

Chapter 1 Introduction

Over the past three decades the Australian sheep industry has shifted its balance from wool to lamb meat production. During this period the lamb industry has focused on improving meat yield through genetic gain by the use of estimated breeding values, but this method has a slow rate of genetic improvement and has the potential danger of adversely affecting meat quality attributes that the market desires (i.e. less fat, larger cuts and improved eating quality). As a consequence there is a greater need for other methods to be considered to improve the efficiency of growth, feed utilisation, carcass size and characteristics while not compromising consumers' demand for quality, tender and lean lamb meat. Alternatives include the naturally occurring *myostatin* g+6723G>A mutation and the inclusion of metabolic modifiers in finishing diets, such as β -adrenergic agonists, known to increase muscle mass and reduce carcass fat. This literature review evaluates the current knowledge on the mechanisms of myostatin on the regulation of skeletal muscle growth and development, and the mechanisms that β -adrenergic agonists use to improve yield of lean meat (from here on referred to as lean).

1.1 Myostatin

Myostatin, formerly known as growth and differentiation factor 8 (GDF8) and a member of the transforming growth factor- β (TGF- β) superfamily, is a secreted protein that negatively regulates embryonic and postnatal skeletal muscle development and growth (McPherron et al., 1997). The gene is highly conserved within many species, and the gene sequence is identical in humans, rats, mice, pigs, chickens and turkeys (Lee, 2004) and almost identical in ruminants: cattle and sheep (Kambadur et al., 1997; Clop et al., 2006). *Myostatin* was initially discovered by a gene target knock-out of GDF8 in mice. The knock-out resulted

in significantly greater skeletal muscle mass associated with increased muscle fibre hyperplasia and hypertrophy; i.e. more and larger cells (McPherron et al., 1997). Following the discovery of *myostatin* in mice, further research identified that the double-muscled phenotype found in Belgian Blue and Piedmontese cattle breeds, Texel sheep, Whippet dogs and, one documented case, a human child, were consequences of mutations in the coding sequences at the *myostatin* locus (Kambadur et al. 1997; McPherron and Lee, 1997; Thomas et al., 2000; Schuelke et al., 2004; Clop et al., 2006; Mosher et al. 2007). It is now well established that myostatin is a regulator of skeletal muscle growth and disruption of the gene results in greater muscle mass which has potential implications for increasing yield of lean in the lamb meat production industry.

1.1.1 Myostatin function

Myostatin regulates embryonic skeletal muscle development by inhibiting the proliferation of myoblast cells during embryogenesis (McPherron et al., 1997; Thomas et al., 2000; Zhu et al., 2000; Taylor et al., 2001; Lee, 2004). Skeletal muscle development and growth initially begins at embryonic development where the mesodermal somite cells become myoblast cells during myogenesis. The myogenenic developmental pathway involves the transformation of mesodermal somite cells to myoblasts (myogenic) which proliferate and progresses through to alignment and fusion, creating a multinucleated myotube (Buckingham et al., 2003; Picard et al., 2002) (Figure 1.1). The fused multinucleated myotube then leads to the development of muscle fibre structure.

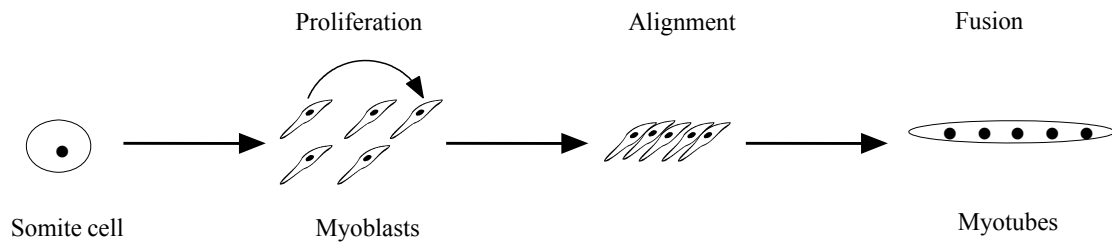
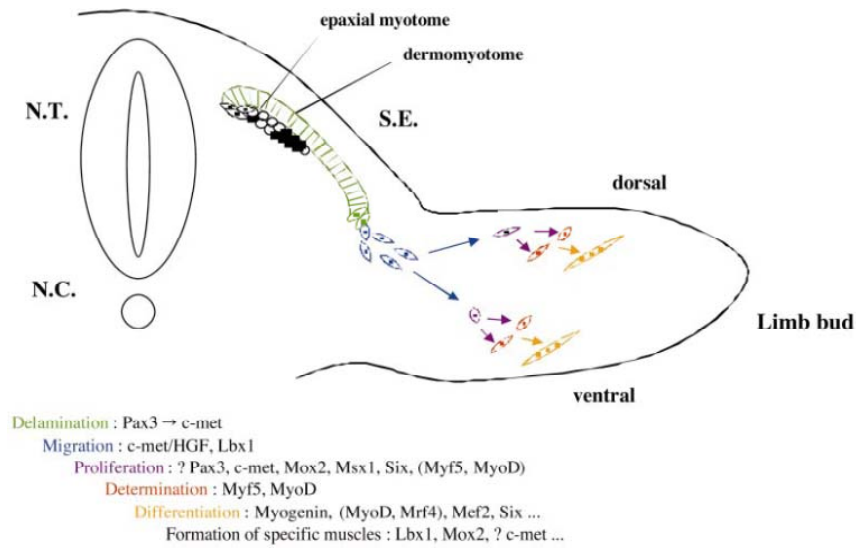


Figure 1.1 Myogenesis. Schematic representation of the initial development of skeletal muscle from the somite cell to myotubes during myogenesis (adapted from Picard et al., 2002).

Prior to myogenesis, the myoblast cells (derived from somite cells) are formed in the embryonic dermomyotome. The dermomyotome is divided into epaxial and hypaxial locations where the somite cells form in either of these two locations. Once the somite cell is formed it delaminates from either location and migrates to a dorsal or ventral location of the ectoderm limb bud (Figure 1.2). The location of the somite cells in the limb bud region ultimately determines the anatomical fate of the myoblast cells. Somite cells in the dorsal region form muscles and the type of muscle formed depends entirely on the region in which delamination occurred (Buckingham et al., 2003). For example, somite cells from the epaxial dermomyotome form the backbone muscles (closest to the neural cord) and somites from the hypaxial region form the remaining muscle in the body. Ventrally located somite cells will become cartilage and bone. Accordingly, the location of the somite cells delamination site ultimately determines the type of skeletal muscle that will be formed.

a)



b)

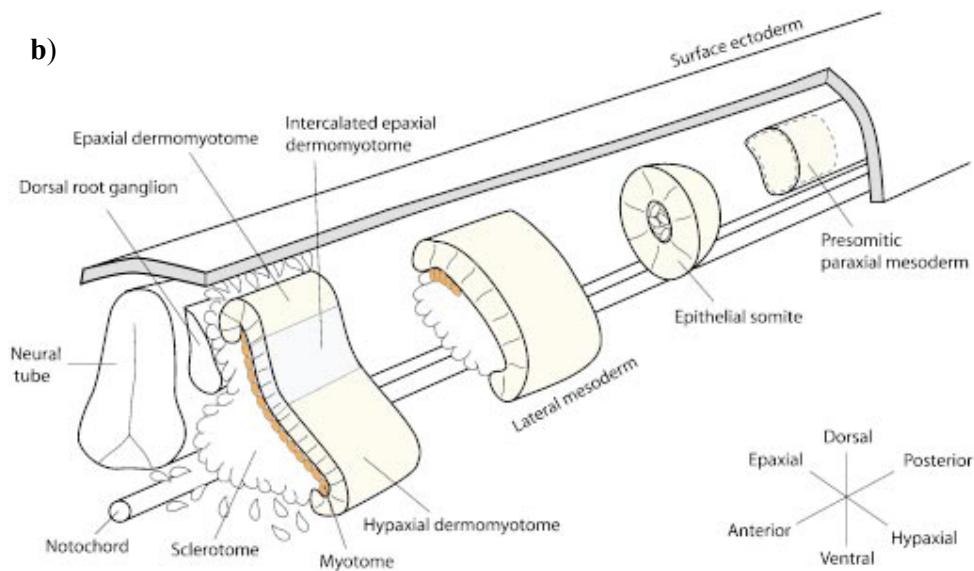


Figure 1.2 Skeletal muscle formation from somite cells, their ultimate location and stages of development. **a)** The different genes involved in each stage of development. Neural tube (NT), neural cord (NC) and surface ectoderm (SE) (Buckingham et al., 2003). **b)** Schematic diagram of posterior to anterior development of somite cells (Buckingham et al., 2003).

1.1.2 Myogenesis regulation factors

The progression of embryonic somite cells to myofibres, through myogenesis, is a tightly orchestrated cell lineage pathway. As outlined earlier, myogenesis involves the delamination, migration, proliferation, determination and differentiation for the formation of skeletal muscles (Figure 1.2). Myogenesis is initiated by extrinsic regulation factors which are produced by the neural, notochord and the surface ectoderm of the embryo (Picard et al., 2002; Linker et al., 2003). At the initial stage of cell lineage is delamination and migration, which is activated by the regulation factors c-met and a tyrosine kinase receptor. C-met is dependent on the transcription factor Pax3 which instructs the developing somite to leave the dermomyotome and migrate to the ectoderm limb region (Buckingham et al., 2003). It is here that myostatin has been detected during these very early stages of development (McPherron and Lee, 1997). As the somite cells leaves the dermomyotome it continues to migrate to the ectoderm limb region where the muscle regulatory factors (MRFs) are activated (MRFs are usually used as markers to identify different stages of development). The MRFs are specific helix-loop-helix transcription factors that produce Myf5 and MyoD, which are associated with the initiation and proliferation of myoblast cells (Chargé and Rudnicki, 2004). Delamination and migration of the somite cells are the first stages of embryonic skeletal muscle growth. Proliferation does not begin until the somite cells have reached their location in the ectoderm limb region.

Once the somite cells have reached the appropriate location they will begin to proliferate as myoblast cells and it is at this stage that myostatin inhibits or regulates myoblast cell proliferation (Thomas et al., 2000; Taylor et al., 2001). Myoblast cells remain in a proliferative state until they differentiate into myotubes which is regulated by a complex pathway of MRFs, p21 and retinoblastoma (Rb) regulatory factors. Myostatin has also been found to have a fundamental role in mediating the regulation of myoblast proliferation

through the cyclin-Cdk2 inactivation of Rb via p21 (Figure 1.3; Thomas et al. 2000; Chargé and Rudnicki, 2004). Once the myoblast cells have proliferated, MRFs and myogenin will determine the alignment and myoblast cells will fuse into multi-nucleated myotubes at the appropriate anatomical muscle location. The multi-nucleated myotubes will ultimately mature into myofibres to form skeletal muscle tissue.

It is not entirely clear how myostatin affects the subsequent roles of the gene transcription factor MyoD and Myf-5 in the myotome compartment of developing somites (expression also continues in adult muscle). Myogenin and MRF4 are required in the differentiation and maturation of myotubes (Thomas et al., 2000) but there are also a number of other regulatory proteins found in the cell cycle that appear to regulate myostatin, and consequently control myoblast proliferation. Thomas et al., (2000) reports that myostatin regulates myofibre embryogenesis synthesis/signalling by up-regulation of p21 which inhibits the cyclin-cdk2 activity preventing the Rb activation (Figure 1.3). Alternatively, inhibition of myostatin increases expression of the cyclin-cdk2, p21, reducing the expression of p21 where Rb accumulates and remains hypophosphorylated resulting in an increase in the number of myoblasts (McCroskery et al. 2003; McFarlane et al. 2011). The function of these regulators in the cell cycle, and their relationship with myostatin, are described below (Figure 1.3).

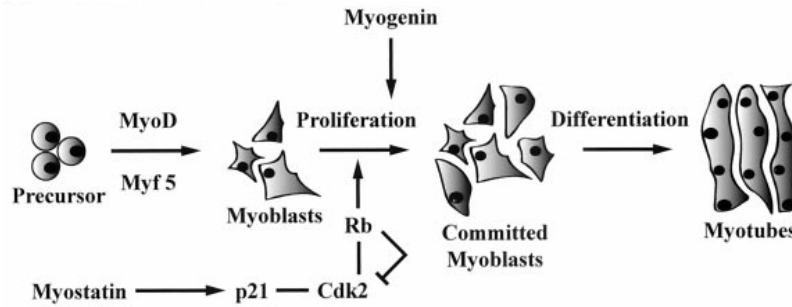


Figure 1.3 The regulation of myoblast proliferation by myostatin orchestrated through p21, cyclin-Cdk2 (Cdk2) and retinoblastoma protein (Rb) (adapted from Thomas et al., 2000).

During embryonic myogenesis the MRFs initiate the migration and proliferation of the myoblast cells. Myostatin then controls the proliferation of the myoblast cells ensuring that muscle size is regulated; therefore, the regulation by myostatin occurs in the cell cycle of the myoblast cells. The cell cycle is a tightly controlled sequence of events regulated by numerous proteins that direct the myoblasts cells through cell cycle to mitosis to produce two identical daughter cells. The cyclin-dependent kinases (cdks) and the cyclin proteins regulate the cells progression through the cell cycle (Schafer, 1998). During the cell cycle the cell goes through a sequence of phases which involve the Gap 1 (G_1) phase, synthesis phase (S phase), Gap 2 (G_2) phase and M (mitosis; Figure 1.4). The G_1 phase is the initial interphase which undertakes growth, normal metabolic functions and prepares for DNA synthesis. The S phase is the DNA replication phase and G_2 is the secondary gap (or growth) phase where the cell prepares for mitosis. Cells in the G_0 phase are not actively cycling (Schafer, 1998).

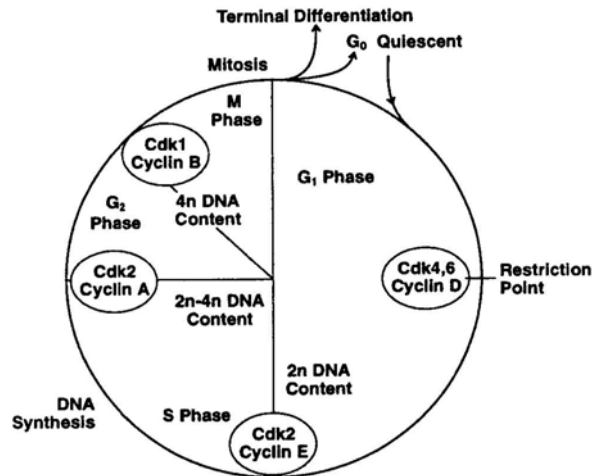


Figure 1.4 Schematic drawing of the cell cycle. The cycle is divided into four phases the Gap 1 (G₁) phase, synthesis phase (S phase), Gap 2 (G₂) phase and Mitosis (M). The cells progress through each phase by regulation of various cdk-cyclin complexes. At M phase the cell can leave the cycle through terminal differentiation or remain in the cycle (Schafer, 1998).

In the cell cycle the cdks and cyclins activate at specific points of the cell cycle for the cell to progress through and inactivation of the cdks and cyclins ultimately prevents mitosis. At the G₁ phase cyclin and cdk initiates transition of the cell to the S phase which is constrained by the cdk inhibitors (CKIs) (Thomas et al., 2000). At each stage of the cell cycle the cyclin cdk complexes have particular tasks that pertain to different CKIs (e.g. p27, p57, p15 and p16); however, it is believed that these CKIs do not have any particular affinity to myostatin (Thomas et al., 2000). Thomas et al., (2000) found that *in vitro* studies of myoblast C₂C₁₂ mice cell cultures treated with myostatin blocked the progression of myoblast cells from the G₁ to S phase which was a consequence of cyclin-Cdk2 inactivation of Rb. The inactivation of Rb is present in a hypophosphorylated form which prevents the cell from migrating from the G₁ phase to the S-phase in the cell cycle (Thomas et al., 2000). Alternatively, a recent study by McFarlane et al., (2011), reported that inhibition of myostatin

increases in Cdk activity increasing the number of myoblast in the G₁ phase of the cell cycle. Therefore, myostatin controls myoblast proliferation by targeting key cell cycle regulators through the inhibition at the G₁ phase of the cell cycle.

1.1.3 Myostatin function on postnatal satellite cells

During the development of myofibres, embryonic somatic cells also give rise to satellite cells. Satellite cells are required for myogenesis during postnatal growth, development and repair of myofibres. Postnatal satellite cells supply myonuclei to promote postnatal muscle growth until maturity where they will then down-regulate to a quiescent state. This is almost like a dormant state, but they progressively down regulate as the muscle tissue approaches maturity, whereupon they lie dormant and ready to activate in the case of injury or disease. In a quiescent state satellite cells position themselves between the basal lamina and the cell membrane and will remain in this state until they are required to produce myoblast cells for repair or to increase muscle (McCroskery et al., 2003).

Satellite cells undertake postnatal myogenesis using similar regulatory transcription factors to those during embryogenesis. However, when satellite cells are in a quiescent state they will continue to produce the regulatory factors Pax7, salimucin CD34 and Myf5. The CD34 regulatory gene is not specifically associated with the satellite cell but is used as a marker to identify satellite cells from other cells (Zammit et al., 2006). The initiation of satellite cells from their quiescent state rapidly produces the transcription factors MRFs, CD34 and Pax7 that commit to the onset and lineage of myogenic differentiation (Zammit et al., 2006). The total number of satellite cells remains constant by self-renewal. The satellite cell is an important contributor to postnatal muscle growth regulation through maintenance and repair. McCroskery et al., (2003) reported that myostatin blocks the activation of

postnatal satellite cells *in vitro* and that myostatin null mice have a greater increase in the actual number and proportion of active satellite cells. As myostatin is an inhibitor of myoblast proliferation during myogenesis it is likely that myostatin does have an effect on the number of postnatal satellite cells, although it is not yet clear what mechanisms myostatin uses to regulate postnatal satellite cells.

1.1.4 Myostatin structure

Myostatin is a member of the TGF- β superfamily and all members share similarity in chemical structure (Figure 1.5). As with other TGF- β superfamily members, the myostatin protein is a precursor protein which is latency associated, having a 375 amino acids signal sequence and two proteolytic processing sites which generate a biologically active molecule (McPherron et al., 1997; Zimmers et al., 2002; Lee, 2004). The precursor protein consists of an N-terminal pro-region peptide and a C-terminal peptide which have a molecular mass of 27,860 and 12,400; respectively (Lee, 2004). The N-terminus peptide is a hydrophobic pro-region that functions as a signal sequence for secretion and is believed to be important for proper folding of the C-terminal domain. The C-terminal (COOH) region of myostatin has nine cysteine residues that form a “cysteine knot” and near this region proteolytically cleaves at the 264-267 amino acid RSRR (Arg-Ser-Arg-Arg) site (McPherron et al., 1997; Lee, 2004; Joulia-Ekaza and Cabello, 2006). The myostatin precursor protein is directed to and translated in the endoplasmic reticulum where the pro-region peptide acts like a chaperone to the dimerisation via a disulphide bond at the C-terminus and the precursor protein appears to be regulated in the *trans*-Golgi by serine protease furin or other members of the proprotein convertase family (Sharma et al., 1999; Lee and McPherron, 2001).

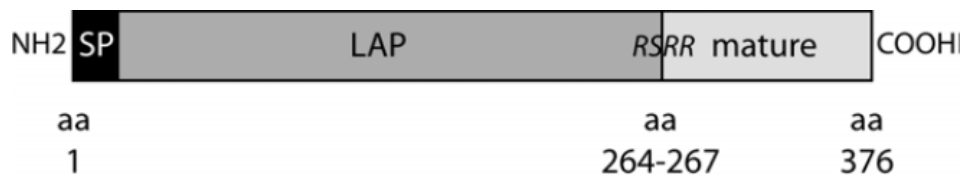


Figure 1.5 Structure of myostatin protein. The mature myostatin protein is categorised as a member of the TGF- β superfamily as the protein has an N-terminal (NH₂) hydrophobic core, latency associated protein (LAP; a pro-region found in all transforming growth factors), a “cysteine knot” at the C-terminal (COOH) which proteolytically cleaves at the RSRR site (adapted Joulia-Ekaza and Cabello, 2006; McFarlane, 2007).

Various studies have reported different active sites of NH₂, latency associated peptides and mature active myostatin protein and it has been reported that the active protein site is either has a monomer or dimer portions of the protein. Oldham et al., (2002) and Hill et al., (2009) reported that the NH₂ peptide was 50 kDa, the latency associated peptide 36 kDa and a monomer mature active protein at the 26 kDa which corresponds to the C-terminal. However, Zimmers et al., (2002) reported that removal of the signal sequence resulted in a 26 kDa NH₂ peptide and a dimer 12.5 kDa peptide.

1.1.5 Other mechanisms through which myostatin may influence cell growth and metabolism

Myostatin is not only present in skeletal muscle but also found circulating in blood in the latent protein form and is activated by synthesis to the BMP1/Tolloid matrix metalloproteinase which releases the protein into its active form (Zimmers et al., 2002; Lee, 2004; Schuelke et al., 2004). Myostatin regulates muscle cell growth and proliferation by

regulating the cell cycle through the activin type IIB cell receptors (ActRIIB). The ActRIIB signals through SMAD transcription factors which activate the GSK-3 β and inhibit P13K/Akt and then signal to the appropriate gene regulators to regulated myoblast proliferation and differentiation (Yang et al., 2007; Matsakas and Patel, 2009). A hypothetical diagram of myostatin regulation through P13K/Akt pathway is outlined in Figure 1.6.

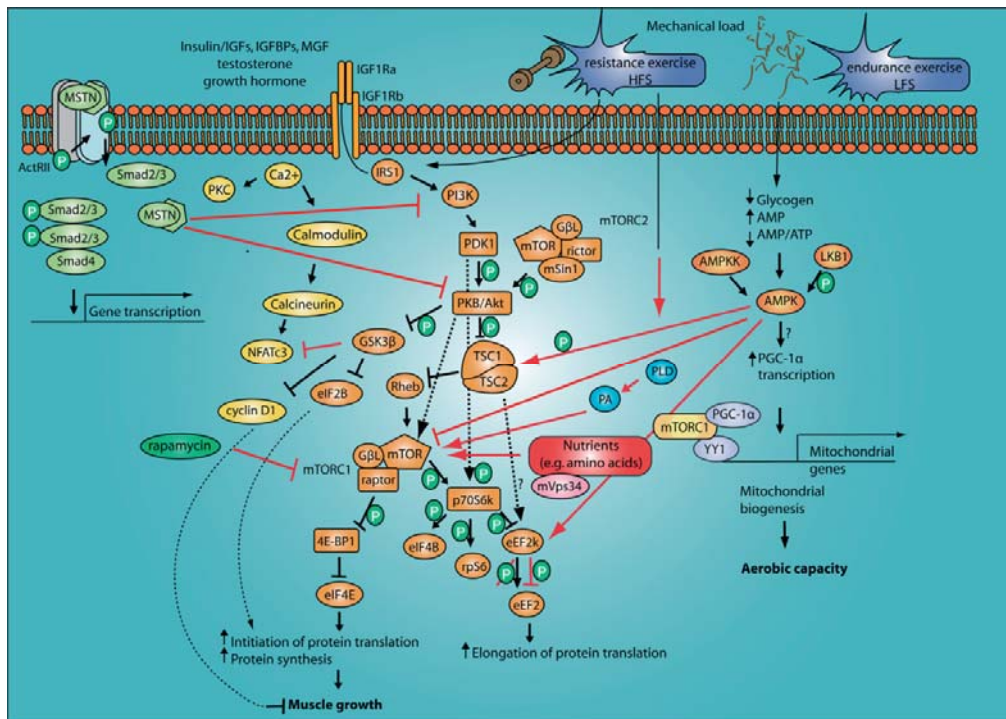


Figure 1.6 Hypothetical schematic diagram of the signalling pathway of myostatin to regulate skeletal muscle protein synthesis (Matsakas and Patel, 2009).

1.1.6 Mutations in the myostatin gene and effects on muscle growth

The discovery of the effect of a *myostatin* (GDF8) knock-out in mice facilitated the identification of different naturally occurring mutations in other species. As outlined earlier the *myostatin* gene, and therefore function, is highly conserved across many species.

Mutations of *myostatin* have been shown to be responsible for the double-muscle phenotype in several cattle breeds (Grobert et al., 1997; Kambadur et al., 1997; McPherron and Lee, 1997), the Texel sheep breed (Clop et al., 2006), Whippet dogs (Mosher et al., 2007) and humans (Schuelke et al., 2004). However, in and within different species *myostatin* mutations have been found to have different effects and modes of function. It is known from chromosomal screening of Texel sheep, displaying the double-muscle phenotype, that the *myostatin* locus is located in chromosome 2 of sheep (Clop et al., 2006). Furthermore, analysis of the gene in other species has established that there are several (seven at last report) naturally occurring mutations at the *myostatin* locus which include premature stop codons, frame shift mutations and point mutations, and are highly conserved at the amino acid residues (Lee, 2004; Schuelke et al., 2004; Clop et al., 2006; Mosher et al., 2007; O'Rourke et al., 2009). Figure 1.7 shows the phenotypic effect that different naturally occurring *myostatin* mutations have on different species.

In cattle, six loss of function mutations have been identified in the coding region of bovine *myostatin* gene and each of these mutations are associated with different cattle breeds, such as the Belgian Blue and Piedmontese, or mixed across breeds. The Belgian Blue and Piedmontese double-muscled cattle have 2 frameshift mutations that lead to premature truncation of the *myostatin* message (Grobert et al., 1997; Kambadur et al., 1997; McPherron and Lee, 1997; Grobet et al., 1998). The Belgian Blue has an 11-bp (821 *del11*) deletion mutation in the third exon and the Piedmontese cattle have a G > A transition, and other cattle breeds with an insertion/deletion at the 419 nucleotide, or three other premature termination codons (Grobert et al., 1997; Kambadur et al., 1997; McPherron and Lee, 1997; O'Rourke et al., 2009). The other cattle breeds' mutations are the insertion/deletion at nucleotide 419 (419 in:del), and premature termination of codons Q204X, E226X and E291X (O'Rourke et al., 2009).

The different forms of mutations can have different phenotypic effects. Not all of the mutations result in extreme muscling like the Belgian Blue, even in sheep the same 3'UTR mutation does not result in double-muscling phenotypes in some genetic backgrounds, although there maybe some hyperplasia affect. Limousin cattle have been identified as having a *myostatin* missense mutation, F94L in exon 1, where homozygous cattle for the variant demonstrated increases in muscle mass but not extreme double-muscled phenotype (Sellick et al., 2007).

The primary characterisation of the muscle fibre type in double muscle cattle breeds is the result of hyperplasia, occurring during foetal development. Double-muscled cattle are reported to have a greater proportion of fast-twitch (type II) muscle fibres (Swatland and Kieffer, 1974; O'Rourke et al., 2009). The extreme double-muscle phenotype is associated with homozygous dell 11 and results in a 20% increase in muscle mass some of which is exhibited during foetal development (Swatland and Kieffer, 1974; Grobert et al., 1997). Animals with this phenotype have a higher meat yield, lower carcass fat, more tender meat and produce a higher proportion of expensive meat cuts (Arthur, 1995). Although the 20% increase in muscle mass is of considerable importance to meat production, there are also considerable negative effects associated with the mutation. Beef cattle with extreme muscling are reported to have a high incidence of dystocia, poor reproductive performance and lower calf survival (Arthur, 1995), which is described in detail later. These negative effects contribute to their restricted use in extensive beef production systems. The objective in the beef meat production system would be to identify *myostatin* mutations that are associated with increases in muscle mass whilst not affecting reproductive performance.

In sheep, the Belgian and New Zealand Texel sheep along with the Charollais sheep are all known carriers of the *myostatin* g+6723G>A mutation (Clöp et al., 2006; Hadjipavlou et al., 2008; Johnson et al., 2009). Clöp et al., (2006) identified and reported that the

g+6723G>A mutation occurred in the 3' untranslated region at the G to A octomorf motif. This octomorf motif is identified by miRNA 1 and miRNA 206 which normally regulate gene silencing. Micro RNAs miRNA 1 and miRNA 206 silence the translation of the sequence by incorporating the use of RISC (ribosome induced silencing complex). The RISC targets mRNA to the ribosome, and is directed by the miRNAs. However, when the fit between the miRNA and mRNA is poor, the RISC is less effective in this targeting, resulting in fewer copies of the translated protein per number of copies of mRNA. It is thought that the g+6723G>A mutation alters the binding of the octomorf recognition site with miRNA 1 and miRNA 206 and thereby reduces the rate of translation into myostatin protein. The g+6723G>A mutation was also found to be present in about approximately 95% of Australian Texel sheep, 10% in the Australian White Suffolk and < 5% in the Australian Poll Dorset sheep (Kijas et al., 2007).

Clop et al., (2006) reported that the 12.5 kDa myostatin protein band could be identified by immunoprecipitation from Texel sheep serum homozygous for the *myostatin* g+6723G>A mutation. The intensity of the band was one third that of the corresponding wild-type animals. This suggests that the *myostatin* g+6723G>A mutation in sheep results in a lower abundance of the circulating active monomer form of myostatin protein. Yet as outlined earlier, Oldham et al., (2006) reported that the latent associated mature myostatin protein is at the 26 kDa band. The reported differences could be attributable to characterisation of mature protein. Hill et al., (2002) reported that myostatin at the 12.5 kDa band is a monomer and identification of myostatin at the 25 to 28 kDa band on gels is a homodimer of the 100 kDa sequence located near the C-terminal end. The bioactive myostatin dimer (25 to 28 kDa band), as discussed earlier, is believed to bind to activin type IIB receptors. It is unclear if the active protein is a dimer or a monomer. It is easier to see the 26 kDa band in gel assays but this may blindside the interpretation. Clop et al., (2006) is the only study that reports differences in

circulating myostatin monomer at the 12.5 kDa band corresponding to mature myostatin in sheep with or without the *myostatin* g+6723G>A mutation. In mice, some researchers have found that the concentration of myostatin measured at the 26 kDa band varies by as much as 50% between adult males and females (McMahon et al., 2003a; Reisz-Porszasz et al., 2003). Temporal variation in myostatin protein concentration in plasma is attributed to sexual dimorphism. Oldham et al., (2009) reported that mature male mice had a lower abundance of mRNA myostatin than female mice once they reached maturity at 32 weeks of age. The reduced production of myostatin for mature male mice could contribute to the attainment of greater skeletal muscle mass. However, in sheep there was no difference found between mRNA myostatin levels in the *m. splenius* and *m. semitendinosus* of castrate males and rams at any age (Mateescu and Thonney, 2005). This suggests that post-translational modification of myostatin protein is an important component of the regulation of myostatin action.



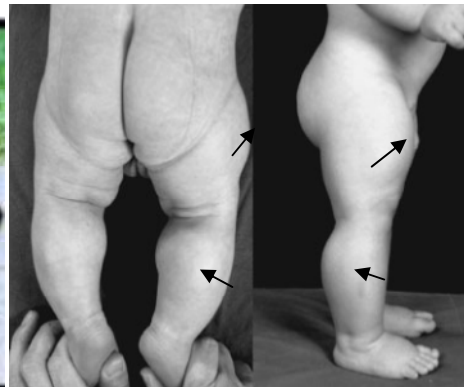
a) Belgian Blue (*b. taurus*)



b) Texel (*a. ovis*)



c) Whippet dog (*s. canis*)



d) Child (*h. sapiens*)

Figure 1.7 Different species with naturally occurring myostatin mutations. **a)** Belgian Blue cattle carriers of the *myostatin* 11-bp deletion mutation (Kambadur et al., 1997); **b)** Texel sheep carriers of the *myostatin* g+6723G>A mutation (Clop et al., 2006); **c)** Whippet dogs carriers of the *myostatin* 2-bp deletion (Mosher et al., 2007); and **d)** a human child carrying a *myostatin* G>A transition mutation, arrow heads pointing to protruding muscle of the thigh and calf (Schuelke et al., 2004).

The presence of homozygote *myostatin* g+6723G>A mutations has been found to be associated with an increase in muscling and a decrease in fatness (Kijas et al., 2007). Johnson et al., (2009) reported that increase in muscle mass is dependent on the number of copies of *myostatin* g+6723G>A mutation(s) the animals are carrying. Sheep heterozygous for the

myostatin g+6723G>A allele have modest increases in lean and decreases in carcass fatness, but this is even greater in lambs homozygous for the *myostatin* g+6723G>A allele. Therefore, the effect may be additive insofar as the response of skeletal protein mass is proportional to the number of copies of the g+6723G>A mutation.

The presence of the naturally occurring *myostatin* mutations reduces the amount of functional myostatin and consequence increases skeletal muscle mass, as discussed in detail above. Reduced production of myostatin protein reduces the negative regulation of the myostatin protein and allows for an increase in skeletal muscle size. The subsequent increase in muscle mass could also have a concurrent effect on other organs, as cattle with the *myostatin* mutation are known to have smaller organs and digestive tract (Arthur, 1995).

Evaluation of meat quality in lambs with the *myostatin* g+6723G>A mutation has been carried out in some studies. Hamelin et al., (2006) reported that Texel sheep (known carriers of the *myostatin* g+6723G>A mutation) had positive effects on the weight of the *m. longissimus dorsi* and *m. semimembranosus* but no difference in the weight of the *m. vastus medialis*. Kijas et al., (2007) found that meat from lambs with the mutation had a negative perception in consumer assessment of eating quality (flavour, tenderness and overall satisfaction), but no difference in shear force, despite a reduction in intramuscular fat content. These authors did not find any association between the mutation and pH decline, ultimate pH, glycogen content, levels of lactate and shear force at time 0, 72 h or 5 days. Masri et al., (2011) suggests that the reduction in perceived tenderness is attributable to a reduction in intramuscular fat; however, intramuscular fat was not measured in their study.

1.1.7 Adverse effects of the *myostatin* mutation

Double-muscled cattle are known to have some adverse production problems such as reduced fertility, dystocia, low calf viability and increased susceptibility to stress (Arthur, 1995). The low fertility is associated with the animals' slower reproductive development which compromises both male and female genitalia, sexual maturity and reduced fertility. Dystocia in double-muscled cows is associated with a narrow pelvic opening which together with the calves' already exhibiting aspects of the double-muscle phenotype at birth results in foeto-pelvic disproportion and calving difficulty (Vissac et al. 1973; Ménéssier 1982). Double muscled cattle are also more susceptible to heat stress where cattle exposed to increased exercise have a faster onset of fatigue and higher metabolic acidosis (Holmes et al. 1973). The onset of fatigue could be attributable to the double-muscled cattle known to have a greater proportion of glycolytic myofibres and smaller hearts possibly contributing to the onset of fatigue. Increases in metabolic acidosis could be attributable to a greater rate of protein accretion which increases metabolism.

There is less information on the effect of the double-muscle phenotype in sheep; however, it is known that Texel sheep, who have a high incidence of the *myostatin* g+6723G>A mutation (> 95%; Kijas et al., 2007), also have a high level of dystocia (Johnston et al., 1999; Carson et al., 1999). Like in double-muscled cattle (above), an increased incidence of dystocia in Texel ewes has been attributed to a narrower width of the pelvis, thereby compromising parturition, in Texel ewes (Grommers et al., 1985; Dawson et al., 2002).

1.1.8 Implications

It is clear that the action of this highly conserved *myostatin* gene is integral to the development, growth and regulation of skeletal muscle. Disruption to the gene is known to have significant implications for livestock production (and also human therapeutics for muscle degenerative diseases). The scope of this review has considered the application of the *myostatin* g+6723G>A mutation in livestock production systems and the potential benefits this mutation would have on improving lean meat yield, feed efficiency and growth rates, and some meat eating quality attributes for the consumer. The following section discusses some preliminary research on the effect the *myostatin* g+6723G>A mutation has on muscle mass in lambs. However, as outlined in the introduction, genetic selection is not the only method to increase muscle growth, the inclusion of exogenous feed additives, such as β -adrenergic agonists, are also reported to improve meat yield in livestock production.

1.2 Preliminary research

1.2.1 Introduction

On the basis of the knowledge gathered from the review of literature it is plausible that the effects of *myostatin* g+6723G>A mutation may have an effect on body composition and as a result increase muscle mass. Considerable evidence indicates that the mechanism of myostatin regulates muscle development during embryogenesis (Thomas et al., 2000; McFarlane et al., 2011) and mutations at the *myostatin* locus, such as the *myostatin* g+6723G>A mutation in sheep has shown increases in muscle mass (Clop et al., 2006; Kijas et al., 2007; Hadjipavlou et al., 2008; Johnson et al., 2009).

Nonetheless there is little knowledge on the differences the mutation has on growth of lambs with the one or two copies of the *myostatin* g+6723G>A mutation. The prevalence of the mutation in the Australian sheep flock is variable were it has been reported by Kijas et al., (2007) that only a few Australian terminal sheep breeds have the mutation present. For example, the Australian White Suffolk breed has around 10% and the Poll Dorset breed less than 1% prevalence of the mutation, whereas Texel sheep have greater than 95% frequency. This is a particularly relevant question to the commercial Australian sheep meat industry as it is predominately comprised of cross-bred lambs derived from Merino or Border Leicester Merino cross dams (both of which are not carriers of the mutation). As a result less than half of all progeny would have one copy of the *myostatin* g+6723G>A mutation.

To determine if an effect of one copy of the *myostatin* g+6723G>A mutation could be observed in cross-bred sheep, a preliminary study was conducted on data collected on cross-bred lambs genotyped for the *myostatin* g+6723G>A mutation. It was anticipated that the presence of the *myostatin* g+6723G>A mutation in cross-bred sheep would be associated with increased lean tissue mass.

1.2.2 Material and Methods

The animals used in this preliminary study were from a study undertaken at the Falkiner Research station, Deniliquin, New South Wales, Australia. A description of the animals used in this experiment and the experimental design has been reported elsewhere (final report to MLA project SG116). Briefly, 452 cross-bred progeny from White Suffolk or Poll Dorset sires bred Merino or Border Leicester Merino cross dams were genotyped to determine their myostatin or carwell (another gene known to increase muscle mass, Banks 1997) genotype. Those lambs that were from Poll Dorset sires (carriers of the carwell gene) were removed from the analysis leaving 161 lambs with same genetic background. Of the remaining 161 lambs there were three genotypes of *myostatin*, two A *myostatin* allele [g+6723G>A *myostatin* mutation (homozygote; *MSTN* A/A)], one A *myostatin* allele (heterozygote; *MSTN* A/G) or no A *myostatin* alleles [homozygote; wild types (*MSTN* G/G)].

DNA extracted from blood samples taken from each lambs was assayed for the *myostatin* g+6723G>A mutation at the SNP in the 3' untranslated region of *myostatin* by real time PCR with primers described by Kijas et al. (2007).

The lambs were slaughtered at a liveweight of 48 kg at a commercial abattoir (Country Fresh, Tamworth, New South Wales, Australia) Country. Once slaughtered the carcasses were transported to the University of New England, Armidale, New South Wales, Australia where composition (total carcass weight, lean and fat) of carcasses was measured using computer aided tomography (CT) X-ray scanning (Picker Ultra Z, Philips Medical Imaging Australia, Sydney NSW). The software program, Autocat, was then used to compute the total weight of lean, fat and bone (Thompson and Kinghorn 1992).

The effects of number of copies of the *myostatin* genotype were analysed as a general linear model using R version 2.10.1 (www.r-project.org).

1.2.3 Results and Discussion

The number of lambs of each genotype and the genotype (*MSTN* A/A, *MSTN* A/G and *MSTN* G/G) on the carcasses CT lean, fat and bone traits is shown in Table 1.1. This study showed that *MSTN* A/A lambs had a 17% increase in carcass lean ($P < 0.05$) and 13% reduction in carcass fat ($P < 0.05$) content than compared to those lambs that were G/G. Sheep with only one copy of the mutation (A/G) had 9% increased carcass lean ($P < 0.05$) and 2.2% less fat ($P > 0.05$) than G/G lambs.

There were far fewer animals ($n = 5$) with two copies of the mutation than with one copy ($n = 72$), yet there was a significant effect of two causative A alleles on lean and more particularly on fat content compared to those lambs with only one copy. The prevalence of two A alleles in an Australian commercial sheep flock will be difficult to increase because the mutation is largely sourced from the terminal sires that are known carriers of the mutation, such as the Texel and a small proportion of White Suffolk and Poll Dorset sires (Kijas et al., 2007), but Merino or Border Leicester Merino cross remain the dominant dam types and the incidence of the A mutation in such ewes is very low. To breed lambs that are carriers for two copies of the mutation both the dam and the sire need to be carriers.

Johnson et al., (2009) reported that the presence of at least one copy of the *myostatin* g+6723G>A mutation increases muscle mass and is maximised when the animal has two copies of the A allele. Yet, Hadjipavlou et al., (2008) wasn't able to detect any difference between genotype for those lambs with the *MSTN* G/G and *MSTN* A/G genotype of Charollais sheep between muscle or fat depth. However, in that study there was a significantly greater muscle depth between the *MSTN* A/A and those lambs with the *MSTN* G/G and *MSTN* A/G genotypes.

Table 1.1 Preliminary results of computer tomography (CT) predicted total carcass weight, carcass lean and carcass fat with differing *myostatin* allelic status (*MSTN* A/A, *MSTN* A/G or *MSTN* G/G) means ($\pm$ SE).

CT derived variable	<i>MSTN</i> A/A	<i>MSTN</i> A/G	<i>MSTN</i> G/G
n	5	72	74
Carcass total weight (kg)	22.7 $\pm$ 0.5 ^a	21.9 $\pm$ 0.3 ^{ab}	20.4 $\pm$ 0.3 ^c
Carcass lean (kg)	15.2 $\pm$ 0.2 ^a	13.9 $\pm$ 0.2 ^b	12.6 $\pm$ 0.2 ^c
Carcass fat (kg)	3.9 $\pm$ 0.2 ^a	4.4 $\pm$ 0.1 ^b	4.5 $\pm$ 0.1 ^b

^{a,b,c} With the same row mean values with different superscripts differ ($P < 0.05$).

Other results reported in the final report to MLA project SG116 has demonstrated that the presence of the *myostatin* g+6723G>A mutation had the greatest effect in the forequarter and loin region, but no significant effect on the leg region (Table 1.2). This suggests that the reduction of the myostatin protein occurs early in myogenesis in the embryo, i.e. to a greater extent during the anterior stage of embryogenesis. This observation lends support to the suggestion of an intrinsic difference in muscle development which occurs through an anterior/posterior gradient during embryonic formation of skeletal muscle (Buckingham et al., 2003).

Table 1.2 Least square means of data derived from the final report of the MLA project SG116 for the shoulder, loin and leg traits measured by CT for lambs with differing *myostatin* allelic status [*MSTN* A/G ($\pm$ sd) and *MSTN* A/A ($\pm$ sd)]. The size and direction relative to the wildtype (0 copies) is shown in this table.

CT derived variable	mean	<i>MSTN</i> A/A	<i>MSTN</i> A/G
Shoulder lean (kg)	4.775	0.279 ± 0.478	0.146 ± 0.25 ***
Loin lean (kg)	3.339	0.386 ± 0.875	0.082 ± 0.187 *
Leg lean (kg)	5.285	0.198 ± 0.33	0.075 ± 0.125

Significance of difference *=0.05, ** = 0.01 ***=0.005

Adapted from final report to MLA project SG116

1.2.4 Conclusion

The results from this preliminary study has shown that lambs which are carriers for the *myostatin* g+6723G>A mutation have more lean tissue and, depending on the number of causative A alleles in an the animal, a general reduction in fatness. Based on the significance of the effect and the numbers provided in this preliminary experiment suggest the subsequent experiments will have sufficient power to detect the expected differences between differing genotypes.

1.3 β -adrenergic agonists

β -adrenergic agonists (β AA) are phenethanolamines which share a similar structure and pharmacological properties with the catecholamines: dopamine, epinephrine and norepinephrine (Beermann, 2002). β -adrenergic agonists have a relatively high affinity to β -adrenergic receptors (β AR). β -adrenergic agonists are metabolic modifiers known to work directly through the cellular activation of β AR leading to an increase in muscle growth and, in some cases, reducing adipose tissue by decreasing lipogenesis and increasing lipolysis (Baker et al., 1984; Mersmann, 1998; Dunshea et al., 2005). β -adrenergic agonists dramatically improve muscle growth in lambs by initiating hypertrophy of muscle fibres (Yang and McElligott, 1989) by repartitioning nutrients into muscle and away from fat (Baker et al., 1984).

Ractopamine hydrochloride is a β AA registered for use in beef cattle and pig finisher diets in South Africa, Mexico, Australia and the USA to improve the efficiency of meat production (Dunshea et al., 2005). The use of ractopamine (RAC) in pig diets has been well documented with the response to its inclusion believed to be dose dependent. Increasing dosage rates has been shown to increase lean muscle accretion; however, there are variable outcomes to reducing fatness (Dunshea et al., 2005). In cattle, RAC increases final liveweight, ADG, efficiency of gain and HCW when included in finisher rations (Schroeder et al., 2004; Walker et al., 2006). There is very little information on the response to including RAC in sheep diets. In sheep, other β -adrenergic agonists (clenbuterol and cimaterol) have been shown to increase muscle mass by up to 40% (Beermann et al., 1987; Mersmann, 1998; Beermann, 2002).

1.3.1 Mechanisms of action of β -adrenergic agonists

Cumulative evidence indicates that β -adrenergic agonists mediate a growth response by signalling through β AR which directly affects both protein synthesis and degradation (Mills, 2002). There are three sub-type types of β AR - β_1 AR, β_2 AR and β_3 AR present on cell surfaces in mostly all muscle and other tissues (Mersmann, 1998). Different types of β AA have different affinity with each of the β AR sub-types. The proportion of the β AR sub-type receptor population varies between tissues (Mersmann, 1998). It also varies between species of animal. This accounts for the variation in the physiological response to the same β AA amongst different animal species. The more prominent β AR are type β_1 AR and β_2 AR which makes understanding the intricate β AA mode of action in different species and tissues type often difficult to interpret (Mersmann, 1998).

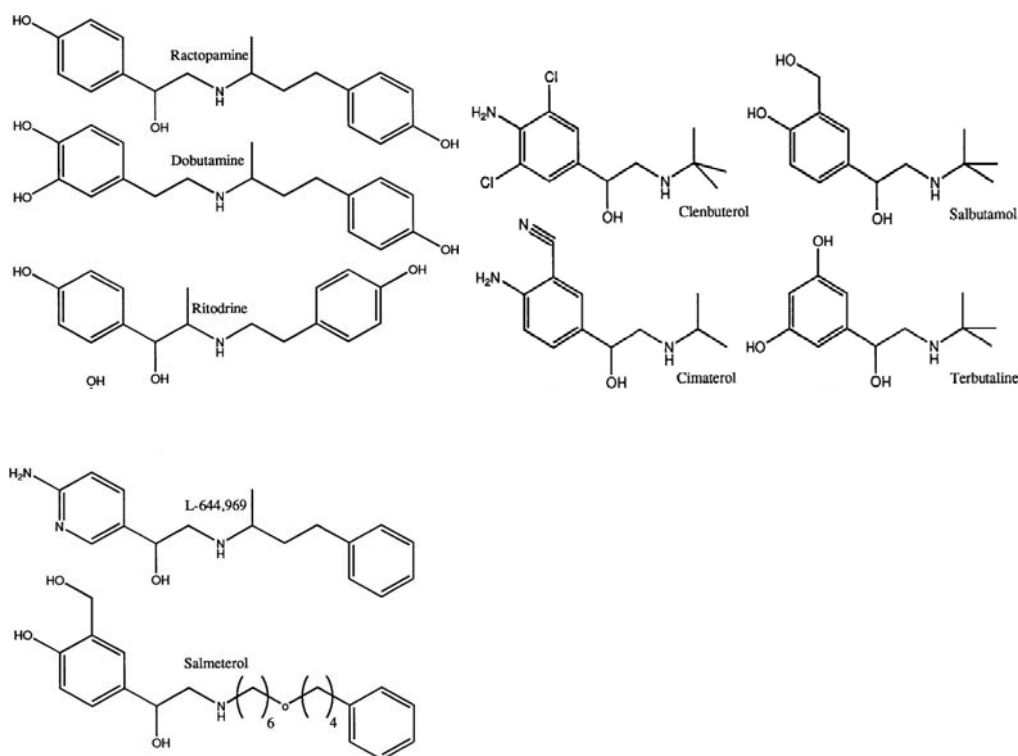


Figure 1.8 Some structures of β -adrenergic agonists (adapted from Smith, 1998).

As Smith, (1998) points out, it is often difficult to understand, or even generalize, the mechanism of action of different β AA compounds. Although β AA have similar chemical structures, their physiological function can differ. Figure 1.8 demonstrates different β AA structures. β ARs activate the G_s protein, which, in turn, modulates cAMP activity by catalysing ATP and subsequent phosphorylation of protein kinase A (PKA). The PKA phosphorylation, which involves the activation or inactivation of many enzymes, triggers transcription factors that bind to DNA and regulate transcription of DNA to mRNA (Mersmann 1998). Table 1.9 is a hypothetical schematic representation showing the signalling and activation of gene transcription mediated by binding of a β AA on its target β AR on the cell membrane.

As the number and size of skeletal muscle cells is determined during embryonic skeletal muscle development; therefore, the inclusion of β AA in the animals diets increases muscle mass by hypertrophy rather than hyperplasia. This occurs because the β AA increases the rate of muscle protein synthesis while at the same time reducing protein degradation, caused by an increase in the rate of transcription of RNA (Mersmann, 1998). The β AA also seem to have a higher affinity with type IIB or IIX muscle fibres (or glycolytic myofibres) rather than type I myofibres (Aalhus et al., 1992; Gonzalez et al., 2007; Strydom et al., 2009). The overall effect of β AA is that they give rise to more glycolytic and larger muscle fibres. However, the response to β AA is more complex than simply increasing cAMP activity to trigger transcription of target genes as there are hormonal and other physiological responses to the numerous tissue cell receptors when β AA are administered (Mersmann, 1998). Therefore, fibre type specificity is determined by the number and types of receptors intrinsic to each fibre type.

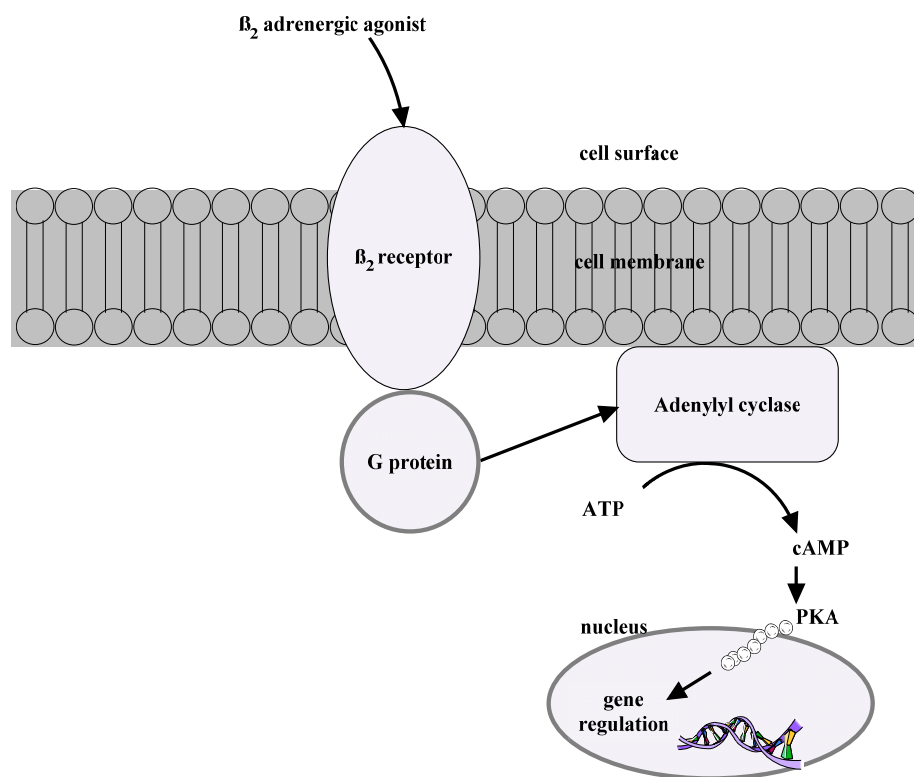


Figure 1.9 Schematic representation of the β -adrenergic agonists mechanism (adapted from Mersmann, 1998 and Anderson, 2006). The β -adrenergic agonists activates the β -adrenergic agonists (in this example it is the β_2 -adrenergic agonists activating the β_2 -adrenergic agonists receptor) where the β -adrenergic agonists receptor activates the Gs protein mediating adenylyl cyclase, where ATP increases cAMP to phosphorylate PKA and as a consequence mediates gene activation.

1.3.2 Adverse and behavioural effects

β -adrenergic agonists are commonly used for treatment of asthma, cardiac and regulation of blood pressure in humans (Mersmann, 1998). Administering β A stimulates the β AR of the bronchial-tracheal musculature which dilates the airways for treatment of asthma. β A compounds are also known to change cardiovascular function by moderating the heart rate. In addition to treating clinical symptoms, the effect of inclusion of β A in the diet of

meat producing animals is to partition nutrients for enhancement of muscle accretion and reduction in fatness.

Inclusion of the β AA clenbuterol in cattle diets has been found to increase the heart rate by 92 to 117% (Sillence et al., 1993). As a result, such increases may potentially have an adverse effect on cardiac function when administered to livestock. In pigs, the inclusion of β AA RAC in their diets was found to make them more alert during the initial weeks of treatments; however, after a few weeks of treatment the pigs were more reticent to move and had increased heart rates if exposed to unfamiliar humans or transport (Marchant-Forde et al., 2003). In the same study the pigs were also found to have a greater concentration of catecholamines than the controls, which is thought to be associated with the down regulation of β AR caused by the continual administration of the RAC.

1.3.3 Ractopamine and animal growth

Ractopamine is a common feed additive used in pig and cattle diets. In pigs, the inclusion of RAC increases the amount and percentage of carcass lean and reduces fat content (Gu et al., 1991). The inclusion of RAC promotes protein deposition; however, it is important that available protein (amino acid supply) is not limited in the diet. Dunshea et al., (1993) reported that dietary RAC is a potent stimulator of protein deposition, yet reduction in carcass fatness is variable. The greater growth and carcass size are facilitated by greater amounts of dietary crude protein which is independent of energy intake (Dunshea et al., 1998).

In cattle, including RAC in beef cattle finisher rations alters metabolism by increasing leanness and muscle accretion whilst decreasing fat accretion (Mersmann, 1998). Yet it was also reported by Mersmann, (1998) that responses to oral administration of β AA in cattle were variable, with increases of muscle mass by up to 10% and reduction in fatness as high as

30%. Other studies have reported that RAC in beef cattle diets increases final liveweight, growth rates, efficiency of gain, carcass weights and dressing percentage when included in cattle finisher rations (Schroeder et al., 2004; Avendaño-Reyes et al., 2006; Walker et al., 2006). Strydom et al., (2009) reported that the inclusion of RAC improved cattle growth rates but had no effect on final liveweight and Quinn et al., (2008) reported that final liveweight, carcass weight and average daily gain were not affected. Yet, other β AA such as clenbuterol and zilpaterol have been reported to increase carcass weights.

The oral administration of RAC was found to increase transcription of the myosin gene in the *longissimus dorsi* muscle which increased myofibrillar protein synthesis (Smith et al., 1989). The growth response to RAC is believed to be dependent on the dosage rate. Schroeder et al., (2004) reported that as the dosage rate increased final liveweight, growth rate (average daily gain) and hot carcass weights increased. Feed efficiency (feed intake to growth) also improved but this was not dependent on the dosage rate. Inclusion of RAC did affect meat eating quality where there were increases in meat colour and the higher dosage rate increased shearforce and reduced tenderness (tougher); however, sensory panels did not detect any difference.

Dunshea et al., (2005) reported on a meta-analysis across a range of species (sheep, cattle and swine) that cimaterol had the greatest effect on reducing intramuscular fat (an indicator of eating quality) and increased shearforce. RAC and salbutamol did not have an effect on these factors. Apart from Schroeder et al., (2004), there are few studies on the effects of meat quality from cattle treated with RAC. Avendaño-Reyes et al., (2006) and Strydom et al., (2009) both reported increases in shearforce values when RAC was included in cattle finisher diets. Quinn et al., (2008) reported RAC had no effect on shearforce; although, the dosage rate in this experiment was lower. Other β AA, such as cimaterol and clenbuterol, have been reported to increase shearforce (Vestergaard et al., 1994; Luño et al.,

1999). The increase in shearforce (or reduction in tenderness) is thought to be associated with changes to the calpain protease system where β AA are associated with increased levels of calpastatin, an inhibitor of muscle protein degradation (Koohmaraie, 1994).

In lambs, the effect of β AA has been reported to be similar to that in cattle. Mersmann, (1998) reported increases in muscle mass as great as 25% and a similar decrease in fatness when β AA were included in lamb diets. Earlier studies have reported that including cimaterol in lambs' diets improved ADG and gain to feed ratio (Beermann et al., 1986; Kim et al., 1987) and increased the weights of *m. biceps femoris*, *m. semimembranosus* and *m. semitendinosus* (Beermann et al., 1987). Other β AA such as; L-665, 969 (Pringle et al., 1993), clenbuterol (Baker et al., 1984; McDonagh et al., 1999) have all reported increases in liveweight or carcass weights. Nourozi et al., (2008) reported on use of terbutaline and metaproterenol in Moghani fat-tailed meat sheep change body composition. They reported evidence of nutrient partitioning, improved feed efficiency, increased carcass weight and reduced tail fatness, but β AA had neither an effect on subcutaneous nor organ fat. There is only one recent study on the effect of RAC in lambs' diets: Masri et al., (2011) found that the final weight, ADG, carcass weight, dressing percentage and muscle area improved with greater levels of β AA (including RAC) in the diet. The differences in response between species, and between different β AA, could be dependent on a wide range of factors such as: age, size, sex, duration of treatment and the potency of the β AA compound.

1.3.4 Implications

Overall, the effect of including β AA in lambs' diets is expected to improve growth and carcass traits. The mechanism that RAC uses to increase muscle mass is through a completely different mechanism to myostatin. Like other β AA, RAC increases muscle mass

through cAMP and myostatin regulates muscle mass through activin type II receptors which regulates myoblast proliferation. Ractopamine is of most interest in this study because it is a compound which is safe to use and registered for use in many countries.

1.4 Aims of this thesis

It is clear from this review of the literature and the preliminary study that the biological effect of variations in the *myostatin* gene and RAC operate through different mechanisms. It is anticipated that if the actions of variation in the *myostatin* locus and dietary RAC are additive they could enhance lamb (meat) production systems through increased muscle mass. There is little knowledge on the differences in growth and muscle fibre characteristics of lambs with the *myostatin* g+6723G>A mutation, and it is not known if the effect of RAC is additive with the *myostatin* mutation in lambs. Therefore, the aim of this thesis was to study aspects of growth, body composition, muscle fibre characteristics and meat quality in sheep heterozygote or homozygote for the *myostatin* g+6723G>A mutation and the interaction with the β -adrenergic agonist, RAC, when included in the diets. In this thesis the approach was to study:

- The interaction between lambs heterozygote for the *myostatin* g+6723G>A mutation and the inclusion of RAC on growth, body composition and differences in muscle fibre characteristics
- The effect of these factors on meat quality (shearforce, intramuscular fat, drip loss, desmin degradation and sarcomere length) traits between heterozygote *myostatin* g+6723G>A mutation lambs and RAC

- The differences in digestibility and efficiency of nutrient and energy utilisation between heterozygote *myostatin* g+6723G>A mutation lambs and RAC
- The differences in *myostatin* protein concentration between homozygote, heterozygote and homozygote wild type lambs for the *myostatin* g+6723G>A mutation and how this may affect postnatal growth and weights
- To exploit differences in nutrient supply to expand potential differences between *myostatin* genotypes and RAC treatment

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Chapter 2 *Myostatin* allelic status interacts with level of nutrition to affect growth, body composition and myofibre characteristics of lambs¹

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Chapter 3 The influence of ractopamine, nutrition and the *myostatin* genotype on objective meat quality traits in lambs¹

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Chapter 4 Effects of nutrition, ractopamine and *myostatin* allelic status on digestibility of feed and protein, fat and energy retention in lambs¹

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Chapter 5 Association between *myostatin* g+6723G>A allelic status and myostatin protein in lambs

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Chapter 6 General Discussion

The objective of this thesis was to further understand methods of improving growth and body composition in lambs to ultimately increase carcass yield without compromising other attributes such as meat quality or feed efficiency. This was done by studying the effect of the naturally occurring *myostatin* g+6723G>A mutation and, in the first experiment, the inclusion of ractopamine (RAC), which is known to increase carcass lean (muscle and connective tissue) and reduce fatness. The major findings and important implications of this thesis are discussed below.

6.1. Growth and body composition

Lambs heterozygous (*MSTN* A/G) for the *myostatin* g+6723G>A mutation (one A allele) have improved growth and increased lean gain, which should contribute to increased lamb meat yield. Preliminary research identified that lambs with at least one A allele had greater carcass lean mass (Chapter 1, Table 1.1 and 1.2). Those lambs homozygous (*MSTN* A/A; two A alleles) for the mutation were found to have more than a 17% increase in lean mass whilst heterozygote's had a 9% increase, with a similar percentage decrease in fatness for both genotypes. These results support the findings of Hadjipavlou et al., (2008), Johnson et al., (2009) and Kijas et al., (2007) who all reported that increases in carcass yield were found to be dependent on the animal's *myostatin* g+6723G>A mutant allelic status, such that each A allele additively increased muscle mass. Therefore, it was anticipated in the first experiment (Chapter 2) that lambs heterozygous for the *myostatin* g+6723G>A mutation (*MSTN* A/G) would have a greater muscle mass than homozygote wild types (*MSTN* G/G).

The results of the first experiment (Chapters 2-4) showed a significant difference in lean gain in the estimated carcass of *MSTN* A/G lambs compared to *MSTN* G/G lambs on high nutrition, and not surprisingly, no difference when fed to maintain weight. Again, this is consistent with the observations of increased muscle mass in lambs, reported by Clop et al., (2006), Kijas et al., (2007), Hadjipavlou et al., (2008), Johnson et al., (2009) and results of our preliminary study (Chapter 1, Table 1.1). A subsequent experiment (Chapter 5) also reported that there is a difference between *myostatin* genotypes as discussed below.

In the second experiment (Chapter 5) lambs homozygous (*MSTN* A/A) for the *myostatin* g+6723G>A mutation had the greatest muscle mass at the beginning and the completion of the feeding experiment and lower fat content (as determined by computer tomography). There was no difference in *MSTN* A/G lambs compared to the *MSTN* G/G lambs at the beginning of the feeding experiment; however, at the completion of the final experiment the *MSTN* A/G lambs had a greater lean mass than *MSTN* G/G lambs, but less than the *MSTN* A/A lambs. The *MSTN* A/A lambs had 5% greater lean mass than *MSTN* G/G lambs, and at slaughter, the *MSTN* A/A lambs had the greatest dressing percentage and greatest primal cut weights.

Together with other reported data (Clop et al., 2006, Hadjipavlou et al., 2008 and Johnson et al., 2009) the results confirm the effect of the *myostatin* g+6723G>A mutation on the muscle phenotype in sheep. Our results and these authors suggest that the muscle phenotype is dose dependent (i.e. two A alleles have a greater effect on muscle growth than one A allele). The final experiment (Chapter 5) also demonstrated that the greater number of myofibres for the *MSTN* A/A lambs (Table 5.8) were most likely established during foetal development (see evidence in Chapter 1). The *myostatin* g+6723G>A mutation, in particular the *MSTN* A/A homozygote lambs, was associated with an increase in the number of muscle fibres, it suggests that the basis for subsequent increased muscle mass was established *in*

utero as the reduction of the myostatin protein during embryogenesis enables greater myofibre proliferation during the early stages of fetal development with greater formation occurring in the anterior region in the developing embryo with less effect in the posterior region. Differences in muscle mass are subsequently expressed (and quantified as differences in weight of muscle) during later stages of development (see Table 1.2), the data presented in Chapter 5 clearly shows that measureable differences in body composition were not present after weaning but were significantly different some 3 months later just prior to slaughter.

6.2 Measurement and role of active myostatin protein

Increases in lean accretion in lambs with the *myostatin* g+6723G>A mutation is thought to be attributed to reduced myostatin protein activity, which increases the rate of myoblast proliferation during myogenesis and establishes a longer latency phase of myoblast fusion (Thomas et al., 2001, Wegner et al., 2000; Chapter 1). It was reported by Clop et al., (2006) that *MSTN* A/A lambs had approximately 66% less myostatin protein than *MSTN* G/G lambs in plasma. Presently there are no reported studies on circulating myostatin protein levels in *MSTN* A/G lambs. The final experiment (Chapter 5) was designed to confirm the response in lean accretion was dose dependent on the A allele and to determine if there was a similar dose dependency in myostatin protein levels, in muscle and plasma. It was to be expected that there would be a difference in myostatin protein concentration and activity as there is an allele dose effect on muscle growth (see Table 1.1).

To evaluate this hypothesis, the final experiment (Chapter 5) was designed to determine the level of myostatin activity in lambs with the three differing *myostatin* allelic status (*MSTN* A/A, *MSTN* A/G and *MSTN* G/G) and four different age time points (1, 4, 16 and 24 weeks of age). The results of this experiment demonstrated that the level of myostatin

protein in both muscle and plasma increased as the lambs' aged, although myostatin in the muscle was less in the *MSTN* A/A at 1 week of age. In particular the muscle myostatin protein concentration was greater in the *MSTN* A/A lambs at 4 and 24 weeks of age compared to the other genotypes. There was no difference in plasma myostatin protein between the three different genotypes. The outcome of this experiment was contrary to the original hypotheses of Chapter 5 and lead to a revision of our understanding of the differences between the protein detection and identification methodology used by Clop et al., (2006) and this experiment (Chapter 5). Lee et al., (2010) recently reported that mice with a *myostatin* deletion which display the double-muscle phenotype have greater levels of circulating myostatin protein than wild type mice. They suggest that regulation of activity and concentration of myostatin protein requires other proteases, and that the relationship between concentration of assayable myostatin protein in muscle and plasma, was not indicative of activity (at least post-natally).

In the second experiment (Chapter 5) the methodology used to detect myostatin differed to the method reported by Clop et al., (2006). Clop et al., (2006) measured the 12.5 kDa monomer of a 303-304 amino acid domain at the C-terminal end of the protein, although it is thought the dimer is the active moiety (McPherron et al., 2010). In the final experiment, attempts were made to identify the active protein at the 12.5 kDa band both as native and by dissociating the dimer, but it was not observed. The active protein (as determined by antibody binding) was consistently identified at the 26 kDa position. Other studies have also reported the myostatin protein at the 26 kDa band (McFarlane et al., 2005, Oldham et al., 2009) or within this vicinity (Reisz-Porszasz et al., 2003).

McFarlane et al., (2005) reported that western blot analysis of myostatin deletion mice had reduced levels of myostatin protein at the 26 kDa band and that wild type mice had a greater level of myostatin protein. To confirm reduction in myostatin protein McFarlane et

al., (2005) used two different myostatin antibodies. The first antibody was the same used in the final experiment (Chapter 5) which was raised 'in-house' as a rabbit anti-myostatin antibody, the other antibody was commercially manufactured by Santa Cruz Biotechnology as a goat anti-myostatin antibody (SC-6884, Santa Cruz Biotechnology, California, USA). Presently, all of the Santa Cruz anti-myostatin antibodies report western blot analysis of myostatin (or mature GDF8) in the vicinity of the 26 kDa band. Consequently, the identification of the myostatin protein at the 26 kDa band is considered to be the identification of the active moiety of the myostatin protein. We repeated these procedures with the naturally occurring *myostatin* g+6723G>A mutation studies reported here, but were unable to detect consistent differences in myostatin protein between genotypes.

The above results could be due to variation in the normal time course of myostatin protein during an animal's development or inability to measure the active moiety of myostatin with the assay conditions. Variation in the role of myostatin during development is possible, for example McPherron, (2010) describes a change in roles from initial effects on myogenesis to a systemic regulator of muscle metabolism. To eliminate the possibility that our results are due to the assay has been initiated by the following work:

- a) Peptide sequencing of the 12.5 kDa region and 26 kDa band by mass spectrometry. The use of mass spectrometry would independently validate the identity of the myostatin protein.
- b) Confirmation of gene expression to identify if there is any difference in mRNA myostatin expression. However, Clop et al., (2006) reported there was no difference in mRNA.
- c) Continue to look for any other single nucleotide polymorphisms that maybe associated with the *myostatin* gene and phenotypic effects.

This work is underway, but unable to be completed in time to report in this thesis.

6.3 Ractopamine

It was anticipated that including RAC in the lambs diets would have two effects. Firstly, it would improve muscle growth by increasing muscle size and change muscle fibre types to more glycolytic myofibres (type II) which is consistent with the known effects of β -adrenergic agonist on muscle. Secondly, (as a consequence of effects on muscle growth) it would lead to increased growth and decreased fatness. The data showed RAC in the lambs' diet improved final liveweight, average daily gain (ADG), increased the proportion of type IIA myofibres whilst decreasing the type IIC myofibres. Ractopamine increased the size of the type I myofibres, but had no effect on hot carcass weight (Chapter 2).

Closer observation, as described in Chapter 4, found that the cause for greater final liveweight and ADG when RAC was administered was reduced digestibility of the diet, and greater retention of feed in the digestive tract. It was also confirmed by computer tomography analysis that RAC significantly increased the gastrointestinal tract weight. However, it is not entirely clear if feed was retained in the rumen or the intestinal tract as the image analysis did not break down the gastrointestinal tract into these parts. The outcome of this study indicates that RAC potentially affects rumen fermentation or it may indicate that gastrointestinal tract β -adrenergic agonist receptor cells are sensitive to RAC stimuli resulting in reduced gut motility.

In pigs, RAC works by partitioning nutrients away from fat and into lean accretion, but understanding the effect of RAC in ruminant diets is complex. In cattle, the response is variable; the inclusion of RAC generally increases HCW but not to the same extent as in pigs. A possible reason for this variable response is that β -adrenergic agonists are phenethanolamines which have a similar structure to the hormones dopamine, epinephrine

and norepinephrine (Beermann, 2002) which are known to increase gram negative bacteria (Walker et al., 2010). *In vitro* studies have also reported that rumen fluid, with RAC added, increased the total amount of gas produced (the authors did not determine the composition of gas(es) produced) (Walker et al., 2010). As previously discussed, the effect of RAC in cattle diets is variable and it is clear that some of these studies have had the same outcome as the sheep experiment in this thesis (increased liveweight without changes in carcass weight). For example: Quinn et al., (2008) reported that including RAC increased final liveweight and ADG without any effect on hot carcass weight; yet Gruber et al., (2007) reported significant differences between all traits (but the data did not reflect the level of significance reported).

The results from these studies indicated that the effects on cattle were potentially the same as those here, but nutrient digestibility was not determined in the cattle experiments. If the results from the sheep studies in this thesis apply generally to ruminants treated with RAC, it is possible that they can potentially have poorer feed digestibility and greater gut fill which in turn leads to measurements showing greater final liveweight and average daily gain but with no effect on HCW or dress percentage. The results from this experiment and other studies demonstrate that the effect of RAC in ruminal microflora activity is poorly understood. Work is currently planned for an *in vitro* study to investigate the effect of RAC on ruminal microflora and gas production (such as methane and proportion of volatile fatty acids) but was not started in time to be reported in this thesis.

Although the inclusion of β -adrenergic agonists in animal diets may be able to meet consumer request for leaner cuts of meat, it is also in direct contrast to consumers' perception about the inclusion of drugs in food products. The use of the naturally occurring *myostatin* mutation, such as the g+6723G>A mutation in sheep, and those generating muscling in cattle, is less likely to be rejected on consumer perception grounds. Natural changes in body

composition to a phenotype with greater muscle mass, and reduced fatness, appeal to the consumer more than the use of growth enhancing drugs in animal diets.

6.4 Interactions between *myostatin*, ractopamine and available nutrition

Results presented here show that the heterozygote (*MSTN* A/G) mutation, RAC and a high plane of nutrition act together to increase growth of muscle. The experiment also demonstrated that poor nutritional background of lambs heterozygous for the *myostatin* mutation may negatively influence their growth rates and myofibre characteristics. The *MSTN* A/G lambs offered greater nutrition, treated with RAC had greater daily growth rates and protein accretion than those non-treated lambs of the same genotype (*MSTN* A/G) or wild type non-carriers (*MSTN* G/G). Restricted nutrition was used as a contrast to determine if the effects of *MSTN* A/G, and RAC were dependent upon nutrient supply. In general this was found to be true, although RAC inclusion affected muscle fibre characteristics in lambs on restricted nutrition it was unable to stimulate growth in the absence of a sufficient nutrient supply.

As discussed in the introduction of this thesis (Chapter 1), myostatin regulates embryonic and postnatal myogenesis by controlling myoblast proliferation and differentiation by signalling through the activin type 2 cell receptors resulting in activation of SMAD transcription factors which then regulate downstream genes (Lee and McPherron, 2001; McFarlane et al., 2011). In contrast, RAC increases muscle mass by binding to β -adrenergic agonist receptors which activate adenylate cyclase and ultimately increase cAMP which triggers target gene transcription factors (Merriman, 1998). The outcome of the first experiment was successful as the combination of the *myostatin* mutation and RAC together increased lean accretion in the carcass, perhaps as a result of the two different mechanisms.

Earlier in the thesis it was postulated that a greater rate of lean accretion, and therefore greater muscle mass, may have been achieved by increasing the dosage rate of RAC. Other studies which have included RAC in lambs diets demonstrated that the response to β -adrenergic agonists are dose dependent (López-Carlos et al., 2010) and the dosage rate used in the first experiment was low (0.4 mg/kg LW). Ractopamine is now registered for use in numerous countries in finisher cattle feedlot diets, yet, the literature on β -adrenergic agonist in cattle diets is conflicting. Two studies clearly demonstrate increases in final liveweight, average daily gain and hot carcass weights (Strydom et al., 2008; Avendaño-Reyes et al., 2006). Ractopamine has been also found to have effects on digestion of feed, as discussed later.

The overlay of nutrition in this experiment demonstrated that *MSTN* A/G lambs with a high nutrient supply improved growth rates compared to *MSTN* G/G lambs. However, nutritional restriction adversely affected the *MSTN* A/G lambs more than the *MSTN* G/G lambs. Hence, sheep heterozygous for the *myostatin* mutation need good nutrition to maximise genetic benefits of increased muscle. The maintenance of greater growth rates in these animals is associated with protein synthesis to protein accretion. Protein accretion is achieved when muscle protein synthesis exceeds the amount of muscle protein degraded (Oddy and Sainz, 2002), both processes are influenced by nutrition and endocrine factors (Reeds, 1986). Increased muscle size can be achieved by using animals with a greater number of muscle fibres (such as the double-muscling cattle, or here *MSTN* A/A lambs) which ultimately contribute to greater muscle mass (Koohmarie et al., 2002). The greater number of muscle fibres have a greater amount of DNA in muscle which directly influences the postnatal regulation of muscle growth determining the rate of amino acid synthesis and degradation. A higher nutrient supply is therefore required for those animals to achieve and possibly maintain more muscle fibres. More fibres can be considered to generate a greater

potential for muscle deposition, which facilitates an increase in muscle size. An example of this can be seen from the data in Table 5.6. *MSTN* A/A lambs had 24% more muscle mass in the loin than *MSTN* G/G. This compares with 48% more muscle fibres (Table 5.8) which suggest that nutrient supply may have limited the expression of the difference between *MSTN* A/A and *MSTN* G/G in this study. This may contribute to differences between studies.

Myofibre characteristics and computer tomography image analysis were used in the first experiment to determine differences between the lambs with differing *myostatin* allelic status, RAC treatment and level of nutrition. There was a strong relationship between feed intake, changes in average daily growth rates and difference in myofibre characteristics (Chapter 2). Lambs offered feed *ab libitum* had greater muscle lean accretion, lower percentage of type IIX myofibres and a greater percentage of type IIA myofibres (less glycolytic cells with more mitochondria). Those lambs on restricted feed intake performed the poorest for growth rate and had a greater percentage of type IIX myofibres. This demonstrates that there are metabolic differences for those animals that are carriers for the *myostatin* g+6723G>A mutation and changes in nutrient supply affect metabolic activity. Therefore, from the perspective of the meat sheep industry, sheep with the *myostatin* mutation may perform comparatively poorly (by contrast to those without the A allele at the g+6723G>A) when limited feed is available, for example during droughts. Nonetheless the higher number of muscle fibres in lambs with the A allele would be expected to respond to improved nutrition at a greater rate than in those without.

Myofibre characteristics were considered in both experiments for two reasons. Firstly, it has been shown that myofibre characteristics change in response to selection using muscle EBVs in lamb (Greenwood et al., 2006b, Gardner et al., 2006 and Gardner et al., 2007). Secondly, given the mechanism of myostatin on muscle fibre development, it was expected that there could be an effect on muscle fibre characteristics (as well on number and size of the

myofibres – see Bünger et al., 2009, Arthur, 1995). The recent studies on sheep with the *myostatin* g+6723G>A mutation have reported only on the difference in lean carcass yield, reduced fatness and meat quality (Clop et al., 2006, Kijas et al., 2007, Hadjipavlou et al., 2008, Johnson et al., 2009). These studies did not report on any effect on myofibre characteristics, the results reported below illustrate that the number of myofibres directly contributes to increases in muscle mass and ultimately carcass yield.

Earlier reports on double-muscled cattle breeds have demonstrated an increase in the number of muscle fibres (hyperplasia) with a greater proportion of glycolytic (fast type) myofibres (Arthur, 1995). In the first experiment (Chapter 2) there were interactions (heterozygote lambs and level of available nutrition) between the three different treatments. In the final experiment (Chapter 5) the *MSTN* A/A lambs had type I myofibres with smaller cross-sectional areas and as a consequence, a greater number of myofibres, supporting the postulate that myostatin regulates myoblast proliferation and its inhibition results in hyperplasia (a greater number of muscle fibres). This result is supported by recent work that reports relationship between eye muscle area, fibre number and cross sectional area in lambs (White et al., 2012).

6.5 Meat quality

A remarkable finding from the first experiment was that RAC reduced shearforce (Chapter 3). It appeared that RAC had a protective effect when carcasses were exposed to cold shortening conditions, which is contrary to the current knowledge of β -adrenergic agonist which are “known” to increase meat toughness. The slaughter and dressing procedure of the carcasses were less than ideal (the lamb carcasses were not electrically stimulated on the kill chain and the chilling rate was faster than intended because the chiller was

understocked – it contained only the carcasses from this experiment), it did demonstrate an unexpected difference. The protective effect that RAC had on the carcasses was confirmed by sarcomere length, lambs not treated with RAC in this experiment had shorter sarcomeres than those treated with RAC (Table 3.5).

It is widely reported that β -adrenergic agonists have a negative effect on meat quality because they reduce post-mortem proteolysis (Dunshea et al., 2005, Koohmaraie et al., 1996). In this study there was no effect on post-mortem proteolysis (Table 3.5). We proposed that the reason RAC protected the *ab libitum* intake lamb carcasses from cold shortening was that those lambs treated with RAC had used a greater proportion of glycolytic store (through the action of β -adrenergic agonists on cAMP; Mersmann, 1998) which would be expected to accelerate pH decline, so that the RAC treated lambs passed through the optimum pH temperature window whereas the other lambs did not. As RAC stimulates cAMP it almost certainly reduced the amount of ATP present at rigor, thereby allowing the actin myosin complex to have reduced overlap and longer sarcomeres at rigor. As the rate of temperature decline is correlated between the extent of ATP and the onset of rigor, higher carcass temperatures enter rigor at higher ATP levels than at lower temperatures (Hertzman et al., 1993). Cold shortening conditions occur when the muscle pH is greater than 6.0 as ATP at rigor is still available for muscle contraction and the temperature is less than 10°C (Pearson and Young, 1963). Figure 6.1 shows the dependence of the rate of pH decline on temperature. Unfortunately, the rate of pH decline and the glycogen present in muscle was not measured during the chilling of the carcasses; as a result, this hypothesis cannot be confirmed.

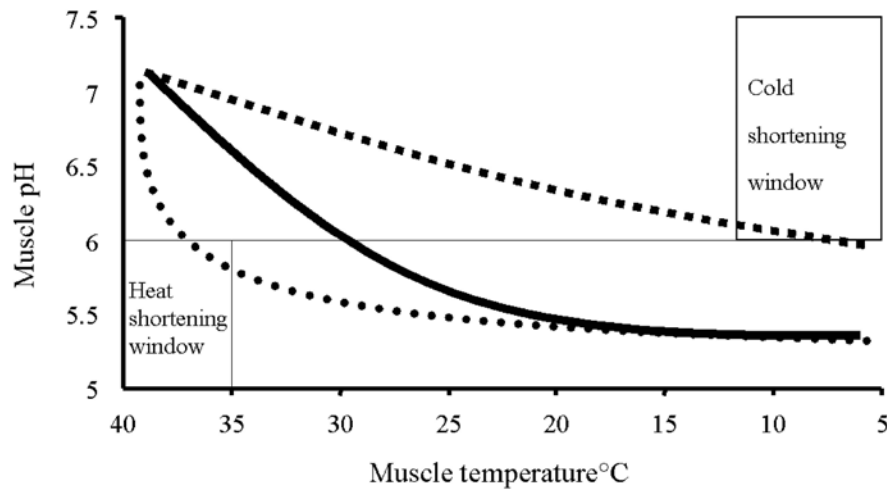


Figure 6.1 Optimal pH/temperature decline window. Solid line is the optimal rate of pH/temperature decline, dashed line cold shortening and dotted line heat shortening (Thompson, 2002).

It was difficult to interpret any effect of the genotype and nutrition on meat quality in the first experiment (Chapter 3) as discussed above. The meat quality measurements on the lambs from the final experiment (Chapter 5) were evaluated elsewhere (Hope, 2011). That work found there were no negative effects of objective and subjective meat quality assessment due to genotype. Taste panel assessment found that the topside from homozygote *myostatin* g+6723G>A mutant (*MSTN* A/A) lambs were more tender than the homozygote wild type (*MSTN* G/G) lambs which is an outcome of these animals having less connective tissue (Hope, 2011). However, there was no difference in intramuscular fat between *MSTN* A/A and *MSTN* G/G lambs. Kijas et al. (2007) reported an association with the *MSTN* A/G mutation and decreased eating quality which was attributed in that report to a decrease in intramuscular fat.

Intramuscular fat (IMF %) is used as one of the key meat quality indicators, because lower levels of IMF % are associated with poor eating quality. There was no difference in IMF % between *MSTN* A/G and *MSTN* G/G lambs in the first experiment (Chapter 2), and as discussed above, in the final experiment (Hope, 2011) associated with this experiment

(Chapter 5). The *MSTN* A/A lambs had a tendency to have lower IMF % in the loin and topside compared to *MSTN* G/G, and no other negative effects on meat quality attributes were seen between the lambs with either genotypes. In the experiment reported by Kijas et al., (2007) poor eating quality was attributable to the lower concentration of IMF % in those lambs with the *myostatin* g+6723G>A mutation. Masri et al., (2010) also concluded that those lambs with the *myostatin* g+6723G>A mutation would have reduced IMF % and therefore poorer eating quality but Masri et al., (2010) never actually measured IMF %. In this thesis, the data suggest that IMF % may not differ between genotype; however, measurement of IMF % on more animals, together with measurement of eating quality for each genotype would give more meaningful information on the relationship between IMF % and myofibre characteristics on objective and subjective assessment of meat eating quality.

6.6 Energetics

It was anticipated that by altering the relative deposition of protein and fat through different myostatin allelic status and RAC that there may also be a change in the efficiency of energy deposition. For example, studies on double-muscled cattle have shown greater muscle mass with a relatively smaller digestive tract which may well be associated with improved efficiency of energy utilisation (Arthur et al., 1995; Fiems et al., 2002). The experiment determined (Chapter 4) that those lambs heterozygous for the *myostatin* g+6723G>A mutation offered *ab libitum* feed intake treated with RAC had greater protein accretion which was an outcome of greater daily growth rates compared to the restricted intake lambs which had significantly lower protein accretion.

The partitioning of energy between fat and protein in growing animals is largely determined by the relationship between energy intake and protein accretion which is critical

to the animal response to changes in body composition and feed efficiency. The inherent capacity of genotype for protein accretion, where faster and more efficient growth is known to influence improvements in growth and nutrient efficiency, allows opportunity for improvement in the animals ability to efficiently utilise nutrients (Campbell et al., 1990). For example: the inclusion of β -adrenergic agonists, and for somatotropin in pigs, cattle and lambs increased the efficiency of amino acid utilisation by 25-50% as a result improved the optimal capacity for protein to improve protein efficiency as a result increasing protein deposition (Etherton and Bauman, 1998).

As has been outlined earlier, it was anticipated that including RAC would have an additive effect on muscle lean accretion with the *MSTN* A/G lambs. The results here demonstrate that lean accretion could improve with a greater RAC dosage rate, yet this could have also be complicated by the effect RAC appears to have on gut motility and possibly on rumen microflora. In this experiment the low dosage rates 0.4 mg RAC/kg liveweight were derived from an earlier study on cattle, where this particular dosage (0.4 mg/kg liveweight) was found to increase final liveweight, ADG and HCW in cattle (Walker et al., 2006). A later study in sheep demonstrated that greater dose rates (0.35, 0.70 and 1.05 mg/kg liveweight) of RAC improved the final liveweight, ADG and HCW (López-Carlos et al., 2010). The results in Chapter 2 and Chapter 4 established that the low dosage rate (0.4 mg/kg liveweight) of RAC did not have any effect on lean accretion or on reducing carcass fat. As a consequence, there was no improvement on energy efficiency for those lambs treated with RAC, if anything it got worse. It is difficult to determine on the basis of this study if increasing the dose rate could lead to beneficial change in energetic efficiency.

It became clear in Chapter 4 that the reason for increase in the ADG and final liveweight, without affecting HCW, was due to lambs having a heavier gastrointestinal tract. These lambs also had reduced DMD indicating that a disproportionate amount of feed may

have been retained in the rumen which was the driver of increasing ADG. As the gastrointestinal tract (with contents) was heavier there could be greater energy requirement for maintenance which in turn would increase the heat production of these animals. The greater intestinal tract may have been an outcome of the β -adrenergic agonists slowing the gastrointestinal tract motility and therefore retaining more feed and reducing DMD. However, the observed reduction in DMD is contradictory as reduced digestibility is usually associated with a greater rumen flow rate.

6.7 Conclusion

Lambs homozygous (*MSTN* A/A) for the *myostatin* g+6723G>A mutation have greater muscle mass, than the single copy heterozygote (*MSTN* A/G) lambs which display greater muscle phenotype than wild type lambs. This thesis demonstrated that the *myostatin* g+6723G>A mutation contributes to an increase in the number of muscle fibres (in particular the *MSTN* A/A lambs) which suggests that the greater muscle mass was established *in utero*, but is expressed during later stages of post-natal development. Therefore, identifying lambs that are carriers for the *myostatin* g+6723G>A mutation would have substantial benefit as a practical method to increase muscle mass in meat sheep breeding programs.

Postnatally there does not appear to be any association between the phenotype and the level of myostatin protein. Myostatin protein concentration increased as the lambs aged but was greater in the homozygous lambs at 4 and 24 weeks of age than any other genotype. It seems that regulation of the myostatin protein in the young animal and adult animals may be acting as a rheostat on muscle growth but also has other effects on muscle metabolism.

Together myostatin and ractopamine on the high level of feed intake additively increased lean protein accretion. The *ab libitum* feed intake heterozygous lambs were also

found to have a lower proportion of type IIX myofibres. The experiment also demonstrated that poor nutritional background of lambs heterozygous for the *myostatin* g+6723G>A mutation may negatively influence their growth rates and myofibre characteristics. For the industry to maximise the opportunity of more fibres available from the myostatin mutation and the additive effect of ractopamine on muscle fibre hypertrophy it's important to ensure adequate nutrients are supplied.

The industry application of selecting animals with the *myostatin* g+6723G>A mutation and manipulating their diet with nutrient partitioning agents, such as ractopamine, demonstrates that there are alternative methods to improving lambs growth (outside the use of EBVs). The combination of the *myostatin* g+6723G>A mutation and ractopamine in lambs' diets offers another potential opportunity to optimise growth and body composition as long as there is satisfactory nutrients available. The outcome of this thesis highlights the relevance of developing a practical method to genotype animals for their *myostatin* allelic status.

6.8 Points for consideration in future research

- This thesis determined that the *myostatin* g+6723G>A mutation influences the number of muscle fibres (hyperplasia) rather than increase muscle fibre size (hypertrophy). The effect of hyperplasia is determined *in utero* where the variation in myostatin concentration is anticipated to influence myogenesis during lamb embryonic and foetal development. Further research is required to evaluate if there is any difference in the level of myostatin protein *in utero*.

- Identification of the active myostatin protein still requires further evaluation. It is unclear if the active protein is a monomer or a dimer. Improving the accuracy of myostatin protein identification and assay method allows for an opportunity to screen animals for their level of myostatin protein to ultimately predict their phenotype.
- Larger scale experimentation on the effect of the *myostatin* g+6723G>A mutation on animal production traits such as stress tolerance, fertility and dystocia (known to have adverse effects in cattle with the double-muscle phenotype) should be undertaken. It remains unclear if breeding animals for this particular mutation would have the same adverse effects that are associated with cattle which have complete knockouts of myostatin.
- The outcome of this thesis, and the results of other studies, indicate that the *myostatin* g+6723G>A mutation improves meat tenderness and eating quality; yet, still requires further evaluation. Meat quality should be evaluated on objective (shearforce), subjective (taste panels), IMF % and other meat quality attributes. It is anticipated that the number of A alleles present will reflect meat quality were it is expected that more A alleles have better meat yield and quality.
- Finally, the unexpected results of changes in nutrient utilisation of lambs treated with ractopamine (such as: reducing dry matter digestibility and increasing feed retention in the intestinal tract) indicates that this β -adrenergic agonist may influence rumen microflora activity. *In vitro* studies on microflora activity would allow for improved knowledge on the effect that this particular β -adrenergic agonist has on ruminants such as sheep.

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Chapter 8 Appendices

Appendix 1 List of peer reviewed scientific publications during candidature

Scientific Journal Manuscripts

Haynes F.E.M., P.L. Greenwood, M.B. McDonagh, and V.H. Oddy. 2012. Myostatin allelic status interacts with level of nutrition to affect growth, composition and myofibre characteristics of lambs. *J. Anim. Sci.* (In press).

Oddy V. H. and F. E. M. Haynes. 2011. Nitrous oxide is no laughing matter – Issues arising from anthropogenic reactive nitrogen use and the role of reducing nitrogen excretion from livestock. *Rec. Adv. An.* 18:173.

Conference abstracts

Milton, F. E., P. L. Greenwood, M. B. McDonagh and V. H. Oddy. 2009. Effect of a myostatin mutation, nutrition and a β -adrenergic agonist (ractopamine) on carcass and meat quality in lambs. XIth International Symposium on Ruminant Physiology. 11:612-613.

Milton F.E., P.L. Greenwood, M.B. McDonagh, and V.H. Oddy. 2009. Effect of myostatin mutant alleles, nutrition and ractopamine on growth of wether lambs. *Rec. Adv. An.* 17: 173.

Haynes, F. E. M., P. L. Greenwood, J. P. Siddell, M. B. McDonagh, and V. H. Oddy. 2010.

Computer Tomography Software Program ‘Osirix’ Aids Prediction of Sheep Body Composition. *Anim. Prod. Aust.* 28: 49.

Haynes F. E. M., P. L. Greenwood, M. B. McDonagh, and V. H. Oddy. 2010. Myostatin heterozygote sheep need good nutrition. *Anim. Prod. Aust.* 2: 3.

Appendix 2 General material and methods

Myofibre characterisation

Muscle sample collection and preparation

Samples of the *m. longissimus dorsi* and *m. semitendinosus*, collected in both experiments (Chapter 2 and Chapter 5), were prepared for immunohistochemistry based on their myosin heavy chain staining characteristics (Greenwood et al., 2006a; Greenwood et al., 2007; Greenwood et al., 2006b; Picard et al., 1998). In both experiments, thirty hours post-slaughter samples of the *m. longissimus dorsi* and *m. semitendinosus* muscle were sampled from the a standardised location, according to the method described by Greenwood et al., (2006a). The *m. longissimus dorsi* myofibre sample location was from the superficial (dorsal) region, adjacent and caudal to the 12/13th rib. The *m. semitendinosus* myofibre sample location was taken from the dorsal, proximal region. The block size was 1 cm³. The cranial end of the tissue sample was mounted onto labelled cork block using gum tragacanth (Sigma-Aldrich, Product no. 3001141483, Castle Hill, New South Wales, Australia) so that the myofibres were perpendicular to the cork block. The mounted samples were immersed in isopentane, cooled to approximately -190°C in liquid nitrogen, prior to storage at -70°C.

Myosin heavy chain immunocytochemistry

Myofibres were prepared for immunocytochemistry by cutting serial cross-sections from the mounted *m. longissimus dorsi* and *m. semitendinosus* sample. The serial, 10 µm thick cross-sections were cut at -25°C using a cryostat microtome (ThermoShandon AS 620 Cryostat SME, Thermotrace Ltd, Noble Park, Victoria, Australia), and placed in sequential order on separate slides, and allowed to dry at room temperature prior to storage at -20°C.

To determine myofibre characteristics, the samples were prepared for immunocytochemistry staining and for classification based on five myofibre types (I, IIC, IIA, IIAx and IIX), according to the myosin heavy chain staining characteristics of three antibodies; anti-type I MHC, anti-type II MHC and anti-type I, IIB and IIX MHC described below (Greenwood et al., 2009; 2006a; 2007; Picard et al., 1998). The slides were removed from -20°C storage, thawed at room temperature and then fixed with 100% acetone. After fixation, a circle was drawn around each tissue sample using a hydrophobic pen to create a well. Within the well the muscle sections were recovered with 0.01 M of phosphate-buffered saline (PBS, pH 7.2) and blocked with 10% non-immune serum solution (Zymed Laboratories, South San Francisco, CA, USA) for 10 min. The samples were incubated in a humid chamber with 50 µl of monoclonal antibody raised against type I MHC (clone WB-MHCs, Novocastra, Newcastle upon Tyne, UK; diluted 1 :100 in PBS) plus rabbit anti-laminin, affinity-isolated antibody (Sigma Chemical Co., St Louis, MO; diluted 1:500 in PBS), type II MHC (clone MY-32, Sigma Chemical Co., St Louis, *Missouri, USA*; diluted 1:1200 in PBS), and types I, IIB and IIX MHC (Clone S5 8H2, Picard et al., (1998); diluted 1:1000 in PBS) for 1 hour at 37°C. Antibodies were detected using a broad spectrum Labelled-[Strept]-Avidin-Biotin amplification system and the substrate chromagen, diaminobenzidine (Zymed Laboratories, South San Fransico, California, USA). The sections were then dehydrated and clarified with graded ethanol's (70, 85, 95 and 100%) and xylene, and mounted using a xylene-based mounting medium and glass cover slips.

The *m. longissimus dorsi* and *m. semitendinosus* myofibres were classified based on positive, negative or intermediate staining with each antibody anti-type I MHC, anti-type II MHC and anti-type I, IIB and IIX MHC, as detailed in Greenwood et al., (2006b, 2007, 2009). Myofibres were classified as types I (slow oxidative), IIC (intermediate or transitional between types I and IIA), IIA (fast oxidative-glycolytic), IIAx (intermediate or transitional

between types IIA and IIX) or IIX (fast glycolytic) as shown in Figure A1. Myofibre types previously classified as types IIAB and IIB were classified as types IIAX and IIX, respectively, due to little or no expression of type IIB MHC in skeletal muscles of ruminants as detailed in Greenwood et al., (2007, 2009). Fields of view were assessed using bright field microscopy (Carl Zeiss Pty Ltd., Göttingen, Germany) with x10 and x20 objectives. The x10 objective was used to identify the location of fields within muscle sections. The x20 objective was then used to classify each myofibre according to antibody staining profiles. Classification was performed using images captured by the software program Axiovision (version AxioVs40 4.8.0.0, Carl Zeiss Pty Ltd., Göttingen, Germany).

The percentage of each type of myofibre in the muscle field was calculated relative to the total number of myofibres assessed. The cross-sectional area of the myofibres was measured using the software program KSRun (version 3.0, Carl Zeiss Pty Ltd., Göttingen, Germany). The relative area comprising each type of myofibre and the average cross-sectional area for all myofibres were derived from the percentage and average cross-sectional area of each myofibre type.

Morphometry

In the first experiment (Chapter 2) the average s.d. between the fields for percentage of myofibre types for each animal was 2.6% for type I, 3.4% for type IIC, 6.1% for type IIA, 2.5% for type IIAX and 6.8% for type IIX. The average s.d. between fields for myofibre type cross-sectional areas were 456 μm for type I, 619 μm for type IIC, 487 μm for type IIA, 665 μm for type IIAX, 487 μm for type IIX and 441 μm for overall average cross-sectional area.

In the second experiment (Chapter 5) the average s.d. between the fields for percentage of myofibre types for each animal was 3.1% for type I, 2.2% for type IIC, 4.8% for type IIA, 2.2% for type IIAX and 6.1% for type IIX. The average s.d. between fields for

myofibre type cross-sectional areas were 367 μm for type I, 563 μm for type IIC, 379 μm for type IIA, 477 μm for type IIX, 350 μm for type IIX and 238 μm for overall average cross-sectional area.

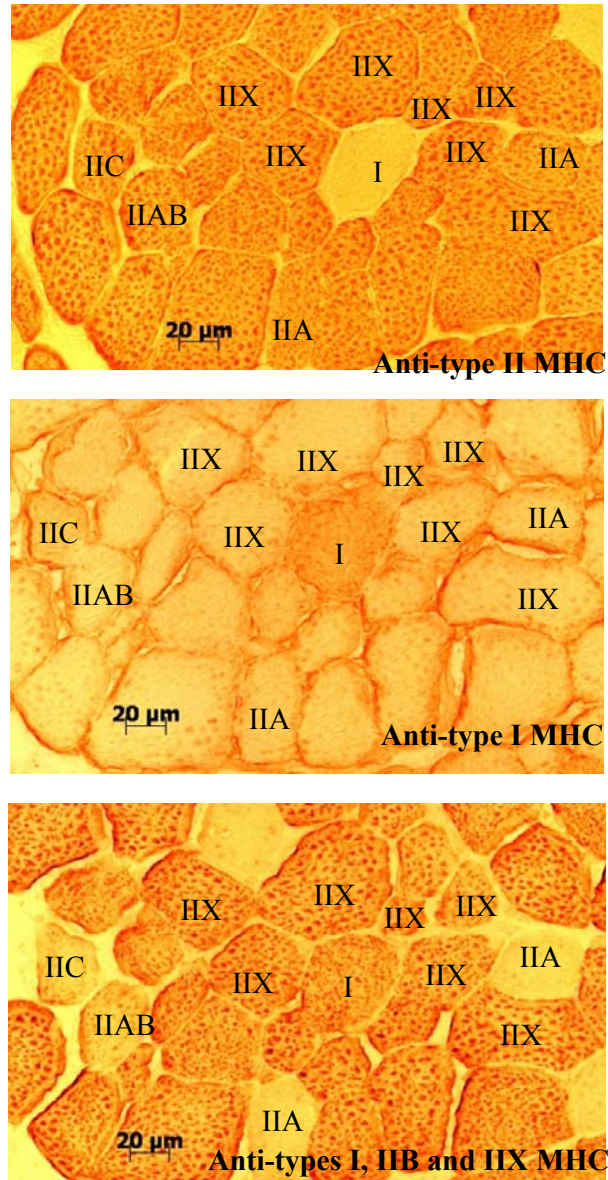


Figure A1 Immunocytochemical staining of myosin heavy chains in sheep *m. longissimus dorsi*. The myofibre types shown are: type I (slow oxidative), IIC (intermediate or transitional between types I and IIA), IIA (fast oxidative-glycolytic), IIX (intermediate or transitional between types IIA and IIX) or IIX (fast glycolytic). Classification of type IIB is derived from positive staining for type 2 MHC antibodies, intermediate staining for type I, IIX MHC antibody and negative staining for type I MHC antibody. Type IIC myofibres were based on positive or intermediate staining for all three antibodies (adapted from Greenwood et al., 2006a).

Computer Tomography

Estimates of the weight of lean, fat and bone in the carcass, and estimates of the weight of lean and fat in the organs and digestive tract were obtained using X-ray computer tomography (CT). Differences in composition and growth were computed at the beginning and completion of each feeding experiment (Chapter 2, Chapter 4 and Chapter 5). Lambs were scanned live and without sedation using a CT scanner, (Picker UltraZ 2000 CT scanner, Philips Medical Imaging Australia, Sydney, New South Wales, Australia) with a 457 mm diameter image as described by Haynes et al., (2010). One lamb in the final experiment (Chapter 5) was given a mild sedative (xylazine hydrochloride, 0.30 ml/50 kg body weight, Ilium Xylazil 20, Troy Laboratories Pty. Ltd., Smithfield, New South Wales, Australia) 10 minutes prior to scanning as the animal was too distressed to collect accurate CT images.

To prevent aspiration pneumonia the lambs were withheld from feed the night prior to scanning, resulting in the lambs being approximately 16 h without feed. At scanning, the lambs were strapped on their backs into a semi-cylindrical cradle and foam pads were used to support the head and neck. The front legs were strapped to the chest and the hind legs restrained in clamps. Each animal had from 54 to 66 (number of scans depended on the size of the animal) serial 5 mm cross-sectional CT images taken at 15 mm intervals, starting at the 2nd cervical vertebra and moving through the torso to finish approximately halfway along the tibia.

To calculate estimated carcass composition total lean (muscle and connective tissue), fat and bone each CT image was manually edited by virtual removal of internal organs and digestive tract (skin was not removed) using an open-source software program, Osirix (Rosset et al., 2004) as shown in Figure A2. Alternatively, to determine the estimated weights of organs (heart, lungs and trachea (pluck), liver, spleen and kidneys) or gastrointestinal tract,

the carcass component of the animal was removed as shown in Figure A2 (organs only). Once the images were modified the weight of lean, fat and bone (carcass estimation only) was calculated using Autocat, as software program developed by Thompson and Kinghorn (1992).

Osirix was used to estimate the eye muscle depth (EMD) and fat depth (C site) which was measured 45 mm from the midline of the 12th/13th rib and the eye muscle area (EMA) (Figure A3). The total soft tissue depth measured 110 mm from midline over the 12/13th rib (GR) as shown in Figure A4.

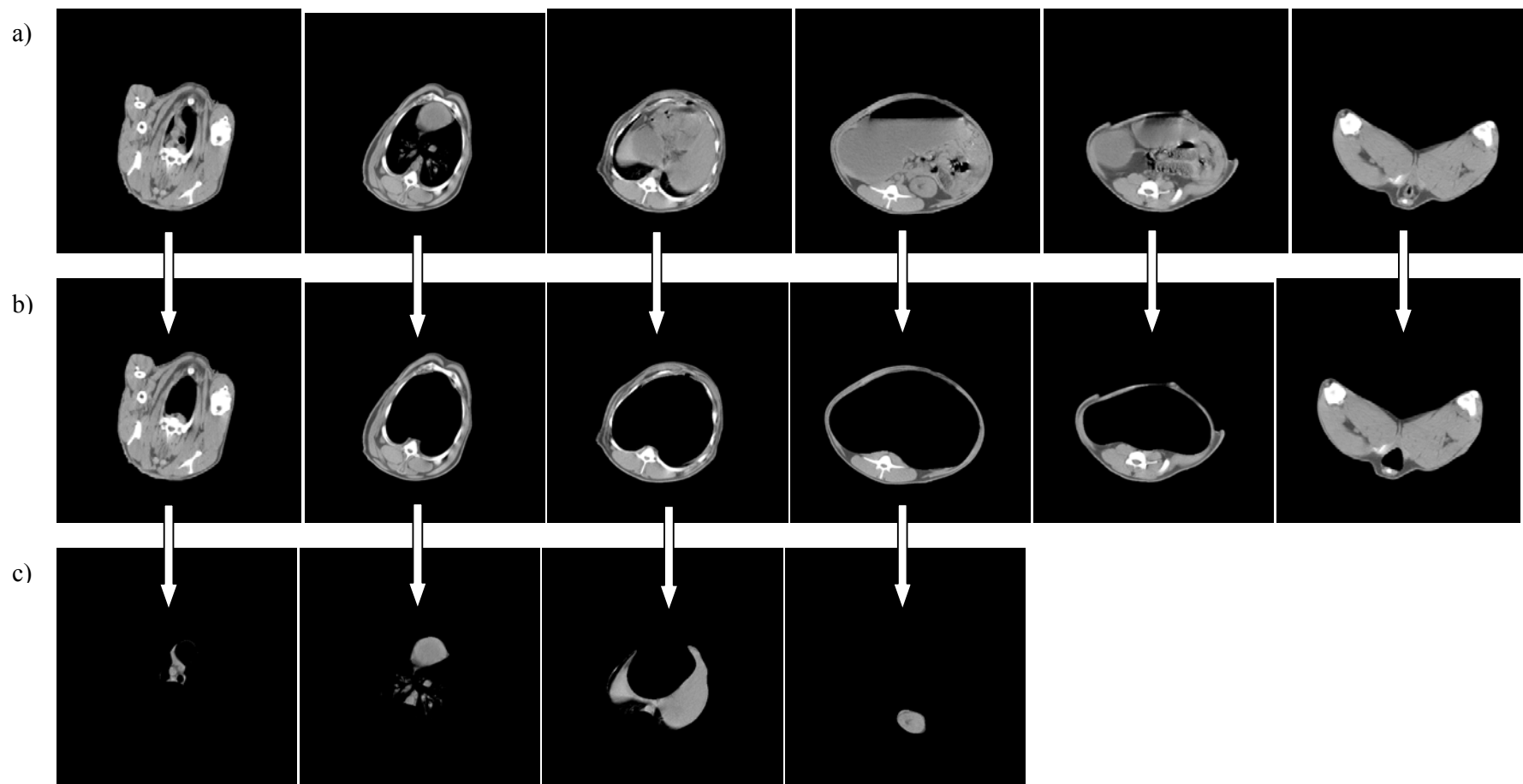


Figure A2. Examples of serial cross-sections computer tomography (CT) images used to estimate lean (muscle and connective tissue), fat or bone. a) whole CT composition, b) carcass composition (without organs and digestive tract), c) organ composition (heart, lungs and trachea (pluck), liver, spleen and kidneys).

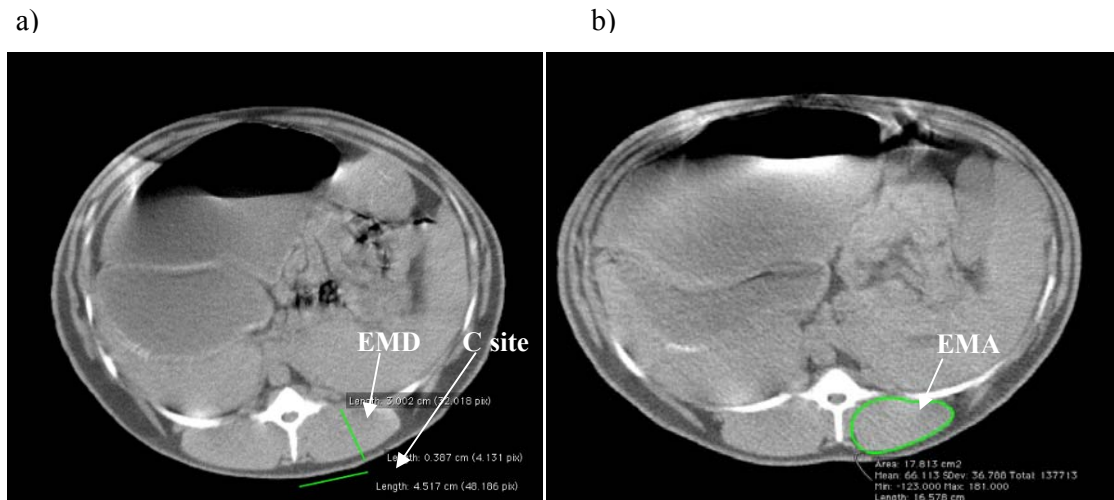


Figure A3. Examples of computer tomography eye muscle and fat depth measurements. a) Eye muscle depth (EMD) and subcutaneous fat depth (C site) were recorded 45 mm from the midline of the 12/13th rib; b) eye muscle area were recorded at the 12/13th rib.



Figure A4. Total soft tissue depth measured 110 mm from midline of the 12/13th rib (GR).

The CT data collected from the animals in experiment 1 (Chapter 2 and 4) have been reported in an abstract describing the method used to predict CT liveweight and CT carcass weight (Haynes et al., 2010). The relationship between the final predicted CT carcass weight and hot carcass weight had an r^2 of 0.96, and between CT carcass weight and hot carcass weight had an r^2 of 0.98 (Haynes et al., 2010), with the difference in intercept between these two relationships attributable to skin and blood in the lambs at the time of scanning, which were not present after slaughter (Figure A5).

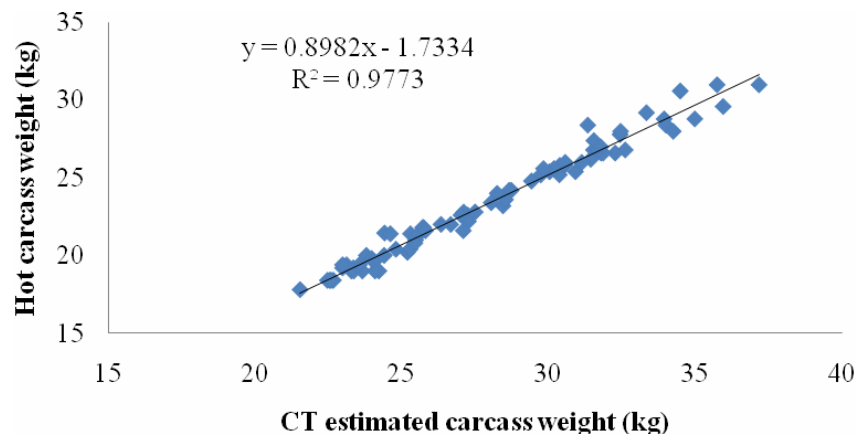


Figure A5. Relationship between estimated CT carcass weight and hot carcass weight (Source Haynes et al., 2010).