlations between all fat depots in all herds, and the lack of consistently high or low fat progeny from any given individual sire, suggest that although there is a large genetic component in fat distribution, the individual fat depots do not have a strong genetic relationship. Therefore, it should be possible to select for or against individual

fat depots independently of the other fat depots. This weak relationship between fat depots has been reported previously in pigs, where correlations between intramuscular fat and two internal fat depots and five subcutaneous fat depots were low (Yang *et al.*, 2010).

Lastly, although there were associations identified between DNA polymorphisms and most fat depots, none of the SNPs were associated with variation in all fat depots. This again indicates that there is a level of genetic independence in the distribution of fat, as was already suggested by the low correlations between fat depots and lack of QTL affecting multiple fat traits. Most of the SNP associations had low or moderate effects on a specific fat depot. However, when the effects of multiple polymorphisms were combined and for some individual SNPs, the overall effect was quite large in many cases. The combined effect was expected, as fat deposition traits are quantitative and therefore, controlled by multiple genes of small effect. Although the SNP effects were influenced by the small population investigated herein, it does suggest that it is possible to make significant improvements in cattle composition through the use of suitable selection techniques, such as marker assisted selection. Furthermore, the identification of these polymorphisms should allow the biology of fat deposition and distribution to be elucidated. This, in turn, will enable the identification of molecular markers suitable for inclusion in a direct method of marker assisted selection, where the causative variant is used for selection instead of whole genome selection which relies on the genetic markers being in strong linkage disequilibrium with the causative mutation (Kruglyak, 1999). The possibility that the linkage disequilibrium may reduce over successive generations or over different breeds limits this approach versus the selection of causative variants. Nevertheless, whichever method is utilised, marker assisted selection for fat traits will help to improve the quantity and quality of beef produced.

Appendices

Appendix A Polymerase Chain Reaction methods

Appendix A.1 Polymerase Chain Reaction mixes

Amplitaq Gold

A standard 25µl reaction mix consisted of 125µM dCTP, 125µM dGTP, 125µM dATP, 125µM dTTP, 2.5pmol forward primer, 2.5pmol reverse primer, 1x Buffer (*Applied Biosystems – Roche*), 2.5mM $MgCl_2$, 0.5 units Amplitaq Gold (*Applied Biosystems – Roche*), 50ng genomic DNA and 13.5ul H_2O.

Gibco Taq

A standard 25µl reaction mix consisted of 125µM dCTP, 125µM dGTP, 125µM dATP, 125µM dTTP, 2.5pmol forward primer, 2.5pmol reverse primer, 1x Buffer (*Invitrogen*), 2.5mM $MgCl_2$ (*Invitrogen*), 0.5 units Gibco Taq (*Invitrogen*), 50ng genomic DNA and 13.5ul H_2O.

KAPATaq

A standard 25µl reaction mix consisted of 125µM dCTP, 125µM dGTP, 125µM dATP, 125µM dTTP, 10pmol forward primer, 10pmol reverse primer, 1x Buffer (1.5mM $MgCl_2$) (*Kapa Biosystems*), 0.5 units KapaTaq (*Kapa Biosystems*), 50ng genomic DNA and 16ul H_2O.

Kapa2G Robust Polymerase

A standard 25µl reaction mix consisted of 125µM dCTP, 125µM dGTP, 125µM dATP, 125µM dTTP, 10pmol forward primer, 10pmol reverse primer, 1x Buffer B (1.5mM $MgCl_2$) (*Kapa Biosystems*), 1x Enhancer 1 (*Kapa Biosystems*), 0.5 units KapaTaq (*Kapa Biosystems*), 50ng genomic DNA and 8.5ul H_2O.

Polymerase Chain Reaction high resolution melt genotyping mixes.

KapaTaq.

A standard 20µl reaction mix consisted of 125µM dCTP, 125µM dGTP, 125µM dATP, 125µM dTTP, 10pmol forward primer, 10pmol reverse primer, 1x Buffer (1.5mM $MgCl_2$) (*Kapa Biosystems*), 0.5 units KapaTaq (*Kapa Biosystems*), 1.95µM Syto® 9 (*Invitrogen*) green fluorescent nucleic acid stain, 50ng genomic DNA and H_2O to 20µl.

Sensimix

A standard 20µl reaction mix consisted of 10µl Sensimix™ (*Quantace Ltd* – 2x HRM mix contains heat activated taq polymerase, reaction buffer, dNTPs, 6mM $MgCl_2$) 2.5pmol forward primer, 2.5pmol reverse primer, 50ng genomic DNA, 1µl EvaGreen™ (*Biotium Inc.*) fluorescent intercalating dye and H_2O to 20µl.

Appendix A.2 Polymerase Chain Reaction programs

Touchdown program reduced the annealing temperature 1°C per cycle for the first 10 cycles. Programs 1 and 2 only differed in the annealing temperature, program 2 is in brackets.

Amplitaq Gold and Gibco

1 cycle: 95°C x 10 minutes initial melt

35 cycles: 95°C x 60 seconds melt

60°C (70°C) x 60 seconds anneal.

72°C x 60 seconds extension

1 cycle: 72°C x 10 minutes final extension

4°C store

KAPATaq and Kapa Robust

As Kapa Robust is not a hot start enzyme the reaction mix was prepared and stored on ice until the thermal cycler had reached the melt temperature of 95°C.

1 cycle: 95°C x 7 minutes initial melt

35 cycles: 95°C x 40 seconds melt

60°C (70°C) x 30 seconds anneal

72°C x 60 seconds extension

1 cycle: 72°C x 10 minutes final extension

4°C store

Amplification for high resolution melt analysis

1 cycle: 95°C x 7 minutes initial melt

35 cycles: 95°C x 30 seconds melt

60°C (70°C) x 30 seconds anneal

72°C x 30 seconds extension

1 cycle: 72°C x 10 minutes final extension

Appendix B Correlation within cohorts of various image analysis results from Davies Gene Mapping Herd

Min – max fleck area			10 – 100mm				10 – 150mm				5 – 100mm				5 – 150mm				All flecks				MA
		Correl with:	fleck area	fleck no.	FA / MA	FN / MA	fleck area	fleck no.	FA / MA	FN / MA	fleck area	fleck no.	FA / MA	FN / MA	fleck area	fleck no.	FA / MA	FN / MA	fleck area	fleck no.	FA / MA	FN / MA	
1996	all	mbusms	0.39	0.36	0.46	0.43	0.37	0.35	0.45	0.43	0.34	0.24	0.41	0.30	0.33	0.24	0.41	0.30	0.28	0.01	0.36	0.08	0.82
		imf	0.16	0.20	0.25	0.30	0.18	0.20	0.27	0.30	0.17	0.19	0.27	0.30	0.19	0.20	0.28	0.31	0.17	0.09	0.28	0.20	
	good	mbusms	0.45	0.33	0.68	0.55	0.42	0.34	0.65	0.56	0.31	-0.02	0.54	0.19	0.30	-0.01	0.54	0.20	0.23	-0.27	0.47	0.01	0.72
		imf	0.51	0.49	0.63	0.59	0.53	0.50	0.64	0.60	0.39	0.14	0.51	0.25	0.43	0.15	0.55	0.26	0.36	-0.05	0.46	0.09	
	best	mbusms	0.54	0.43	0.77	0.68	0.52	0.43	0.73	0.68	0.48	0.18	0.74	0.52	0.47	0.19	0.70	0.52	0.40	-0.19	0.65	0.29	0.68
		imf	0.56	0.55	0.71	0.71	0.56	0.56	0.69	0.71	0.50	0.28	0.67	0.49	0.50	0.28	0.65	0.50	0.46	0.17	0.60	0.42	
1997H	all	mbusms	0.14	0.16	0.29	0.32	0.14	0.16	0.29	0.32	0.12	0.11	0.31	0.30	0.07	-0.01	0.17	0.04	0.08	0.01	0.28	0.15	0.88
		imf	0.15	0.14	0.40	0.39	0.19	0.15	0.46	0.40	0.09	0.01	0.38	0.28	-0.05	-0.18	0.13	-0.05	0.07	-0.06	0.37	0.17	
	good	mbusms	0.26	0.28	0.40	0.43	0.24	0.28	0.37	0.43	0.23	0.20	0.41	0.37	0.26	0.20	0.34	0.22	0.19	0.11	0.41	0.30	0.86
		imf	0.21	0.17	0.49	0.45	0.25	0.18	0.54	0.46	0.15	0.05	0.48	0.36	0.03	-0.11	0.22	0.01	0.13	-0.04	0.52	0.30	
	best	mbusms	0.32	0.37	0.54	0.59	0.30	0.37	0.50	0.58	0.30	0.26	0.57	0.52	0.30	0.19	0.38	0.20	0.22	0.06	0.54	0.35	0.85
		imf	0.28	0.28	0.59	0.57	0.35	0.29	0.66	0.59	0.21	0.08	0.58	0.41	0.10	-0.07	0.28	0.05	0.19	-0.02	0.60	0.36	
1997S	all	mbusms	0.15	0.10	0.35	0.32	0.17	0.10	0.38	0.32	0.08	-0.05	0.34	0.24	0.10	-0.05	0.36	0.24	0.00	-0.23	0.31	0.04	0.89
		imf	0.00	-0.07	0.30	0.25	0.02	-0.07	0.32	0.26	-0.06	-0.17	0.31	0.25	-0.05	-0.17	0.32	0.25	-0.12	-0.28	0.32	0.18	
	good	mbusms	0.44	0.39	0.60	0.62	0.44	0.39	0.63	0.63	0.41	0.31	0.63	0.63	0.41	0.31	0.65	0.64	0.35	0.20	0.66	0.63	0.88
		imf	0.35	0.21	0.63	0.56	0.33	0.20	0.63	0.56	0.27	0.06	0.62	0.53	0.25	0.06	0.63	0.53	0.14	-0.08	0.60	0.50	
	best	mbusms	0.68	0.64	0.71	0.73	0.71	0.65	0.74	0.74	0.67	0.55	0.74	0.75	0.69	0.55	0.75	0.75	0.63	0.47	0.78	0.78	0.89
		imf	0.68	0.58	0.80	0.77	0.69	0.58	0.81	0.77	0.61	0.39	0.79	0.73	0.62	0.39	0.80	0.73	0.48	0.22	0.78	0.69	
1998H	all	mbusms	0.13	0.07	0.31	0.26	0.13	0.07	0.31	0.26	0.10	0.04	0.30	0.25	0.10	0.04	0.30	0.25	0.05	-0.13	0.28	0.14	0.77
		imf	0.25	0.18	0.42	0.37	0.25	0.18	0.42	0.37	0.21	0.13	0.41	0.35	0.21	0.13	0.41	0.35	0.16	0.01	0.40	0.30	
	good	mbusms	0.15	0.02	0.46	0.27	0.14	0.02	0.45	0.27	0.17	0.12	0.47	0.39	0.16	0.12	0.47	0.39	0.11	0.01	0.39	0.20	0.79
		imf	0.05	0.01	0.44	0.38	0.04	0.01	0.43	0.38	0.05	0.04	0.46	0.42	0.05	0.04	0.45	0.42	0.03	-0.01	0.43	0.33	
	best	mbusms	0.46	0.37	0.88	0.84	0.46	0.37	0.88	0.84	0.44	0.36	0.86	0.77	0.44	0.36	0.86	0.77	0.45	0.40	0.85	0.76	0.67
		imf	0.28	0.31	0.69	0.77	0.28	0.31	0.69	0.77	0.36	0.39	0.77	0.81	0.36	0.39	0.77	0.81	0.37	0.39	0.77	0.75	
1998S	all	mbusms	-0.28	-0.33	-0.04	-0.08	-0.24	-0.32	0.00	-0.07	-0.29	-0.31	-0.02	0.00	-0.26	-0.31	0.02	0.00	-0.29	-0.37	0.02	-0.09	0.84
		imf	-0.23	-0.34	0.11	-0.01	-0.21	-0.33	0.12	0.00	-0.26	-0.34	0.12	0.06	-0.24	-0.34	0.13	0.06	-0.30	-0.43	0.12	-0.08	
	good	mbusms	-0.04	-0.19	0.31	0.08	0.05	-0.17	0.39	0.10	-0.05	-0.16	0.34	0.19	0.03	-0.15	0.41	0.20	-0.06	-0.26	0.34	0.03	0.64
		imf	-0.14	-0.25	0.23	0.06	-0.08	-0.24	0.27	0.07	-0.12	-0.17	0.33	0.29	-0.08	-0.17	0.36	0.30	-0.18	-0.38	0.26	-0.04	
	best	mbusms	0.07	-0.23	0.49	0.06	0.23	-0.20	0.61	0.11	0.03	-0.17	0.42	0.12	0.15	-0.16	0.52	0.14	0.04	-0.35	0.46	-0.07	0.75
		imf	0.06	-0.18	0.45	0.14	0.27	-0.14	0.66	0.21	0.03	-0.12	0.42	0.20	0.20	-0.10	0.58	0.23	0.12	-0.22	0.56	0.11	

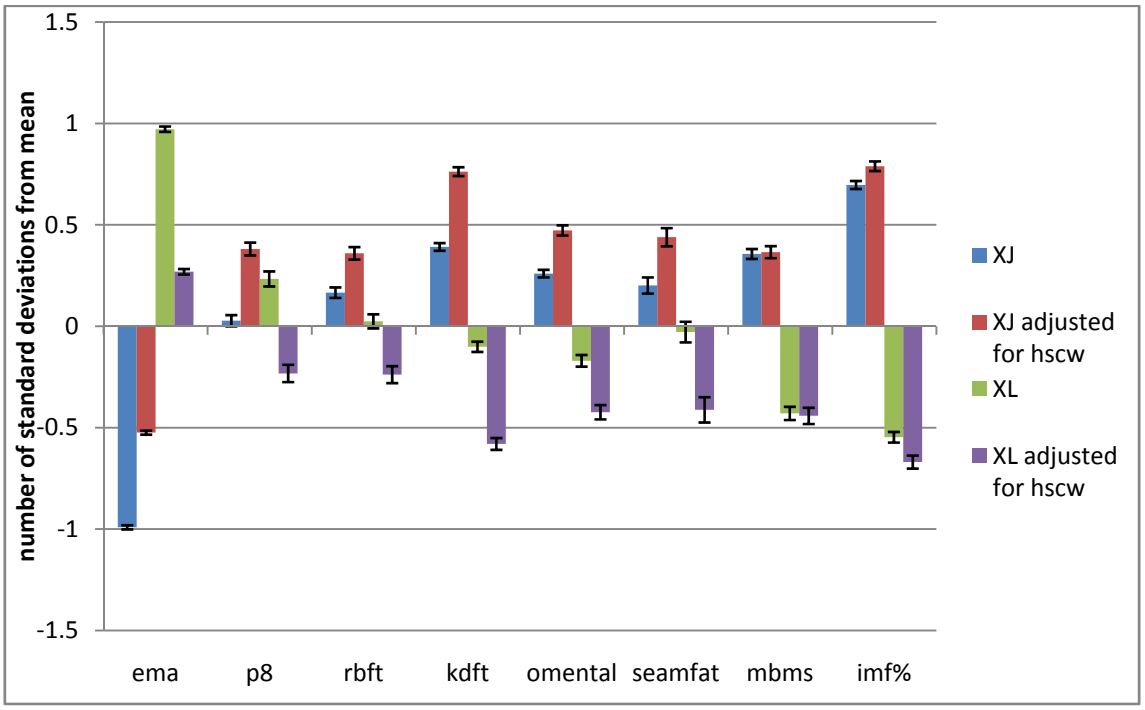
Appendix C Comparison of correlations between marble score and image analysis with differing thresholds and parameters in separate marble score ranges.

			MSAMB				IMF%			
	marble score range	n	fleck area	FA/MA	fleck number	FN/MA	fleck area	FA/MA	fleck number	FN/MA
All flecks	**350 - 390**	**36**	0.12	0.18	0.04	0.03	0.13	0.29	0.14	0.32
	400 - 480	**74**	-0.06	0.01	-0.09	-0.02	0.31	0.55	0.17	0.43
	490 - 520	**32**	0.11	0.05	0.09	0.00	0.48	0.63	0.40	0.57
	530 - 610	**32**	0.49	0.49	0.33	0.25	0.56	0.75	0.38	0.53
	620 - 830	**33**	-0.08	-0.11	0.10	0.09	0.04	0.36	-0.24	0.14
> 5mm	**350 - 390**	**36**	0.14	0.19	0.12	0.16	0.15	0.24	0.24	0.37
	400 - 480	**74**	-0.04	0.01	0.00	0.08	0.33	0.46	0.39	0.59
	490 - 520	**32**	0.13	0.10	0.25	0.24	0.44	0.55	0.39	0.51
	530 - 610	**32**	0.51	0.50	0.52	0.49	0.59	0.71	0.56	0.67
	620 - 830	**33**	-0.14	-0.16	0.10	0.08	0.15	0.37	0.15	0.46
< 100mm	**350 - 390**	**36**	0.08	0.13	0.04	0.03	0.14	0.29	0.14	0.32
	400 - 480	**74**	-0.07	-0.01	-0.09	-0.02	0.32	0.57	0.17	0.43
	490 - 520	**32**	0.11	0.05	0.09	0.00	0.48	0.63	0.40	0.57
	530 - 610	**32**	0.49	0.49	0.33	0.25	0.56	0.75	0.38	0.53
	620 - 830	**33**	-0.08	-0.11	0.10	0.09	0.04	0.36	-0.24	0.14
< 150mm	**350 - 390**	**36**	0.12	0.18	0.04	0.03	0.13	0.29	0.14	0.32
	400 - 480	**74**	-0.06	0.01	-0.09	-0.02	0.31	0.55	0.17	0.43
	490 - 520	**32**	0.11	0.05	0.09	0.00	0.48	0.63	0.40	0.57
	530 - 610	**32**	0.49	0.49	0.33	0.25	0.56	0.75	0.38	0.53
	620 - 830	**33**	-0.08	-0.11	0.10	0.09	0.04	0.36	-0.24	0.14
5 - 100mm	**350 - 390**	**36**	0.09	0.14	0.12	0.15	0.16	0.25	0.24	0.37
	400 - 480	**74**	-0.05	-0.01	0.00	0.07	0.35	0.50	0.39	0.59
	490 - 520	**32**	0.13	0.10	0.25	0.24	0.44	0.55	0.39	0.51
	530 - 610	**32**	0.51	0.50	0.52	0.49	0.59	0.71	0.56	0.67
	620 - 830	**33**	-0.14	-0.16	0.10	0.08	0.15	0.37	0.15	0.46

Appendix D Correlations between image analysis fleck characteristics.

	Fleck number	FA / EMA	fleck area	FN / EMA	average fleck area	average eccentricity of fleck	average orientation	average ellipticity	average normalised ellipticity	average X coord of fleck	average Y coord of fleck	Average of dist from centre	ave dist from centre / EMA	MSAMB	IMF%
Fleck number	1														
FA / EMA3	0.74	1													
fleck area	0.86	0.91	1												
FN / EMA3	0.87	0.84	0.75	1											
average fleck area	0.12	0.61	0.59	0.10	1										
average eccentricity of fleck	-0.21	-0.29	-0.21	-0.31	-0.08	1									
average orientation	0.03	-0.05	-0.04	0.03	-0.14	-0.11	1								
average ellipticity	0.08	0.55	0.53	0.06	0.95	-0.19	-0.09	1							
average normalised ellipticity	-0.08	0.23	0.19	-0.05	0.51	-0.33	0.02	0.68	1						
average X coord of fleck	-0.10	-0.16	-0.12	-0.15	-0.09	-0.02	-0.04	-0.07	0.06	1					
average Y coord of fleck	0.16	0.13	0.17	0.10	0.11	-0.06	-0.16	0.09	0.02	-0.15	1				
Average of dist from centre	0.44	0.14	0.40	0.12	0.08	0.08	0.11	0.10	0.01	0.01	0.16	1			
ave dist from centre / EMA3	-0.29	0.04	-0.25	0.07	-0.06	-0.10	0.06	-0.02	0.13	-0.02	-0.06	-0.03	1		
MSAMB	0.44	0.47	0.46	0.47	0.18	-0.26	0.05	0.14	0.00	-0.03	0.09	0.09	-0.02	1	
IMF%	0.50	0.61	0.50	0.65	0.18	-0.20	-0.01	0.14	0.03	-0.12	0.01	-0.05	0.13	0.49	1

Appendix E Comparison of fat depots and ema with and without carcass weight as covariate



Appendix F SNP effects: cohort + breed + sire + SNP

SNP	EMA	fat %	fattobn	hscw	imf%	kidfat	marbam	mbms	mbusms	meltpt	omental	p8am	rbft	seam fat	desat	elong	mufa	sfa
ACACA7	0.228	0.134	0.171	0.488	0.176	0.855	0.239	0.058	0.018	0.682	0.262	0.305	0.442	0.06	0.746	0.333	0.289	0.239
ALDH8A10	0.356	0.959	0.978	0.632	0.316	0.604	0.004	0.069	0.127	0.786	0.591	0.99	0.427	0.619	0.979	0.869	0.953	0.863
ALDH8A15	0.817	0.273	0.143	0.932	0.505	0.152	0.354	0.13	0.059	0.933	0.998	0.664	0.945	0.356	0.423	0.921	0.221	0.268
ALDH8A16	0.161	0.694	0.992	0.654	0.259	0.488	0.007	0.311	0.397	0.833	0.849	0.982	0.358	0.722	0.851	0.686	0.631	0.515
APM1-1	0.435	0.266	0.144	0.372	0.161	0.064	0.108	0.048	0.216	0.49	0.772	0.458	0.792	0.813	0.382	0.108	0.086	0.086
ATP2B432	0.427	0.38	0.712	0.084	0.304	0.791	0.437	0.131	0.244	0.238	0.842	0.327	0.974	0.617	0.717	0.485	0.648	0.875
ATP2B433	0.978	0.465	0.575	0.131	0.088	0.392	0.334	0.068	0.081	0.183	0.976	0.152	0.907	0.9	0.008	0.336	0.539	0.242
BCMO113	0.125	0.807	0.742	0.006	0.105	0.55	0.4	0.792	0.707	0.295	0.846	0.208	0.861	0.38	0.233	0.111	0.747	0.539
BCMO14	0.616	0.137	0.082	0.542	0.209	0.033	0.532	0.483	0.229	0.735	0.041	0.003	0.548	0.176	0.89	0.136	0.475	0.431
BCMO17	0.641	0.319	0.279	0.031	0.155	0.231	0.113	0.522	0.798	0.51	0.508	0.815	0.405	0.176	0.285	0.443	0.965	0.954
BCMO18	0.798	0.166	0.151	0.793	0.119	0.009	0.34	0.465	0.502	0.133	0.206	0.021	0.096	0.006	0.456	0.13	0.905	0.969
EDG1-UTR	0.562	0.484	0.6	0.939	0.466	0.764	0.21	0.063	0.048	0.854	0.782	0.194	0.651	0.006	0.649	0.665	0.516	0.518
ENO3-11	0.678	0.892	0.86	0.609	0.813	0.221	0.949	0.641	0.866	0.247	0.919	0.597	0.593	0.509	0.13	0.39	0.467	0.451
ENO3-5	0.605	0.07	0.143	0.957	0.886	0.642	0.559	0.293	0.52	0.059	0.38	0.937	0.422	0.921	0.103	0.474	0.076	0.088
ESR1 - 2	0.017	0.072	0.138	0.211	0.815	0.274	0.293	0.58	0.389	0.535	0.015	0.133	0.127	0.444	0.637	0.482	0.315	0.527
ESR1-14	0.553	0.266	0.125	0.4	0.15	0.615	0.042	0.089	0.074	0.354	0.498	0.797	0.449	0.229	0.977	0.987	0.963	0.939
LAMIN-1	0.237	0.006	0.013	0.31	0.212	0.444	0.999	0.366	0.479	0.045	0.283	0.078	0.82	0.367	0.257	0.828	0.141	0.09
NCOA7-1	0.754	0.309	0.327	0.78	0.046	0.514	0.893	0.548	0.781	0.039	0.509	0.881	0.397	0.707	0.82	0.523	0.867	0.892
NCOA7-1a	0.858	0.091	0.181	0.724	0.116	0.295	0.83	0.415	0.712	0.732	0.45	0.285	0.328	0.941	0.96	0.354	0.961	0.874
NCOA7-1b	0.857	0.213	0.28	0.743	0.1	0.512	0.708	0.808	0.916	0.138	0.484	0.815	0.657	0.786	0.601	0.249	0.832	0.953

Appendix F continued.

SNP	EMA	fat %	fattobn	hscw	imf%	kidfat	marbam	mbms	mbusms	meltpt	omental	p8am	rbft	seam fat	desat	elong	mufa	sfa
NCOA7-2	0.303	0.652	0.765	0.543	0.26	0.413	0.174	0.207	0.045	0.947	0.46	0.482	0.213	0.682	0.602	0.407	0.817	0.802
NCOA7-3	0.591	0.817	0.858	0.384	0.128	0.273	0.606	0.676	0.378	0.258	0.904	0.844	0.963	0.491	0.178	0.412	0.646	0.559
NCOA7-4	0.689	0.724	0.75	0.551	0.11	0.397	0.584	0.746	0.695	0.226	0.793	0.685	0.845	0.289	0.101	0.389	0.425	0.398
PPARa-4	0.6	0.255	0.337	0.211	0.704	0.067	0.918	0.822	0.467	0.079	0.999	0.955	0.873	0.003	0.932	0.067	0.338	0.258
PPARG-2	0.601	0.943	0.96	0.522	0.931	0.274	0.888	0.835	0.844	0.184	0.989	0.372	0.651	0.367	0.026	0.928	0.023	0.017
PPARG-6	0.389	0.604	0.474	0.336	0.706	0.794	0.658	0.39	0.898	0.574	0.922	0.915	0.649	0.716	0.311	0.571	0.124	0.231
TEK-1	0.113	0.31	0.199	0.16	0.552	0.015	0.416	0.498	0.377	0.132	0.653	0.073	0.827	0.287	0.043	0.806	0.131	0.119
TEK-2	0.904	0.542	0.586	0.541	0.162	0.652	0.408	0.1	0.123	0.129	0.451	0.656	0.46	0.41	0.709	0.352	0.939	0.982
TEK-3	0.174	0.507	0.354	0.498	0.106	0.756	0.615	0.389	0.414	0.249	0.918	0.66	0.257	0.175	0.464	0.585	0.983	0.894
TEK1-4	0.303	0.938	0.936	0.607	0.639	0.91	0.436	0.171	0.359	0.304	0.676	0.589	0.34	0.943	0.526	0.434	0.486	0.427
TEK1-5	0.085	0.689	0.81	0.378	0.338	0.095	0.412	0.302	0.288	0.467	0.002	0.626	0.899	0.087	0.726	0.426	0.603	0.702
TEK1-6	0.615	0.849	0.801	0.419	0.519	0.98	0.851	0.609	0.767	0.73	0.777	0.385	0.667	0.34	0.979	0.58	0.83	0.884
TEK-7	0.184	0.752	0.767	0.906	0.457	0.709	0.612	0.677	0.594	0.937	0.966	0.863	0.139	0.238	0.429	0.558	0.823	0.892

Appendix G SNP effects: cohort + breed + sire + hscw + SNP

SNP	imf%	kidfat	marbam	mbms	mbusms	omental	p8	rbft	seam fat
ALDH8A10	0.292	0.756	0.004	0.071	0.130	0.544	0.959	0.354	0.672
ALDH8A15	0.497	0.105	0.354	0.132	0.061	0.988	0.573	0.925	0.380
ALDH8A16	0.241	0.601	0.007	0.32	0.406	0.901	0.994	0.287	0.674
APM1-1	0.182	0.128	0.109	0.044	0.205	0.894	0.334	0.683	0.91
ATP2B432	0.338	0.552	0.438	0.128	0.250	0.840	0.664	0.948	0.361
ATP2B433	0.102	0.366	0.334	0.066	0.081	1.00	0.251	0.790	0.931
BCMO113	0.126	0.087	0.401	0.792	0.703	0.581	0.121	0.969	0.792
BCMO14	0.220	0.034	0.536	0.475	0.225	0.04	0.004	0.583	0.267
BCMO17	0.192	0.615	0.113	0.484	0.768	0.596	0.939	0.500	0.331
BCMO18	0.119	0.009	0.342	0.459	0.500	0.177	0.021	0.095	0.006
EDG1-UTR	0.456	0.681	0.21	0.064	0.049	0.726	0.18	0.671	0.003
ENO3-11	0.838	0.124	0.948	0.629	0.857	0.826	0.439	0.677	0.576
ENO3-5	0.865	0.6	0.56	0.295	0.522	0.467	0.935	0.419	0.898
ESR1 - 2	0.839	0.341	0.297	0.569	0.377	0.014	0.171	0.175	0.424
ESR1-14	0.137	0.459	0.043	0.091	0.076	0.613	0.946	0.558	0.177
LAMIN-1	0.240	0.652	0.999	0.350	0.460	0.235	0.164	0.782	0.537
NCOA7-1	0.050	0.586	0.894	0.543	0.786	0.444	0.867	0.368	0.813
NCOA7-1a	0.125	0.382	0.832	0.408	0.705	0.506	0.307	0.304	0.971
NCOA7-1b	0.105	0.607	0.709	0.813	0.921	0.434	0.669	0.584	0.793
NCOA7-2	0.281	0.57	0.176	0.201	0.044	0.491	0.443	0.236	0.596
NCOA7-3	0.123	0.436	0.607	0.668	0.367	0.896	0.65	0.886	0.353
NCOA7-4	0.106	0.510	0.585	0.75	0.694	0.739	0.503	0.757	0.209
PPARa-4	0.756	0.165	0.919	0.814	0.463	0.772	0.742	0.757	0.006
PPARG-2	0.917	0.408	0.889	0.834	0.839	0.916	0.316	0.702	0.335
PPARG-6	0.732	0.594	0.66	0.400	0.908	0.811	0.861	0.765	0.629
TEK-1	0.561	0.045	0.423	0.510	0.388	0.845	0.067	0.922	0.437
TEK-2	0.179	0.474	0.406	0.095	0.117	0.456	0.773	0.526	0.405
TEK-3	0.100	0.877	0.658	0.386	0.413	0.903	0.639	0.293	0.216
TEK1-4	0.615	0.740	0.439	0.170	0.356	0.783	0.738	0.267	0.871
TEK1-5	0.306	0.041	0.425	0.302	0.286	0.001	0.816	0.780	0.138
TEK1-6	0.487	0.833	0.856	0.598	0.762	0.783	0.511	0.606	0.437
TEK-7	0.456	0.727	0.615	0.675	0.591	0.961	0.821	0.149	0.251

Appendix H SNP effect: cohort + BOD + sire + BOD.mstn + SNP + SNP.mstn – SNP effect

SNP	EMA	fat %	fattobn	hscw	imf%	kidfat	marbam	mbms	mbusms	meltpt	omental	p8	rbft	seam fat	desat	elong	mufa	sfa
ACACA7	0.438	0.371	0.221	0.56	0.399	0.839	0.754	0.155	0.054	0.437	0.233	0.573	0.375	0.197	0.591	0.41	0.232	0.181
ALDH8A10	0.277	0.841	0.985	0.591	0.347	0.707	0.023	0.051	0.118	0.654	0.589	0.977	0.384	0.401	0.933	0.821	0.884	0.795
ALDH8A15	0.682	0.185	0.111	0.898	0.485	0.2	0.507	0.123	0.052	0.833	0.995	0.67	0.895	0.261	0.457	0.911	0.22	0.26
ALDH8A16	0.126	0.827	0.963	0.651	0.374	0.626	0.053	0.249	0.349	0.837	0.861	0.895	0.392	0.8	0.742	0.552	0.46	0.399
APM1-1	0.437	0.145	0.125	0.436	0.121	0.088	0.203	0.035	0.178	0.559	0.687	0.517	0.882	0.878	0.43	0.111	0.099	0.104
ATP2B432	0.618	0.929	0.908	0.078	0.437	0.782	0.935	0.242	0.403	0.166	0.738	0.2	0.805	0.752	0.473	0.427	0.798	0.967
ATP2B433	0.926	0.545	0.552	0.089	0.132	0.381	0.97	0.11	0.124	0.117	0.969	0.088	0.959	0.985	0.003	0.318	0.483	0.189
BCMO113	0.086	0.676	0.442	0.012	0.044	0.555	0.241	0.673	0.625	0.367	0.842	0.196	0.784	0.154	0.298	0.13	0.531	0.363
BCMO14	0.854	0.22	0.101	0.456	0.28	0.036	0.259	0.531	0.27	0.717	0.042	0.004	0.459	0.209	0.864	0.163	0.508	0.479
BCMO17	0.148	0.455	0.303	0.026	0.136	0.231	0.223	0.417	0.726	0.435	0.509	0.835	0.373	0.119	0.219	0.483	0.893	0.908
BCMO18	0.758	0.139	0.119	0.782	0.233	0.012	0.539	0.559	0.659	0.125	0.23	0.022	0.111	0.012	0.47	0.157	0.913	0.976
EDG1-UTR	0.414	0.311	0.404	0.877	0.512	0.787	0.362	0.083	0.054	0.884	0.842	0.119	0.579	0.011	0.706	0.682	0.547	0.541
ENO3-11	0.678	0.592	0.695	0.573	0.761	0.244	0.951	0.61	0.828	0.351	0.937	0.654	0.553	0.347	0.143	0.49	0.451	0.424
ENO3-5	0.522	0.176	0.331	0.982	0.873	0.782	0.846	0.368	0.6	0.078	0.416	0.993	0.484	0.993	0.165	0.484	0.1	0.105
ESR1 - 2	0.074	0.267	0.225	0.304	0.749	0.237	0.582	0.309	0.169	0.391	0.017	0.22	0.147	0.565	0.634	0.508	0.344	0.539
ESR1-14	0.529	0.553	0.319	0.385	0.143	0.609	0.03	0.053	0.052	0.319	0.554	0.966	0.648	0.274	0.946	0.942	0.986	0.917
LAMIN-1	0.196	0.033	0.038	0.201	0.383	0.595	0.847	0.445	0.626	0.039	0.276	0.118	0.787	0.561	0.248	0.837	0.131	0.089
NCOA7-1	0.34	0.683	0.598	0.569	0.086	0.744	0.944	0.703	0.84	0.058	0.547	0.97	0.6	0.893	0.732	0.527	0.898	0.911
NCOA7-1a	0.732	0.321	0.334	0.476	0.181	0.432	0.967	0.487	0.83	0.768	0.459	0.336	0.489	0.925	0.868	0.369	0.99	0.924

Appendix H continued.

SNP	EMA	fat %	fattobn	hscw	imf%	kidfat	marbam	mbms	mbusms	meltpt	omental	p8	rbft	seam fat	desat	elong	mufa	sfa
NCOA7-1b	0.845	0.514	0.532	0.74	0.186	0.488	0.951	0.795	0.841	0.15	0.531	0.664	0.637	0.836	0.443	0.247	0.733	0.89
NCOA7-2	0.164	0.786	0.967	0.604	0.138	0.38	0.049	0.115	0.025	0.96	0.567	0.371	0.196	0.621	0.751	0.377	0.828	0.843
NCOA7-3	0.626	0.603	0.641	0.417	0.162	0.301	0.422	0.849	0.504	0.316	0.895	0.711	0.988	0.481	0.149	0.474	0.616	0.505
NCOA7-4	0.684	0.502	0.528	0.56	0.131	0.503	0.376	0.861	0.814	0.282	0.777	0.536	0.905	0.276	0.087	0.428	0.413	0.371
PPARa-4	0.713	0.212	0.241	0.157	0.752	0.107	0.906	0.887	0.551	0.086	0.996	0.859	0.873	0.019	0.883	0.074	0.304	0.247
PPARG-2	0.225	0.998	0.987	0.385	0.946	0.318	0.516	0.861	0.936	0.205	0.998	0.455	0.789	0.55	0.03	0.928	0.025	0.018
PPARG-6	0.418	0.403	0.444	0.336	0.661	0.901	0.26	0.38	0.944	0.742	0.866	0.878	0.788	0.603	0.287	0.532	0.108	0.197
TEK_1	0.27	0.605	0.53	0.727	0.595	0.784	0.391	0.634	0.605	0.949	0.93	0.77	0.148	0.314	0.367	0.526	0.738	0.826
tek1	0.05	0.18	0.154	0.143	0.309	0.021	0.063	0.568	0.354	0.113	0.652	0.056	0.854	0.327	0.027	0.698	0.121	0.111
TEK1-4	0.192	0.761	0.65	0.606	0.541	0.937	0.592	0.092	0.262	0.334	0.743	0.506	0.301	0.833	0.552	0.461	0.422	0.371
TEK1-5	0.126	0.897	0.563	0.351	0.537	0.112	0.684	0.212	0.205	0.483	0.003	0.501	0.785	0.041	0.643	0.423	0.658	0.725
TEK1-6	0.338	0.722	0.705	0.242	0.544	0.952	0.678	0.511	0.663	0.829	0.757	0.591	0.523	0.51	0.962	0.57	0.87	0.896
tek2	0.963	0.651	0.582	0.322	0.2	0.629	0.824	0.144	0.174	0.199	0.513	0.745	0.439	0.67	0.646	0.311	0.951	0.997
TEK3-4	0.254	0.367	0.19	0.386	0.177	0.828	0.423	0.466	0.543	0.274	0.9	0.519	0.297	0.309	0.391	0.534	0.963	0.907

Appendix I SNP effect: cohort + BOD + sire + BOD.mstn + SNP + SNP.mstn – SNP:mstn interaction

SNP	EMA	fat %	fattobn	hscw	imf%	kidfat	marbam	mbms	mbusms	meltpt	omental	p8	rbft	seam fat	desat	elong	mufa	sfa
ACACA7	0.72	0.582	0.49	0.915	0.83	0.523	0.601	0.988	0.946	0.092	0.186	0.787	0.431	0.767	0.608	0.454	0.296	0.319
ALDH8A10	0.189	0.478	0.604	0.301	0.317	0.13	0.136	0.438	0.42	0.374	0.442	0.744	0.724	0.966	0.263	0.485	0.888	0.863
ALDH8A15	0.408	0.526	0.226	0.29	0.397	0.432	0.826	0.858	0.852	0.129	0.735	0.454	0.159	0.859	0.601	0.479	0.604	0.517
ALDH8A16	0.705	0.628	0.517	0.527	0.966	0.704	0.61	0.489	0.531	0.146	0.905	0.766	0.791	0.824	0.169	0.787	0.38	0.249
APM1-1	0.175	0.375	0.523	0.371	0.929	0.354	0.282	0.49	0.429	0.095	0.343	0.219	0.991	0.959	0.111	0.888	0.202	0.247
ATP2B432	0.363	0.226	0.111	0.048	0.368	0.648	0.186	0.058	0.131	0.245	0.12	0.394	0.217	0.364	0.06	0.023	0.224	0.211
ATP2B433	0.578	0.145	0.09	0.048	0.157	0.7	0.229	0.013	0.017	0.274	0.029	0.439	0.566	0.088	0.094	0.058	0.184	0.15
BCMO113	0.233	0.132	0.171	0.082	0.76	0.578	0.925	0.839	0.811	0.321	0.817	0.631	0.533	0.19	0.27	0.902	0.458	0.475
BCMO14	0.211	0.49	0.224	0.094	0.93	0.609	0.644	0.811	0.918	0.368	0.661	0.054	0.514	0.83	0.794	0.619	0.277	0.278
BCMO17	0.834	0.272	0.316	0.151	0.44	0.662	0.543	0.194	0.281	0.683	0.942	0.71	0.339	0.495	0.757	0.677	0.826	0.895
BCMO18	0.725	0.821	0.586	0.787	0.151	0.387	0.589	0.57	0.432	0.902	0.401	0.095	0.72	0.889	0.953	0.145	0.745	0.829
EDG1-UTR	0.687	0.274	0.235	0.89	0.018	0.917	0.171	0.133	0.031	0.569	0.727	0.985	0.466	0.496	0.052	0.604	0.253	0.287
ENO3-11	0.031	0.12	0.1	0.285	0.272	0.072	0.046	0.358	0.652	0.965	0.802	0.263	0.385	0.026	0.754	0.229	0.716	0.77
ENO3-5	0.349	0.785	0.699	0.995	0.893	0.637	0.78	0.955	0.845	0.038	0.412	0.849	0.953	0.933	0.641	0.313	0.499	0.556
ESR1 - 2	0.008	0.958	0.61	<0.001	0.822	0.181	0.988	0.995	0.913	0.571	0.78	0.47	0.11	0.038	0.273	0.683	0.239	0.287
ESR1-14	0.894	0.372	0.585	0.536	0.994	0.161	0.447	0.917	0.906	0.907	0.227	0.355	0.553	0.469	0.557	0.139	0.984	0.989
LAMIN-1	0.745	0.06	0.081	0.464	0.903	0.002	0.375	0.873	0.841	0.898	0.304	0.106	0.002	0.746	0.691	0.985	0.767	0.848
NCOA7-1	0.549	0.886	0.915	0.674	0.818	0.873	0.55	0.31	0.041	0.087	0.127	0.385	0.126	0.732	0.436	0.464	0.332	0.405
NCOA7-1a	0.64	0.841	0.674	0.857	0.913	0.807	0.701	0.389	0.2	0.065	0.262	0.505	0.061	0.529	0.983	0.516	0.723	0.731

Appendix I continued.

SNP	EMA	fat %	fattobn	hscw	imf%	kidfat	marbam	mbms	mbusms	meltpt	omental	p8	rbft	seam fat	desat	elong	mufa	sfa
NCOA7-1b	0.366	0.854	0.848	0.742	0.664	0.879	0.908	0.265	0.073	0.059	0.136	0.857	0.051	0.777	0.28	0.993	0.455	0.469
NCOA7-2	0.52	0.934	0.875	0.637	0.462	0.454	0.589	0.816	0.925	0.151	0.314	0.629	0.761	0.88	0.385	0.441	0.429	0.255
NCOA7-3	0.152	0.283	0.037	0.177	0.442	0.623	0.567	0.805	0.643	0.166	0.027	0.71	0.041	0.553	0.487	0.165	0.181	0.21
NCOA7-4	0.205	0.328	0.08	0.391	0.393	0.812	0.372	0.572	0.407	0.146	0.03	0.7	0.05	0.543	0.444	0.153	0.134	0.152
PPARa-4	0.745	0.923	0.977	0.201	0.556	0.563	0.862	0.984	0.693	0.149	0.878	0.102	0.841	0.325	0.381	0.4	0.801	0.78
PPARG-2	0.015	0.82	0.744	0.041	0.814	0.713	0.714	0.088	0.186	0.44	0.225	0.926	0.794	0.659	0.34	0.513	0.077	0.073
PPARG-6	0.489	0.886	0.706	0.861	0.697	0.195	0.667	0.218	0.104	0.525	0.018	0.847	0.267	0.864	0.315	0.808	0.306	0.188
TEK-1	0.802	0.814	0.671	0.301	0.512	0.17	0.187	0.331	0.263	0.271	0.151	0.865	0.717	0.579	0.29	0.208	0.279	0.35
TEK-2	0.035	0.251	0.14	0.882	0.777	0.152	0.995	0.742	0.363	0.614	0.384	0.82	0.86	0.974	0.618	0.987	0.379	0.378
TEK-3	0.121	0.763	0.637	0.49	0.476	0.857	0.553	0.79	0.633	0.81	0.281	0.995	0.887	0.973	0.859	0.266	0.958	0.991
TEK1-4	0.956	0.699	0.446	0.316	0.642	0.983	0.491	0.624	0.87	0.262	0.012	0.823	0.411	0.411	0.32	0.276	0.662	0.52
TEK1-5	0.594	0.448	0.472	0.922	0.75	0.037	0.705	0.475	0.59	0.222	0.496	0.835	0.633	0.574	0.402	0.331	0.133	0.134
TEK1-6	0.726	0.196	0.223	0.207	0.553	0.833	0.373	0.93	0.784	0.162	0.268	0.644	0.647	0.747	0.125	0.13	0.296	0.391
TEK-7	0.027	0.873	0.734	0.771	0.44	0.969	0.448	0.908	0.725	0.452	0.136	0.928	0.817	0.915	0.701	0.151	0.937	0.958

Appendix J SNP interactions within gene.

	EMA	fat %	fattobn	hscw	imf%	kidfat	marbam	mbms	mbusms	omental	p8am	rbft	seam fat
						ALDH8							
10 x 15													
10 x 16													
15 x 16			0.061										0.014
						ATP2B4							
32 x 33											0.037		
						BCMO							
113 x 18		<0.001	0.001	0.034			0.011				0.019		
14 x 18			0.024						0.067		0.025	0.009	
14 x 113							0.003					0.064	
17 x 18					0.005								
						ENO3							
5 x 11	0.01					0.064							
						ESR1							
2 x 4				0.077									
						NCOA7							
1 x 3		0.03	0.079										
2 x 3													0.068
						PPARG							
2 x 6													
						TEK							
_1 x 14	0.041	0.073											
_1 x 16											0.055		
_1 x tek1								0.098					
_1 x tek2										0.081			
14 x 16		0.096	0.058								0.028	0.015	
14 x 34								0.063					
14 x tek1						0.084							
15 x 16	0.082				0.075								
15 x tek2			0.011	0.024							0.001		
34 x tek1				0.044		0.074							
34 x tek2		0.076											
tek1 x tek2							0.093				0.054		

Appendix K Interactions between genes associated with variation in fat depots, P-values

	EMA	fatpc	fattobn	hscw	imf%	kidfat	marbam	mbms	mbusms	omental	P8	ribfat	Seam fat
tek1*TEK15*EDGUTR													
TEK15*EDGUTR					0.03								
tek1*TEK15*EDGUTR						0.06							
tek1*TEK15*PPARG2*PPARG6													
TEK15*PPARG2			0.07										
tek1*TEK15*PPARG2				0.01								0.06	
tek1*TEK15*ACACA7													
tek1*ACACA7	0.02												
TEK15*ACACA7						0.01	0.01	0.06	0.03	0.01			
tek1*TEK15*ALDH8A10*ALDH8A15*ALDH8A16													
tek1*ALDH8A10	0.07										0.01		
tek1*TEK15*ALDH8A10											0.05		
tek1*TEK15*ALDH8A15	0.04												
TEK15*ALDH8A10						0.07							
TEK15*ALDH8A15		0.01	0.05									0.04	
tek1*TEK15*APM11													
TEK15*APM11	0.01	0.01	0.07										
tek1*APM11				0.07	0.03							0.01	
tek1*TEK15*ATP2B432*ATP2B433													
tek1*ATP2B432		0.00	0.00	0.05							0.01		
tek1*ATP2B433								0.03					0.06
tek1*TEK15*ATP2B432													0.05
tek1*TEK15*ENO35*ENO311													
tek1*TEK15*ENO311											0.05		
tek1*ENO35*ENO311								0.03	0.00				0.05
TEK15*ENO35*ENO311		0.02	0.05										

Appendix K continued.

	EMA	fatpc	fattobn	hscw	imf%	kidfat	marbam	mbms	mbusms	omental	P8	ribfat	Seam fat
tek1*TEK15*BCMO14*BCMO17*BCMO18*BCMO113													
tek1*BCMO14				0.06							0.02		
tek1*BCMO17	0.02												
tek1*BCMO113			0.06										
tek1*BCMO14*BCMO113						0.03	0.05				0.01		
tek1*BCMO17*BCMO18										0.05			
tek1*BCMO17*BCMO113							0.04	0.06		0.01			
tek1*BCMO18*BCMO113							<0.001	0.03	0.02				
tek1*TEK15*BCMO17								0.07					
tek1*TEK15*BCMO14*BCMO17				0.04									
TEK15*BCMO14		0.03	0.01								0.01		
TEK15*BCMO17			0.05										
TEK15*BCMO18					0.04								
TEK15*BCMO113		0.06	0.05								0.00		
tek1*TEK15*ESR114*ESR12													
tek1*TEK15*ESR114						0.02							
tek1*ESR12	0.03			0.02									
tek1*TEK15*LAMIN													
tek1*TEK15*NCOA71A*NCOA71B*NCOA72													
tek1*NCOA71B*NCOA72									0.02		0.06		
TEK15*NCOA72							0.02						
TEK15*NCOA71A						0.04				0.06			
TEK15*NCOA71B*NCOA72												0.07	
tek1*TEK15*NCOA73*NCOA74*NCOA7_1													
tek1*TEK15*NCOA73*NCOA7_1				0.03		0.01							

Appendix K continued.

	EMA	fatpc	fattobn	hscw	imf%	kidfat	marbam	mbms	mbusms	omental	P8	ribfat	Seam fat
tek1*TEK15*NCOA71A*NCOA71B*NCOA72 continued													
tek1*TEK15*NCOA73				0.00									
tek1*NCOA7_1						0.01							
TEK15*NCOA73*NCOA7_1	0.01												
TEK15*NCOA7_1						0.07					0.02		
TEK15*NCOA73							0.06						
tek1*TEK15*PPARA													
ENO35*ENO311*LAMIN													
ENO35*ENO311*LAMIN								0.04					
ENO311*LAMIN	0.04												
PPARG2*PPARG6*ACACA7													
PPARG2*ACACA7							0.04						
PPARG2*PPARG6*ALDH8A10*ALDH8A15*ALDH8A16													
PPARG2*ALDH8A10*	0.02								0.04				
PPARG2*PPARG6*ALDH8A10	0.01					0.01				0.06			
PPARG2*ALDH8A16		0.05											
PPARG2*PPARG6*ALDH8A16				0.07									<0.001
PPARG2*PPARG6*ALDH8A10*ALDH8A15		0.02	0.05										
PPARG2*ALDH8A10*ALDH8A15		0.05	0.02	0.03									
PPARG6*ALDH8A10		0.05					0.01				0.04		
PPARG2*PPARG6*APM11													
PPARG2*PPARG6*APM11				0.03	0.04	0.03							
PPARG6*APM11								0.02	0.00				0.06
PPARG2*PPARG6*ATP2B432*ATP2B433													
PPARG2*ATP2B432													0.04

Appendix K continued.

	EMA	fatpc	fattobn	hscw	imf%	kidfat	marbam	mbms	mbusms	omental	P8	ribfat	Seam fat
PPARG2*PPARG6*ATP2B432*ATP2B433 continued													
PPARG2*ATP2B433		0.01	0.02						0.04				
PPARG2*PPARG6*BCMO14*BCMO17*BCMO18*BCMO113													
PPARG2*BCMO14											0.02		
PPARG2*BCMO17			0.05	0.05						0.03			
PPARG6*BCMO14				0.03									
PPARG6*BCMO18				0.07									
PPARG2*PPARG6*BCMO18										0.02	0.07		0.04
PPARG2*PPARG6*BCMO113				0.03	0.04								
PPARG2*BCMO17*BCMO113													0.01
PPARG2*BCMO18*BCMO113													0.00
PPARG6*BCMO17*BCMO18										0.00			
PPARG2*PPARG6*EDGUTR													
PPARG2*PPARG6*EDGUTR						0.03		0.01	0.00				
PPARG2*PPARG6*ENO35*ENO311													
PPARG2*ENO35		0.05	0.03		0.01								
PPARG2*ENO311										0.04			0.01
PPARG2*ENO35*ENO311				0.04								0.04	
PPARG6*ENO35*ENO311					0.06								
PPARG2*PPARG6*ESR114*ESR12													
PPARG2*ESR12					0.01								
PPARG2*PPARG6*ESR12	0.05										0.05		
PPARG6*ESR114						0.04							
PPARG6*ESR12						0.06							
PPARG2*PPARG6*ESR114*ESR12	<0.001	0.02											

Appendix K continued.

	EMA	fatpc	fattobn	hscw	imf%	kidfat	marbam	mbms	mbusms	omental	P8	ribfat	Seam fat
PPARG2*PPARG6*LAMIN1													
PPARG2*LAMIN1		0.01	0.02		0.05			0.06	0.03				
PPARG6*LAMIN1	0.02												
PPARG2*PPARG6*LAMIN1						0.04							
PPARG2*PPARG6*NCOA71A*NCOA71B*NCOA72													
PPARG2*NCOA71A												0.06	
PPARG2*PPARG6*NCOA71B								0.02	0.01				
PPARG2*PPARG6*NCOA72						0.03						0.05	
PPARG2*PPARG6*NCOA71A*NCOA72									0.06	0.02			
PPARG2*PPARG6*NCOA71B*NCOA72		0.02	0.02	0.03									
PPARG2*NCOA71A*NCOA72						0.05							
PPARG6*NCOA71A													0.06
PPARG2*PPARG6*NCOA73*NCOA74*NCOA7_1													
PPARG2*NCOA74	0.03			0.05									
PPARG2*NCOA73*NCOA7_1	0.04												
PPARG2*PPARG6*NCOA73			0.05		0.05							0.03	
PPARG2*PPARG6*NCOA7_1											0.06	0.01	
PPARG2*PPARG6*NCOA73*NCOA7_1				0.01									
PPARG2*PPARG6*PPARA													
PPARG6*PPARA	0.04							0.01	0.01				0.01
ESR114*ESR12*NCOA71A*NCOA71B*NCOA72*NCOA73													
ESR114*NCOA71A							0.05	0.04	0.03				0.04
ESR114*NCOA71B						0.06							0.06
ESR114*NCOA72	0.03												0.05
ESR114*ESR12*NCOA71B		0.03											
ESR114*NCOA71B*NCOA72											0.03		0.05

Appendix K continued.

	EMA	fatpc	fattobn	hscw	imf%	kidfat	marbam	mbms	mbusms	omental	P8	ribfat	Seam fat
ESR114*ESR12*NCOA71A*NCOA71B*NCOA72*NCOA73 continued													
ESR114*NCOA71B*NCOA73													0.04
ESR114*NCOA72*NCOA73													0.00
ESR114*ESR12*NCOA71A*NCOA712												0.01	
ESR114*NCOA71A*NCOA71B*NCOA72*NCOA73*								0.04					
ESR12*NCOA71A													0.04
ESR12*NCOA72	0.06												
ESR12*NCOA73													<0.001
ESR12*NCOA71A*NCOA73						0.07						0.04	
ESR12*NCOA71B*NCOA72					0.04					0.03			
ESR114*ESR12*ACACA7													
ESR114*ACACA7							0.03						
ESR12*ACACA7													0.05
ESR114*ESR12*ACACA7							0.03						
ESR114*ESR12*ALDH8A10*ALDH8A15*ALDH8A16													
ESR114*ALDH8A10	0.02												
ESR114*ALDH8A10*ALDH8A15		0.02	0.06										
ESR114*ALDH8A16			0.04	0.07									0.05
ESR114*ESR12*ALDH8A15											0.04		
ESR12*ALDH8A10					0.04			0.04					
ESR12*ALDH8A16										0.00			
ESR114*ESR12*APM1													
ESR114*ESR12*ATP2B432*ATP2B433													
ESR12*ATP2B433								0.06					
ESR114*ESR12*ATP2B433								0.03	0.03				

Appendix K continued.

	EMA	fatpc	fattobn	hscw	imf%	kidfat	marbam	mbms	mbusms	omental	P8	ribfat	Seam fat
ESR114*ESR12*ATP2B432*ATP2B433 continued													
ESR114*ESR12*ATP2B432											0.03	0.02	
ESR114*ATP2B432												0.06	
ESR114*ATP2B433													0.00
ESR114*ESR12*BCMO14*BCMO17*BCMO18*BCMO113													
ESR114*BCMO14							0.03						
ESR114*BCMO17		0.02	0.01							0.05			
ESR114*BCMO18			0.05	0.03	0.07						0.06		0.00
ESR114*BCMO14*BCMO17	0.06												
ESR114*BCMO14*BCMO113				0.02			0.06						
ESR114*ESR12*BCMO17*BCMO113							0.07						
ESR114*BCMO17*BCMO113										0.01			0.02
ESR114*ESR12*BCMO14											0.07		
ESR114*BCMO18*BCMO113											0.04		0.02
ESR12*BCMO14				0.01								0.04	
ESR12*BCMO17				0.05									
ESR12*BCMO18							0.02						
ESR12*BCMO14*BCMO113				0.02									
ESR114*ESR12*EDGUTR													
ESR114*ESR12*EDGUTR					0.04								
ESR114*ESR12*ENO35*ENO311													
ESR114*ENO35*ENO311	0.02	0.00	0.01		0.04		<0.001		0.03				0.01
ESR12*ENO311							0.05	0.04			0.05	0.01	
ESR12*ENO35		0.02											
ESR114*ESR12*Lamin1													
ESR12*Lamin1	0.07	0.03				0.05							

Appendix K continued.

	EMA	fatpc	fattobn	hscw	imf%	kidfat	marbam	mbms	mbusms	omental	P8	ribfat	Seam fat
					ESR114*ESR12*PPARA								
ESR114*PPARA													<0.001
ESR12*PPARA	0.03						0.01	0.01	<0.001				0.00
					ACACA7*ALDH8A10*ALDH8A15*ALDH8A16								
ACACA7*ALDH8A15		0.03	0.00										
ACACA7*ALDH8A16										0.03			
ACACA7*ALDH8A10*ALDH8A15			0.07										
ACACA7*ALDH8A15*ALDH8A16					0.05								
					ACACA7*APM1								
ACACA7*APM1						0.03							
					ACACA7*ATP2B432*ATP2B433								
ACACA7*ATP2B433													0.00
					ACACA7*BCMO14*BCMO17*BCMO18*BCMO113								
ACACA7*BCMO14*BCMO17	0.03			0.02									
ACACA7*BCMO14*BCMO113				0.02									
ACACA7*BCMO18*BCMO113										0.04			
ACACA7*BCMO17*BCMO113				0.02	0.07	0.02					0.02		
					ACACA7*EDGUTR								
ACACA7*ENO35*ENO311													
ACACA7*ENO35					0.03								
ACACA7*ENO311				0.01								0.04	
					ACACA7*LAMIN								
ACACA7*LAMIN	0.07												
					ACACA7*NCOA71A*NCOA71B*NCOA72*NCOA73*NCOA74*NCOA7_1								
ACACA7*NCOA74	0.04												
ACACA7*NCOA71A*NCOA72									0.04				

Appendix K continued.

	EMA	fatpc	fattobn	hscw	imf%	kidfat	marbam	mbms	mbusms	omental	P8	ribfat	Seam fat
ACACA7*NCOA71A*NCOA71B*NCOA72*NCOA73*NCOA74*NCOA7_1 continued													
ACACA7*NCOA71A*NCOA72*NCOA73	0.03					0.02							
ACACA7*NCOA71B*NCOA73		0.04											0.02
ACACA7*NCOA72*NCOA73	0.00												
ACACA7*NCOA71B													0.03
ACACA7*PPARA													
ACACA7*PPARA	0.04									0.05			
EDGUTR*ALDH8A10*ALDH8A15*ALDH8A16													
EDGUTR*ALDH8A10										0.06			
EDGUTR*ALDH8A10*ALDH8A15		0.01	0.01										
EDGUTR*APM1													
EDGUTR*APM1		0.02									0.01		
EDGUTR*ATP2B432*ATP2B433													
EDGUTR*ATP2B433													0.00
EDGUTR*BCMO14*BCMO17*BCMO18*BCMO113													
EDGUTR*BCMO17*BCMO18	0.03												0.02
EDGUTR*BCMO18				0.04						0.04			
EDGUTR*BCMO14*BCMO113		0.05						0.03	0.03				
EDGUTR*BCMO17*BCMO113				0.02		0.07							
EDGUTR*BCMO14*BCMO17									0.03				
EDGUTR*ENO35*ENO311													
EDGUTR*ENO311	0.04									0.02			
EDGUTR*ENO35*ENO311	0.03	0.04					0.05	0.04		<0.001			
EDGUTR*LAMIN													

Appendix K continued.

	EMA	fatpc	fattobn	hscw	imf%	kidfat	marbam	mbms	mbusms	omental	P8	ribfat	Seam fat
					EDGUTR*PPARA								
EDGUTR*PPARA													0.002
					ALDH8A10*ALDH8A15*ALDH8A16*BCMO14*BCMO17*BCMO18*BCMO113								
ALDH8A10*BCMO14						0.03							
ALDH8A10*BCMO18				0.05							0.04	0.01	
ALDH8A10*ALDH8A15*BCMO113		0.01	0.00	0.03									
ALDH8A10*BCMO113											0.07		
ALDH8A10*BCMO14*BCMO113					0.06								0.04
ALDH8A10*BCMO17*BCMO113							0.01						
ALDH8A10*BCMO18*BCMO113		0.04	0.02				0.03						0.06
ALDH8A10*ALDH8A15*BCMO17													0.02
ALDH8A14*BCMO18							0.05				0.05	0.06	
ALDH8A15*BCMO14				0.04									
ALDH8A15*BCMO14*BCMO113				0.05									
ALDH8A15*BCMO17*BCMO113													0.06
ALDH8A16*BCMO17										0.05			
ALDH8A16*BCMO18									0.07	0.06			
ALDH8A16*BCMO113					0.07					0.06			0.05
					ALDH8A10*ALDH8A15*ALDH8A16*APM1								
ALDH8A10*APM1									0.03				
ALDH8A15*APM1			0.01										
ALDH8A16*APM1	0.00					0.06							
ALDH8A10*ALDH8A15*APM1					0.06								
					ALDH8A10*ALDH8A15*ALDH8A16*ATP2B432*ATP2B433								
ALDH8A10*ATP2B432*ATP2B433		0.04	0.07										
ALDH8A10*ATP2B432							0.03						

Appendix K continued.

	EMA	fatpc	fattobn	hscw	imf%	kidfat	marbam	mbms	mbusms	omental	P8	ribfat	Seam fat
ALDH8A10*ALDH8A15*ALDH8A16*ATP2B432*ATP2B433 continued													
ALDH8A15*ATP2B432				0.00							0.01		0.06
ALDH8A16*ATP2B432	0.03												
ALDH8A10*ALDH8A15*ALDH8A16*ENO35*ENO311													
ALDH8A10*ENO35*ENO311							0.05	0.04	0.01	0.07	0.02	0.06	
ALDH8A15*ENO35		0.05	0.04										
ALDH8A10*ENO311		0.04				<0.001				0.02			
ALDH8A15*ENO311		0.02	0.01			0.03							
ALDH8A10*ALDH8A15*ENO35		0.05	0.04										
ALDH8A16*ENO35*ENO311				0.05									
ALDH8A10*ENO35										0.00			
ALDH8A10*ALDH8A15*ALDH8A16*LAMIN													
ALDH8A15*LAMIN		0.00	0.00										
ALDH8A16*LAMIN						0.04							0.04
ALDH8A10*ALDH8A15*LAMIN							0.05						
ALDH8A10*ALDH8A15*ALDH8A16*NCOA71A*NCOA71B*NCOA72													
ALDH8A10*NCOA72											0.03		
ALDH8A10*NCOA71A*NCOA72													0.05
ALDH8A15*NCOA71A													0.03
ALDH8A15*NCOA71B					0.07								
ALDH8A15*NCOA71B*NCOA72											0.01		
ALDH8A16*NCOA71A					0.05	0.00							0.01
ALDH8A16*NCOA72										0.01			
ALDH8A10*ALDH8A15*ALDH8A16*NCOA73*NCOA74*NCOA7_1													
ALDH8A10*NCOA7_1											0.02		
ALDH8A10*NCOA73							0.01						

Appendix K continued.

	EMA	fatpc	fattobn	hscw	imf%	kidfat	marbam	mbms	mbusms	omental	P8	ribfat	Seam fat
ALDH8A10*ALDH8A15*ALDH8A16*NCOA73*NCOA74*NCOA7_1 continued													
ALDH8A10*ALDH8A15*NCOA7_1						0.03							
ALDH8A15*NCOA7_1					0.05				0.03				0.02
ALDH8A15*NCOA73													0.03
ALDH8A16*NCOA7_1					0.04	0.01							0.04
ALDH8A10*ALDH8A15*ALDH8A16*PPARA													
ALDH8A15*PPARA		0.05											0.05
ALDH8A16*PPARA												0.05	
APM1*ATP2B432*ATP2B433													
APM1*ATP2B433													0.01
APM1*BCMO14*BCMO17*BCMO18*BCMO113													
APM1*BCMO14				0.06									
APM1*BCMO14*BCMO113				0.03									
APM1*BCMO17								0.04					
APM1*BCMO17*BCMO18													0.02
APM1*BCMO17*BCMO113										0.04		0.03	
APM1*BCMO18*BCMO113	0.02												
APM1*BCMO113													0.01
APM1*ENO35*ENO311													
APM1*ENO35*ENO311	0.03												
APM1*ENO35	0.00							<0.001	0.00				
APM1*ENO311		0.06											
APM1*LAMIN													

Appendix K continued.

	EMA	fatpc	fattobn	hscw	imf%	kidfat	marbam	mbms	mbusms	omental	P8	ribfat	Seam fat
					APM1*NCOA71A*NCOA71B*NCOA72								
APM1*NCOA71A	0.03												0.05
APM1*NCOA71A*NCOA72	0.04												
APM1*NCOA71B*NCOA72					0.03						0.01		
APM1*NCOA73*NCOA74*NCOA7_1													
APM1*NCOA73*NCOA7_1		0.05			0.04		0.00						
APM1*NCOA74	0.07			0.04									
					APM1*PPARA								
					ATP2B432*ATP2B433*BCMO14*BCMO17*BCMO18*BCMO113								
ATP2B432*BCMO14			0.03								<0.001		
ATP2B432*BCMO14*BCMO17				0.06									
ATP2B432*BCMO17*BCMO113													0.02
ATP2B432*BCMO113				0.01	0.05								
ATP2B432*ATP2B433*BCMO17		0.02	0.02										0.00
ATP2B433*BCMO17				0.06									
ATP2B433*BCMO18													0.05
					ATP2B432*ATP2B433*LAMIN								
ATP2B433*LAMIN		0.02	0.05										
					ATP2B432*ATP2B433*NCOA71A*NCOA71B*NCOA72								
ATP2B432*NCOA72		0.06		0.06									
ATP2B432*NCOA71A*NCOA72									0.05				
ATP2B432*NCOA71B*NCOA72					0.03			0.04					
ATP2B433*NCOA71A*NCOA72						0.02							
ATP2B432*ATP2B433*NCOA73*NCOA74*NCOA7_1													
ATP2B432*NCOA73*NCOA7_1					0.05		0.01	0.04	0.02				
ATP2B432*NCOA74				0.01									

Appendix K continued.

	EMA	fatpc	fattobn	hscw	imf%	kidfat	marbam	mbms	mbusms	omental	P8	ribfat	Seam fat
						ATP2B432*ATP2B433*ENO35*ENO311							
ATP2B432*ATP2B433*ENO35		0.00	0.01	0.06		0.01		0.05	0.06				0.01
ATP2B432*ENO35*ENO311			0.06										
ATP2B433*ENO311													0.01
						ATP2B432*ATP2B433*PPARA							
ATP2B433*PPARA													0.01
						BCMO14*BCMO17*BCMO18*BCMO113*ENO35*ENO311							
BCMO14*ENO311			0.01							0.04	0.00		0.04
BCMO14*BCMO17*ENO35*ENO311		0.03	0.03										
BCMO14*BCMO113*ENO35	0.02	0.07					0.06						
BCMO14*BCMO113*ENO311						0.05							
BCMO17*BCMO113*ENO35							0.05						
BCMO18*ENO311							0.02						
BCMO113*ENO311				0.04		0.06							
BCMO113*ENO35*ENO311							0.04	0.05					
						BCMO14*BCMO17*BCMO18*BCMO113*LAMIN							
BCMO14*BCMO17*LAMIN		0.01	0.04										0.03
BCMO14*LAMIN							0.04				0.02		
BCMO17*BCMO18*LAMIN		0.02	0.03								0.04		
BCMO18*LAMIN							0.03						
						BCMO14*BCMO17*BCMO18*BCMO113*PPARA							
BCMO14*PPARA									0.05				
BCMO14*BCMO17*PPARA								0.03	0.02				
BCMO17*PPARA	0.02												
BCMO18*PPARA				0.05			0.04						
BCMO113*PPARA													0.05

Appendix K continued.

	EMA	fatpc	fattobn	hscw	imf%	kidfat	marbam	mbms	mbusms	omental	P8	ribfat	Seam fat
BCMO14*BCMO17*BCMO18*BCMO113*NCOA71A*NCOA71B*NCOA72													
BCMO14*NCOA71B	0.04								0.05				
BCMO14*NCOA72											0.01		
BCMO14*BCMO17*NCOA71B		0.04			0.05		0.03		0.05			0.04	
BCMO14*BCMO17*NCOA72											0.00		
BCMO14*BCMO113*NCOA71A*NCOA72					0.03			0.01	0.01				
BCMO14*BCMO113*NCOA71A													0.05
BCMO14*NCOA71A*NCOA72													0.00
BCMO17*BCMO113*NCOA71A		0.06	0.05				0.03						
BCMO17*BCMO113*NCOA71B		0.06	0.06										0.00
BCMO17*NCOA71B						0.06							
BCMO17*NCOA71A*NCOA72							0.06			0.05	0.03		
BCMO17*NCOA71A								0.05					
BCMO17*NCOA71B*NCOA72											0.02		
BCMO17*BCMO18*NCOA72											0.01		
BCMO18*NCOA71A													0.00
BCMO18*NCOA71B										0.06			
BCMO113*NCOA71B													0.04
BCMO113*NCOA71A*NCOA72		0.03	0.03										
BCMO14*BCMO17*BCMO18*BCMO113*NCOA73*NCOA74*NCOA7_1													
BCMO14*NCOA7_1	0.03												0.03
BCMO14*BCMO17*NCOA73							0.02						
BCMO14*BCMO17*NCOA73*NCOA7_1							0.03						
BCMO14*BCMO113*NCOA73	0.04	0.06				0.01							
BCMO17*NCOA7_1				0.04									
BCMO14*BCMO113*NCOA73*NCOA71		0.02	0.04			0.04							
BCMO17*BCMO18*NCOA73					0.04						0.06		
BCMO17*BCMO113*NCOA7_1			0.05		0.04								0.01

Appendix K continued.

	EMA	fatpc	fattobn	hscw	imf%	kidfat	marbam	mbms	mbusms	omental	P8	ribfat	Seam fat
BCMO14*BCMO17*BCMO18*BCMO113*NCOA73*NCOA74*NCOA7_1 continued.													
BCMO17*BCMO18*NCOA7_1						0.03							
BCMO17*NCOA73												0.05	
BCMO17*NCOA73*NCOA7_1					0.02			0.02				0.02	
BCMO17*BCMO113*NCOA73*NCOA7_1												0.05	
BCMO18*NCOA7_1													0.05
BCMO18*BCMO113*NCOA73				0.06							0.06		0.01
BCMO18*NCOA73							0.01						
ENO35*ENO311*NCOA73*NCOA74*NCOA7_1													
ENO35*NCOA7_1				0.03	0.00								
ENO35*NCOA73*NCOA7_1							0.04						
ENO35*ENO311*NCOA7_1		0.02	0.01										
ENO35*ENO311*NCOA73*NCOA7_1	0.00												
ENO311*NCOA7_1	0.02										0.01		0.04
ENO311*NCOA74				0.05									
ENO311*NCOA73*NCOA7_1							0.02						
ENO35*ENO311*NCOA71A*NCOA71B*NCOA72													
ENO35*NCOA71A		0.04			0.00				0.03				
ENO35*NCOA72								0.02	0.01				
ENO35*NCOA71A*NCOA72						0.04				0.03			
ENO35*NCOA71B*NCOA72	0.01												
ENO35*ENO311*NCOA71A		0.02	0.01										
ENO35*ENO311*NCOA71B							0.04						
ENO35*ENO311*NCOA72						0.06				0.04	0.02		
ENO311*NCOA71A						0.05					0.06		
ENO311*NCOA72							0.06						
ENO311*NCOA71A*NCOA72						0.02							

Appendix K continued.

	EMA	fatpc	fattobn	hscw	imf%	kidfat	marbam	mbms	mbusms	omental	P8	ribfat	Seam fat
ENO35*ENO311*PPARA													
ENO35*PPARA													0.02
LAMIN*NCOA71A*NCOA71B*NCOA72													
LAMIN*NCOA71A												0.06	
LAMIN*NCOA71A*NCOA72													0.03
LAMIN*NCOA71B							0.07						
LAMIN*NCOA71B*NCOA72					0.00						0.04		
LAMIN*NCOA72		0.04	0.05			0.01							
LAMIN*NCOA73*NCOA74*NCOA7_1													
LAMIN*NCOA7_1												0.06	
LAMIN*NCOA73*NCOA7_1							0.00	0.01	0.06				
LAMIN*NCOA73*NCOA74*NCOA7_1													
LAMIN*NCOA73*NCOA74*NCOA7_1													
LAMIN*NCOA73*NCOA74*NCOA7_1													
LAMIN*PPARA													
LAMIN*PPARA							0.06						
PPARA*NCOA71A*NCOA71B*NCOA72													
PPARA*NCOA71A				0.01							0.04		0.05
PPARA*NCOA71A*NCOA72									0.06				0.01
PPARA*NCOA71B									0.03				
PPARA*NCOA71B*NCOA72	0.00												0.01
PPARA*NCOA72										0.05			0.06
PPARA*NCOA73*NCOA74*NCOA7_1													
PPARA*NCOA73													0.05
PPARA*NCOA73*NCOA7_1													0.00
PPARA*NCOA7_1				0.03								0.05	

Appendix L *Myostatin* F94L variant genotype effect on muscle and fat traits (F probabilities) Davies Gene Mapping herd.

	cohort+BOD+sire+BOD.MSTN	cohort+BOD+sire+MSTN
hscw	0.035	0.017
ema	<0.001	<0.001
p8am	<0.001	<0.001
rbft	0.117	0.059
kidfat	0.308	0.22
omental	0.758	0.813
marbam	0.023	0.018
mbms	0.093	0.054
mbusms	0.117	0.055
imf%	0.021	0.008
seam fat	<0.001	<0.001
fat %	<0.001	<0.001
fattobn	<0.001	<0.001
meltpt	0.323	0.19
desat	0.468	0.677
elong	0.886	0.754
mufa	0.806	0.901
sfa	0.77	0.872

Hot standard carcass weight (hscw), eye muscle area (ema), rump fat depth at P8 (p8am), rib fat depth (rft), channel fat (kdft), omental fat (omental), marble score (marbam, mbms, mbusms), intramuscular fat (imf%), intermuscular fat (seamfat), total fat% (fat%), fat to bone ratio (fattobn), melting point (mltpt), desaturation index (desat), elongation index (elong), mono-unsaturated fatty acids – percent of triacylglyceride (mufa), saturated fatty acids – percent of triacylglyceride (sfa).

Appendix M Primers for sequencing and genotyping

Gene / Primer	Forward	Reverse
	Sequencing Primers	
ACACA		
5'UTR	GCTGGATGGCATCACCGACTT	GGCTCTGCTGCGTCTTCCTT
5'UTRa	AGGACTTAGAAAGGGCTTAAA	GAAACTGTCCGCAGGAAG
Exon 1	CTCTGTGGCGAACTCTGTCT	GCTGCTGCTAAGTCGCTTC
Exon 3	AGCCAAGAACACTAGATTTCA	GCCCTATCAGTCACTGGA
Exon 11	TTTGTTCTTTGCCCTTGTAAC	GGTTGCTATTTCCGTCTCCA
Exon 42	TGAGCAAACCCAAAGAAGAAA	TCTAGTGCAACCCAAGGAATG
Exon 56	TCTAAAGCTCCGCTGATCCAG	CGGGATTGGAGAGACAGCAG
APM1		
Exon 1	GCTGATGGTGGTAACTGGTG	GTGCTTCTTTCTCTGCCCTAC
Exon 2	AAAGGAGGGAGCCAAGAGAG	GGAAGGTGGTGTGGGTAGAA
Exon 3a	GAGAGAAAATGCCCTGGATG	AACTGGTCGTGGGTGAAGAG
Exon 3b	GTCACTGTCCCCAATGTTCC	TCTGCCACTTAATGCCTGTG
UTR	GTACTCTTCGCCACCCTATGA	CGACAGAAGACGGGACCAA
UTR Promoter region	TCCCGTCTTCTGTCGCTGTT	CACTTCACTGACCCCAACCTT
EDG1		
1	CTGTGTGTTTCTGCCGCAGAT	CACCGCAGCTTCCTCTATCCC
2	TTCCCCCAAGAAATGTGAGTT	CCCGCCAACAGGTCTGA
3	GACATCATCGTCCGGCATTAT	GGCGGCTGCGAGTCCTG
4	TCTGCGGGAAGGGAGTATGT	TGGGGGTGGGAGGAGTTGTCT
5	CCTGCTCCTGCTCGACGTG	ACACACTCACTTGCGTTGGAA
6	GCACTGAGCCAAAGGTCTAGC	GGGCACAAGAAGATGGTATT
7	GGCTGAAATCTGCATAAGGA	ACAGAAATTTGCCTTTAACTT
UTR	ATATCCCACTGAGACCTGTTA	GAAGGCAATGGTGTATATCA
ENO3		
Exon 1	TTAGGCTGTAGTGGGCACTTG	CCTCACTTTTCCTGCTCCTCA
Intron 1	AGACTCAGAGCCCAGCGAAGA	CCAAATCCCTTGCCCTTTAAG
Exon 2	CGCCTGCCACTTTGGTCTG	CGGGAGGAATTGGAAGATCAC
Exon 3	CATGCCACATCCACTCTTCTC	GCAAAGCAGAGGGTTCAGAT
Exon 4/5	CCACGGGAAGAGACCTGAT	AACCACTGGGCACTGAGTAAG
Exon 6	CCTGAGGTCTGGTTAGGATTATTTC	GAAGGCTGGATGGCAGTAGGA
Exon 6a	CGCTCTACCGACACATTG	AACATTGACATGGCACTTACT
Exon 7	TTCTGGCAAGTTTATTGAACC	ACTTCCCATTGCGATAGAAC
Exon 8/9	GGAAGGACGCCACCAATGTG	CACTCACGCCTGGATGGATTC
Exon 9/10	GATGACTGGGCTACCTGGAC	AAACTCAGACACTGGGCAAAG
Exon 10/11/12	GAACTTTACCCTCCCAACACA	CGGGTTCCACATCCACTC
ESR1		
Promoter 1	GGAGGCACGGCAACAG	TTACACAAAGAACCCTTACGG
Promoter 2	AACATTTCTGGAAAGACGCT	TTTTGAAAGTCAGTGATGCCT
Promoter 2a	GCCAAGTGCCCTGCCTACTG	GCTATCCCAAACACCTGACGG
Promoter 2b	CAGTTTTGGCTTCATAGTAA	AAAGTTAAACCTGCTCAAGTA
Promoter 3	ACTGTCCATGATTATAGGTGC	GCCAGGCTCCAATCTATCT
Promoter 4	GGGAGAATCTCGGAAGATCG	ACTTGGTCATGGTCATGAGCG
Promoter 4a	AGTTCTGGTTGCATAGTCCGT	GTGCCAGGCTCCAATCTATC

Appendix M continued

Gene / Primer	Forward	Reverse
ESR1 continued		
Promoter 4b	GATGCCTGCGATCAGTCT	GCGGCGTTGAAGTCGT
5'UTR	TAGTCCGTGGAAAGCAT	AAGGCATGACAAAGGTG
5'UTR A	GGCATCATTGGGAAATAGACT	AATGGCAGTGGAGAGTAAGG
5'UTR B	CCATCCAATAACGCTGACTT	ACAGATGACTTCGTTTCCGTA
5'UTR C	CTTTCAGGACCCGTAGG	AACTCTGATACAACCGCTACT
5'UTR D	GTCAGGTGTTTGGGATAGCA	CGTATAGGAAGAGCCGAGTTT
5'UTR E	CTGGTTGCATAGTCCGTGGAA	CCGCTGCTGGATAGAGGCT
5'UTR F	GATGCCTGCGATCAGTCTCTGC	GAGCTGCGGGCGGTTCAG
5'UTR G	TCTATCCAGCAGCGGCAAGTA	AGCTGCGGGCGGTTCA
5'UTR H	CCAGATCCAAGCCAACGAG	TTTGCCACCACCATAGTCACC
Exon 1	GCCTGGAGTGATGTTGAA	GGAAAGACTTGCGAGGAA
Exon 3 and 4	GGTGTAGGCAGTCCATTTTT	CCAGCTATTGAAATCCTCTTC
Exon 5	CAAGAAGGACACAAAAAGGACAG	ACCACCTCCCACACATCTTGA
Exon 6 and 7	GAAATTGGTGAATGCTAC	CAGATTTGTAGGGTGAAT
HS5	TGACTAGACGAACCTTTGTTGG	GAGGCATCAGGCAGACAGTT
HS6	AATCTTTTTCCATCTTGC	TGACAGAGGAATCAAAAT
Exon 19	CTGGAAACTGGCTCACACAC	AGAACAACCCTTCCCCTGAC
Exon 20	TGTCCTGGGGCTTTTCTT	TGAATCCTCGTGCTGAGT
Exon 1b	GGAGAGCGGTACTTAAAGTTGG	GGACTCAAAGCACCTTACGTG
3'UTR	CTCTCTGCCTTTGCTACC	AGTCGCTCAGTCGTGTCT
RNA 3	GCCTTGACTAGACGAACCTTT	GCGAACTCAATTACCCTACCT
RNA 4	GGGGACTGAATAAGACATC	CCTCCATAAGTCATCTACAAA
RNA 6	CCTTCCTCAGCGTTTCAG	GGAAGTGGGTGGACAATC
Lamin A/C		
5'UTR	CTTGGATCTCTGAGCAGGT	TCGATGTAGACAGCCAAGC
5'UTR	GCCTGAAAGAACATCCCTTA	CAACAGCAAATTGGGTAGTTA
5'UTR	CTCAGGCAGGCTCTTTTT	AGTAGGCACAAAGCAAGG
5'UTRa3	GCTCTTTTTCCGACTGCT	CCTTTTCCTCCCCATAGT
5'UTRb3	CTGAGATGCGGCTTTGAATG	ATAGGTCTAGGGAATGGGAG
5'UTRc3	CTTGGATCTCTGAGCAGG	AGCTCCTGTAGGTCTTCC
Exon 1	AGCAGTCTCTGTCCTTCG	CGGGAGGGAGGAAACTAT
Exon 1/Intron 1	GGGGCAGAAGATAGGAAA	TAGAAGGGGTAAAGGGCA
Intron 1	CCAGCAGTCTCTGTCCTT	CTTGAACTCCTCTCGCAC
Intron 1a	CAAAGTGCGAGAGGAGTT	GCTGCCAAGAAAGTTCCA
Intron 1b	CTTTCTCTCCCCACACAT	CCCTAGTCCCTTTACACA
Intron 1A	TCATCAGGGCCAAGTAGGAG	CCAGACATGCAGGGAGTAAAG
Intron 1B2	GCTGGGCTTGTGGTTGATAG	TTTTCCCTCTCTCCCC
Intron 1B3	AGGGCTAGGAGTCTGGCT	GGTCTCCCTCCTTCTTGG
Exon 2	AGGGTGGAGAAGCAGGAAAG	AGATGGAAATGGCTTGTGGA
Exon 2/Intron 2	AGGCTCTGCTCAACTCCAA	TCAACCATCAGCTCCCAAC
Intron 2a	GTGCTTCCTCCTTTGCT	AACTGCTGGCTTCTGGT
Intron 2b	TCACCGCCACATCTACTCA	GTCCAGCACCTCCCTTTAT
Intron 2C	TCCCTCGTGTTCCTTCTG	TTCCCACCTCGCTGTAGA
Exon 3	TCATTAGCACAGCAACGATACA	TACTGCTCCACCTGGTCCTC

Appendix M continued

Gene / Primer	Forward	Reverse
Lamin A/C continued		
Intron 3	TGGGTGCTGAAAGATGTC	CCGAATAGGTCTTCTCCA
Exon 4	GTGAGACCAAACGCCGCCA	GGACCCAAGAAGGACATCGC
Exon 5	TCCCCGAGATGTCCTGTG	TTCTTACTCAGTCTACGCTCC
Exon 5a	AGCGATGTCCTTCTTGGGT	AGTCCTGGACCAGCAAACA
Intron 5b	CGGAGCGTAGACTGAGTAA	CCTATCTCCTCTGCCACAA
Intron 5c	GGTAGCCATTCCCTTCTCCA	TCTGAAGGTCCCCGAGTATG
Exon 6	GTGGTGGGGGTGGTGTGT	CTGCGAGGTGGGACTAGG
Exon 7	CTCCGCCTGTCCCCTAGT	AGATACCCACACTTGCCCTG
Intron 7	GGTCGGGTTAGGGAGAAGA	TAGGTCAGCAGGGGGTCAT
Exon 8	AGTGCCTGTAGACGTAGCTG	GTTCTGAGCCTTCCACACCA
Exon 9	CAGGTGGTGACGGTGAGTG	TCTAACCCCCACCCCTCCT
Exon 9a	ATGACCCCCTGCTGACCTA	GCGTGATCTGCCCTTAACC
Intron 9	TGGCCTCTGACTGGACAA	TGCCACTAGCTCTGTCCT
Exon 10	TTGGAGGACAGAGCTAGTGGCA	AGGGCAGCGAGTGAAGTTCCAA
Intron 10a	TCTGCTCCATCACCACCAC	CGTCTGTCCTCCCTTCTCT
Intron 10b	GGTAAGGAAGGGAGTGGGA	GGGGGAAGGGTCAAGAAGA
Intron 10c	GCTCCTGTTTCCTGCCTGT	GGTAGCTGCGAGTGACTGTG
Intron 10/Exon 11	GCTCCTGTTTCCTGCCTGT	AACCCTCCCTTCCCCTCTT
Exon 11	AGCGGTCAGTCCCAGACTC	AGAAGGCTCTCCCTCCCAC
Exon 12	AGAGCCTTCTCTTCCGCA	TGGGCATGAGGTGAGGAA
3'UTRa	TCTCCCCGTCTCTCTTCT	GTAAAAGCAGCCCCTCTC
3'UTRb	CCTTTTCTCCCTGCTTCC	CTTTCTTCCCCCTCCTCT
3'UTRc	GAATGAGGCGGGAGGTAGA	AGGACACCCACCAACAAAGA
NCOA7		
Exon 1	TTCTAAAAGGGCTGTGCTGAT	GGACACGACTGAGCGACTT
Exon 2	AGACAGGCAAATCGACAGAAA	CCTGCTGAATGTCTCCACCT
Exon 3	TTCCCCCACCTTTCTCTCTT	ATGCCCCATCACGTCTTTAG
Exon 4	TGTCATTAGTTGAGAAAAGAAACCA	GCATTTGCTGTTAAAACACATTCTA
Exon 5	AAACCCAAGATCCCCGTACT	GGAAAGACACAAGCAGAAGAAGA
Exon 6a	TGGTTCTTTATTTTGCCACTTTT	TCCCTTCAGTTCAGCATCAC
Exon 6b	AAGGTGGAATGGACAAAAGAGA	GCAAGAATGACCAAAATGTGAA
Exon 7	TGCTGTGGTGTTGGTTTTTATC	GGGTAGCACTTGGGGTTTAG
Exon 8	GTCAATTTTTCCCCTGTGAAC	CCATCCATTGTGCTCCTAAC
Exon 9	GCCACACTGCGTTAAATTCTG	TGATGGGGGAAGTACAAGAGG
Exon 10	CATAAAATACCCATGCTTTC	GACATGCTCCATTTTCTTTT
Exon 11	GGCTCTCACTCTCTCAACCA	AGCTAGACCTTTCCCACACC
Exon 11/12 version 2	GCCTCCTTTTCATCTTTCC	GGGACCTCTGCATTTAGC
Exon 12	CTGTGGTTGGAGCGGTAGA	AAGCAAAATGGTGAAAACATCTC
Exon 13	CAAATGAGGGACCACCTGAA	TGCTGTGCACTTTCAATTTTTC
PPARG		
5'UTR1	ATGGCATCATCAACTCAATGG	AGACTGACACGGATAGGTTGG
5'UTR2	GCCACTTGTGTGATAGGAG	ACCCTTGGTTGAATCTCTAGT
Exon 1	TAGAGAATCTGGATCGCTGTG	CGCAAGAGCAGCAAGTTAAG
Exon 2	CAGGGCTGTCTGTAGGACGTT	TTCCCGACCCAGGTATCAA

Appendix M continued

Gene / Primer	Forward	Reverse
NCOA7 continued		
Exon 3	TGCCAAATACAGCCCATAGAA	TGCGAAGGTGGAATTAGATCA
Exon 4	CCATTGACGGAACGTGTC	TCACACTTAAAGCCGAACAAC
Exon 5	GGAAACTGCCGAAGTATCCAC	CCGACTCTTGGCAACCCTAC
Exon 6	GTATGCCATTGAAAGCTAGAA	GGGAAAGTGCGGTAAGTG
Exon 7	TTGCCTGCTATCTGCTTACCT	GGGGTGGGGAACACAAC
TEK 1		
5'UTR1	CGACTAAAGTGACGCAGCA	CCACAGGAAACGGCAGA
5'UTR2	AGTGTTTGAACCCAGGTCTAT	CTAGGAGGGATTTCATGGTTA
5'UTR3	TATCCCCACCTAAAACTTACA	TAAAGGCGTTTGGTATCAG
Exon 1 + 5'	CCGGTCATCAGCACATACCA	GAGCCCAGAGTCACGGTTAAA
Exon 1	TCTTGCCTCTGACTTGTAAAC	CCCATCACTCTCAGCATAAA
Exon 2	TGGGGAATGGAGAATGAGTAA	GTGAAAGGGGTGTATATGAGG
Exon 3	GCCCAGGACTGTCTCGT	TCCAGTATTCTTGCCTACAGA
Exon 4	ATTGATTGGTTGGCAAGGTTA	CTAGTGCCCTCGCTGTAA
Exon 5	TTATGTGGCAGCCTGTATGGG	CGCGAAGAGTCAGACACGAC
Exon 5 (2)	GTCTTTATCATGTTCCGTTAC	CGAAGAGTCAGACACGAC
Exon 6	TCATTGATGTGAACACCGT	CCCAAATCTTGTCTGTATCTG
Exon 7	GTGGCCTGGTAAGTGCTTAT	AGAACAGATGGTAGGGCAAT
Exon 8	CCATTTCAGCATTGAGACCGT	GGAAAGGGAGAGGGCTAGT
Exon 9	CTCCCAAACACACAACTACAA	AAGCCACACCCAAGTTTAC
Exon 10	CGGGTAAGTGCGTTGAATCT	GCCCTGGACTTCCCTGTTACT
Exon 11	TAATTGAGTTTTTCCCCCTAA	TTCTCTGTCCCCGTGGAT
Exon 12	GTGGGCTGCCGTCTATGG	TATATATTGCGGGTCACTAAA
Exon 13	GGCCTCAATACTAACAGTAAG	GCTGATGGGCTAAGTTC
Exon 14	ATCAATTCTTTGGCACTTAGC	ATGCCCTTCAACTGACAATA
Exon 15	CAGTCGTGCCCAACTCTTA	TCCATCTAACATCATGCCAAT
Exon 16	GGTTGGGCTCCTGGTAACT	TGGGTATCTTTGGGCATTACT
Exon 17	AGGGAGCCTGCTGTTCGGTAG	GGATTCTGCCAAACGACCCAC
Exon 18	GCCTTTCATGGTCATTGGA	AGGAAACCTCGTTGATTCACT
Exon 19	CACCTCTGACAGCCACGAAGT	GGCCACCTCCTCGGATCTA
Exon 20	GCTCTGTGTAAGACCCAATCC	CACCGAATCTGAATGTTACCC
Exon 21	CACGGGAGCACGGTCTGTA	TCCTGCCCATTGCCTAACAGT
Exon 22	ACCCAAAGTAGGCACTAGAT	GACAGATTTAAGATTCCGTCT
Exon 23	GGCAACCCACTCCAGTATTCT	CGCTGAGCATCAAGGTATGAA
	High Resolution Melt Primers	
ACACA SNP1	GGGACACAGGTTTGAATGAT	TTGCATGACACCTCCAGTATC
ACACA SNP7	GGAACAAAATTCTGCATGTAA	AAGTGATGTCATTGCCAATAA
APM1 UTRA/C	GGCTCTTCTGTCTGTGGGAT	GATTGGGCTTCTCAGGTGGT
EDG1 SNP 1	TATTGGTGTCTGTTTAGTATG	CATTGTCAGAAACCTTTTA
EDG1 SNP 1	AGGTGAAAACTGACTCGG	AAAGTGCCAAAGATAGATGTT
EDG1 UTR	AGGAACTATTATTTACCCATA	AAGAGAATGGAGCAACC
ENO3 SNP 10	CCCTCTCCACCTCAAAC	GGTGACAATGACAAAAGTTC
ENO3 SNP 11	AGAACTTTTGTCATTGTCAC	GATGTGTTGGGAGGGTA

Appendix M continued

Gene / Primer	Forward	Reverse
ENO3 SNP 5	CTGTAGTAAGTGCCATGTCAA	ATTTTATTCCTCAGAGCCTC
ENO3 SNP 8	TCCATCCAGGCGTGAGTG	TCAGACCTGGAGTTAGTGGACA
ESR1 SNP 1	TGTTAATGGATTCACTGGGTTTT	ATGCTTCCCTTGTCACTGGT
ESR1 SNP 2	GGCTACGCAAGTGCTATGA	ACTCCATTCCCACAGTTACCA
ESR1 SNP14a	ATGGAAGTCTTTTTATGGATG	TCCAGAAGACATGATAAGGTT
ESR1 SNP14b	CTTTTTCCATCTTGCCTTC	CATGATAAGGTTCACATTCA
Lamin SNP1	CAGTCTCACCGCCACATC	AACTGCTGGCTTCTGGTCT
NCOA7 SNP1	ACTCCTTTTTCTACAGAATTTGAATG	TCAAAAATAATCACCCAAGTCAA
PPARG SNP2a	CCCGATGGTTGCAGATTATA	TGTCTTCACCACAGCCATTAG
PPARG SNP2b	TGAAGCTCCAAGAGTACCAAA	TCACCACAGCCATTAGTCCT
PPARG SNP6a	CCCAACTCTCCACTGTATTC	TATATCTGGTCCATCGACATT
TEK1 SNP4	CGAGGGCATCAATCCA	ACTGACCCCATCTCATTGTTA
TEK1 SNP4	AGTGTTTATCCCATACTCCCA	CTGACCCCATCTCATTGTTA
TEK1 SNP4C	TACAAGCATTGGACTGATCTT	GGCTGGAAAGGTCAAATAC
TEK1 SNP4D	TTATACAAGCATTGGACTGAT	GTTCATAACACTGACCCCA
TEK1 SNP5	CCTCTGACCGCTAAAATGC	AAATCAAACCCAGGGCTATGT
TEK1 SNP5	AAATGCCTTTTGGATGTTCTC	CTCAAAGCCACACCCAAGT
TEK1 SNP6	CCCCAAAGGTGATCGACAC	CGCCAAGCCTCATAGTGATTA
TEK1 SNP6	TTCTTCCAAAGCCCTTGAATG	CGCCAAGCCTCATAGTGATT
	Allele Specific PCR primers – ASP primer = *	
EDG1	GAATAACACATTAAAAAATACTCT	GTATAAAAAAATAATAATAATTGAAG*****
ENO3 SNP11x	AGAACTTTTGTCATTGTCACG*****	GATGTGTTGGGAGGGTAAA
ENO3 SNP8x	TCCATCCAGGCGTGAGTGC	TCAGACCTGGAGTTAGTGGACAAC*****
ESR1	CCTGCCATGATGTCCTTGTC*****	CCACCTCCCACACATCTTG
LMNA	ACTAGAGTCAGCCACCAAGA*****	GCACCTTTCTTAGATCTTCAT
PPARG - C allele	CCTTTCTATGATGGATGACC	AGCATTTACCCTACTGTGG*****
PPARG - G allele	ATTGGACTAAGCTCTCGTG*****	CAGCCAGATTCTTTACCAC
	Lightcycler Primers	
APM1-1a	GGGTATTGGGCAACCTAGA	CCTCCACCCTGCCAGTACTTT
APM1 UTR 5' region	TGTGTCTTCTGTATTGGCATA	GGCTTCTCAGGTGGTACAAG
EDG1 UTR	AGGAACTATTATTTACCCATA	AAGAGAATGGAGCAACC
ESR1 exon 19	GGCACTGTCTCAGTATCACC	TTCCAGAGACCTCAGAGTGCT
NCOA7-1a	AAGGGTGCAGGGCTATCCAT	TGCTGCTGACTTCCGGTAGAG
NCOA7-1b	CGGAAGTCAGCAGCACTAGA	AAGCCTCACCTGATTATCCAT
NCOA7-2	AGCTGATTGTAGTTAGTCCA	TCATTTTATCTTCGATTCTTA
NCOA7-3	CATGTGGAAGGCAGACGA	ACTCCTCACTCTATGGGTTTG
NCOA7-4	AGGTATGTTGGCGTCCTGAT	GAGAGCAGCCTGATCCTTTCT

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