GENETIC VARIATION IN THE MYOSTATIN GENE (*MSTN*) AND ITS ASSOCIATION WITH LAMB GROWTH AND CARCASS TRAITS IN NEW ZEALAND ROMNEY SHEEP

JIN HAN

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by Jin Han

Myostatin (MSTN), which is also known as growth and differentiation factor 8 (GDF8), has been reported to act as a negative regulator of myogenesis during skeletal muscle development. It has also been implicated in the regulation of adipocyte function and in controlling the structure and function of tendons. Variation in the myostatin gene (*MSTN*) has been associated with differences in muscularity in certain breeds of sheep.

Polymerase chain reaction - single strand conformational polymorphism (PCR-SSCP) analysis coupled with nucleotide sequencing was used to assess variation in *MSTN* in New Zealand (NZ) sheep. In this study, a total of 218 sheep from a range of breeds were screened for variation across an extended region spanning nucleotide c.-1199 in the promoter region to c.*1813 in the 3'UTR. The breeds investigated included dual-purpose breeds for meat and wool production (Coopdale, Coopworth, Corriedale, Perendale and NZ Romney sheep), meat breeds (Dorper, Dorset Down, Poll Dorset, Suffolk and Texel sheep), a wool breed (Merino) and some NZ cross-bred sheep.

A total of 28 nucleotide substitutions were identified in these breeds across the region investigated. Of these substitutions, one (c.101G>A) is located in a coding region and results in an amino acid substitution of glutamic acid (Glu) with glycine (Gly) at codon 34. The other nucleotide substitutions are located within non-coding regions. Ten substitutions at positions c.-959C>T, c.-784A>G, c.373+563A>G, c.373+607A>G, c.374-654G>A, c.374-54T>C, c.748-54T>C, c.*83A>G, c.*455A>G and c.*709C>A are reported for the first time in this study. These substitutions suggest ovine *MSTN* is highly variable and that a potential exists for such variation to affect *MSTN* activity, and thus to be used as a gene-marker to help with breeding selection for muscle yield.

Based on the genetic variation found in this study, a strategic PCR-SSCP typing approach was used to define the haplotypic diversity of *MSTN*. Eight haplotypes (designated H1-8) of *MSTN* spanning an extended region including the 5'UTR, coding regions, intronic regions and 3'UTR were characterised in the different breeds. Certain haplotypes (e.g. H2 and H3) were found with high frequencies whereas others (e.g. H8) were less common. Two nucleotide substitutions, c.101G>A and c.*83A>G specifically defined haplotypes H7 and H8 respectively in this study and have not been reported previously.

Of the various breeds studied, the NZ Romney is the most common breed in NZ. Thus the association of *MSTN* with meat production in NZ Romney is of utmost interest to the NZ sheep industry. Associations between the various *MSTN* haplotypes and variation in lamb growth and carcass traits in 1376 NZ Romney lambs from 19 sire-lines were investigated. The lambs were selected from both North Island and South Island farms over four consecutive years from 2006 to 2009.

Five extended *MSTN* haplotypes (H1, H2, H3, H5 and H7) were identified in these NZ Romney sheep while H4, H6 and H8 were not found. Of these five haplotypes, H1 carrying *MSTN* c.*1232A is reported for the first time in NZ Romney sheep.

Using a Restricted Maximum Likelihood (REML) mixed-model approach in both half-sib and pooled-data analyses, H1 was associated with an increase in birth weight, tailing weight and draft weight in lambs, and had a significant effect on loin yield, leg yield, total yield and proportion loin yield from lamb carcasses. Breeding for an increased number of lambs carrying H1 may therefore lead to an improvement in lamb growth and meat yield, and thus underpin increased productivity and improved economic returns in the NZ sheep industry.

Haplotype H2 in lambs was associated with an increase in draft weight and an earlier drafting age, together with an increase in loin, shoulder and total yield of lean meat. H3 was associated with an increase in growth rate to weaning, leg yield and total yield of lean meat. The presence of haplotype H5 in lambs was associated with a decrease in loin yield, shoulder yield and total yield of lean meat yield. The association of haplotype H7 with an increase in hot carcass weight and a decrease in leg yield was identified for the first time in this study. This suggests that H7 may have a possible effect on growth but not meat yield.

In this context, it would seem advantageous to promote the selection of NZ Romney for haplotypes H2 and H3 and not H5 and H7.

Pleiotropic effects of *MSTN* c.*1232A were also investigated. A total of 79 NZ Romney lambs derived from a single sire-line that was heterozygous for c.*1232G>A were investigated. A factorial REML mixed-model was used in the half-sib data analyses. A significant association was found between *MSTN* c.*1232 genotype and gender with the *MSTN* c.*1232AG genotype being more common in ewe lambs. Thus, *MSTN* may also affect other physiological activities during embryonic development. This may offer an explanation for the abnormal gender ratio effect observed in this study. It is important for the livestock industry to balance the benefit of increased birth weight, lamb growth and carcass yield against potential pleiotropic effects caused by *MSTN* c.*1232A.

The work presented in this thesis suggests that the variation in ovine *MSTN* in NZ Romney is associated with differences in lamb growth and carcass traits, and thus meat production. It also suggests that using *MSTN* as a marker-assisted selection tool for improved carcass traits in NZ Romney is a future possibility.

Keywords: Myostatin (MSTN), Myostatin gene (*MSTN*), GDF8, nucleotide substitution, genetic variation, haplotype, PCR, SSCP, sheep, muscularity, growth, carcass trait, gene-marker

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ABBREVIATIONS

$	New Zealand dollar
µg	microgram
µL	microlitre
µm	micrometre
µM	micromolar
aa34	amino acid 34
ARE	androgen response element
AP1	activator protein 1
β	beta
bHLH	basic helix-loop-helix
B×H	Beltex × Huyang
BMP	bone morphogenetic protein
BSE	bovine spongiform encephalopathy
bp	base pair
c34	codon 34
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
dTTP	deoxythymidine triphosphate
dsRNA	double-stranded RNA
DPO	Dual Purpose Overall
E. coli	*Escherichia coli*
EDTA	ethylene diaminetetra-acetic acid
EU	European Union
FLRG	follistatin-like related gene
FMD	foot and mouth disease
G	gap phase
GASP	growth/differentiation factor-associated serum protein
Gfi-1B	growth factor independence 1 zinc-finger protein
GRE	glucocorticoid response element

GDF8	growth differentiation factor 8
H	haplotype
HGF	hepatocyte growth factor
H-W	hot carcass weight
IGF-1	insulin-like growth factor 1
LAP	Latency Associated Protein
LIF	leukaemia inhibitory factor
MF	Meat Chinese Merino Fine Wool
mM	millimolar
MPMF	Meat and Multi-prolific Chinese Merino Fine Wool
Mstn$^{-/-}$	*MSTN* "knock-out" mice
Mstn$^{-/-}$/F66	follistatin transgenic mice with a *Mstn*$^{-/-}$ background
MEF2	myocyte enhancer factor 2
MYOG	myogenin
MSTN	myostatin gene
MSTN	myostatin protein
MRF4	muscle regulatory factor 4
MTBF	muscle-specific Mt binding site
ng	nanogram
nm	nanometre
nt	nucleotide
NZ	New Zealand
OAR2	Chromosome 2
Oct-1	Octamer-binding factor 1
PCR	polymerase chain reaction
PRE	progesterone response element
QTL	quantitative trait loci
Rb	retinoblastoma
REML	Restricted Maximum Likelihood
RNA	ribonucleic acid
rpm	revolutions per minute
RSRR	Arg-Ser-Arg-Arg
RXXR	Arg-X-X-Arg
SIL	Sheep Improvement Limited

SNP	single nucleotide polymorphism
SSCP	single strand conformational polymorphism
SP	single peptide
SRF	serum response factor
STN	semitendinosus
TATA	TATA box
TGF-β	transforming growth factor-β
Tris	tris(hydroxymethyl)aminomethane
TSE	transmissible spongiform encephalopathy
UTR	un-translated region
U	unit
UV	ultraviolet
VIAscan®	video imaging analyses
wt	weight

AMINO ACID RESIDUE ABBREVIATIONS

A	Ala	alanine
C	Cys	cysteine
D	Asp	aspartic acid
E	Glu	glutamic acid
F	Phe	Phenylalanine
G	Gly	glycine
H	His	histidine
I	Ile	isoleucine
K	Lys	lysine
L	Leu	leucine
M	Met	methionine
N	Asn	asparagine
P	Pro	proline
Q	Gln	glutamine
R	Arg	arginine
S	Ser	serine
T	Thr	threonine
V	Val	valine
W	Trp	tryptophan
Y	Try	tyrosine
X		any amino acid

Chapter 1

Literature review

1.1 Introduction

New Zealand (NZ) is unique amongst developed nations with approximately two-thirds of the primary sector export revenue being derived from animal production (Figure 1.1 A). Its farms, which are based on a pasture-fed livestock production system, produce predominantly dairy products and red meat. The latter has become the country's second largest export earner (Figure 1.1 A). In recent years, the total value of red meat exports has grown and the NZ Red Meat Export Strategy states that the industry aims to double the annual red meat export earnings by 2025 (MIA, 2010). In 2010, total red meat exports (including co-products) were worth NZ$5.7 billion (MIA, 2010). Thus red meat exports have been, and will continue to be, a critically important part of the NZ economy.

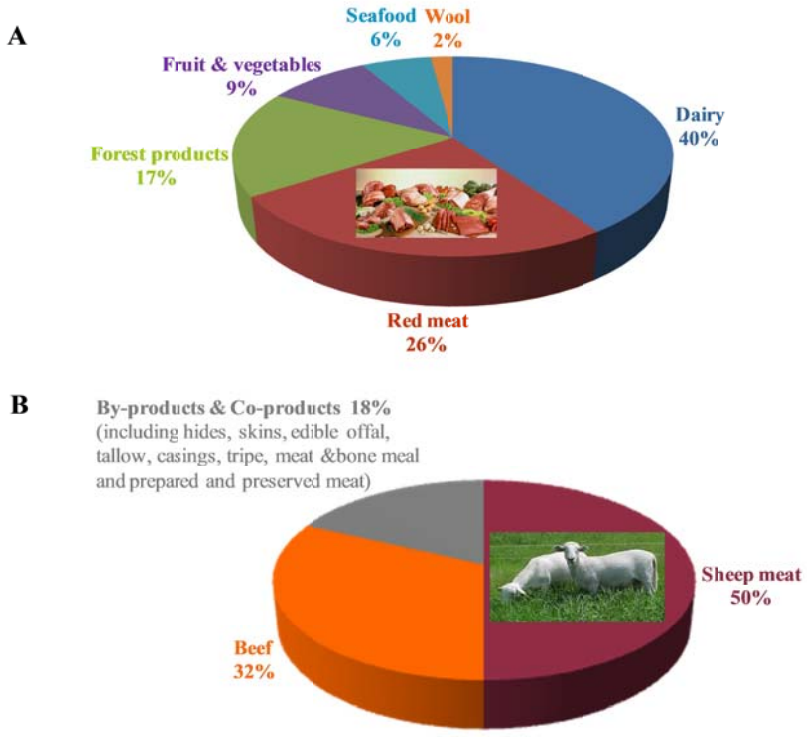

Figure 1.1 (A) New Zealand primary sector export revenue and (B) red meat export revenue in the year ended June 2010 (MIA, 2010)

This literature review consists of (i) an overview of the importance of sheep meat production and its contribution to the NZ economy; (ii) a discussion of the challenges facing the NZ sheep meat industry and (iii) a discussion of the use and value of genetic testing in improving sheep meat production. The biology of skeletal muscle growth and development will be described, along with the physiological function of the myostatin protein (MSTN) in skeletal muscle growth and development. This will include a discussion of the myostatin gene "knock-out" mouse model and the role of MSTN in growing farm animals. Finally, genetic variation in the myostatin gene (*MSTN*) and its association with carcass composition and muscle traits will be discussed in various species including cattle, pigs, chickens, humans, dogs, fish and sheep.

Sheep meat production and its contribution to the NZ economy

Sheep meat exports are the largest contributor to the value of NZ's red meat exports, followed by beef (Figure 1.1 B). In 2010, a total of 372,862 tonnes of sheep meat were exported, which accounted for 50% of the total red meat exports (Figure 1.1 B). This sheep meat had a value of NZ$2.8 billion (MIA, 2010). As a sheep exporter, NZ contributes to approximately 40% of the global sheep meat export trade by volume, and it is the largest exporter of lamb and mutton in the global market.

NZ sheep meat is exported to over 90 countries around the world (MIA, 2010). The European Union (EU) is currently NZ's largest market, particularly for high value products. It accounted for 47% of NZ sheep meat exports by volume and 57.5% by value at NZ$1.61 billion in 2010 (MIA, 2010). North America and Asia were NZ's second and third largest sheep meat export markets, accounting for 14% and 13% of total export value respectively. The Middle East was a region where sheep meat exports grew significantly to NZ$212 million in 2010. The EU will likely remain the largest high-value export market for the foreseeable future, but there are emerging market opportunities in regions such as China and India, where demand is increasing (Beef + Lamb NZ, 2009).

New Zealand's red meat is in demand because consumers appreciate its attributes and quality. Over the last few decades, the NZ red meat industry has changed significantly from exporting frozen whole carcasses, to exporting fresh "chilled" shelf-ready products that have been tailored for individual clients and markets. Currently, lamb carcasses are processed into over thirty main product cuts, enabling the products to be sold into a wide-

range of markets, some of which offer high returns for premium cuts. Less developed countries tend to purchase low value meat cuts for traditional dishes (Beef + Lamb NZ, 2009).

The NZ Romney is the most important contributor to NZ sheep meat production

In NZ, there are over 30 sheep breeds which can be classified as dual-purpose breeds for both meat and wool production (e.g. Coopdale, Coopworth, Corriedale, NZ Romney and Perendale), meat breeds (e.g. Dorper, Poll Dorset, Suffolk and Texel), or wool breeds (Merino). These categories are not mutually exclusive. The NZ Romney sheep constitutes about half of the sheep population nationwide and it is therefore the most important contributor to NZ sheep meat production (Beef + Lamb NZ, 2011a). The Coopworth (Romney x Border Leicester) and Perendale (Romney x Cheviot) are the second and third most numerous dual-purpose breeds in the country.

Today's "NZ Romney" is a vastly different breed when compared with the original Romney Marsh sheep from Kent in the south of England. The original Romney Marsh sheep were introduced into NZ in the 1860s, and have been developed since 1910 to become the most popular breed in the country (Meadows, 2008). The sheep have been bred into a dual-purpose sheep breed, with what are described as "easy-care" characteristics, superior mothering abilities, survival ability and high meat-yielding characteristics (Romney NZ Inc, 2011). The NZ Romney has demonstrated an ability to thrive over a wide range of environments from fertile lowland to hard hill country, and in both islands of NZ.

Challenges facing the NZ sheep meat industry

Although NZ is ranked as a world leader in sheep meat production and exporting, it has faced many challenges such as quota restrictions and trade barriers in major export markets. There have also been internal difficulties, such as a recent chronic decline in sheep numbers, the impacts of adverse seasons and the conversion of some sheep farms into dairy farms and forestry blocks.

With the world's population predicted to grow by a further three billion people by 2025, it is apparent that new ways are required to produce the 70% more food required to meet the growing global food demand. Consequently, sheep production research has focused on

innovative agricultural technologies, new farming practices involving more efficient animal production systems, alternative breeding selection tools and various novel farm management approaches. More inventive processing, packaging and distribution methods have also been introduced. The application of contemporary research findings to NZ production systems has also contributed significantly to the development of the NZ sheep meat industry.

Genetic improvement to advance sheep meat production

Meat producers are paid primarily on the weight of carcasses, with some adjustment applied according to the grade of the carcass, yield of lean tissue, export market demand and the strength of the NZ dollar.

Red meat is derived from sheep muscles, and the growth and development of those muscles can be influenced by several factors such as gender, breed, physiological responses and variation in feed supply. In recent years, use of genetic evaluation tools such as breeding values to describe genetic merit, has had a great impact on the NZ sheep industry. Accordingly, genetic gain has been made in lamb growth performance and carcass characteristics, and particularly after the establishment of Sheep Improvement Limited (SIL) in 1999 (Newman *et al.*, 2009). The SIL Dual Purpose Overall (DPO) index is based on various performance traits such as growth, meat, reproduction, survival and wool production and is a favoured selection tool for NZ Romney breeders. Selection based on the DPO index contributes to the overall improvement of the NZ sheep industry.

Genetic selection is viewed as a long term solution for genetic improvement in livestock species. The improvement of farm animals for meat production by genetic selection requires that strategies are in place to increase muscle growth, whilst retaining leanness and meat quality at slaughter. Ideally, *post-mortem* carcass and meat characteristics need to be predicted and selected in the live animal, as this will allow the early selection of desirable breeding stock and thus increase genetic gain for desirable traits. One approach to improving genetic selection is the use of gene-markers to identify sheep carrying traits of economic importance.

One gene that has been targeted already for use as a gene-marker is *MSTN*. The product, myostatin protein (MSTN) of this gene, regulates muscle growth (McPherron & Lee, 1997). It is deemed responsible for the “double-muscling” phenotype seen in cattle

(Kambadur *et al.*, 1997), a phenotype that has been identified as a heritable physiological characteristic since the early 1900's (Arthur, 1995). In this thesis, emphasis will be placed on identifying genetic diversity in *MSTN* across various NZ sheep breeds and how variation in *MSTN* can potentially be used as a gene-marker in selecting breeding stock for meat production.

1.2 Skeletal muscle growth and development

Meat provides a good source of dietary protein in many different cultures. In general, it consists of skeletal muscle, connective tissue and fat (Lawrie & Ledward, 2008). Skeletal muscles' primary component is muscle fibres and these are surrounded by connective tissue. Muscle mass is largely determined by the number of muscle fibres and the size of those fibres. It is important to understand the underlying physiological mechanisms that determine muscle growth and development, as muscle typically constitutes over 40% of the lean body weight of livestock.

Prenatal muscle growth and development

During the embryonic development of muscle, myoblasts develop from myogenic precursor cells. These precursor cells are derived from the mesoderm which originates from the somites (epithelial spheres of the paraxial mesoderm, Figure 1.2). Specification of the mesodermal precursor cells to enter the myogenic lineage requires the up-regulation of the muscle-specific basic helix-loop-helix (bHLH) transcriptional activators MyoD and/or Myf5 (Cossu & Biressi, 2005). These MyoD and/or Myf5 positive myogenic cells are able to proliferate and divide to establish a pool of myoblasts.

In response to differentiation signals, myogenin (MYOG) and muscle regulatory factor 4 (MRF4), which belong to the bHLH family, assist myoblast differentiation (Molkentin & Olson, 1996; Olson, 1992). Proliferating myoblasts withdraw from the cell cycle at the first gap phase (G1), to become terminally differentiated myocytes. These subsequently begin to express muscle-cell specific proteins, such as the myosin heavy chain and muscle creatine kinase. Finally, mono-nucleated myocytes fuse to form multinucleated myotubes (Figure 1.2). These mature into muscle fibres (Asakura *et al.*, 2002; Olson, 1992; Rehfeldt *et al.*, 2000).

Figure 1.2 removed due to copyright

Figure 1.2 Schematic diagram depicting the physiological mechanisms involved in embryonic skeletal muscle formation at the cellular level in mouse (Cossu & Biressi, 2005). Stages of skeletal muscle development include determination, proliferation, differentiation and maturation. Determination ends with the formation of the myotome from the dermo-myotome. Embryonic myoblasts differentiate to form secondary fibres that surround the primary fibres.

During myogenesis, the primary myofibres that are formed during the initial stages of myoblast fusion provide a framework for the development of a larger population of smaller secondary myofibres. This occurs through a second wave of differentiation of the foetal myoblasts. The final phase, which determines the actual number of muscle fibres, occurs during the terminal differentiation and fusion of proliferating myoblasts into primary and secondary myotubes and myofibres (Schiaffino & Reggiani, 1996). Heterogeneous populations of myoblasts separately give rise to successive generations of muscle fibres. It is therefore, an increase in muscle fibre number (hyperplasia) prenatally that is the critically important determinant of the ultimate size of muscles.

The myoblast populations that do not differentiate and form fibres, are found just outside the myofibres' cell membranes. These are termed muscle satellite cells. These satellite cells are able to divide and serve as the source of new myonuclei. They contribute to the growth of the fibres during postnatal growth and also participate in muscle regeneration and repair processes (Heslop *et al.*, 2001; Schultz, 1996).

Postnatal muscle growth and development

During postnatal muscle growth, increases in skeletal muscle mass are mainly due to an increase in muscle fibre size. This is called hypertrophy, as opposed to hyperplasia which occurs prenatally. Although the number of muscle fibres largely determines the amount of

postnatal muscle growth, the total count of muscle fibres typically remains the same in postnatal muscle growth (Luff & Goldspink, 1970; Wegner *et al.*, 2000).

It has been suggested that there might be an increase in fibre numbers shortly after birth (Rehfeldt *et al.*, 2000) and that this is possibly a result of maturation and elongation of the existing myotubes during the initial postnatal muscle growth period (Ontell & Kozeka, 1984). Thus, the ability to manipulate foetal muscle fibre number would have significant consequences for the postnatal growth of an animal (Fahey *et al.*, 2005).

In the context of meat production, the most efficient animals are those that gain weight and reach a mature body size rapidly and with minimal fat deposition during the postnatal muscle growth period. It is therefore important to both be able to understand muscle growth and development, and to manipulate the processes to improve production. As muscle fibre number and size during both muscle pre- and postnatal development are in part determined by the genetic makeup of the animal, a better understanding of the genetics of muscle biology should allow improvement in meat production.

Genetic selection of livestock based on having a larger mature body size or rapid lean-tissue growth can lead to increased body weight. Typically selection for muscle growth or increased body weight leads to increases in myoblast and/or satellite cell proliferation and muscle fibre formation as indicated by higher myonuclear numbers and total muscle protein accretion (Rehfeldt *et al.*, 2002). According to the principles of skeletal muscle growth, higher proliferation rates contribute both to the formation of higher muscle fibre numbers prenatally and to the accumulation of more myofibre nuclei postnatally. The potential for lean-tissue growth of an animal may therefore largely depend on both the number of prenatally formed muscle fibres and the postnatal rate of muscle fibre hypertrophy (Larzul *et al.*, 1997)

1.3 MSTN and muscle growth and development

Myostatin (MSTN), also known as growth and differentiation factor 8 (GDF8), acts as a negative regulator of muscle growth. It was first identified as an important member of the transforming growth factor-β (TGF-β) superfamily, when a two to three-fold increase in skeletal muscle mass was found in *MSTN* "knock-out" mice ($Mstn^{-/-}$) compared with wild-type mice (McPherron *et al.*, 1997). This *MSTN* "knock-out" model will be discussed in

more detail after a general discussion of the role of MSTN in muscle growth and development.

The role of MSTN in muscle growth and development

It was concluded from earlier research into TGF-β activity, that this growth factor inhibited the proliferation of both early and late myoblasts (Cusella-De Angelis *et al.*, 1994) and foetal and postnatal satellite cells (Hathaway *et al.*, 1994). Upon realisation that the TGF-β inhibitory activity was mediated by what became known as MSTN (McPherron *et al.*, 1997), its influence on both myoblast proliferation and differentiation during muscle growth was extensively studied using *in vitro* C_2C_{12} skeletal muscle bioassay cell-line systems (Joulia *et al.*, 2003; Ríos *et al.*, 2002; Taylor *et al.*, 2001; Thomas *et al.*, 2000). This is summarised in Figure 1.3.

Figure 1.3 removed due to copyright

Figure 1.3 A model for the role of MSTN in muscle growth adapted from (Thomas *et al.*, 2000). (A) During embryonic myogenesis, Myf5 and MyoD commit cells to the myoblast lineage. These myoblasts then migrate and proliferate. In response to MSTN signalling, p21 is up-regulated, inhibiting cyclin-E·Cdk2 activity, which causes Rb inactivation and G1 arrest. Thus, myoblast number and hence fibre number following differentiation is regulated (limited). (B) In the absence of functional MSTN, the signal for p21 up-regulation is lost and Rb remains in a hyper-phosphorylated form, resulting in deregulated (increased) myoblast proliferation and self-renewal of satellite cells, which ultimately leads to increased muscle fibre number.

The role of MSTN in myoblast proliferation

During embryonic myogenesis, Myf5 and MyoD activated precursor cells are committed to the myoblast lineage prior to their proliferation. In response to MSTN signalling, a cyclin-dependent kinase inhibitor p21 is up-regulated, and it then inhibits cyclin-E·Cdk2 and retinoblastoma (Rb) protein activity. This subsequently can lead to the arrest of myoblasts in the G1 phase and hence, inhibit their proliferation (Thomas *et al.*, 2000) and the proliferation of satellite cells (McCroskery *et al.*, 2003). Experimentally, when C_2C_{12} bovine myoblasts were incubated with varying concentrations of MSTN in *vitro*, there was a decrease in the proliferation of myoblasts with increasing MSTN levels (See Figure 1.3 A) and *vice versa* (Thomas *et al*., 2000). It was concluded that myoblast number, myofibre number and subsequent muscle differentiation were regulated by MSTN.

In contrast, when MSTN was absent during myogenesis (Figure 1.3 B), the signal for p21 up-regulation is lost and Rb remains in a hyper-phosphorylated form. This results in deregulated (increased) myoblast proliferation, which leads to increased muscle fibre number (Thomas *et al.*, 2000). The absence of MSTN and thus p21 also causes the normally quiescent satellite cells to re-enter the cell cycle, and proliferate and fuse with existing muscle fibres, which leads to increased muscle fibre size (McCroskery *et al.*, 2003; Tobin & Celeste, 2005). A study by Joulia *et al*. (2003) confirmed that inhibition of endogenous MSTN stimulated myoblast proliferation in combination with an increased myoblast apoptotic rate.

The inhibitory effect of MSTN on cell proliferation was also investigated using human recombinant MSTN in a mouse skeletal muscle C_2C_{12} cell culture system (Taylor *et al.*, 2001). Both a recombinant full-length (375-amino acid) and an abbreviated (110-amino acid) carboxy-terminal protein fragment of MSTN inhibited cell proliferation in both myoblasts and myotubes. The full-length recombinant MSTN produced a greater inhibition of DNA synthesis in myotubes than in myoblasts (Taylor *et al.*, 2001).

This is at variance with Thomas *et al*. (2000) who showed that the growth inhibitory effect of MSTN did not increase myotube formation, but was only active on myoblasts. Taylor *et al*. (2001) also reported that neither protein had significant effects on apoptosis, which is in contrast to the later work of Joulia *et al*. (2003), which suggested murine MSTN over-expression strongly reduces low-mitogen-induced apoptosis. The discrepancy may be due to variable levels of gene expression in the *in vitro* model systems used to produce the

recombinant MSTN. That is, the expression system may not be capable of appropriate processing, post-translational modification and folding, with the result being that the three dimensional structure of recombinant MSTN is not equivalent to that produced *in vivo*, in animal cells (Taylor *et al*., 2001). Regardless, the ability of MSTN to regulate myoblast proliferation (Kirk *et al.*, 2000; Thomas *et al.*, 2000) and self-renewal of satellite cells (McCroskery *et al.*, 2003), has been widely studied and confirmed.

The role of MSTN in myoblast differentiation

Muscle differentiation and subsequent myotube formation depends on the expression and activity of muscle transcription factors, as well as on the reversal of the G0/G1 cell cycle arrest phase (Gu *et al.*, 1993). Although initially there was controversy over the growth inhibitory effect of MSTN on myotube formation (Thomas *et al.*, 2000), more recent studies have demonstrated the negative effect of MSTN on myoblast differentiation (Joulia *et al.*, 2003; Ríos *et al.*, 2002; Taylor *et al.*, 2001).

Joulia *et al*. (2003) revealed that MYOG is an important target of MSTN. MSTN-stimulated cell cycle withdrawal was associated with reduced expression of the myogenic regulatory factors Pax-3, Myf5 and MyoD (Amthor *et al.*, 2006). Over-expression of *MSTN* cDNA could reversibly inhibit myogenic differentiation by down-regulating mRNA levels of MyoD and MYOG, as well as the activity of their downstream target, creatine kinase (Ríos *et al.*, 2002). In contrast, inhibition of MSTN leads to the elevated expression of MyoD and thus increased formation of muscle fibres (Oldham *et al.*, 2001). The MSTN-deficient condition enhanced the frequency of release of the G0/G1-arrested cells, and consequently stimulated myoblast differentiation (Joulia *et al.*, 2003).

Further detail about the action of MSTN during myogenesis *in vivo*, was revealed by comparing "double-muscled" (Belgian Blue) cattle and "normal-muscled" cattle (Hereford x Friesian) at different gestational stages (Oldham *et al.*, 2001). *MSTN* mRNA levels peaked at the onset of secondary fibre formation and were greater in "normal-muscled" than "double-muscled" cattle. This suggests that MSTN may not be the primary regulator during the on-going development and maturation of secondary myofibres (Oldham *et al.*, 2001).

It has also been suggested that MSTN may affect myoblasts differentially by stimulating the induction of terminal differentiation processes and fusion of early myoblasts, while

down-regulating the proliferation of late myoblasts (Oldham *et al.*, 2001). A disruption of an intracellular/autocrine feedback loop mechanism was proposed when a relatively higher level of *MSTN* mRNA was detected in the "double-muscled" Belgian Blue cattle foetuses throughout myogenesis (Oldham *et al.*, 2001).

MSTN "knock-out" mouse models

The *MSTN* "knock-out" model studies have provided compelling evidence of the biological activity of MSTN during muscle growth and development. In one *MSTN* "knock-out" mouse model, the mature C-terminal region (active portion of the MSTN peptide) was deleted via either replacement with a neo cassette (McPherron *et al.*, 1997), or by flanking it with a *cre-loxP* sequence (Grobet *et al.*, 2003; Lin *et al.*, 2002; Welle *et al.*, 2007), thus rendering it dysfunctional and increasing muscle mass.

When *Mstn*$^{-/-}$ ("knock-out") mice were first generated by McPherron *et al*. (1997), it was found that the homozygous mutant *Mstn*$^{-/-}$ mice were both viable and fertile, and that their body weights were about 30% larger than their wild-type littermates (Figure 1.4 A). Individual muscles, including facial, upper limb (Figure 1.4 B and C), lower limb and pectoral muscles from homozygous *Mstn*$^{-/-}$ mice weighed 2-3 times more than those from their wild type littermates aged 3-6 months (McPherron *et al.*, 1997). Muscle fibre numbers in the *Tibialis cranialis* muscle were 86% higher in the mutant mice than wild type littermates, while the diameter of the *Tibialis cranialis* and *gastrocnemius* muscle were 7% and 22% respectively larger in *Mstn*$^{-/-}$ mice than the wild-type (McPherron *et al.*, 1997). This increase in muscle mass throughout the body was derived from a combination of muscle fibre hyperplasia and muscle fibre hypertrophy, again demonstrating the role of MSTN as a potent and specific negative regulator of skeletal muscle growth (Ríos *et al.*, 2002; Taylor *et al.*, 2001; Thomas *et al.*, 2000).

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Figure 1.4 Comparison of (A) a wild type and *MSTN* "knock-out" (*Mstn*$^{-/-}$) skin-on mouse; and comparison of skin-off upper limb muscle of (B) wild-type and (C) *Mstn*$^{-/-}$ mouse

The effects of the MSTN suppression are not limited to developing muscle. Welle *et al*. (2007) demonstrated that MSTN also inhibited development in mature muscle growth using a *cre-loxP* system. In this work, muscle mass increased approximately 25% within three months after *MSTN* mRNA expression was reduced to less than 1% of normal levels in 4-month-old *Mstn*$^{-/-}$ mice. This was due to muscle fibre hypertrophy as a result of myonuclear domain volume expansion. A similar increase in muscle mass but accompanied by a reduction in the size of internal organs was reported by Lin *et al*. (2002) in 1-month-old *Mstn*$^{-/-}$ mice, using a *cre-loxP* system. Both the increase in muscle mass and decrease in mass of internal organs (lung, liver and heart) were also observed in 2-month old mice (Grobet *et al.*, 2003). An explanation for the decrease in the mass of internal organs in *Mstn*$^{-/-}$ mice, may be due to a direct effect of the reduced concentration of circulating MSTN, or it may reflect an indirect physiological adaptation of the organism to the increased muscle mass (Grobet *et al.*, 2003).

Further studies have been carried out to understand the regulatory role of MSTN and its association with the increased muscle mass in *Mstn*$^{-/-}$ mice. Recent work by Lee (2007b) revealed that an excessive body size and four-fold increase in muscle mass (including the *pectoralis*, *triceps*, *quadriceps* and *gastrocnemius* muscle) were displayed in follistatin transgenic mice with a *Mstn*$^{-/-}$ background (*Mstn*$^{-/-}$ /F66) compared with the wild-type mice. It is known that follistatin is capable of regulating MSTN activity by blocking its binding to ACT RIIB in the signalling pathway during muscular development (Lee & McPherron, 2001). The quadrupling of muscle growth in these mice demonstrates that follistatin and other follistatin-like related proteins can exert their effects on regulating muscle fibre growth by targeting other ligands in addition to MSTN. The effect of blocking these other ligands is comparable in magnitude to that resulting from the loss of MSTN

(Lee, 2007b). This led Lee (2007a) to suggest that MSTN may not be the sole regulator of enhancing muscle mass in the *Mstn*$^{-/-}$ mice, and that other ligand(s) may cooperate with MSTN and ultimately target the TGF-β signalling pathway. Potential candidate ligands include activin (Winters & Moore, 2007), bone morphogenetic proteins (BMPs) (Gamer *et al.*, 1999), and activin type II receptors (Lee *et al.*, 2005). With all these possible alternative mechanisms, considerable effort could be justified in investigating strategies to modulate MSTN activity in animals of economic importance.

In parallel with the muscular effects, MSTN may also have effects on adipocyte function (Lin *et al.*, 2002; McPherron & Lee, 2002). A 70% decrease in the mean total body fat was found in *Mstn*$^{-/-}$ mice compared with wild-type mice, and despite the fact that normal energy intake and body temperature appeared to be maintained (McPherron & Lee, 2002). *Mstn*$^{-/-}$ mice were also found to have decreased leptin expression and secretion resulting from a decrease in adipogenesis (Lin *et al.*, 2002). Although MSTN was able to inhibit adipogenic differentiation in both 3T3-L1 and C3H 10T1/2 cell cultures (Kim *et al.*, 2001; Rebbapragada *et al.*, 2003), the mechanism of how MSTN acts on adipocytes *in vivo* is not fully understood. Further experiments are required to elucidate the precise mechanism by which MSTN regulates fat metabolism, including whether it directly inhibits the differentiation of adipocytes (Kim *et al.*, 2001), or indirectly influences the release of a hypothetical secondary messenger from muscles (Mauvais-Jarvis *et al.*, 2000).

Other effects have also been observed in *Mstn*$^{-/-}$ mice. For example, in addition to increased muscular mass, there is an increase in bone mineral density (Hameric, 2003), and also an increase in the susceptibility of muscle fibres to contraction-induced injury associated with having smaller, brittle and hypo-cellular tendons (Mendias *et al.*, 2008). Moreover, an increased isometric force on muscles was also observed in *MSTN* null *mdx* mice; a murine model of Duchenne muscular dystrophy (Bogdanovich *et al.*, 2002). These findings may provide an opportunity for future investigations on chronic muscle diseases such as muscular dystrophy and cachexia in humans, and specifically by inhibiting MSTN activity.

MSTN structure, signalling pathway and expression

The structure of *MSTN* in different species

MSTN was first isolated, cloned and characterised in mice (McPherron *et al*., 1997). This was achieved by aligning the known TGF-β family members and using the conserved regions to design primers for PCR amplification of the gene. Subsequently, *MSTN* was located and mapped in a large number of different species. It was found to be located on chromosome 1 (C1.1) in mice (McPherron *et al.*, 1997), chromosome 2 (2q32.2) in humans (Schuelke *et al.*, 2004), chromosome 2 (2q14-q15) in cattle (Grobet *et al.*, 2003), chromosome 2 in sheep (Clop *et al.*, 2006), chromosome 15 in pigs (Sonstegard *et al.*, 1998), chromosome 18 in horses (Lowe & Eddy, 1997) and chromosome 37 in dogs (NCBI GenBank Accession Number NW876304). Sequence alignment of *MSTN* from different species revealed an extraordinarily high level of conservation through evolution, which is consistent with MSTN having a conserved function (McPherron *et al.*, 1997). The structure of *MSTN* in various mammalian species is summarised in Figure 1.5.

In sheep, molecular analysis has shown that *MSTN* consists of three exons and two introns (Clop *et al.*, 2006). There are 508 (373 coding), 374 and 1893 nucleotides (381 coding) in exons 1, 2 and 3 respectively and 1833 and 2030 nucleotides in introns 1 and 2 respectively. Exon 1 and 3 comprise part of the 5' un-translated region (UTR, 135 nucleotides) and 3'UTR (1512 nucleotides) respectively (Clop *et al.*, 2006).

In the *MSTN* promoter region, there is high homology between sheep and other species including the bovine, porcine, human and mouse genes (95.8, 86.9, 80.2 and 67.7% respectively) (Du *et al.*, 2005). Several putative transcriptional response elements and muscle growth response elements, such as androgen response elements (ARE) and myocyte enhancer factor 2 (MEF2) elements, are found in the *MSTN* promoter of animals (Du *et al*., 2005) and humans (Ma *et al.*, 2001) (Figure 1.5). Important motifs including three TATA boxes, one CAAT box and eight putative E-box transcriptional response elements have been identified in the sheep promoter (Du *et al.*, 2007a; Du *et al.*, 2007b; Du *et al.*, 2005). These motifs are potential binding sites for bHLH transcription factors including MyoD and Myf5 (Salerno *et al.*, 2004).

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Figure 1.5 Comparative structure of mouse, human, sheep, bovine and pig *MSTN* and promoter regions adapted from (Du *et al.*, 2005; Grisolia *et al.*, 2009; Rodgers & Garikipati, 2008). Exons are boxed with open reading frames in *white* and un-translated regions in *grey*. Exon sizes are indicated within the boxes, intron sizes in italics. The locations of transcriptional response elements such as myocyte enhancer factor 2 (MEF2) binding site, muscle-specific Mt binding site (MTBF), growth factor independence 1 zinc finger protein (Gfi-1B) binding site, progesterone response element (PRE), androgen response element (ARE) and glucocorticoid response element (GRE) within each gene's promoter region (c.-1211 to c.1) are indicated by the shapes shown in the key. Putative E-boxes and some of the TATA boxes (TATA) are numbered.

The structure and proteolytic processing of MSTN

MSTN is synthesized as a 376 amino acid pro-peptide in mice with its amino acid sequence containing all the hallmark characteristics of other members of the TGF-β superfamily (McPherron *et al.*, 1997). The pro-peptide has a secretion signal peptide (SP), a pro-peptide pro-proteolytic processing site region and a carboxy-terminal active peptide, containing a novel pattern of cysteine residues which is highly conserved across species (Figure 1.6).

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Figure 1.6 Structure and the proteolytic processing of MSTN adapted from (Joulia-Ekaza & Cabello, 2006; Rodgers & Garikipati, 2008). (A) Prepro-MSTN consists of three domains which are the signal peptide (SP), the pro-peptide region, which will be cleaved by protease digestion at the RSRR site during maturation and the active peptide at the C-terminal; (B) The N-terminal signal peptide is removed during the first proteolytic process; (C) The pro-peptide is cleaved by a furin class protease at a conserved RXXR (R, arginine; X, any amino acid) epitope, and the resulting peptides dimerize via disulphide linkages at the cysteines indicated, producing mature MSTN. The Latency Associated Protein (LAP) sequesters MSTN dimers in a latent complex and can prohibit receptor activation.

The generation of biologically active MSTN involves two proteolytic cleavages of the pre-proprotein (Joulia-Ekaza & Cabello, 2006; Lee, 2004; McPherron *et al.*, 1997; Rodgers & Garikipati, 2008) (Figure 1.6). The first cleavage removes the 24 amino acid signal peptide (SP) necessary for targeting the peptide to the secretary pathway (Figure 1.6 A to B). The second cleavage occurs at a putative proteolytic Arg-X-X-Arg (RXXR) processing site and results in the formation of a N-terminal pro-peptide and a 109 amino acid C-terminal active peptide. This N-terminal pro-peptide or Latency Associated Protein (LAP) plays an important role in the proper folding of C-terminal dimers into a cystine knot structure (Figure 1.6 B to C). This cystine knot structure is critical in determining the biological activity of MSTN (McPherron *et al.*, 1997), and is a common hallmark of all TGF-β superfamily proteins (Galat, 2011). The amino acid sequence that targets the mature

MSTN for secretion consists of a core of hydrophobic amino acids close to the N-terminal pro-peptide and the RXXR proteolytic processing site is located close to the C-terminal end (Thomas *et al.*, 2000).

MSTN amino acid sequences have been predicted or resolved in various species including mouse, rat, human, baboon, cattle, pig, sheep, chicken, turkey and zebrafish (McPherron & Lee, 1997). The high level of amino acid conservation across species suggests that the function of MSTN is likely to have been conserved (McPherron & Lee, 1997; McPherron *et al.*, 1997). Except for zebrafish, MSTN amino acid sequences in the putative proteolytic Arg-Ser-Arg-Arg (RSRR) processing site and the C-terminal region are identical, with only one to three amino acid differences observed between the species. The zebrafish MSTN sequence is divergent and is only 88% identical to that in other species (McPherron & Lee, 1997). This is possibly expected, given the longer evolutionary history of fish which may have resulted in greater genetic variations in *MSTN* and greater variation in its function in fish, than in mammals (Lee, 2004).

The MSTN signalling pathway

Upon activation from the MSTN latent complex, the MSTN C-terminal dimer (mature protein) is capable of binding to receptors and activating signal transduction pathways in target cells (Joulia-Ekaza & Cabello, 2006, 2007; Kollias & McDermott, 2008). This is shown in Figure 1.7.

The mature protein dimer is capable of binding to activin type II receptors (mainly ACT RIIB *in vitro*) on the surface of many tissues and muscle cell lines (Lee & McPherron, 2001). Binding of MSTN to ACT RIIB can be inhibited by several proteins such as follistatin (Lee & McPherron, 2001), follistatin-like related gene protein (FLRG) (Hill *et al.*, 2002) and growth/differentiation factor-associated serum protein (GASP)-1 (Hill *et al.*, 2003). When MSTN binds to the activin type II receptor, an intracellular pathway is activated (Lee & McPherron, 2001). This involves trans-phosphorylation of the type I receptor (activin-like kinase 4 or 5) and phosphorylation of the receptor-regulated downstream proteins Smad2 and Smad3. These are intracellular signal transducers involved in MSTN signalling (Lee & McPherron, 2001). The co-Smad4 protein also potentiates this signalling (Zhu *et al.*, 2004). A competent transcription factor is formed and this is capable of entering the nucleus and regulating transcription.

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Figure 1.7 Secretion, activation and activity of MSTN adapted from McNally (2004), Lee and McPherron (2001) and Joulia-Ekaza and Cabello (2006)

1.4 Natural genetic variation in *MSTN* and its association with muscular phenotypes in various species

Over a decade has passed since MSTN was first identified and its biological function as a negative regulator of muscle growth was discovered (McPherron *et al.*, 1997). Since then, variations in *MSTN* have been found to be associated with the "double-muscled" phenotype in various species including mice (McPherron *et al.*, 1997), cattle (Bellinge *et al.*, 2005; Kambadur *et al.*, 1997; McPherron *et al.*, 1997), humans (Schuelke *et al.*, 2004), dogs (Mosher *et al.*, 2007), chickens (McFarland *et al.*, 2007; Zhang *et al.*, 2011), pigs (Stinckens *et al.*, 2005; Stinckens *et al.*, 2008) and sheep (Clop *et al.*, 2006; Johnson *et al.*, 2009; Kijas *et al.*, 2007; Hickford *et al*., 2010). The presence of variation in *MSTN* in other species and its association with variation in muscling, suggests that further naturally occurring variation in *MSTN* in sheep, could also affect muscle traits including skeletal muscle growth and meat yields.

In the following section, previous studies of genetic variation in *MSTN* and the association with increased muscle mass in species such as human and dogs, and the major commercial meat producing animals including cattle, sheep, pigs, chickens and fish will be reviewed.

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Figure 1.8 "Double-muscled" phenotypes and underlying genotype in cattle and sheep. (A) "Double-muscled" Belgian Blue cattle (McPherron & Lee, 1997); (B) "Double-muscled" Texel sheep (Clop *et al*., 2006).

MSTN variation and muscular phenotypes in other species

The identification of "double-muscled" cattle prompted a comprehensive investigation into the existence of a *MSTN* homologue in other species renowned for increased muscularity. Here is a brief synopsis of our understanding of *MSTN* in humans, dogs and other meat producing species such as fish, chickens and pigs. These studies have provided evidence of the effect of *MSTN* variation on increased muscle mass in various species, and also have provided valuable information for further study of genetic variation in ovine *MSTN* and its effect on meat production in sheep.

In humans, Schuelke *et al.* (2004) reported a "loss-of-function" *MSTN* variant g.IVS1+5 in a German child. This splice-site nucleotide transition on the first intron-exon boundary leads to a 108bp mis-splicing insertion and adds a single lysine residue followed by a premature termination codon. This causes a muscular hypertrophic phenotype.

In a whippet dog with a muscular hypertrophic phenotype, a two-nucleotide deletion in the exon 3 coding region was identified which led to creation of a premature stop codon at amino acid 313 (Mosher *et al.*, 2007). Dogs heterozygous for the mutation are more muscular and run faster than wild-type dogs (Mosher *et al.*, 2007; Shelton & Engvall, 2007).

In fish, *MSTN* varies among different fish species (Maccatrozzo *et al.*, 2001; Maccatrozzo *et al.*, 2002; Terova *et al.*, 2006). However, the function of MSTN in fish is poorly understood. Nevertheless, transgenic zebrafish expressing the MSTN pro-domain exhibited an increased number of myofibres in skeletal muscle (Xu *et al.*, 2003). Acosta *et al*. (Acosta *et al.*, 2005) also reported the generation of "giant zebrafish" by injecting double-stranded *MSTN* RNA (dsRNA) to inhibit MSTN function. These studies suggest that MSTN plays a major role during myogenesis and does contribute to muscle growth in fish (Vianello *et al.*, 2003). Skeletal muscle in fish constitutes a higher percentage of total body weight than in mammals. A small improvement in muscle growth would provide enormous benefits to the fish industry.

In chickens, polymorphisms in *MSTN* were reported in two different chicken lines selected for body weight and egg production (Baron *et al.*, 2002). The genetic variation c.234G>A in exon 1 coding region of *MSTN* was associated with a significantly higher body weight in Bian chicken (Zhang *et al.*, 2011). Pleiotropic effects of *MSTN* on muscle growth, along with other traits such as increased mortality, have been also described in broiler chickens (Ye *et al.*, 2007).

In pigs, approximately twenty nucleotide substitutions in the *MSTN* sequence have been detected in various breeds (Guimaraes *et al.*, 2007; Jiang *et al.*, 2002; Li *et al.*, 2002; Stinckens *et al.*, 2008). Studies have revealed associations between porcine *MSTN* variation and various production traits, with an emphasis being placed on variation in the coding (Li *et al.*, 2002) and promoter region (Guimaraes *et al.*, 2007; Stinckens *et al.*, 2008; Yu *et al.*, 2007). The findings in Belgian Piétrain pigs, a pig breed with a similar muscular phenotype to the "double-muscled" cattle, suggest that genetic variation in the promoter region of *MSTN*, may alter the binding of factors to *cis*-acting elements (Stinckens *et al.*, 2008; Yu *et al.*, 2007), which subsequently influences *MSTN* mRNA levels and muscle growth (Guimaraes *et al.*, 2007). However, the mechanism by which *MSTN* influences muscle growth in pigs is still unclear, and further studies are needed.

The "double-muscled" phenotype in cattle and meat production

The visibly distinct muscular hypertrophy, commonly known as "double-muscling", was first described in cattle breeds in 1807 (Culley, 1807). Since then, there has been at least

two hundred years of breeding for the "double-muscled" trait. A 20-25% increase in muscle mass has been shown in "double-muscled" cattle indicating that MSTN has a significant effect on cattle muscularity (Grobet *et al*., 1997; Kambadur *et al*., 1997) and primarily because of muscle fibre hyperplasia (Wegner *et al.*, 2000). This effect has also contributed significantly to animal growth rates, meat yield and carcass value (Ménissier, 1982b). The "double-muscled" trait has been widely described in a variety of cattle breeds after the effect was initially identified in Belgian Blue (Figure 1.8 A), Piedmontese and Asturiana de los Valles cattle (Dunner *et al.*, 1997; Grobet *et al.*, 1997; Kambadur *et al.*, 1997; McPherron & Lee, 1997; Smith *et al.*, 1997).

The role of *MSTN* variation in these "double-muscled" cattle has been studied extensively (Arthur, 1995; Bellinge *et al.*, 2005) and the gene is now known to be highly variable (details refer to Appendix A). Initial studies into bovine *MSTN* have focused on single nucleotide substitutions, with an emphasis on identifying genetic variation within the coding regions. To date, nine variations affecting the amino acid sequence have been identified to be responsible for "double-muscled" phenotypes in cattle (Dunner *et al.*, 2003; Esmailizadeh *et al.*, 2008; Grisolia *et al.*, 2009; Grobet *et al.*, 1998; Grobet *et al.*, 1997; Kambadur *et al.*, 1997). Six of these are disruptive variations and include nt821(del11), nt419del7-ins10, Q204X, E226X, C313Y and E291X. The other three mis-sense variations include S105C, D182N and F94L.

Multiplex genotyping using the disruptive variations as genetic marker(s) to identify "double-muscled" cattle, has been applied in the cattle industry with the aim of increasing meat production (Di Stasio & Rolando, 2005; Karim *et al.*, 2000). However, the disruptive variations identified in the coding regions do not account for all of the inherited variation in muscle mass in "double-muscled" cattle (Bellinge *et al.*, 2005; O'Rourke *et al.*, 2009). What is more, the contribution of *MSTN* variation in the non-coding regions to variation in muscle mass remains largely unknown. Gene expression and subsequent metabolic functions are affected by both coding and non-coding regions of a gene (Babbitt *et al.*, 2010; Zavolan *et al.*, 2002). Therefore effort has been made to explore the contribution effect of both "disruptive" and "non-disruptive" polymorphisms to variation in muscularity in cattle in recent studies. Haplotypes across an extended region of bovine *MSTN* have been inferred or defined (Dunner *et al.*, 2003; Miranda *et al.*, 2002; O'Rourke *et al.*, 2009). Haplotyping provides more insights on genetic diversity and association studies compared

with single polymorphism studies, particularly for complex traits, where variations outside of the tested region may also affect gene activity (Davidson, 2000; Hayes & Goddard, 2001).

The twenty different bovine haplotypes identified by Miranda *et al*. (2002) and Dunner *et al*. (2003) confirmed the occurrence of a high level of haplotypic diversity in European cattle breeds, and this may increase when more cattle breeds are tested. In order to examine the association effect of non-disruptive *MSTN* polymorphisms on muscularity, six tag variations across an extended region of *MSTN* [tag 1 and 2 are located in the promoter region, tag 3 is in intron 1, tag 4 is in nt821(del11) in exon 3, tag 5 and 6 are in 3'UTR] were used by O'Rourke *et al*. (2009). Eleven haplotypes were defined in Angus cattle. A significant association between *MSTN* haplotype and eye muscle area was revealed in this study, which indicated that non-disruptive polymorphisms, which had not been previously implicated in "double-muscled" phenotype, were strongly associated with variation in muscle mass in cattle. These results provided valuable evidence for an effect of extended *MSTN* haplotypes that had not been reported previously, especially when the haplotype spans an extended region of the gene. Further studies to ascertain haplotypic diversity in bovine *MSTN* and to quantify its effect on muscle mass are arguably needed.

In addition to the improving meat yield characteristic, "double-muscled" cattle also have other "advantages" such as having less fat and more tender meat compared with normal cattle (Ménissier, 1982a; Shahin & Berg, 1985). With consumers' demanding lean meat, the reduced adipocyte size observed in subcutaneous and internal fat depots in "double-muscled" cattle (Smet *et al.*, 2000) should satisfy the future needs of consumers for leaner meat (Martínez *et al.*, 2010). In addition, tenderness, which is one of the most important meat factors affecting the consumer acceptance of meat, reaches a satisfactory level in "double-muscled" cattle. The latter has been attributed to the reduced intramuscular collagen (connective tissue) relative to the myofibrillar proteins in the muscles from such cattle (Uytterhaegen *et al.*, 1994).

Despite these advantages, other pleiotropic effects of *MSTN* may be disadvantageous, including reduced fertility, calving difficulties and high mortality rates (Casas *et al.*, 2004; Wiener *et al.*, 2002). Although there are no data available on the number of stillborn calve and consequential economic loss, it has been suggested that the effect on calving difficulty

in “double-muscled” cattle has been underestimated. In order to maximise the benefits associated with the “double-muscled” syndrome, while minimizing the birth problems, use of a “double-muscled” terminal sire breeding system has been suggested; a system in which normal females are mated to “double-muscled” sires and all progeny are slaughtered for commercial production (Arthur, 1995).

A more recent investigation (Esmailizadeh *et al.*, 2008) has suggested that *MSTN* F94L appears to be an ideal candidate for genotype-assisted breeding stock selection, since cattle carrying the *MSTN* F94L variation have increased muscle mass with a reduced fat content. The birth weights of the calves are similar to those from normal animals. Thus the presence of the F94Lvariation will ultimately benefit cattle breeding by providing “double-muscled” cattle with increased muscularity and without any calving difficulties.

The “double-muscled” phenotype in sheep and meat production

It has been shown that genetic variation in *MSTN* explains the “meatiness” or the “double-muscled” phenotype of Texel sheep (Figure 1.8 B). In the following section, genetic variation in ovine *MSTN* and its effect on meat yield in sheep will be summarised.

To date 78 nucleotide substitutions (for details refer to Appendix B) have been identified in various sheep breeds (Boman & Våge, 2009; Boman *et al.*, 2009; Boman *et al.*, 2010; Clop *et al.*, 2006; Gan *et al.*, 2008; Heaton *et al.*, 2007; Hickford *et al.*, 2010; Kijas *et al.*, 2007; Sjakste *et al.*, 2011; Zhou *et al.*, 2008) illustrating that ovine *MSTN* is polymorphic. This variability is similar to the variation described in cattle (Dunner *et al.*, 2003; Grobet *et al.*, 1998; Smith *et al.*, 2000).

In contrast to the findings in cattle, where genetic variation often occurs within the coding regions (Appendix A), there are only three variations reported in the ovine *MSTN* coding region (Appendix B). These include: c.101G>A which results in a mis-sense substitution of Glutamic acid → Glycine at amino acid 34 (aa34/codon 34/c34) (Zhou *et al.*, 2008); c.120insA which results in a premature stop codon at aa49 and leads to a completely non-functional MSTN due to the bioactive carboxy-terminal end of the protein not being produced (Boman & Våge, 2009) and c.960delG which results in a disruption of the reading frame from aa320 to a pre-mature stop codon at aa359 (Boman *et al.*, 2009). c.120insA and c.960delG are associated with increased muscle mass and reduced carcass

fat in the Norwegian Spælsau (Boman & Våge, 2009) and Norwegian White breeds (Boman *et al.*, 2009), respectively.

There have been intensive studies on genetic variations of *MSTN* in Texel sheep (Clop *et al.*, 2006; Gan *et al.*, 2008; Johnson *et al.*, 2005; Kijas *et al.*, 2007; Marcq *et al.*, 2002; Walling *et al.*, 2001). A nucleotide substitution in the 3'UTR called *MSTN* c.*1232G>A [also known as g.6223G>A, and originally designated incorrectly as g+6723G>A (Hickford *et al.*, 2009)] was initially thought to be specific to the Texel breed (Clop *et al.*, 2006). Examination of the sequences flanking c.*1232 G>A revealed that *MSTN* c.*1232A created an eight-nucleotide octamer motif (ACATTCCA) that was the binding site for miR-1 and miR-206 (Clop *et al*., 2006). Binding of the miRNAs result in the translational inhibition of MSTN synthesis in skeletal muscle and, consequently, contributes to muscular hypertrophy. Further study of these miRs suggested that miR-206 may be fibre-type I specific and may have a role in the regulation of skeletal muscle hypertrophy (McCarthy & Esser, 2007). Decreased miR-1 expression during muscle hypertrophy may promote the activity of other genes such as c-Met, hepatocyte growth factor (HGF), insulin-like growth factor 1 (IGF-1), serum response factor (SRF) and leukaemia inhibitory factor (LIF) (McCarthy & Esser, 2007). All these genes are known to be involved in muscle growth, particularly IGF-1, which is also a potential target of miR-1, and has a well-established role in skeletal muscle hypertrophy (McCarthy & Esser, 2007).

MSTN c.*1232G>A has been utilized as a gene-marker to assist in the selection of sheep for improved muscle growth and carcass yields (Hadjipavlou *et al.*, 2008; Johnson *et al.*, 2009; Kijas *et al.*, 2007), and has been marketed under the name MyoMAX® and MyoMAX GOLD® (Catapult Systems Ltd, Pfizer Animal Health, Dunedin, NZ), or T+ Muscling (Lincoln University Gene-Marker Laboratory). The presence of *MSTN* c.*1232G>A has been also identified in sheep breeds other than Texel, and has been associated with increased muscle mass and decreased fatness in Charollais sheep (Hadjipavlou *et al.*, 2008), White Suffolk, Poll Dorset and Lincoln sheep (Kijas *et al.*, 2007) and Norwegian White Sheep (Boman *et al.*, 2010).

Clop *et al*. (2006) reported that the offspring of Belgian Texel inheriting the *MSTN* c.*1232A had heavier hindquarter yields than those carrying *MSTN* c.*1232G. Kijas *et al.* (2007) also demonstrated that the presence of *MSTN* c.*1232A had a significant

association with increased eye muscle depth, loin and forequarter yields and decreased carcass and intramuscular fat in Australian White Suffolk, Poll Dorset and Lincoln sheep. This nucleotide substitution was also studied by Hadjipavlou *et al*. (2008) who reported that *MSTN* c.*1232G>A increased muscle depth at the third lumbar vertebra in commercial Charollais sheep. A more recent study by Johnson *et al*. (2009) also reported that *MSTN* c.*1232A was associated with increased muscling and reduced fat traits in NZ Texel sheep. Furthermore, maximum benefit was achieved if the NZ Texel sheep carried two copies of the *MSTN* c.*1232A variation (Johnson *et al.*, 2009). In this NZ work, the increase in the size of the *M. longissimus* muscle was associated with increased eye muscle width and total area, but not eye muscle depth (Johnson *et al.*, 2009). This contrasts previous studies with *MSTN* c.*1232A which showed there were increases in eye muscle depth, but not width (Hadjipavlou *et al.*, 2008; Kijas *et al.*, 2007). Nevertheless, all trials have provided evidence of an increase in the total area of the *M. longissimus* muscle.

In sheep, a haplotype approach has been attempted to define the contribution effect of *MSTN* on carcass traits. Kijas *et al*., (2007) defined twenty haplotypes using nucleotide substitutions that included *MSTN* c.-41C>A, c.748-575C>A and c.*1232 G>A, along with two microsatellites *BM81124* and *BULGE20* that flank *MSTN*. Four of the haplotypes (Hap1, Hap2, Hap6 and Hap13), which do not carry c.*1232A, also had significant associations with muscling and fatness.

Gan *et al*., (2008) predicted twelve haplotypes (named Haplo1-12) using the PHASE program when four nucleotide substitutions including *MSTN* c.-41C>A, c.-781G>A, c.-956T>C and c.*1232G>A were selected for the study. Two haplotypes (Haplo2 and Haplo8) were suggested to be associated with the "double-muscled" trait. Haplo1-4 were found mainly in meat breeds [Beltex (Australia), Beltex × Huyang (B×F), Meat and Multi-prolific Chinese Merino Fine Wool (MPMF), Meat Chinese Merino Fine Wool (MF) and Dorper (South Africa)] and Haplo7 and Haplo9 dominated the non-meat breeds (Huyang and Kazak sheep) (Gan *et al.*, 2008).

In NZ, a recent trial by Hickford *et al*. (2010) revealed five different sequences (accession nos. FJ858196 – FJ858200, named A-E) in the intron 1 region of ovine *MSTN* in NZ Romney sheep. In this study, they reported the association of B with increased loin yield and proportion loin yield (loin yield divided by total yield expressed as percentage) and

association of A with decreased leg, loin and total yield of lean meat. Sjakste *et al*. (2011) defined five haplotypes (named Hap 1-5) in Latvian Darkhead sheep, but at least ten haplotypes of *MSTN* intron 1 were suggested by Sjakste *et al*. (2011) when the results of their work and that reported by Hickford *et al*. (2010), were taken together.

In contrast to the disadvantageous pleiotropic effect of *MSTN* with calving difficulties reported in "double-muscled" cattle, there are no reported negative phenotypes in sheep associated with *MSTN*. Kijas *et al*. (2007) reported that c.*1232A was possibly associated with a decreased in eating quality. This is due to the increased muscularity and reduced intramuscular fat, which may affect consumer preference. However, *MSTN* c.*1232A has been reported to have no effect on elevated shear force or other meat quality traits such as juiciness and flavour. Therefore, the potential pleotropic effects of *MSTN* and whether genetic variation in *MSTN* affects meat quality or other important traits warrants further investigation.

Previous research has revealed the high frequency of 90% of the *MSTN* c.*1232G>A in Texel sheep (Clop *et al.*, 2006; Kijas *et al.*, 2007). This suggests that there is a limitation on any further improvements in genetic gain in this breed by selecting for this variation (Kijas *et al.*, 2007) as the A-containing variant is nearly fixed in the population. Therefore, identifying if there is other variation in different sheep breeds is required before studies can be conducted to use the variations to capture meat yield benefits.

An extensive study of *MSTN* in a diverse range of common NZ sheep breeds is, therefore, essential. It might also be expected that if an increased number and diversity of sheep and breeds are studied, then further genetic variation may be found, including sequences that are unique to a breed, or at least very uncommon in other breeds. This would add new and valuable information on our understanding of the genetic diversity of ovine *MSTN* and provide a research platform for further investigation of its effect on growth and carcass traits.

A few studies have been attempted to define the haplotype diversity of ovine *MSTN* by using several nucleotide substitutions in a defined region (Hickford *et al.*, 2010; Sjakste *et al.*, 2011). However, the results only partially reflect the haplotype structure of *MSTN* due to the fact that only small fragments were screened in the reported studies. Other attempts have used SNPs along with microsatellite markers (Kijas *et al.*, 2007), or SNPs and

haplotype predicting software (Gan *et al.*, 2008) to characterise haplotype diversity across a larger region of *MSTN*. Extended haplotype diversity could be more informative than a single or several nucleotide substitution(s), especially when the extended haplotype covers large regions of a gene. Determination of the number of extended haplotypes of ovine *MSTN* and how they affect muscle growth in NZ sheep breeds has not been studied comprehensively.

In this thesis, polymerase chain reaction (PCR) coupled with a single strand conformational polymorphism (SSCP) analysis approach will be used to investigate haplotypic diversity in *MSTN* in various NZ sheep breeds. PCR-SSCP method is based on the relationship between the electrophoretic mobility of a single stranded DNA and it folded conformations, which in turn reflects the nucleotide sequence. It is a reliable, reproducible and effective analytical method for the detection of deletions, insertions, or rearrangement in PCR amplified DNA sequence (Hayashi, 1991; Konstantinos *et al.*, 2008). The approach was previously used by Hickford *et al.* (2010) who identified five haplotypes by targeting a 414bp fragment of the gene.

Given the highly variable characteristic of *MSTN*, where 78 nucleotide substitutions have already been identified (details refer to Appendix B), and given the sensitivity of the PCR-SSCP analytical method (Hayashi, 1991), it is conceivable that more haplotype diversity will be uncovered when an enlarged region of the ovine *MSTN* is investigated.

1.5 Aims of this study

1) To ascertain the extent of variation in ovine *MSTN* using polymerase chain reaction-single strand conformational polymorphism (PCR-SSCP) and sequencing analyses;

2) To define the extended haplotypes spanning the *MSTN* coding sequences in a range of NZ sheep breeds;

3) To investigate if associations exist between *MSTN* haplotypes and various growth and carcass traits in NZ Romney sheep;

Chapter 2

Investigating the extent of genetic variation and number of haplotypes of *MSTN* in New Zealand sheep breeds

Most studies to date have investigated specific regions within the *MSTN*. Little effort has been made to comprehensively characterise variation across an extended region of *MSTN* in a wide variety of commercial sheep breeds. This is important, as it is now generally accepted that regions up- and down-stream of genes, and in both the coding and non-coding sequences can affect gene expression (Babbitt *et al.*, 2010; Zavolan *et al.*, 2002). Little seems to be known about naturally occurring variation outside of the coding sequence in ovine *MSTN*, or of its effect on gene activity. There are also still parts of the coding region of the gene that are not well characterised, particularly in different breeds.

On the basis of the genetic variation identified, further clarification of the extent of haplotypic variation in different breeds would be invaluable in gaining a better understanding of MSTN function and its potential effect on growth and carcass characteristics. This is important as various features including the presence of alternative start and termination sites, alternative intron splice sites, variation in the intronic regions, and variation in other upstream and downstream sequences may affect the expression and function of the gene.

Until such time as a comprehensive knowledge of *MSTN* variation across an extended gene region and in a large variety of breeds of sheep is acquired, it would be difficult to ascertain how this variation might be used to improve sheep meat production.

The origin of any given breed may reflect the amount and type of variation found in *MSTN* (Kijas *et al.*, 2009; Peter *et al.*, 2006). For example, 41 of the nucleotide substitutions observed by Gan *et al*. (2008) in seven pure-bred and cross-bred Chinese sheep populations were not found by Clop *et al*. (2006) in twelve European sheep breeds (details refer to Appendix B). Genetic variation in the Asian populations would therefore be likely

to cluster separately from breeds of European origin, as are typically also found in Australia, New Zealand, South Africa and North America (Kijas *et al.*, 2009).

In NZ, although the majority of the sheep are largely derived from European breeding stock, the level of genetic variation in *MSTN* in these NZ sheep breeds could be different from their counterparts in Europe as a consequence of founder effects, genetic selection, cross-breeding and the introgression of new genetics (Tapio *et al.*, 2003). This makes it even more important to investigate the overall profile of genetic variation of *MSTN* in various common NZ sheep breeds, especially those that form the basis of NZ's lamb export industry.

Various studies have demonstrated associations between variation in *MSTN* and variation in muscling and other meat quality traits in sheep. Such studies have typically relied on the use of individual SNPs (Boman & Våge, 2009; Boman *et al.*, 2009; Johnson *et al.*, 2009), or limited numbers of SNPs in a discrete part of the gene (Hickford *et al.*, 2010; Sjakste *et al.*, 2011). These SNPs are often assumed to describe diversity spanning the whole gene. However, this is likely to weaken these studies, as genetic variation outside of the analysed region, which may be of consequence to gene function, may not be adequately marked, and/or intragenic meiotic recombination may result in greater diversity than revealed by a single nucleotide or group of SNPs.

Other approaches that have used SNPs along with microsatellite markers (Kijas *et al.*, 2007), or SNPs and haplotype predicting software (Gan *et al.*, 2008) have attempted to characterise haplotypic diversity across a larger region of *MSTN*. However, these approaches may also lack the necessary precision and therefore resolving all, or as much as possible, of the haplotypic diversity of *MSTN* is still critically important; before associations with growth or carcass characteristics can be properly investigated.

In this chapter, an examination of genetic variation in ovine *MSTN* across a range of common sheep breeds in NZ was taken initially. Polymerase chain reaction (PCR) amplification coupled with single strand conformational polymorphism (SSCP) analysis and DNA sequencing was used to describe variation across the *MSTN* gene. Subsequently, an effort was made to resolve haplotypes within an extended region of ovine *MSTN*. The PCR-SSCP method was used to investigate the occurrence of extended haplotypes in both

coding and non-coding regions and that may be functionally involved in gene function or expression.

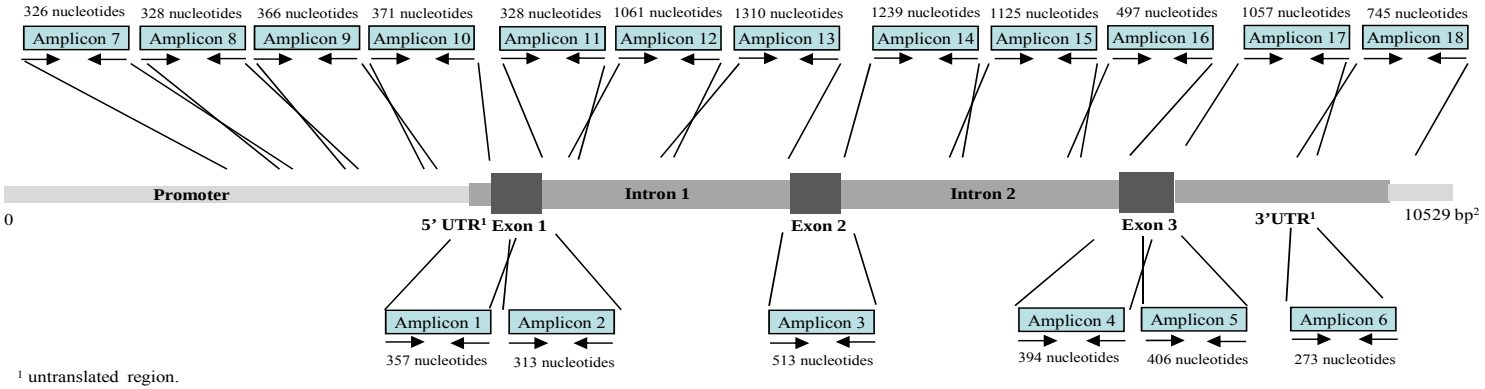
2.1 Materials and methods

Sheep investigated and DNA samples collected

A total of 218 sheep, selected from a variety of common breeds in NZ including NZ Romney (n= 72), Coopworth (n=12), Corriedale (n=12), Dorper (n=12), Perendale (n=12), Suffolk (n=12), Merino (n=12), Dorset Down (n=19), Coopdale (n=7), Poll Dorset (n=21), Texel (n=10) and other cross-bred sheep (n=17), were used to identify genetic variation in ovine *MSTN*. Of these twelve sheep breeds, the NZ Romney, Perendale, Coopworth, Corriedale and Coopdale are considered dual-purpose breeds for both meat and wool production. The Dorper, Poll Dorset, Suffolk, Dorset Down, and particularly the Texel are classified as meat sheep breeds, while the Merino is considered to be a wool breed. Blood samples were collected onto FTA cards (Whatman, Middlesex, UK) and genomic DNA was purified using a two-step procedure (Zhou *et al.*, 2006).

PCR amplifications

Eighteen sets of PCR primers (Figure 2.1, Primer sets 1-18) spanning ovine *MSTN* were designed, based on a NCBI GenBank gene sequence (accession number DQ530260, Clop *et al*., 2006). These eighteen sets of primers were overlapping and included both shorter amplicons that could be used for PCR-SSCP screening (primer sets 1-11) and larger amplicons that could be used subsequently for direct sequencing (primer sets 12-18).



[1] untranslated region.
[2] The GenBank accession number is DQ530260.

Amplicon region	Amplicon size (bp)	Forward primer (5'-3')/ Reverse primer (5'-3')	PCR Annealing Temp (°C)	Polyacrylamide Gel Concentration (%)	SSCP Electrophoresis Condition	Number of SSCP pattern obtained	GenBank Accession number
Amplicon 1	357	acacttgtctcatcaaagttg/tatggcttctagtcttgagg	58	8	180 V for 19 h at 7.5°C	3	JN572923 - JN572925
Amplicon 2	313	gtaatccatgcttgtggagac/acactagaacagcagtcag	58	12	280 V for 19 h at 7.5°C	2	JN856457 & JN856458
Amplicon 3	513	ctaaatatgcacacattattcc/gcatgttatttttcagtaatcact	59	11	390 V for 19 h at 7.5°C	2	JN856459 & JN856460
Amplicon 4	394	actcttctttccttttccatac/cacagcgatctactaccatg	59	14	390 V for 19 h at 7.5°C	1	JN856461
Amplicon 5	406	gtactcctacaaagatgtctc/agaattgctttcattcacctg	60	11	390 V for 19 h at 7.5°C	2	JN856462 & JN856463
Amplicon 6	273	aattagttgattaaatagtggt/acaatttgtaagataccatcag	55	14	390 V for 19 h at 7.5°C	2	JN856464 & JN856465
Amplicon 7	326	atccctgccaggagtctg/tacaaagaggatattgtcagc	60	14	390 V for 19 h at 7.5°C	2	JN856466 &JN856467
Amplicon 8	328	gtcattctaagttattctaagatc/agaaaatacagatttattcagg	60	14	390 V for 19 h at 7.5°C	1	
Amplicon 9	366	atcacaatcttttcatttaagtc/ttgttacagtcaagggtgag	56	14	320 V for 19 h at 10°C	1	
Amplicon 10	371	aagaagtagtcaaatgaatcag/caacaagcagcataaataggt	61	8	390 V for 19 h at 4°C	2	
Amplicon 11	328	acggaaacggtcattacca/attaagctgtgaaaacataaac	59	11	150 V for 22 h at 10°C	5	JN856468 - JN856472
Amplicon 12	1061	atatgctaatgagactgaaag/acactgtctttagggtcag	58	-	-	-	JN856473 - JN856476
Amplicon 13	1310	actattgttgagagtacctg/aagtttcagagatcggattc	59	-	-	-	JN856477 & JN856478
Amplicon 14	1239	agagcattgatgtgaagac/tcatttggggagttaataac	55	-	-	-	JN856479 & JN856480
Amplicon 15	1125	ctatgcttgtatttacttctg/ctaatcattttgtttatgtcac	54	-	-	-	JN856481 & JN856482
Amplicon 16	497	tgtcatccattagtatattcag/attcacattctccagagcag	56	-	-	-	JN856483 & JN856484
Amplicon 17	1057	acagtatatgaactaaaagag/attatacagccatcacgaac	56	-	-	-	JN856485 - JN856487
Amplicon 18	745	ttaaatagtggtcttaaaactc/agaacttgtaactctaggac	56	-	-	-	JN856488 & JN856489

Figure 2.1 The amplicons and primer sets for PCR-SSCP and sequencing analysis in ovine *MSTN* (These primer sets were designed based on the ovine *MSTN* sequence GenBank Accession No. DQ530260)

PCR amplifications were performed in 20 µL reactions containing the genomic DNA on a 1.2 mm diameter disc of FTA paper, 0.25 µM of each primer, 150 µM dNTPs (Eppendorf, Hamburg, Germany), 2.5 mM $MgCl_2$, 0.5 U *Taq* DNA polymerase (Qiagen, Hilden, Germany) and 1 x the reaction buffer supplied with the enzyme. Amplification was carried out in an iCycler (Bio-Rad Laboratories, Hercules, CA, USA).

For the primer sets 1-11, the thermal profile consisted of denaturation at 94°C for 2 mins, followed by 35 cycles of 94°C for 30 s, annealing for 30 s at either 55°C, 56°C, 58°C, 59°C, 60 °C or 61°C depending on the primer set used (see Figure 2.1) and extension at 72°C for 30 s, followed by a final extension step at 72°C for 5 mins. The annealing step for primer set 3 was for 40 s in each cycle.

For primer sets 12-18, the thermal profile consisted of denaturation at 94°C for 2 mins, followed by 38 cycles of 94°C for 30 s, annealing at either 54°C, 55°C, 56°C, 58°C or 59°C for the various regions (see Figure 2.1) for 30 s and extension at 72°C for 40 s, with a final extension step at 72°C for 5 mins.

The amplicons from the various PCR reactions were visualized by electrophoresis in 1% agarose (Quantum Scientific, Queensland, Australia) gels using 1 x TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM Na_2EDTA), containing 200 ng/mL ethidium bromide. A 2 µL aliquot of PCR product was added to 2 µL of loading dye (0.2% bromophenol blue, 0.2% xylene cyanol, 40% (w/v) sucrose) and the gels were run at a constant 10 V/cm for 10 min, prior to visualization by UV transillumination at 254 nm.

Detection of genetic variation using SSCP and clone screening

For amplicons 1-11, SSCP was used to ascertain the nucleotide differences present in any given amplicon. A 0.7 µL aliquot of each PCR product was mixed with 7 µL of loading dye (98% formamide, 10 mM EDTA, 0.025% bromophenol blue, 0.025% xylene-cyanol). After denaturation at 95°C for 5 min, samples were rapidly cooled on wet ice and then loaded on 16 x 18 cm, acrylamide: bisacrylamide (37.5:1, Bio-Rad) gels. Electrophoresis was performed using Protean II xi cells (Bio-Rad), at different gel percentages and running conditions as listed in Figure 2.1, and in 0.5 x TBE buffer. Gels were silver-stained according to the method of Sanguinetti *et al.* (1994).

To reveal sequence variation in the amplicons produced by primer sets 1-11, DNA samples representative of different PCR-SSCP patterns were amplified using *Pwo* Super Yield DNA polymerase (Roche Applied Science, Mannheim, Germany), according to the conditions listed in Figure 2.1. The amplicons were ligated into the pCR 4 Blunt-TOPO vector (Invitrogen, Carlsbad, CA, USA). A 2 μL aliquot of the ligation mixture was used to transform competent *E. coli* cells (One Shot INVαF' Invitrogen), following the manufacturer's instructions. Twelve insert positive colonies for each transformation were picked and incubated overnight in Terrific broth (Invitrogen) at 37°C, in a shaking (225rpm) rotary incubator.

Clones were screened using a clonal PCR-SSCP approach (Zhou & Hickford, 2008). Only those clones, for which the SSCP patterns matched those of the corresponding genomic DNA, were selected for subsequent DNA sequencing.

Plasmid DNA from selected clones was extracted using a QIAprep Spin Miniprep kit (Qiagen), and sequenced in both directions at the Lincoln University DNA typing facility using the M13 forward and reverse primers. Identical sequences obtained from at least three clones, from three separate colonies, were subjected to further sequence analysis. Sequence alignment, translation and comparisons were performed using DNAMAN (Version 5.2.10, Lynnon BioSoft, Vaudreuil, Canada).

Detection of genetic variation using direct sequencing

Sheep that produced simple PCR-SSCP patterns with any of primer sets 1-11, suggesting these sheep were homozygous in that region, were subjected to direct sequencing using amplicons derived from primer sets 12-18 in breeds in Texel, Merino, Dorset Down, Poll Dorset and other cross-bred sheep. DNA from each amplicon was extracted using a MinElute^{TM} PCR Purification Kit (Qiagen) and sequenced by the Lincoln University DNA Sequencing Facility using the same PCR primers to prime the sequencing reactions. Each purified amplicon was sequenced in triplicate, in both directions, using the forward and reverse PCR primers. Identical sequences obtained from the triplicated reactions were subjected to subsequent sequence alignment, translation and other comparisons using DNAMAN (Version 5.2.10, Lynnon BioSoft, Vaudreuil, Canada).

Identification of *MSTN* extended haplotypes

Four sets of PCR primers were used to amplify regions (amplicons 1, 5, 6 and 11) of *MSTN* as described in Figure 2.1. These amplicons were subjected to SSCP genotyping and specific sequences identified for each variant.

The combinations of different PCR-SSCP patterns enabled the identification of extended ovine *MSTN* haplotypes. To resolve these extended haplotypes, sheep homozygous for one PCR-SSCP pattern (and sequence) in a region of the gene, were then genotyped in another region of the *MSTN*, to ascertain whether they were homozygous or heterozygous in that second region. If heterozygous in the second region, then two haplotypes, spanning the first and second regions, could be defined. Utilization of this process across amplicons 1, 5, 6 and 11 specifically, enabled the extended haplotypes of *MSTN* in the 218 sheep examined in detail to be ascertained.

2.2 Results

Nucleotide substitutions identified by the sequencing screening test

A total of eighteen sets of primers were used to produce eighteen amplicons spanning a genomic region of 8,003bp. This included the ovine *MSTN* promoter, 5'UTR, three coding regions, two introns and the 3'UTR (Figure 2.1). Sequences derived from each amplicon were submitted to GenBank with the accession number listed in Figure 2.1.

A total of 28 nucleotide substitutions were identified using PCR-SSCP coupled with sequencing analysis (Table 2.1) (Note: recommended nucleotide numbering DNA reference sequence nomenclature is used in this thesis as per www.hgvs.org/mutnomen/recs-DNA.html#number, dated 25 July 2011. Nucleotide coordinates are based on ovine GenBank sequence DQ530260). Of these 28 substitutions, three were located in the promoter region, three in the 5'UTR, eleven in intron 1, five in intron 2, and five in the 3'UTR (Table 2.1). One exon 1 substitution (c.101G>A) results in an amino acid substitution of glutamic acid (Glu) with glycine (Gly) at codon 34 (c34, Table 2.1). Ten substitutions at positions c.-959C>T, c.-784A>G, c.373+563A>G, c.373+607A>G, c.374-654G>A, c.374-54T>C, c.748-54T>C, c.*83A>G, c.*455A>G and c.*709C>A are newly reported.

Table 2.1 Nucleotide substitutions found within ovine *MSTN* in various NZ sheep breeds (The recommended nucleotide numbering nomenclature is used: www.hgvs.org/mutnomen/recs-DNA.html#number, dated 25 July 2011. The nucleotide coordinates are based on NCBI GenBank accession number DQ530260. Substitutions that are bolded are newly identified. Symbol x marks whether the substitution was identified in the breed).

Breed	Nucleotide substitutions																														
	Promoter			5'UTR			Exon 1	Intron 1												Intron 2					3'UTR						
								c.373+									c.374-			c.747+	c.748-										
	c.-1129 C>T	**c.-959 C>T**	**c.-784 A>G**	c.-41 A>C	c.-38 C>T	c.-31 delT	c.101 G>A	18 T>G	241 C>T	243 A>G	246 C>T	249 C>T	259 T>G	323 T>C	**563 A>G**	**607 A>G**	**654 G>A**	**54 T>C**	164 G>A	810 T>C	575 C>A	568 T>C	**54 T>C**	**c.*83 A>G**	**c.*455 A>G**	**c.*709 C>A**	c.*1232 G>A	c.*1316 A>G				
Coopworth						x		x	x	x		x	x																		
Corriedale				x	x	x	x	x	x	x	x	x	x					x													
Coopdale						x		x	x	x		x	x																		
NZ Romney				x		x	x	x	x	x		x	x																		
Perendale						x			x	x																					
Dorper					x	x		x	x	x	x		x					x													
Dorset Down	x	x			x	x		x	x	x	x		x			x		x	x	x	x			x							
Poll Dorset						x		x		x		x	x											x							
Suffolk						x		x	x	x		x	x																		
Texel						x																			x		x				
Merino	x	x		x		x	x	x	x	x			x			x		x	x	x	x			x							
NZ cross-bred	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x		x	x	x	x	x	x	x	x		x	x	x				

Different SSCP patterns detected using PCR-SSCP

Of the 218 sheep genotyped, haplotypic variation could be defined based on the PCR-SSCP patterns detected for amplicons 1, 5, 6 and 11 (Figure 2.2). These four amplicons encompass the 5'UTR, coding sequence, intron splice sites and 3'UTR and generated three patterns for amplicon 1 (named *A*, *B* & *C*), two patterns for amplicon 5 (named *A* & *B*), two patterns for amplicon 6 (named *A* & *G*) and five patterns for amplicon 11 (named *A*, *B*, *C*, *D* and *E*), respectively. In these regions, the substitutions included: c.-41A>C, c.-38C>T in the 5'UTR region of *MSTN*; c.101G>A in Exon 1; c.373+18T>G, c.373+241C>T, c.373+243A>G, c.373+246C>T, c.373+249C>T and c.373+259T>G in intron 1; and c.*83A>G and c.*1232G>A in the 3'UTR region.

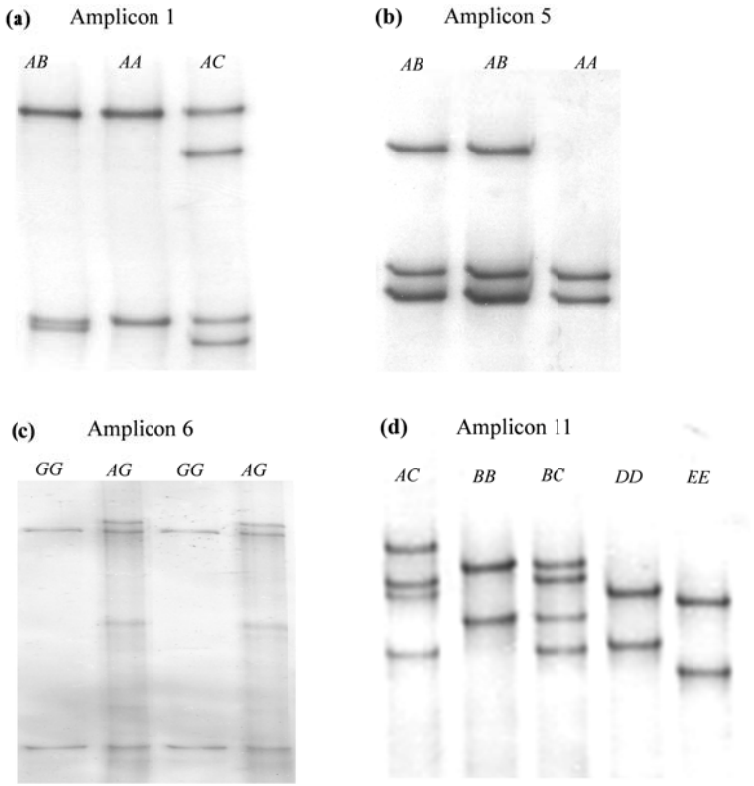


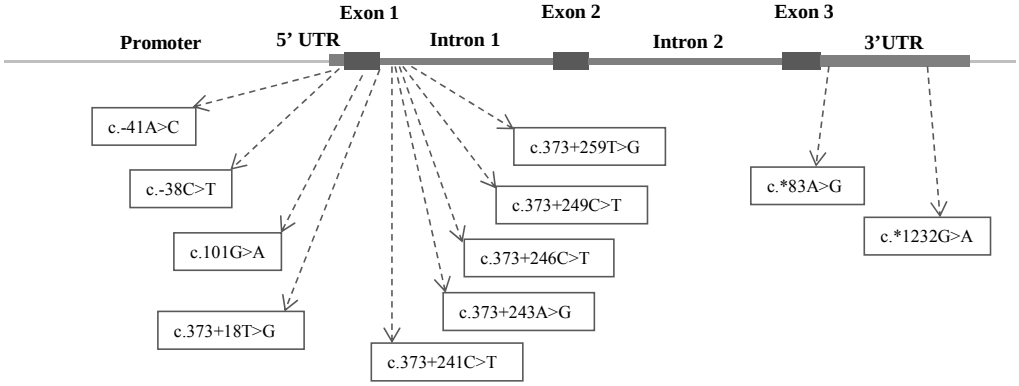
Figure 2.2 PCR-SSCP patterns detected for amplicons 1, 5, 6 and 11. (a) three banding patterns (*A*, *B* and *C*) were detected for amplicon 1; (b) two banding patterns (*A* and *B*) were detected for amplicon 5; (c) two banding patterns (*A* and *G*) were detected for amplicon 6; and (d) five banding patterns (*A*, *B*, *C*, *D* and *E*) were detected for amplicon 11.

Eight extended haplotypes identified in ovine *MSTN*

Haplotypic diversity was characterised using the various PCR-SSCP patterns detected for amplicons 1, 5, 6 and 11. Based on this typing, eight extended haplotypes (designated H1-

8), with a total of eleven nucleotide substitutions were defined. These are summarised in Figure 2.3.

The nucleotide substitutions detected included: c.-41A>C, c.-38C>T in the 5'UTR region of *MSTN*; c.101G>A in Exon 1; c.373+18T>G, c.373+241C>T, c.373+243A>G, c.373+246C>T, c.373+249C>T and c.373+259T>G in intron 1; and c.*83A>G and c.*1232G>A in the 3'UTR region (Figure 2.3).



Haplotype	Amplicon 1				Amplicon 11							Amplicon 5		Amplicon 6	
	Nucleotide			SSCP	Nucleotide						SSCP	Nucleotide	SSCP	Nucleotide	SSCP
	c.-41	c.-38	c.101	pattern	c.373+18	c.373+241	c.373+243	c.373+246	c.373+249	c.373+259	pattern	c.*83	pattern	c.*1232	pattern
H1	A	T	A	*A*	G	T	G	T	T	G	*E*	G	*A*	A	*A*
H2	A	T	A	*A*	G	T	G	T	T	G	*E*	G	*A*	G	*G*
H3	A	T	A	*A*	T	C	A	T	T	T	*A*	G	*A*	G	*G*
H4	A	T	A	*A*	T	T	G	T	T	T	*C*	G	*A*	G	*G*
H5	A	T	A	*A*	T	T	A	T	C	T	*B*	G	*A*	G	*G*
H6	A	C	A	*B*	T	T	A	C	T	T	*D*	G	*A*	G	*G*
H7	C	T	G	*C*	G	T	G	T	T	G	*E*	G	*A*	G	*G*
H8	A	T	A	*A*	G	T	G	T	T	G	*E*	A	*B*	G	*G*

Figure 2.3 Eight extended haplotypes identified in ovine *MSTN*

Extended haplotypes of *MSTN* in NZ sheep

The frequencies of the eight extended haplotypes identified in the various sheep breeds investigated are given in Table 2.2. Of the eight haplotypes identified, haplotype H1 carrying *MSTN* c.*1232G>A was only present in Texel sheep and NZ cross-bred sheep. Haplotype H2 was the most common haplotype with a high frequency in all the meat, wool and dual-purpose sheep breeds studied. Haplotype H3 was common in dual-purpose and wool breeds, but not in the meat breeds, except the Dorper and Suffolk sheep. Haplotype 7 was only present in NZ Romney, Corriedale and Merino sheep, but not in the meat breeds. H8 was only detected in NZ cross-bred sheep.

Table 2.2 The frequency of the eight haplotypes in the various sheep breeds studied

Breed	Number of sheep investigated	Frequencies of haplotypes identified (%)								Breed Description
		H1	H2	H3	H4	H5	H6	H7	H8	
Coopworth	12		54.17	8.33		37.50				Dual-purpose breed
Corriedale	12		66.67	4.17	8.33	12.50	4.17	4.17		Dual-purpose breed
Coopdale	7		71.43	14.29		14.29				Dual-purpose breed
NZ Romney	72		63.19	22.22		13.89		0.69		Dual-purpose breed
Perendale	12		87.50	12.50						Dual-purpose breed
Dorper	12		50.00	12.50	20.83		16.67			Meat breed
Dorset Down	19		76.32	2.63			21.05			Meat breed
Poll Dorset	21		95.83			4.17				Meat breed
Suffolk	12		58.33	16.67	12.50	12.50				Meat breed
Texel	10	56.25	43.75							Meat breed
Merino	12		63.64	9.09	9.09			18.18		Wool breed
NZ cross-bred	17	3.85	69.23	3.85	3.85	7.69	3.85	3.85	3.85	Cross-bred

2.3 Discussion

To better understand the distribution of natural genetic variation across ovine *MSTN*, this investigation focused on exploring genetic diversity of *MSTN* in twelve different commercial sheep breeds representing specific meat or wool breeds, or dual-purpose sheep breeds in NZ. An extended region spanning nucleotide c.-1199 from the promoter region to c.*1813 in the 3'UTR of ovine *MSTN* was investigated using PCR-SSCP analysis. This method allows detection of variation in the genomic DNA, through the variable mobility in polyacrylamide gels of separated single-strands of denatured PCR amplicons (Hayashi, 1991). This technique provides a reliable, sensitive and cost-effective measure for the detection of variation in genomic DNA (Hayashi, 1991), and has enabled previously un-described variation in *MSTN* to be identified in this work.

In this study, the terms "nucleotide substitution" and "genetic variation" have been used to describe the differences identified in gene sequences. It is believed that these terms are more precise than using the phrases "mutation" or "SNP" to define variation in genetic sequences (Cotton, 2002). The latter two phrases have caused confusion historically when describing genetic changes or variations as the phrase "mutation" usually has a negative connotation and refers to nucleotide changes that cause deleterious effects (Condit *et al.*, 2002). "SNP" stands for single nucleotide polymorphism, but is typically used to refer to a single nucleotide change. The combination of a number of nucleotide changes can create polymorphism. Accordingly the terms "nucleotide substitution" and "genetic variation" are used preferentially as they are considered to be more accurate and neutral.

Of the total of twenty eight nucleotide substitutions identified across the *MSTN* region studied (Table 2.1), eighteen have been reported previously. These include c.-1129C>T (Clop *et al.*, 2006) in the promoter; c.-41A>C (Clop *et al.*, 2006; Gan *et al.*, 2008; Kijas *et al.*, 2007; Sjakste *et al.*, 2011), c.-38C>T (Clop *et al.*, 2006; Sjakste *et al.*, 2011) and c.-31delT (Boman & Våge, 2009; Boman *et al.*, 2009; Du *et al.*, 2005; Sjakste *et al.*, 2011; Zhou *et al.*, 2008) in the 5'UTR; c.101G>A (Zhou *et al.*, 2008) in the Exon 1 coding region; c.373+18 T>G (Clop *et al.*, 2006; Gan *et al.*, 2008; Hickford *et al.*, 2010; Kijas *et al.*, 2007; Sjakste *et al.*, 2011; Zhou *et al.*, 2008), c.373+241C>T (Hickford *et al.*, 2010; Sjakste *et al.*, 2011), c.373+243A>G (Clop *et al.*, 2006; Gan *et al.*, 2008; Hickford *et al.*, 2010; Sjakste *et al.*, 2011), c.373+246C>T and c.373+249C>T (Clop *et al.*, 2006; Hickford *et al.*, 2010; Sjakste *et al.*, 2011), c.373+259T>G and c.373+323T>C (Clop *et al.*, 2006; Gan *et al.*, 2008; Hickford *et al.*, 2010; Sjakste *et al.*, 2011) in the intron 1; c.747+164G>A (Gan *et al.*, 2008), c.748-810T>C (Gan *et al.*, 2006), c.748-575C>A (Clop *et al.*, 2006; Gan *et al.*, 2008; Kijas *et al.*, 2007) and c.748-568T>C (Clop *et al.*, 2006; Gan *et al.*, 2008) in the intron 2; and c.*1232G>A (Boman & Våge, 2009; Boman *et al.*, 2010; Clop *et al.*, 2006; Gan *et al.*, 2008; Hadjipavlou *et al.*, 2008; Hickford *et al.*, 2009; Johnson *et al.*, 2005; Kijas *et al.*, 2007; Takeda *et al.*, 2010) and c.*1316A>G (Heaton *et al.*, 2007) in the 3'UTR. These substitutions together with the ten newly reported substitutions, suggest ovine *MSTN* is highly variable, and indicate that the potential exists for the variation to affect MSTN activity.

Substitution c.101G>A is the only genetic variation identified in a coding region (exon 1). It is located in the pro-peptide domain of ovine *MSTN* (McPherron *et al.*, 1997). This variation leads to an amino acid substitution of Glu with Gly at codon 34 and was also observed by Zhou *et al*. (2008) in NZ Romney sheep. This result is in contrast to a statement made previously, that no genetic variation occurs in the ovine *MSTN* coding region (Clop *et al.*, 2006; Cockett *et al.*, 2001; Marcq *et al.*, 2002). The non-detection of the variation in the coding region in the abovementioned reports is likely because they looked at a limited number of sheep and/or used less sensitive typing methods (Zhou *et al.*, 2008). The Glu at codon 34 in *MSTN* is highly conserved across species (McPherron & Lee, 1997), although a comparable nucleotide substitution (nt267A>G in exon 1 of cattle) was reported by Dunner *et al*. (2003). This substitution does not however lead to an amino acid change in cattle *MSTN*. It therefore appears that c.101G>A is unique to sheep (Zhou *et al.*, 2008). Glu and Gly are different amino acids with Glu having a charged side chain,

whereas there is an uncharged aliphatic side chain on Gly (Zubay, 1998). This amino acid difference may affect the inhibitory function of the MSTN during muscle growth and subsequently affect meat yield in sheep.

In the promoter region, some motifs are important for MSTN transcriptional regulation (Du *et al.*, 2007a; Du *et al.*, 2007b). The newly reported substitution c.-784A>G is located at the binding site of the Octamer-binding factor 1 (Oct-1) motif (TGCAATTTTCATATG) in the promoter region. Oct-1 has been reported to be one of the ubiquitously expressed members of the POU-domain gene families and these have a wide variety of functions related to the development of an organism (Sturm *et al.*, 1988). It is possible that this variation may be involved in *MSTN* transcriptional regulation via the Oct-1 mediated pathway during animal development, particularly as *MSTN* expression has been detected in different organs, such as heart (Sharma *et al.*, 1999), spleen (Helterline *et al.*, 2007), brain (Ng *et al.*, 2007) and mammary glands (Ji *et al.*, 1998) and in a variety of animals.

Motifs including some putative muscle growth response elements such as activator protein (AP1) and glucocorticoid response elements (GRE) have also been found in the ovine *MSTN* promoter region, and possibly play a role in the regulation of the sheep MSTN function (Du *et al.*, 2007b; 2005). This leads to the idea that genetic variation in the promoter may affect the transcriptional regulation of *MSTN* (Du *et al.*, 2007a; Du *et al.*, 2007b; Du *et al.*, 2005) or *MSTN* mRNA stability (Cieślak *et al.*, 2003), and thus have influence on skeletal muscle development. Further studies may be needed to clarify the regulatory roles of this promoter variation.

Of the eleven substitutions detected in ovine *MSTN* intron 1, variation at position c.373+18 T>G is very close to the splice donor site. This variation was also found in Texel sheep (Clop *et al.*, 2006) and Latvian Darkhead sheep (Sjakste *et al.*, 2011). A similar splice site variation, identified as g.IVS1+5 (nucleotide position c.373+5G>A), has also been identified in a child with a muscular hypertrophy phenotype (Schuelke *et al.*, 2004). Genetic variation located at intronic boundary regions may affect mRNA splicing, and subsequently affect the amino acid sequence produced from the transcript (Zavolan *et al.*, 2002). It results in a splicing error by changing the core donor site (Wang & Burge, 2008) and thus influenced the pre-mRNA secondary structure during skeletal muscle development (Sjakste *et al.*, 2011). This could then influence the MSTN function (Hiller *et al.*, 2007) and subsequently contribute to the increased muscle mass phenotype in sheep.

Characterisation of the extent of *MSTN* variation is thought to be essential for further assessment of association between the genetic diversity of *MSTN* and variation in meat or carcass traits. Use of fewer or less well characterised nucleotide variations in a limited number of sheep from any given breed could confound the benefit of *MSTN* variation to sheep meat production. On the basis of the genetic variations identified in this study, further clarification of the extent of extended haplotypes of ovine *MSTN* would be valuable to gaining a better understanding of *MSTN*'s structure and function.

In this study, four regions encompassing the 5'UTR, entire coding sequence and 3'UTR were used to define the extended haplotype diversity of *MSTN* due to the constraints of funding, time and availability of the experimental method. These four regions are considered very important as they contain various features including the presence of alternative start and termination sites, alternative intron splice sites, variation in the intronic regions, and variation in the UTR which may affect the expression and function of genes (Babbitt *et al.*, 2010; Zavolan *et al.*, 2002), and hence also be important in the biology of MSTN. This approach has only provided a description of haplotypic diversity in the regions typed and may not be able to reveal the full extent of the *MSTN* haplotype diversity. It is also conceivable that greater haplotype diversity may exist if all 28 nucleotide substations were used to construct extended *MSTN* haplotype.

Although the structure of haplotypes could also have been generated using long-range gene sequencing, resolution of heterozygous sequences may be challenging using electropherogram traces, especially if insertions and deletions have occurred (Dmitriew & Rakitov, 2008), as might be expected especially in non-coding DNA.

The cost of sequencing the entire *MSTN* gene (approximately 10k bp in length) is decreasing, but it is still an expensive option and currently impractical for industrial commercial DNA testing purposes. In contrast, PCR-SSCP allows the detection of haplotypes directly using a strategic typing approach. It also enables the typing of a large number of samples, while also easily resolving heterozygous individuals.

Based on the sequencing of the eight haplotypes in this study, the eleven nucleotide substitutions found in the defined regions are more than the genetic variations analysed in previous reports (refer to Appendix C for haplotype comparison between this study and the reports of Gan *et al.*, 2008; Hickford *et al.*, 2010; Kijas *et al.*, 2007 and Sjakste *et al.*,

2011). Of the substitutions, c.-41A>C (Gan *et al.*, 2008; Kijas *et al.*, 2007; Sjakste *et al.*, 2011) and c.-38C>T (Sjakste *et al.*, 2011) in the region upstream of the ATG codon may affect transcription. Nucleotide substitutions c.373+18T>G, c.373+241C>T, c.373+243A>G, c.373+246C>T, c.373+249C>T and c.373+259T>G located in intron 1 region, were as described previously by Hickford *et al.* (2010) and Sjakste *et al*. (2011). The phenotypic consequence of c.* 1232G>A has been well documented in Texel sheep and this variation has been used previously to define *MSTN* haplotypes by Gan *et al*. (2008) and Kijas *et al*. (2007).

Haplotype H7 and H8 carry the nucleotide substitutions c.101A>G and c.*83G>A respectively are reported here for the first time. Substitution c.101G>A is the only variation identified in the coding region and leads to the amino acid substitution of Glu with Gly at codon 34. This has been reported previously in NZ sheep by Zhou *et al*. (2008). The substitution of c.*83A>G in the 3'UTR may create microRNA targets that bind to miRNAs. This may suppress or block *MSTN* mRNA translation and lead to different phenotypes in sheep.

Of the eight haplotypes identified, H3, H4, H5 and H6 had identical nucleotide sequences from c.373+18 to c.373+259 when compared with haplotypes B, E, C and D respectively as reported by Hickford *et al*. (2010). Haplotype A reported by Hickford *et al*. (2010) was further divided into four different haplotypes; H1, H2, H7 and H8. This was likely to be because only a small fragment of intron 1 (414 bp in length) was investigated by Hickford *et al*. (2010) and the genetic variations defining H1, H2, H7 and H8 were not located in this small region. This suggests that the technique developed in this chapter has advanced our understanding of genetic diversity in ovine *MSTN*.

All of the eight *MSTN* haplotypes identified here carry nucleotide c.373+101T. This is consistent with the work reported by Hickford *et al*. (2010) in other NZ sheep breeds, but contrasts with the results of Sjakste *et al*. (2011), who reported that haplotypes Hap 1-5 identified in Latvian Darkhead sheep carry nucleotide c.373+101C. Although the nucleotide substitution c.373+101T>C was reported in a Romanov x Texel F2 population (Clop *et al.*, 2006), this variation was not identified in the current study, or by Hickford *et al*. (2010) or Sjakste *et al*. (2011). This genetic divergence presumably reflects breed differences.

It is difficult to cross-match the haplotypes identified in this chapter with those identified by Kijas *et al*. (2007) and Gan *et al*. (2008), with the exception of the variation at c.-41and c.*1232. Different haplotyping approaches were used in the above mentioned studies. Kijas *et al*. (2007) reported twenty haplotypes based on three nucleotide substitutions, c.-41A>C, c.784-573A>C and c.*1232G>A and two flanking microsatellites BM81124 and BULGE20. However, the reported haplotypes may not represent functionally significant variation in *MSTN*, especially given the highly variable nature of microsatellite markers (Vali *et al.*, 2008) and the absence of evidence that they affect *MSTN* expression. Gan *et al*. (2008) used four nucleotide substitutions (c.-956C>T, c.-781G>A, c.-41C>A, c.*1232G>A) and haplotype predicting software called PHASE to define haplotypes of *MSTN*. The reliability of this software however depends on marker density and missing genotype data imputation. The limitation of the software is that it can only accommodate a small number of samples (Browning, 2008). PCR-SSCP was therefore chosen as the haplotyping method for this study and because large number of samples can easily be analysed.

Haplotype H1 carrying c.*1232A had a substitution c.-41A which differentiates it from haplotype 4 (nucleotide c.-41C linked with c.*1232A) as described by Kijas *et al*. (2007). Both of these haplotypes were never-the-less described by Gan *et al*. (2008).This likely reflects differences in the breeds investigated, but it also suggests that further investigation of haplotypic diversity across various breeds is needed. Taking into account the various haplotypes identified in the current study and those reported by Sjakste *et al*. (2011), Hickford *et al*. (2010), Gan *et al*. (2008) and Kijas *et al*. (2007), it could be concluded that at least fourteen haplotypes of *MSTN* are distinguishable in sheep. It is also conceivable that even more *MSTN* haplotypes may exist, if more samples across more sheep breeds were to be analysed.

The detection of haplotype H1 carrying *MSTN* c.*1232A in Texel sheep in the current study was expected, because the presence of this particular nucleotide substitution and its effect on muscular hypertrophy has been well documented in this breed (Clop *et al.*, 2006; Hadjipavlou *et al.*, 2008; Johnson *et al.*, 2009; Kijas *et al.*, 2007). However, the frequency of *MSTN* c.*1232A in Texel sheep in the current study (56.25%) was lower than reported previously, where frequencies of up to 85-95% have been reported (Clop *et al.*, 2006; Hadjipavlou *et al.*, 2008; Johnson *et al.*, 2009; Kijas *et al.*, 2007). This is likely to be the

result of only a few Texel sheep being tested and the majority of these Texels being heterozygous for *MSTN* c.*1232A>G.

Haplotype H1 was also observed in NZ cross-bred sheep with a low frequency of 3.85%. One explanation is that some of these sheep have been cross-bred with Texel sheep in an effort to increase meat yield and thus H1 was introduced into their genetic profile. The introgression of *MSTN* c.*1232A from Texel into other sheep breeds has been suggested by Kijas *et al.* (2007) when the pedigree of the various animals were analysed. Alternatively, it might be speculated that c.*1232A was initially present in all breeds, but has subsequently been lost or bred against in many breeds. *MSTN* c.*1232A has been reported in other breeds such as Charollais sheep in Britain with frequency of 30% (Hadjipavlou *et al.*, 2008), and in White Suffolk, Poll Dorset and Lincoln sheep in Australia with low frequencies of approximately 10% (Kijas *et al.*, 2007). However, haplotype H1 was not identified in Poll Dorset sheep in the current study. This is presumably because limited numbers of samples were typed. This supports the contention that haplotype frequency may only reveal information about the typed samples and not about the whole breed. It is suggested that further phylogenetic analyses are needed to ascertain further diversity in *MSTN* and the frequency of these haplotypes in various sheep breeds.

Of the various commercial sheep breeds tested in the current study, H2 was found to be the most common haplotype with the highest frequency compared with other haplotypes. This is consistent with the findings reported by Hickford *et al*. (2010) and Sjakste *et al*. (2011). Therefore, H2 might be more closely related to the notional ancestral haplotype. Haplotype H3 was present in the dual-purpose sheep breeds and Merinos in the current study which is in agreement with the findings of Hickford *et al*. (2010). In contrast, H8 is the least common haplotype and was only identified in NZ cross-bred sheep. The presence of different haplotypes with different frequencies as reported in this study probably can only be taken to reflect the sheep that were sampled for typing, as opposed to being truly representative of the breed. However, it still provides valuable information on the genetic diversity of *MSTN* in these breeds. Until a more complete understanding of the broad genetic history of breeds is known, and all the possible haplotypes in those breeds are defined, it will be difficult to explain the likely origin of different haplotypes.

The study of genetic variation in *MSTN* across a range of NZ sheep breeds in the current study, taken together with reports of *MSTN* variation in mammalian species with "double-muscled" phenotype, would be fundamental for a full understanding of ovine *MSTN* and its contribution to meat production. However, because the sheep selected for this analysis were not randomly selected and, consequently, are not representative of the breed as a whole, it would not be appropriate to discuss the presence or absence of any given nucleotide substitution for these or any other breeds based solely on this information. This approach would also assume that there are "pure" or "true to type" breeds and that the sheep selected for study in this chapter, have this character. This is also unsupportable as a contention.

It was noted that four haplotypes H2, H3, H5 and H7 were identified in NZ Romney sheep when a small sample size was used in this preliminary trial. The Romney is the most popular sheep breed in New Zealand (Meadows, 2008), and plays a significant role in sheep meat production and thus the economic value of the NZ sheep industry. The development of the *MSTN* haplotyping system in this chapter allows further investigation of the relationship between *MSTN* haplotype and variation in lamb growth and carcass traits in a large number of NZ Romney sheep (Chapter 3).

Chapter 3
Association of *MSTN* haplotypes with growth and carcass traits in New Zealand Romney sheep

In New Zealand's sheep production systems, carcass weight is a major determinant of carcass value, but other carcass traits like lean meat yield are gaining in importance. Lamb growth rates and carcass traits are therefore key determinants of the economic value of lamb. In respect of the latter carcass traits, very little is known about the underlying genetics of carcass to carcass variation, especially what affects the ratios of lean meat to fat, or to bone, in a carcass. Improved understanding of the factors that affect carcass traits will enable us to further improve the value of carcasses.

Variation in *MSTN* is already being used commercially in the sheep industry to increase lamb carcass weight and muscle yield (Pfizer Animal Health, Dunedin, NZ; Lincoln University Gene-Marker Laboratory). This is based on the detection of genetic variation in the 3'UTR of *MSTN* where it has been shown that c.*1232A is associated with improved carcass traits (Clop *et al.*, 2006; Hadjipavlou *et al.*, 2008; Johnson *et al.*, 2009). However, the high frequency of *MSTN* c.*1232A present in Texel sheep may limit its value in contributing to any further genetic gain. This leads to the questions of whether any other variation in *MSTN* affects carcass traits and whether such variation might therefore be of further value to improving carcass characteristics.

In this respect, Kijas *et al*. (2007) demonstrated that four haplotypes not carrying *MSTN* c.*1232A had significant associations with variation in muscling and fatness. In addition, a recent trial with NZ Romney sheep revealed three haplotypes (A, B and C) of *MSTN* defined by variation in the exon 1- intron 1 region, and with the presence of haplotypes A and B being associated with variation in lean meat production (Hickford *et al.*, 2010). Given the eight extended haplotypes of ovine *MSTN* described in Chapter 2, it would therefore be reasonable to expect that this variation might also affect carcass characteristics.

The NZ Romney sheep investigated in this study, are from the most common maternal breed in NZ and, with their crosses (the Perendale and Coopworth), constitute more than

half of the total NZ sheep flock (Beef + Lamb NZ, 2011a). If the haplotypes described in Chapter 2 can be detected in NZ Romney sheep, then they may affect carcass traits and consequently could be used as genetic markers to improve the selection of breeding stock.

Thus, in this chapter, the PCR-SSCP approach used to define the haplotypes was used to investigate haplotypic diversity in a large number of NZ Romney lambs that had been slaughtered and for which records were available describing a variety of traits including lamb growth and carcass characteristics. Upon haplotyping, various statistical analyses were used to determine the relationship between the *MSTN* haplotype and variation in the lamb growth and carcass traits.

3.1 Materials and methods

Animals

The Romney sheep selected for this study were part of the Ancare-Merial Romney NZ Saleable Meat Yield Trial. This trial has been run over seven consecutive years from 2006 to 2012 at "Gleneyre", Oxford, North Canterbury, NZ and "Osborne" farm, Ashhurst, NZ.

For this study, the lambs were selected from both North Island and South Island farms over four consecutive years from 2006 to 2009. Nineteen NZ Romney sires and their progeny were studied (see Table 3.2), and all were ranked in the top 20% of Romney rams in the Sheep Improvement Limited (SIL – a Division of Beef + Lamb NZ, Wellington, NZ) DPO index.

Each ram was single-sire mated to a group of randomly selected commercial (non-stud) NZ Romney ewes ($n \approx 40$-60). Ewes varied in age from 2-7 years, with each ewe identified to the sire-group by a numbered plastic ear tag. These ewes were kept in one mob until pregnancy scanning, and then the single-bearing ewes were drafted off from the multiple-bearing ewes to enable a differential feeding regime to be used. The ewes were not pre-lamb shorn. Prior to lambing, ewes were set-stocked at approximately 12 per hectare.

A blood sample for DNA extraction was collected onto an FTA card (Whatman BioScience, Middlesex, UK) from the nineteen rams at mating and the ewes at pregnancy scanning. A small notch was taken out of the end of an ear and several drops of whole blood were collected onto a labelled FTA card (Whatman BioScience). The FTA card was allowed to air dry and was stored in darkness at room temperature until needed.

Data collection

During lambing, all lambs (n = 1376) were tagged within 12 hours of birth using ear tags that carried a unique identification number. At tagging, birth date, birth rank (i.e. single or multiple birth), birth weight, gender and dam number were recorded. Lambing dates varied and extended over the period from 19 August to 23 September of each year (Table 3.2).

All of the ewes and lambs were brought together at tailing (approximately 3 weeks after birth) and remained together until weaning. All lambs were tailed with a rubber ring, ear-marked and weighed. A blood sample for DNA extraction was collected onto an FTA card.

Weaning occurred at about three months after birth. At weaning, all lambs were weighed with the weight of each individual lamb recorded against tag number. Pre-weaning growth rate was calculated as the difference between weaning weight and birth weight divided by age in days (expressed in grams/day). At weaning, lambs were separated according to gender. Only male lambs were used in the subsequent meat yield investigations and female lambs were retained as flock replacements.

At weaning, those lambs weighing 36 kg and over were drafted and transported to the Alliance Group Limited meat processing plants. Two subsequent drafts occurred at approximately four weekly intervals. At the second draft, male lambs were drafted at weights over 36 kg and draft three consisted of all the remaining male lambs regardless of weight (ranged from 30.5 to 50.5 kg). Draft age and weight for each male lamb were recorded.

Hot carcass weights (H-W) were measured directly at slaughter. H-W is the weight in kilograms of the carcass components minus the pelt, head and gut. Other carcass data including loin yield, shoulder yield, leg yield, total yield, proportion loin yield, proportion shoulder yield and proportion leg yield were subsequently calculated using video imaging analyses (VIAScan® Sastek, Hamilton, QLD Australia). Loin yield, shoulder yield and leg yield is the percentage of lean tissues as a proportion of the H-W. Total yield is the sum of the leg, loin and shoulder yield for any given carcass. The proportion yield of leg, loin or shoulder is the yield of the specific area divided by the total yield, expressed as a percentage.

Of the 1376 lambs born, growth, blood sample and genotype data were collected for 1206 lambs. Of these, 582 were male lambs sent to the meat processing plant. Of these male lambs, 79 were excluded from the dataset as only partial meat processing plant data were available as a result of either tags being mistakenly removed or the presence of carcass faults that required trimming. Sample numbers therefore vary in the different analyses undertaken below.

Genotyping of *MSTN* using PCR-SSCP

Blood samples from all the lambs, sires and ewes were collected on FTA cards and were purified using a two-step procedure described by Zhou *et al*. (2006). Four fragments of *MSTN*; amplicon 1, amplicon 5, amplicon 6 and amplicon 11 were amplified using the PCR primers described in Chapter 2. The amplicons were subjected to SSCP analyses according to the methods described in Chapter 2. All SSCP gels were silver-stained according to the method of Sanguinetti *et al*. (1994) and the results were compared with the banding pattern standards described in Chapter 2, to identify haplotypes.

Statistical analyses

GenStat (Version 12.2, VSN International Ltd, Hemel Hempstead, UK) was used to analyse the data. Pearson correlation coefficients were calculated to test the strength of the linear correlations between the various lamb growth and carcass traits. Restricted Maximum Likelihood (REML) linear mixed-models were used to calculate and test correlations between the lamb growth data and carcass trait data.

REML mixed-model analyses were used to assess sire and birth rank effects on the means of the lamb growth and carcass data. In brief, for each lamb growth variable, factors including birth rank, gender, sire-line (sire-line had a significant effect on lamb birth weight, lamb pre-weaning growth variables and carcass traits, refer to Appendix D and E), were factored into the models. For lamb carcass traits, gender was excluded from the model, as only males were slaughtered. In the model that assessed the effect of haplotype on weaning weight; weaning age was included in the model as a co-variate. Draft weight was included as a co-variate in the models testing haplotype effect on draft age and the various carcass traits.

This investigation into the association of *MSTN* haplotype with lamb growth and carcass traits was limited by estimating the haplotype effect independently of sire-line. In addition, the sire-line effects were confounded with farm-related effects such as environment, different farm/year and stock management, as individual sires were only used on one farm. Thus the farm and year were removed from the model although the correction for sire would accommodate year-to-year variability as the different sires were only used for a single year.

The association of growth and carcass traits with haplotype differences in the *MSTN* gene was assessed using both half-sib analyses (within family) and pooled-data analyses (across family).

Half-sib (segregation) analyses

A half-sib (segregation) analysis was performed for those lines that were sired by a ram heterozygous for *MSTN*. For each sire-line, progeny which typed the same as the sire and dam were removed from the data set because the segregation of the paternal haplotype could not be determined. REML mixed-model analyses were performed to ascertain the effect of the sire-inherited haplotype, sire-line, gender and birth rank (single or multiple) on each of the lamb growth variables (birth weight, tailing weight, weaning weight, weaning age, growth rate to weaning, draft weight and draft age). Animal growth data were available for all progeny, whereas carcass trait data (hot carcass weight, loin yield, shoulder yield, leg yield, total yield, proportion loin yield, proportion shoulder yield and proportion leg yield) were available only for male lambs.

In the model that assessed the effect of sire-inherited haplotype on weaning weight; weaning age was included in the model as a co-variate. Draft weight was included as co-variate in the models testing the sire-inherited haplotype effect on the draft age and the various carcass traits.

With sire-line Fernvale 1106.02, REML cross classification mixed-model were used to explore the association between genotype and gender.

Only main effects were tested. All non-significant effects were removed from the model and only main effects are reported. T-tests were used to compare the means of the sire-inherited variation.

Pooled-data analyses

In the pooled-data analyses, REML mixed-model analyses was used to assess whether the presence (or absence) of a particular haplotype in a sheep's genotype was associated with various lamb growth and carcass traits. For each trait, a factorial REML was performed for each *MSTN* haplotype observed in the population. For each lamb growth variable, factorial REML analyses incorporating haplotype presence or absence as factors along with birth rank, gender, sire-line and any appropriate co-variates were undertaken. For lamb carcass traits, gender was excluded from the model. For each trait, a factorial REML containing all of the haplotypes as factors along with other co-variates were also tested.

In the model that assessed the effect of haplotype on weaning weight, weaning age was included in the model as a co-variate. Draft weight was included as co-variate in the models testing haplotype effect on draft age and various carcass traits.

REML mixed-model analyses were also performed to assess the association of each *MSTN* genotype present in the progeny with birth weight, tailing weight, weaning weight, growth rate to weaning, draft weight, draft age and the various carcass traits. For each trait, factorial REML analyses were performed for each *MSTN* haplotype observed in the population. Genotype, birth rank, gender and sire-line were included as factors in each lamb growth model. Again, because only males were sent for slaughter, gender was excluded as a factor in carcass trait model. In the model that assessed the effect of genotype on weaning weight, weaning age was included in the model as a co-variate. Draft weight was included as co-variate in the models testing genotype effect on draft age and various carcass traits. Only main effects were tested.

The data were unbalanced with respect to genotype and sire group, and accordingly the order in which terms are entered into the model for analyses will affect the deviance (and therefore the significance) associated with that term. In the analyses used, the genotype distribution among the progeny was studied and the effect of adding genotype before adding sire group to the model was investigated. Adding sire group effect first removes all genotype effects.

To facilitate understanding of the effects of the genotypes on lamb growth and carcase traits, the trait means with 95% confidence intervals are displayed in graphs.

Unless otherwise indicated, all P-values were considered statistically significant when $P < 0.05$ and trends were noted when $0.05 < P < 0.10$.

3.2 Results

Correlations between lamb growth and carcass traits

Pearson correlation coefficients between the lamb growth data and various carcass traits evaluated are listed in Table 3.1. Lamb birth weight, tailing weight, weaning weight and growth rate to weaning were significantly correlated ($P < 0.05$).

Lamb birth weight was not significantly correlated with loin yield, total yield or proportion loin yield. Draft weight was highly correlated with loin yield and proportion loin yield ($P \leq 0.001$), but not shoulder yield, leg yield, total yield or proportion shoulder yield. Hot carcass weight was highly correlated with loin yield, shoulder yield, total yield, proportion loin yield and proportion shoulder yield ($P \leq 0.001$). Total carcass lean meat yield was highly correlated with shoulder yield, leg yield and loin yield ($P \leq 0.001$), as might be expected.

Haplotype and genotype frequencies in NZ Romney sheep

The 19 rams studied were haplotyped and revealed to have five of the haplotypes including H1, H2, H3, H5 and H7 (Table 3.2).

Five *MSTN* haplotypes including H1, H2, H3, H5 and H7 were identified in the lambs investigated. H1 and H7 carried the nucleotide substitutions c.*1232A and c.101A respectively. Haplotypes H4, H6 and H8 were not found. The frequencies for H1, H2, H3, H5 and H7 across all the lambs typed were 2.79%, 53.13%, 24.55%, 15.19% and 4.34%, respectively (Figure 3.1). Genotypes (frequency in brackets) H1H2 (3.23%), H2H2 (34.08%), H2H7 (4.89%), H3H1 (0.83%), H3H2 (26.87%), H3H3 (4.48%), H3H5 (5.89%), H3H7 (1.16%), H5H1 (0.42%), H5H2 (16.34%), H5H5 (0.83%) and H5H7 (1%) were also observed (Figure 3.2).

Table 3.1 Correlation coefficients for comparisons between lamb growth and carcass traits.

	Tailing weight (kg)	Weaning weight (kg)	Growth rate to weaning (g/day)	Draft weight (kg)	Draft age (days)	Hot carcass weight (kg)	Loin yield[1]	Shoulder yield[1]	Leg yield[1]	Total yield[2]	Proportion loin yield[3]	Proportion shoulder yield[3]	Proportion leg yield[3]
Birth weight (kg)	0.304***	0.483*	0.3874*	0.179**	-0.378	0.345*	-0.100	0.102*	-0.143	-0.064	-0.094	0.250***	-0.194
Tailing weight (kg)		0.640**	0.519**	0.187***	-0.213	0.365**	0.157***	0.286***	0.060	0.185***	0.035	0.165***	-0.223
Weaning weight (kg)			0.890***	0.308***	-0.596	0.562***	0.116*	0.348***	0.012	0.173***	-0.037	0.305***	-0.305
Growth rate to weaning (g/day)				0.236***	-0.767	0.423***	0.100*	0.291***	0.004	0.143**	-0.022	0.258***	-0.265
Draft weight (kg)					0.148**	0.650***	0.169***	0.038	-0.067	0.042	0.270***	-0.003	-0.222
Draft age (days)						-0.076	0.073	-0.103	0.059	0.014	0.124**	-0.184	0.098*
Hot carcass weight (kg)							0.2414***	0.3119***	-0.0675	0.1669***	0.2190***	0.2547***	-0.4606
Loin yield[1]								0.525***	0.694***	0.863***	0.627***	-0.375	-0.115
Shoulder yield[1]									0.494***	0.780***	-0.174	0.491***	-0.390
Leg yield[1]										0.888***	-0.010	-0.454	0.504***
Total yield[2]											0.149**	-0.161	0.052
Proportion loin yield[3]												-0.487	-0.305
Proportion shoulder yield[3]													-0.683

*0.01<P≤0.05, **0.001<P≤0.01, ***P≤0.001;

[1] Lean meat yield expressed as a percentage of hot carcass weight; [2] Total yield is the sum of the loin, shoulder and leg yield;

[3] The proportion yield of leg, loin or shoulder is the yield of the specific area divided by the total yield.

Table 3.2 Sires used to assess the association of *MSTN* haplotype with sheep growth and carcass traits

Sires	Location	Lambing date	*MSTN* genotype	Number of progeny (n)
Doughboy 45/04	South Island	24 Aug - 14 Sep, 2006	H2H2	80
Longridge 626/02	South Island	23 Aug - 12 Sep, 2006	H3H5	74
Mana 83/04	South Island	25 Aug - 13 Sep, 2006	H2H5	85
Mana 90/01	South Island	20 Aug - 20 Sep, 2006	H2H3	92
Offord 414/01	South Island	27 Aug - 15 Sep, 2006	H2H7	84
Clifton Downs 497.04	South Island	24 Aug - 22 Sep, 2007	H2H2	88
Fernvale 1106.02	South Island	30 Aug - 22 Sep, 2007	H1H5	92
Sudeley 181.06	South Island	30 Aug - 19 Sep, 2007	H2H3	81
Holly Farm 398.05	North Island	15 Aug - 1 Sep, 2008	H2H3	65
Paka-iti 617.04	North Island	14 Aug - 1 Sep, 2008	H2H5	25
Waio 408.07	North Island	15 Aug - 6 Sep, 2008	H2H2	46
Doughboy 41/06	South Island	28 Aug - 20 Sep, 2008	H2H5	64
Fernvale 671/06	South Island	29 Aug - 20 Sep, 2008	H2H2	71
View Hill 480/04	South Island	29 Aug - 20 Sep, 2008	H2H3	71
Trigg 6448/07	North Island	9 Aug - 26 Aug, 2009	H1H2	32
Doughboy 167/07	South Island	2 Sep - 23 Sep, 2009	H2H3	81
Gatton Park 114/08	South Island	2 Sep - 22 Sep, 2009	H2H2	86
Offord 55/05	South Island	5 Sep - 22 Sep, 2009	H2H7	68
Ram Hill 468/06	South Island	2 Sep - 22 Sep, 2009	H3H5	91
				Total = 1376

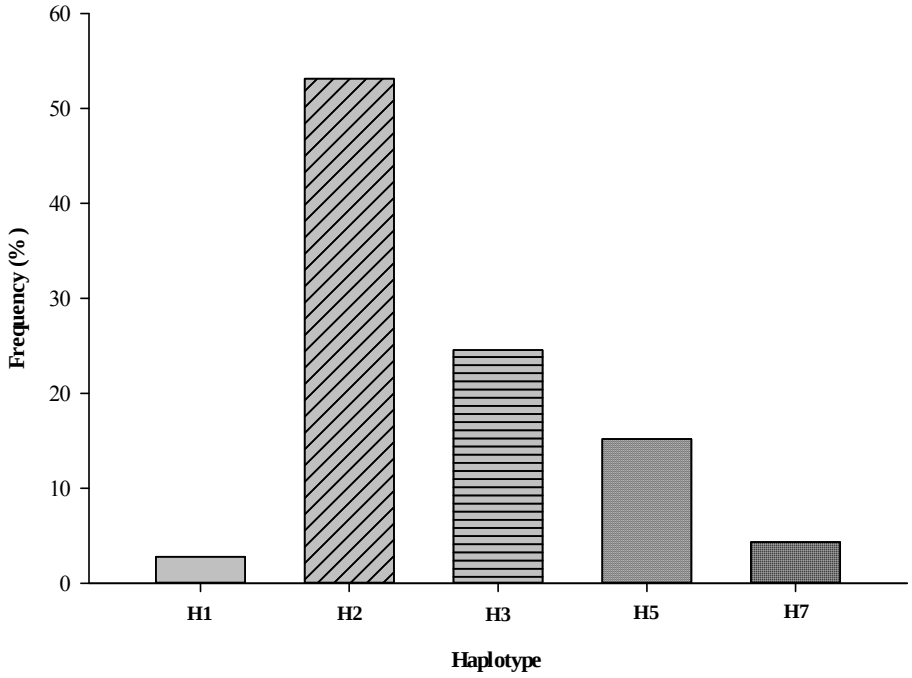


Figure 3.1 Haplotype frequencies of ovine *MSTN* in the NZ Romney sheep

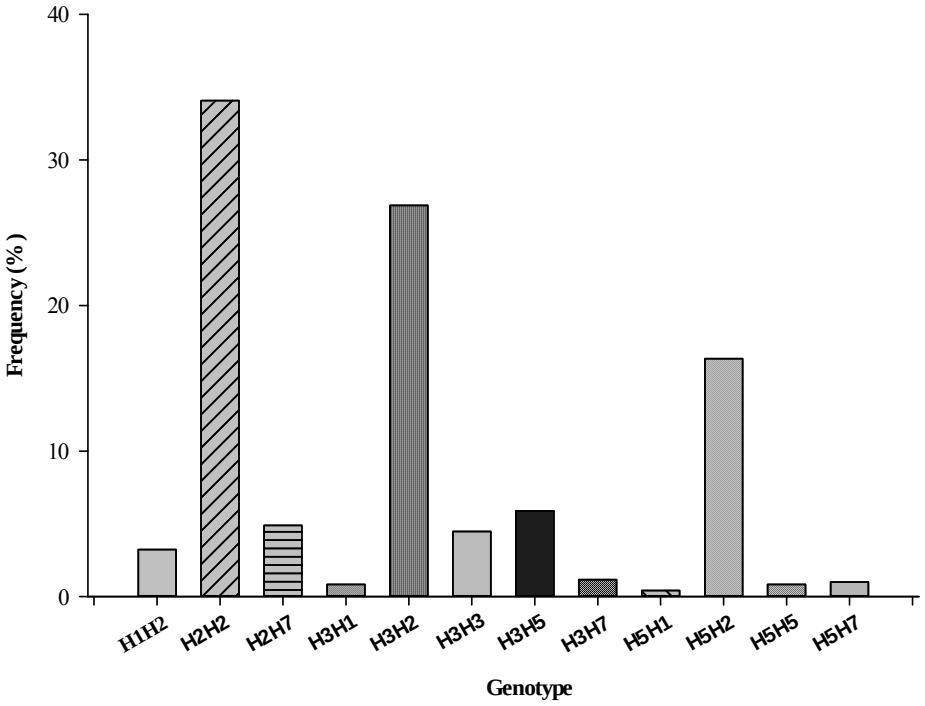


Figure 3.2 Genotype frequencies of ovine *MSTN* in the NZ Romney sheep

Effect of haplotype on lamb growth and carcass traits

Half-sib data analyses

Of the eleven sire-lines selected for half-sib analysis, the paternally inherited *MSTN* haplotypes had a significant effect on the lamb growth and carcass traits in four sire-lines; Mana 83/04, Fernvale 1106.02, Sudeley 181.06 and Doughboy 41/06 (Table 3.3 and Table 3.4).

In the Fernvale 1106.02 sire-line, progeny inheriting H1 from the sire had a higher mean birth weight (P=0.029), tailing weight (P=0.021), draft weight (P=0.035) (Table 3.3), loin yield (P=0.001), shoulder yield (P=0.025), leg yield (P=0.015) and total yield (P=0.002) (Table 3.4) than progeny inheriting haplotype H5.

In the sire-line Doughboy 41/06, progeny inheriting H2 from the sire had a higher draft weight (P=0.024) with an earlier draft age (P=0.006), than progeny inheriting H5 (Table 3.3). Progeny inheriting H2 also had a higher loin yield (P=0.012), shoulder yield (P=0.009) and total yield (P=0.008, Table 3.4).

In the Sudeley 181.06 sire-line, progeny inheriting H3 had a higher proportion loin yield and a lower proportion shoulder yield than progeny inheriting H2, and they also tended to have a higher loin yield (P=0.055, Table 3.4).

In the Mana 83/04 sire-line, progeny inheriting H2 had a higher proportion shoulder yield (P=0.004) than H5, whilst H5 had a higher proportion leg yield (P=0.015, Table 3.4).

No paternally inherited *MSTN* haplotype was found to be associated with the mean weaning age (Table 3.3), or hot carcass weight (Table 3.4), in any of the sire-lines examined.

Table 3.3 Overall means for lamb growth data within half-sib families

Sire-line	Paternal haplotype inherited	Birth weight (kg)		Tailing weight (kg)		Weaning weight (kg)		Weaning Age (days)		Growth rate to weaning (g/day)		Draft weight (kg)		Draft age (days)	
		n	Mean[1] ± SEMs	n	Mean[1] ± SEMs	n	Mean[1] ± SEMs	n	Mean[1] ± SEMs	n	Mean[1] ± SEMs	n	Mean[1] ± SEMs	n	Mean[1] ± SEMs
Longridge 626/02	H3	40	4.88 ± 0.20	39	14.89 ± 0.48	40	29.31 ± 0.99	40	89.51±1.24	40	267.72 ± 10.43	18	42.48 ± 0.83	18	130.35 ± 5.67
	H5	26	4.90 ± 0.22	25	14.93 ± 0.50	25	27.67 ± 1.12	25	90.79±1.42	25	248.09 ± 11.85	14	41.82 ± 0.93	14	131.94 ± 6.04
Mana 83/04	H2	38	5.20 ± 0.20	37	15.59 ± 0.56	35	30.08 ± 1.01	35	89.66±1.28	35	272.77 ± 10.71	14	39.72 ± 0.92	14	123.08 ± 6.16
	H5	28	5.35 ± 0.21	27	15.53 ± 0.61	27	29.09 ± 1.01	27	88.60±1.37	27	260.03 ± 11.56	11	40.90 ± 1.05	11	130.62 ± 6.53
Mana 90/01	H2	34	5.14 ± 0.20	33	15.45 ± 0.58	34	28.27 ± 1.02	34	89.07±1.29	34	251.39 ± 10.81	15	40.42 ± 0.89	15	128.97 ± 5.87
	H3	36	4.86 ± 0.20	36	15.40 ± 0.57	33	28.70 ± 1.03	33	88.78±1.30	33	259.63 ± 10.89	16	41.92 ± 0.88	16	133.47 ± 5.75
Fernvale 1106.02	H1	**36**	**5.31 ± 0.20***	**35**	**16.18 ± 0.67***	33	32.09 ± 1.01	35	90.54±1.27	34	293.65 ± 10.68	**13**	**43.32 ± 0.96***	13	118.48 ± 6.14
	H5	**34**	**4.85 ± 0.20***	**34**	**14.93 ± 0.50***	33	30.87 ± 1.01	34	88.84±1.26	33	283.47 ± 10.64	**15**	**40.56 ± 0.89***	15	120.62 ± 5.76
Sudeley 181.06	H2	33	5.00 ± 0.19	34	15.57 ± 0.65	33	30.37 ± 0.94	34	92.34±1.18	33	279.06 ± 9.96	18	40.34 ± 0.81	18	127.86 ± 5.02
	H3	25	4.92 ± 0.22	25	15.13 ± 0.71	33	31.48 ± 1.09	25	91.88±1.37	25	291.44 ± 11.48	10	41.09 ± 1.09	10	122.95 ± 6.61
Holly Farm 398.05	H2	22	4.52 ± 0.23	n.d.	n.d.	33	24.21 ± 1.41	22	115.88±1.49	20	225.98 ± 14.93	8	39.64 ± 1.36	8	175.54 ± 7.24
	H3	6	4.92 ± 0.38	n.d.	n.d.	33	25.00 ± 2.01	6	114.22±2.37	6	227.25 ± 21.30	3	40.41 ± 2.02	3	156.72 ± 10.88
Paka-iti 617.04	H2	7	4.07 ± 0.35	n.d.	n.d.	33	23.13 ± 1.91	7	116.16±2.21	7	221.19 ± 20.21	4	41.67 ± 2.47	2	155.08 ± 13.07
	H5	7	4.40 ± 0.35	n.d.	n.d.	33	24.37 ± 2.04	7	117.00±2.35	6	231.01 ± 21.55	3	37.56 ± 2.11	3	179.70 ± 11.02
Doughboy 41/06	H2	22	5.24 ± 0.22	n.d.	n.d.	33	34.27 ± 1.14	22	87.49±1.43	21	318.09 ± 12.02	**8**	**41.05 ± 1.25***	**8**	**103.95 ± 7.13****
	H5	25	5.01 ± 0.22	n.d.	n.d.	33	32.19 ± 1.14	25	89.60±1.43	22	298.76 ± 12.00	**12**	**37.42 ± 1.00***	**12**	**126.88 ± 6.39****
View Hill 480/04	H2	29	5.32 ± 0.21	n.d.	n.d.	33	**36.00 ± 1.06**	29	86.36±1.33	**29**	**337.68 ± 11.18**	12	41.04 ± 1.04	12	95.17 ± 6.30
	H3	28	5.49 ± 0.21	n.d.	n.d.	33	**38.01 ± 1.06**	28	86.68±1.35	**27**	**358.32 ± 11.19**	13	41.96 ± 0.99	13	101.45 ± 6.05
Doughboy 167/07	H2	32	6.20 ± 0.21	32	15.32 ± 0.66	33	38.00 ± 1.07	32	87.93±1.38	**30**	**348.63 ± 11.30**	**16**	**42.94 ± 0.90**	**16**	**99.46 ± 5.84**
	H3	30	6.19 ± 0.21	30	14.89 ± 0.48	33	36.27 ± 1.05	30	89.33±1.33	**30**	**328.94 ± 11.05**	**13**	**40.68 ± 0.97**	**13**	**111.16 ± 6.01**
Ram Hill 468/06	H3	32	6.21 ± 0.19	31	14.56 ± 0.64	33	36.59 ± 1.03	32	90.46±1.31	28	329.85 ± 10.84	13	42.64 ± 0.98	14	105.92 ± 6.11
	H5	40	6.15 ± 0.19	40	14.69 ± 0.62	33	36.42 ± 1.01	40	91.75±1.29	33	331.21 ± 10.67	17	42.73 ± 0.85	18	107.62 ± 5.32

[1]For each half-sib anaysis: predicted mean values were caculated from a REML mixed-model that included sire-line, birth rank and gender as fixed factors;
*P <0.05, **P<0.01, 0.05<P<0.10 (approaching significance) in bold;
n.d. = no data

Table 3.4 Overall means for carcass traits data within half-sib families

Sire-line	Paternal haplotype inherited	Means[1] ± SEMs								
		n	Hot carcass weight (kg)	n	Loin yield[2]	Shoulder yield[2]	Leg yield[2]	Total yield[3]	Proportion loin yield[4]	Proportion shoulder yield[4]	Proportion leg yield[4]
Longridge 626/02	H3	17	16.91 ± 0.43	15	14.17 ± 0.20	16.02 ± 0.27	21.07 ± 0.29	51.38 ± 0.57	0.276 ± 0.002	0.313 ± 0.003	0.410 ± 0.003
	H5	15	16.89 ± 0.46	15	14.00 ± 0.21	16.10 ± 0.27	20.57 ± 0.30	50.54 ± 0.57	0.275 ± 0.002	0.320 ± 0.003	0.404 ± 0.003
Mana 83/04	H2	11	17.11 ± 0.49	11	14.00 ± 0.23	**16.51 ± 0.30**	19.92 ± 0.32	50.54 ± 0.67	0.275 ± 0.002	**0.329 ± 0.004****	**0.394 ± 0.003***
	H5	8	17.05 ± 0.54	8	14.02 ± 0.27	**15.79 ± 0.33**	20.36 ± 0.38	50.42 ± 0.78	0.278 ± 0.003	**0.315 ± 0.004****	**0.405 ± 0.004***
Mana 90/01	H2	15	17.40 ± 0.44	14	14.13 ± 0.21	16.49 ± 0.27	20.92 ± 0.30	51.72 ± 0.59	0.273 ± 0.002	0.321 ± 0.003	0.405 ± 0.003
	H3	17	17.36 ± 0.43	16	14.22 ± 0.20	16.58 ± 0.26	20.64 ± 0.29	51.63 ± 0.55	0.275 ± 0.002	0.323 ± 0.003	0.400 ± 0.003
Fernvale 1106.02	H1	13	17.88 ± 0.45	**13**	**15.23 ± 0.21*****	**16.97 ± 0.28***	**22.15 ± 0.30***	**54.55 ± 0.61****	**0.279 ± 0.002**	0.313 ± 0.004	0.406 ± 0.003
	H5	15	17.33 ± 0.43	**15**	**14.22 ± 0.19*****	**16.28 ± 0.26***	**21.15 ± 0.28***	**51.90 ± 0.57****	**0.274 ± 0.002**	0.316 ± 0.003	0.408 ± 0.003
Sudeley 181.06	H2	18	16.95 ± 0.37	**16**	**14.52 ± 0.19**	16.64 ± 0.23	21.18 ± 0.27	52.57 ± 0.55	**0.276 ± 0.002***	**0.318 ± 0.003****	0.404 ± 0.003
	H3	10	16.63 ± 0.49	**9**	**15.12 ± 0.25**	16.31 ± 0.31	21.89 ± 0.35	53.55 ± 0.74	**0.282 ± 0.003***	**0.306 ± 0.004****	0.409 ± 0.004
Holly Farm 398.05	H2	7	17.79 ± 0.56	7	14.39 ± 0.29	17.48 ± 0.34	21.60 ± 0.40	53.82 ± 0.83	0.267 ± 0.003	0.327 ± 0.004	0.404 ± 0.004
	H3	2	18.72 ± 0.97	2	13.96 ± 0.53	16.74 ± 0.59	20.54 ± 0.75	51.73 ± 1.56	0.270 ± 0.005	0.326 ± 0.008	0.402 ± 0.007
Paka-iti 617.04	H2	2	17.95 ± 0.97	2	14.68 ± 0.53	17.50 ± 0.59	21.08 ± 0.75	53.60 ± 1.56	0.274 ± 0.005	0.328 ± 0.008	0.396 ± 0.007
	H5	3	18.52 ± 0.82	3	14.65 ± 0.43	17.51 ± 0.49	21.13 ± 0.61	53.47 ± 1.27	0.274 ± 0.004	0.329 ± 0.006	0.395 ± 0.006
Doughboy 41/06	H2	8	17.84 ± 0.53	**8**	**15.81 ± 0.27***	**18.30 ± 0.32****	22.57 ± 0.38	**57.07 ± 0.78****	0.277 ± 0.003	0.322 ± 0.004	0.399 ± 0.004
	H5	11	17.33 ± 0.48	**11**	**14.92 ± 0.23***	**17.31 ± 0.29****	21.82 ± 0.33	**54.32 ± 0.67****	0.274 ± 0.002	0.320 ± 0.004	0.403 ± 0.003
View Hill 480/04	H2	12	17.75 ± 0.47	12	14.56 ± 0.22	17.33 ± 0.28	21.30 ± 0.31	53.44 ± 0.64	0.273 ± 0.002	0.326 ± 0.004	0.399 ± 0.003
	H3	13	17.98 ± 0.45	13	14.55 ± 0.21	17.83 ± 0.27	21.40 ± 0.29	54.08 ± 0.61	0.269 ± 0.002	0.331 ± 0.003	0.397 ± 0.003
Doughboy 167/07	H2	15	17.75 ± 0.44	15	14.39 ± 0.19	16.85 ± 0.27	21.11 ± 0.27	52.66 ± 0.57	0.273 ± 0.002	0.322 ± 0.003	0.404 ± 0.003
	H3	13	18.48 ± 0.45	13	14.23 ± 0.21	16.85 ± 0.27	20.89 ± 0.30	52.33 ± 0.61	0.272 ± 0.002	0.324 ± 0.003	0.403 ± 0.003
Ram Hill 468/06	H3	14	16.82 ± 0.45	13	14.15 ± 0.22	16.78 ± 0.28	21.50 ± 0.31	52.67 ± 0.61	0.268 ± 0.002	0.321 ± 0.004	0.410 ± 0.003
	H5	18	17.32 ± 0.39	17	14.32 ± 0.19	16.69 ± 0.24	21.28 ± 0.27	52.49 ± 0.54	0.273 ± 0.002	0.320 ± 0.003	0.407 ± 0.003

[1] For each half-sib anaysis: predicted mean values were caculated from a REML mixed-model that included sire-line as a fixed factor, birth rank and gender as random factors;

[2] Lean meat yield expressed as a percentage of hot carcass weight; [3] Total yield is the sum of the leg, loin and shoulder yield;

[4] The proportion yield of leg, loin or shoulder is the yield of the specific area divided by the total yield expressed as a percentage.

*P <0.05, **P<0.01, ***P<0.001, 0.05<P<0.10 (approaching significance) in bold;

Effect of *MSTN* c.*1232A on lamb gender

A significant association was found between *MSTN* c.*1232 genotype and gender (P = 0.046) with *MSTN* c.*1232AG genotype being more common in ewe lambs (Table 3.5).

Table 3.5 An association between ovine *MSTN* c.*1232G>A and gender

	Number of progeny		P value
	GG (n=43)	AG (n=36)	
Female	19	24	**0.046**
Male	24	12	

Pooled-data analyses

REML factorial mixed-model analyses revealed that the presence (or absence) of *MSTN* haplotype H1, H2, H3, H5 and H7 in a lamb's genotype was associated with various animal growth and carcass variables (Table 3.6 and Table 3.7). There was a confounding effect between sire-line and haplotype, which means that the estimate of the haplotype effect depends on whether the sire-line effect is added to the model before, or after, adding the haplotype. In the current analyses, sire-line was fitted after the haplotype term in order to evaluate the haplotype effect. The number of tested haplotypes in each sire-line differs considerably (Appendix F). It should be noted that the data are not balanced, and the standard errors of differences in Table 3.6 and Table 3.7, have a marked covariance term.

The presence of H1 was associated with an increased tailing weight, growth rate to weaning (Table 3.6), hot carcass weight, loin yield, leg yield, total yield and proportion loin yield, but a decreased proportion shoulder and leg yield (Table 3.7). The presence of H2 was associated with an increased proportion shoulder yield (Table 3.7), but a decreased weaning weight (Table 3.6), leg yield and a decreased proportion leg yield (Table 3.7). The presence of H3 was associated with decreased proportion shoulder yield but an increased proportion leg yield (Table 3.7). The presence of H7 was only associated with increased proportion shoulder yield (Table 3.7), but tended to be associated with decreased tailing weight (Table 3.6), loin yield, leg yield and total yield (Table 3.7) and with earlier draft age (Table 3.6).

Haplotype H1 remained associated with an increased tailing weight (Table 3.6), hot carcass weight, loin yield, leg yield, total yield and proportion loin yield, and a decreased

proportion shoulder and leg yield (Table 3.7), when other haplotypes (i.e. H2, H3 and H5) were introduced into the model. In comparison the presence of H2 and H3 was associated with a decreased tailing weight with an earlier draft age (Table 3.6) when other haplotypes were introduced into the model. H3 was also associated with an increased hot carcass weight, and a decreased proportion shoulder yield (Table 3.7) when other haplotypes (i.e. H1, H2, H5 and H7) were introduced into the model. The presence of H7 was associated with an increased hot carcass weight, decreased leg yield (Table 3.7) and tended to be associated with a decreased tailing weight, draft weight (Table 3.6), loin yield and total yield (Table 3.7), when H2, H3 and H5 were introduced into the model.

MSTN haplotype had no effect on mean birth weight or draft weight in the pooled-data analyses (Table 3.6).

Effect of genotype on lamb growth and carcass traits

MSTN genotype was found to have an effect on mean growth rate to weaning (Figure 3.3 A), weaning weight (Figure 3.3 B), draft age (Figure 3.3 C), loin yield (Figure 3.3 D), leg yield (Figure 3.3 E), total yield (Figure 3.3 F), proportion shoulder yield (Figure 3.3 G) and proportion leg yield (Figure 3.3 H). Genotype H5H1 was associated with the highest mean values of growth rate to weaning, loin yield, leg yield and total yield compared with other genotypes. Genotype H1H2 was associated with the highest mean values of weaning weight compared with other genotypes. Genotype H2H2, H3H2, H3H3 and H3H5 were associated with significantly higher mean growth rate to weaning, leg yield and total yield than H5H5, whereas H5H5 was associated with a decreased growth rate to weaning, loin yield, leg yield, total yield and a later draft age compared with other genotypes.

No association was found between the *MSTN* genotype and mean birth weight, tailing weight, draft weight, shoulder yield and proportion loin yield (data not shown).

The means of the traits have quite different standard errors and accordingly, pair-wise components would be unwieldy and confusing. Thus, graphs of the means (Figure 3.3 A-H) with 95% confidence intervals were considered a better statistical tool than pair-wise components to demonstrate the effects of different genotypes on the various traits.

Table 3.6 Association of haplotype with various lamb growth traits (pooled-data analyses)

Haplotype being assessed	Other haplotypes in model	Mean[1] ± Std Error				P value[2]
		Haplotype absent	n	Haplotype present	n	
Birth weight (kg)						
H1	none	5.08 ± 0.10	1149	5.26 ± 0.19	54	0.187
H2	none	5.10 ± 0.12	177	5.09 ± 0.10	1025	0.409
H3	none	5.07 ± 0.10	725	5.12 ± 0.10	474	0.380
H5	none	5.09 ± 0.10	909	5.07 ± 0.11	294	0.941
H7	none	5.09 ± 0.10	1118	4.98 ± 0.15	84	0.257
H1	H2, H3, H5	5.08 ± 0.10	1149	5.28 ± 0.20	54	0.167
H2	H1, H3, H5, H7	5.13 ± 0.15	177	5.13 ± 0.15	1025	0.584
H3	H1, H2, H5, H7	5.11 ± 0.14	725	5.16 ± 0.15	474	0.673
H5	H1, H2, H5, H7	5.13 ± 0.14	909	5.14 ± 0.16	294	0.817
H7	H2, H3, H5	5.10 ± 0.10	1118	4.98 ± 0.16	84	0.310
Tailing weight (kg)						
H1	**none**	**14.40 ± 0.25**	**833**	**15.32 ± 0.56**	**36**	**0.006**
H2	none	14.71 ± 0.32	133	14.39 ± 0.25	735	0.245
H3	none	14.46 ± 0.26	519	14.36 ± 0.27	346	0.211
H5	none	14.43 ± 0.25	659	14.43 ± 0.29	210	0.986
H7	none	14.44 ± 0.25	791	14.26 ± 0.41	78	0.436
H1	**H2, H3, H5**	**14.52 ± 0.26**	**833**	**15.42 ± 0.62**	**36**	**0.017**
H2	**H1, H3, H5, H7**	**15.06 ± 0.43**	**133**	**14.53 ± 0.44**	**735**	**0.035**
H3	**H1, H2, H5, H7**	**14.93 ± 0.41**	**519**	**14.66 ± 0.45**	**346**	**0.037**
H5	H1, H2, H5, H7	14.84 ± 0.39	659	14.75 ± 0.48	210	0.276
H7	H2, H3, H5	14.58 ± 0.27	791	14.14 ± 0.41	78	**0.073**
Weaning weight (kg)						
H1	none	30.16 ± 0.48	1100	30.48 ± 0.94	53	0.873
H2	**none**	**30.74 ± 0.59**	**170**	**30.10 ± 0.48**	**982**	**0.041**
H3	none	30.10 ± 0.49	698	30.31 ± 0.51	451	0.364
H5	none	30.15 ± 0.49	879	30.26 ± 0.54	274	0.340
H7	none	30.15 ± 0.48	1071	30.56 ± 0.78	81	0.415
H1	H2, H3, H5	30.41 ± 0.51	1100	30.71 ± 1.01	53	0.822
H2	H1, H3, H5, H7	30.95 ± 0.75	170	30.33 ± 0.77	982	**0.064**
H3	H1, H2, H5, H7	30.62 ± 0.71	698	30.66 ± 0.77	451	0.881
H5	H1, H2, H5, H7	30.64 ± 0.69	879	30.64 ± 0.81	274	0.919
H7	H2, H3, H5	30.40 ± 0.51	1071	30.57 ± 0.79	81	0.260
Growth rate to weaning (g/day)						
H1	**none**	**290.3 ± 1.1**	**1097**	**290.6 ± 2.2**	**53**	**0.020**
H2	none	290.2 ± 1.4	170	290.2 ± 1.1	979	0.919
H3	none	290.5 ± 1.2	694	289.6 ± 1.2	450	0.174
H5	none	290.3 ± 1.1	876	290.3 ± 1.3	274	0.984
H7	none	290.3 ± 1.1	1068	291.3 ± 1.8	81	0.311
H1	H2, H3, H5	290.1 ± 1.2	1097	289.9 ± 2.4	53	0.032
H2	H1, H3, H5, H7	290.7 ± 1.8	170	290.2 ± 1.8	979	0.637
H3	H1, H2, H5, H7	290.9 ± 1.7	694	289.9 ± 1.8	450	0.195
H5	H1, H2, H5, H7	290.6 ± 1.6	876	290.2 ± 1.9	274	0.785
H7	H2, H3, H5	290.1 ± 1.2	1068	290.8 ± 1.9	81	0.173

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Haplotype being assessed	Other haplotypes in model	Mean[1] ± Std Error				P value[2]
		Haplotype absent	n	Haplotype present	n	
Draft weight (kg)						
H1	none	41.07 ± 0.49	549	42.77 ± 1.16	20	0.170
H2	none	40.91 ± 0.64	79	41.18 ± 0.49	489	0.579
H3	none	41.05 ± 0.50	357	41.32 ± 0.54	209	0.376
H5	none	41.22 ± 0.51	429	40.99 ± 0.56	140	0.606
H7	none	41.15 ± 0.49	523	41.06 ± 0.83	46	0.983
H1	H2, H3, H5	40.94 ± 0.53	549	42.92 ± 1.23	20	0.112
H2	H1, H3, H5, H7	41.68 ± 0.86	79	42.29 ± 0.87	489	0.209
H3	H1, H2, H5, H7	41.73 ± 0.81	357	42.23 ± 0.88	209	0.21
H5	H1, H2, H5, H7	41.88 ± 0.78	429	42.09 ± 0.92	140	0.764
H7	H2, H3, H5	41.03 ± 0.53	523	41.06 ± 0.86	46	0.786
Draft age (days)						
H1	none	130.6 ± 2.5	546	130.7 ± 5.8	20	0.771
H2	none	129.0 ± 3.2	81	130.8 ± 2.4	484	0.061
H3	none	129.8 ± 2.5	353	132.0 ± 2.7	210	0.082
H5	none	130.1 ± 2.5	425	131.7 ± 2.8	141	0.933
H7	none	130.7 ± 2.4	520	127.5 ± 4.2	46	**0.065**
H1	H2, H3, H5	130.0 ± 2.6	546	133.9 ± 6.2	20	0.330
H2	**H1, H3, H5, H7**	**129.2 ± 4.3**	**81**	**133.8 ± 4.3**	**484**	**0.004**
H3	**H1, H2, H5, H7**	**129.3 ± 4.0**	**353**	**133.6 ± 4.4**	**210**	**0.004**
H5	H1, H2, H5, H7	129.6 ± 3.9	425	133.3 ± 4.6	141	0.241
H7	H2, H3, H5	130.2 ± 2.6	520	129.1 ± 4.3	46	0.433

[1]Predicted means; [2]P value derived from a REML mixed-model with haplotype presence/absence and birth rank, gender and sire-line fitted as fixed factors ($P \leq 0.05$ were shaded and bolded, $0.05 < P < 0.1$ in bold).

Table 3.7 Association of haplotype with various carcass traits across family (pooled-data analyses)

Haplotype being assessed	Other haplotypes in model	Mean[1] ± Std Error				P value[2]
		Haplotype absent	n	Haplotype present	n	
Hot carcass weight (kg)						
H1	**none**	**16.64 ± 0.27**	**532**	**17.90 ± 0.64**	**19**	**0.005**
H2	none	16.78 ± 0.34	76	16.65 ±0.28	474	0.418
H3	none	16.58 ± 0.28	341	16.84 ± 0.30	207	0.174
H5	none	16.71 ± 0.28	412	16.58 ± 0.31	139	0.716
H7	none	16.65 ± 0.28	507	17.00 ± 0.43	44	**0.051**
H1	**H2, H3, H5**	**16.68 ± 0.29**	**532**	**18.06 ± 0.69**	**19**	**0.004**
H2	H1, H3, H5, H7	17.51 ± 0.47	76	17.65 ± 0.47	474	0.452
H3	**H1, H2, H5, H7**	**17.41 ± 0.45**	**341**	**17.76 ± 0.48**	**207**	**0.045**
H5	H1, H2, H5, H7	17.51 ± 0.43	412	17.65 ± 0.50	139	0.411
H7	**H2, H3, H5**	**16.66 ± 0.29**	**507**	**17.06 ± 0.44**	**44**	**0.030**
Loin Yield[3]						
H1	**none**	**14.19 ± 0.11**	**514**	**14.92 ± 0.26**	**19**	**<0.001**
H2	none	14.34 ± 0.15	74	14.22 ± 0.11	458	0.126
H3	none	14.21 ± 0.11	330	14.27 ± 0.12	200	0.748
H5	none	14.27 ± 0.11	398	14.13 ± 0.13	135	0.455
H7	none	14.24 ± 0.11	489	13.98 ± 0.19	44	**0.070**
H1	**H2, H3, H5**	**14.24 ± 0.12**	**514**	**14.92 ± 0.28**	**19**	**<0.001**
H2	H1, H3, H5, H7	14.51 ± 0.19	74	14.38 ± 0.20	458	0.455
H3	H1, H2, H5, H7	14.44 ± 0.18	330	14.45 ± 0.20	200	0.729
H5	H1, H2, H5, H7	14.49 ± 0.17	398	14.40 ± 0.21	135	0.463
H7	H2, H3, H5	14.29 ± 0.12	489	13.98 ± 0.19	44	**0.072**
Shoulder Yield[3]						
H1	none	16.88 ± 0.11	514	17.15 ± 0.27	19	0.639
H2	none	16.86 ± 0.15	74	16.90 ± 0.11	458	0.670
H3	none	16.93 ± 0.12	330	16.86 ± 0.12	200	0.117
H5	none	16.96 ± 0.12	398	16.77 ± 0.13	135	0.280
H7	none	16.88 ± 0.11	489	17.14 ± 0.19	44	0.798
H1	H2, H3, H5	16.87 ± 0.12	514	16.98 ± 0.28	19	0.891
H2	H1, H3, H5, H7	17.04 ± 0.20	74	17.02 ± 0.20	458	0.577
H3	H1, H2, H5, H7	17.08 ± 0.19	330	16.98 ± 0.20	200	**0.075**
H5	H1, H2, H5, H7	17.12 ± 0.18	398	16.95 ± 0.21	135	0.225
H7	H2, H3, H5	16.85 ± 0.12	489	17.08 ± 0.20	44	0.754
Leg Yield[3]						
H1	**none**	**20.94 ± 0.16**	**514**	**21.57 ± 0.37**	**19**	**<0.001**
H2	**none**	**21.19 ± 0.21**	**74**	**20.95 ± 0.16**	**458**	**0.035**
H3	none	20.93 ± 0.16	330	21.08 ± 0.17	200	0.263
H5	none	21.06 ± 0.16	398	20.78 ± 0.18	135	0.970
H7	none	20.97 ± 0.16	489	20.84 ± 0.27	44	**0.097**
H1	**H2, H3, H5**	**21.02 ± 0.17**	**514**	**21.47 ± 0.39**	**19**	**<0.001**
H2	H1, H3, H5, H7	21.28 ± 0.27	74	21.00 ± 0.28	458	0.188
H3	H1, H2, H5, H7	21.13 ± 0.26	330	21.15 ± 0.28	200	0.592
H5	H1, H2, H5, H7	21.27 ± 0.25	398	21.02 ± 0.30	135	0.946
H7	**H2, H3, H5**	**21.06 ± 0.17**	**489**	**20.82 ± 0.27**	**44**	**0.048**

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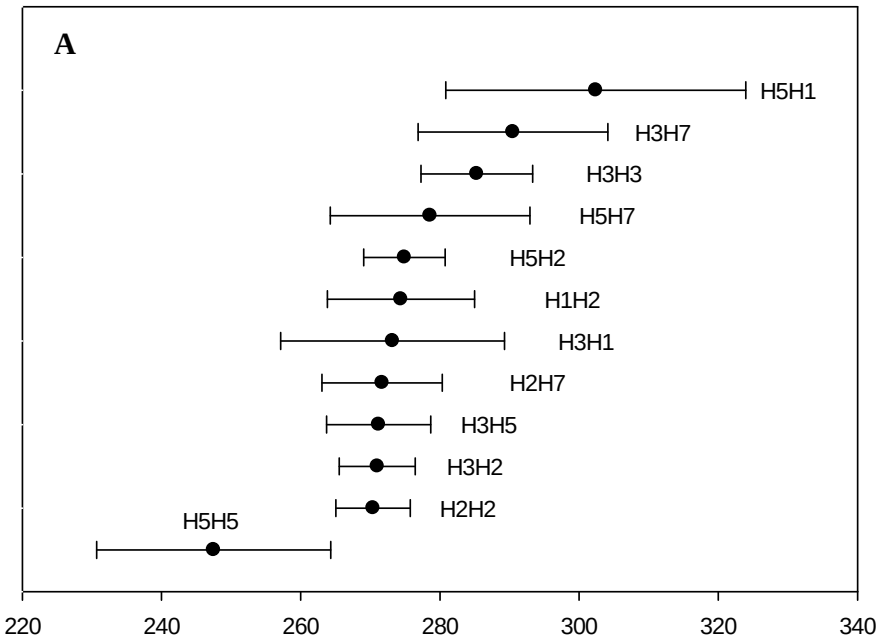
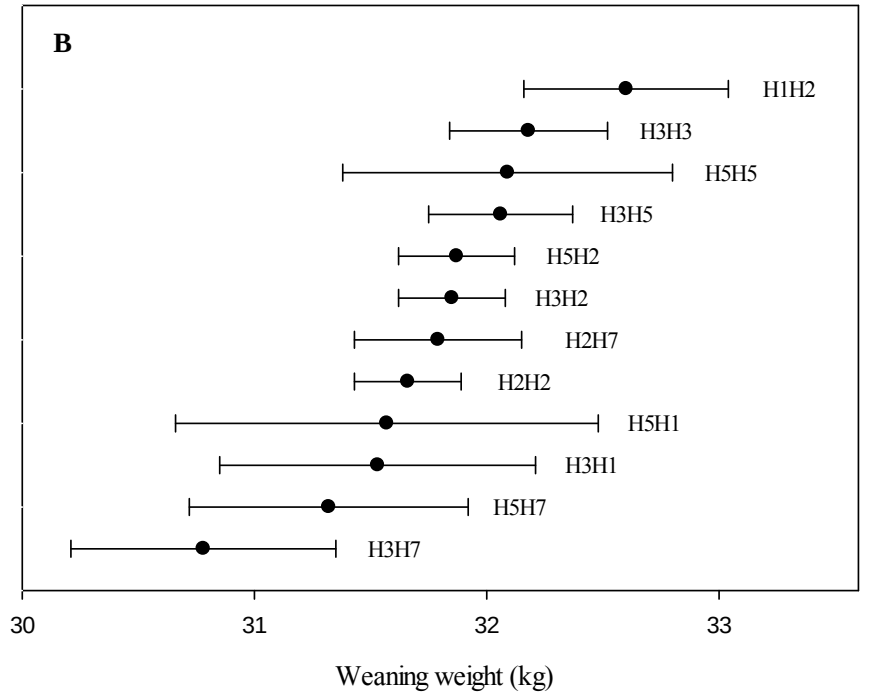
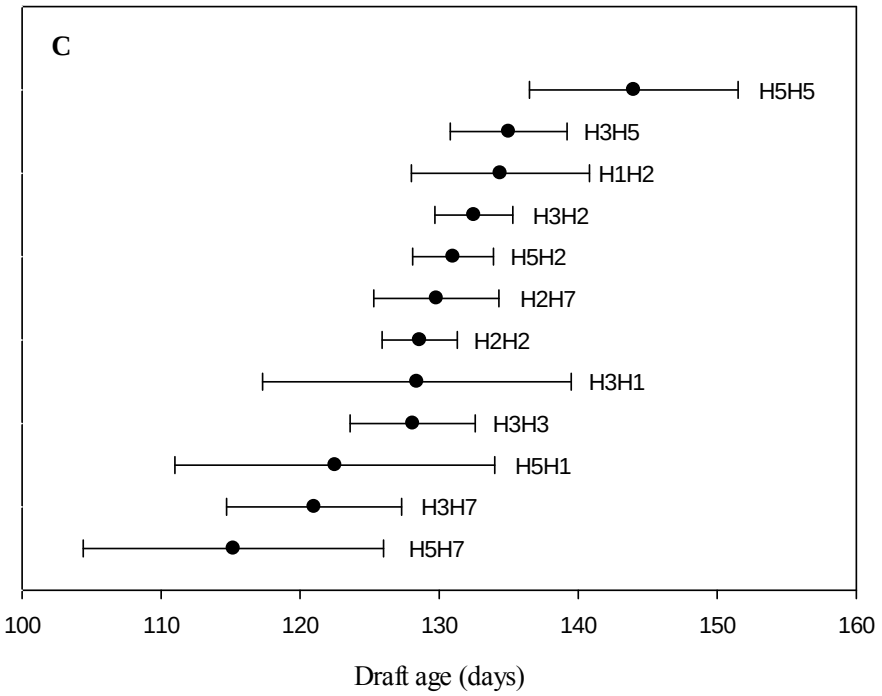
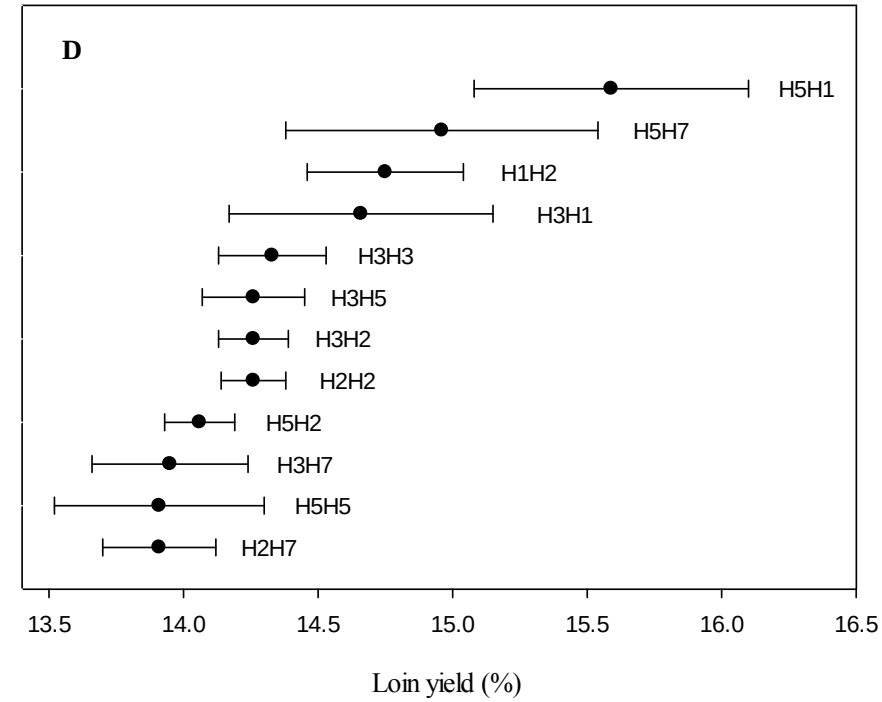
Haplotype being assessed	Other haplotypes in model	Mean[1] ± Std Error				P value[2]
		Haplotype absent	n	Haplotype present	n	
Total Yield[4]						
H1	**none**	**52.01 ± 0.32**	**514**	**53.73 ± 0.75**	**19**	**<0.001**
H2	none	52.34 ± 0.42	74	52.07 ± 0.32	548	0.245
H3	none	52.07 ± 0.32	330	52.22 ± 0.35	200	0.982
H5	none	52.28 ± 0.33	398	51.69 ± 0.36	135	0.719
H7	none	52.10 ± 0.32	489	51.94 ± 0.54	44	0.148
H1	**H2, H3, H5**	**52.10 ± 0.34**	**514**	**53.47 ± 0.79**	**19**	**<0.001**
H2	H1, H3, H5, H7	52.84 ± 0.56	74	52.49 ± 0.56	548	0.308
H3	H1, H2, H5, H7	52.68 ± 0.52	330	52.65 ± 0.57	200	0.705
H5	H1, H2, H5, H7	52.90 ± 0.50	398	52.43 ± 0.59	135	0.669
H7	H2, H3, H5	52.19 ± 0.34	489	51.87 ± 0.55	44	**0.065**
Proportion loin yield[5]						
H1	**none**	**0.2725 ± 0.0011**	**514**	**0.2777 ± 0.0027**	**19**	**0.013**
H2	none	0.2731 ± 0.0015	74	0.2728 ± 0.0011	458	0.542
H3	none	0.2726 ± 0.0012	330	0.2730 ± 0.0013	200	0.668
H5	none	0.2726 ± 0.0012	398	0.2731 ± 0.0013	135	0.130
H7	none	0.2730 ± 0.0011	489	0.2686 ± 0.0020	44	0.151
H1	**H2, H3, H5**	**0.2727 ± 0.0012**	**514**	**0.2792 ± 0.0029**	**19**	**0.006**
H2	H1, H3, H5, H7	0.2742 ± 0.0020	74	0.2741 ± 0.0020	458	0.721
H3	H1, H2, H5, H7	0.2737 ± 0.0019	330	0.2745 ± 0.0021	200	0.299
H5	H1, H2, H5, H7	0.2736 ± 0.0018	398	0.2747 ± 0.0022	135	0.107
H7	H2, H3, H5	0.2733 ± 0.0012	489	0.2692 ± 0.0020	44	0.386
Proportion shoulder yield[5]						
H1	**none**	**0.3249 ± 0.0015**	**514**	**0.3196 ± 0.0035**	**19**	**<0.001**
H2	**none**	**0.3224 ± 0.0020**	**74**	**0.3248 ± 0.0015**	**458**	**0.029**
H3	**none**	**0.3254 ± 0.0015**	**330**	**0.3232 ± 0.0016**	**200**	**0.023**
H5	none	0.3246 ± 0.0015	398	0.3247 ± 0.0017	135	0.252
H7	**none**	**0.3243 ± 0.0015**	**489**	**0.3303 ± 0.0026**	**44**	**0.022**
H1	**H2, H3, H5**	**0.3240 ± 0.0016**	**514**	**0.3180 ± 0.0038**	**19**	**<0.001**
H2	H1, H3, H5, H7	0.3227 ± 0.0026	74	0.3247 ± 0.0026	458	0.581
H3	**H1, H2, H5, H7**	**0.3247 ± 0.0025**	**330**	**0.3228 ± 0.0027**	**200**	**0.034**
H5	H1, H2, H5, H7	0.3239 ± 0.0024	398	0.3236 ± 0.0028	135	0.216
H7	**H2, H3, H5**	**0.3232 ± 0.0016**	**489**	**0.3294 ± 0.0026**	**44**	**0.048**
Proportion leg yield[5]						
H1	**none**	**0.4027 ± 0.0014**	**514**	**0.4023 ± 0.0032**	**19**	**0.010**
H2	none	0.4045 ± 0.0018	74	0.4025 ± 0.0013	458	**0.053**
H3	**none**	**0.4020 ± 0.0014**	**330**	**0.4038 ± 0.0015**	**200**	**0.031**
H5	none	0.4029 ± 0.0014	398	0.4022 ± 0.0016	135	0.999
H7	none	0.4027 ± 0.0013	489	0.4013 ± 0.0023	44	0.229
H1	**H2, H3, H5**	**0.4034 ± 0.0015**	**514**	**0.4024 ± 0.0034**	**19**	**0.008**
H2	H1, H3, H5, H7	0.4029 ± 0.0024	74	0.4010 ± 0.0024	458	0.390
H3	H1, H2, H5, H7	0.4015 ± 0.0023	330	0.4025 ± 0.0025	200	0.131
H5	H1, H2, H5, H7	0.4024 ± 0.0022	398	0.4015 ± 0.0026	135	0.964
H7	H2, H3, H5	0.4035 ± 0.0015	489	0.4015 ± 0.0024	44	0.194

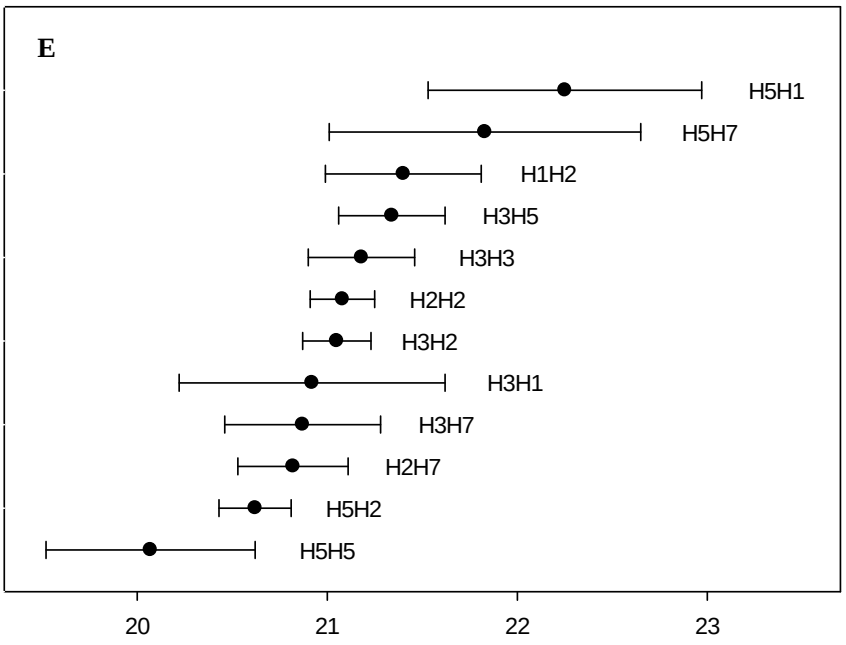
[1]Predicted means; [2]P value derived from a REML mixed-model with haplotype presence/absence and birth rank and sire-line fitted as fixed factors (P≤0.05 were shaded and bolded, 0.05<P<0.1 in bold); [3] Lean meat yield expressed as a percentage of hot carcass weight; [4]Total yield is the sum of the leg, loin and shoulder yield; [5]The proportion yield of loin, shoulder or leg is the yield of the specific area divided by the total yield.


A
H5H1
H3H7
H3H3
H5H7
H5H2
H1H2
H3H1
H2H7
H3H5
H3H2
H2H2
H5H5
220
240
260
280
300
320
340
Growth rate to weaning (g/day)


B
H1H2
H3H3
H5H5
H3H5
H5H2
H3H2
H2H7
H2H2
H5H1
H3H1
H5H7
H3H7
30
31
32
33
Weaning weight (kg)


C
H5H5
H3H5
H1H2
H3H2
H5H2
H2H7
H2H2
H3H1
H3H3
H5H1
H3H7
H5H7
100
110
120
130
140
150
160
Draft age (days)


D
H5H1
H5H7
H1H2
H3H1
H3H3
H3H5
H3H2
H2H2
H5H2
H3H7
H5H5
H2H7
13.5
14.0
14.5
15.0
15.5
16.0
16.5
Loin yield (%)


E
H5H1
H5H7
H1H2
H3H5
H3H3
H2H2
H3H2
H3H1
H3H7
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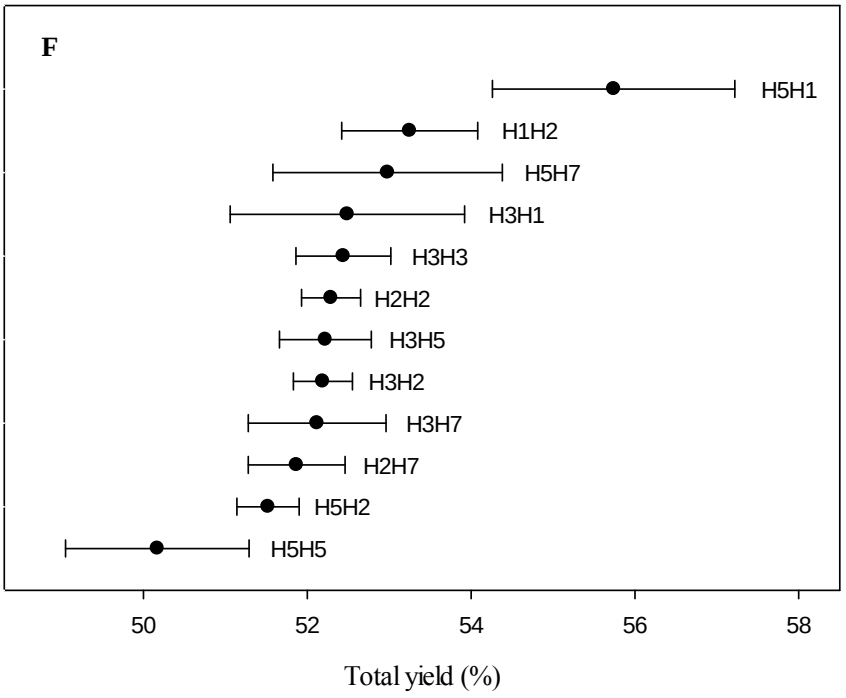
H5H2
H5H5
20
21
22
23
Leg yield (%)


F
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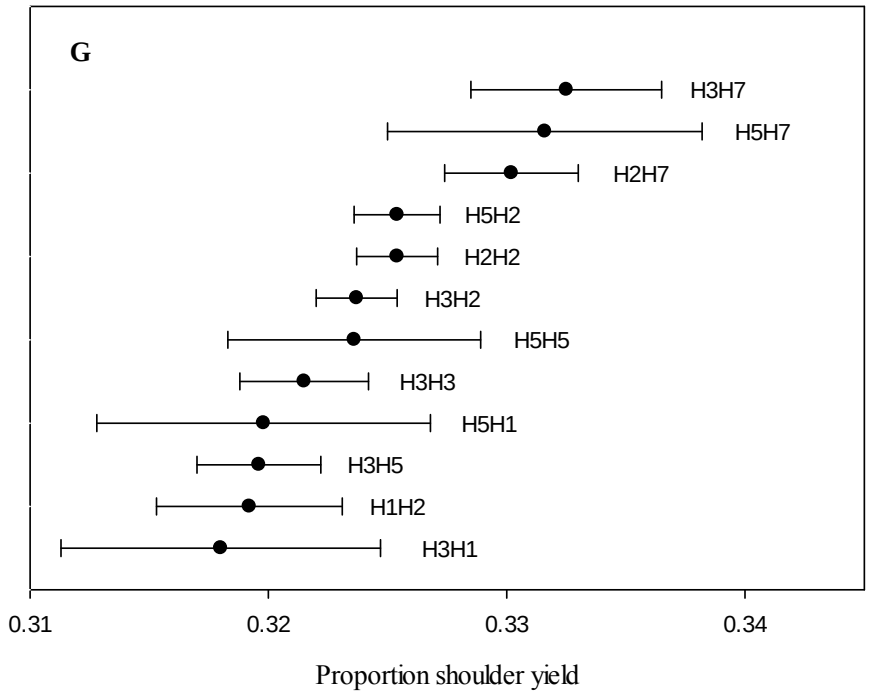
H1H2
H5H7
H3H1
H3H3
H2H2
H3H5
H3H2
H3H7
H2H7
H5H2
H5H5
50
52
54
56
58
Total yield (%)



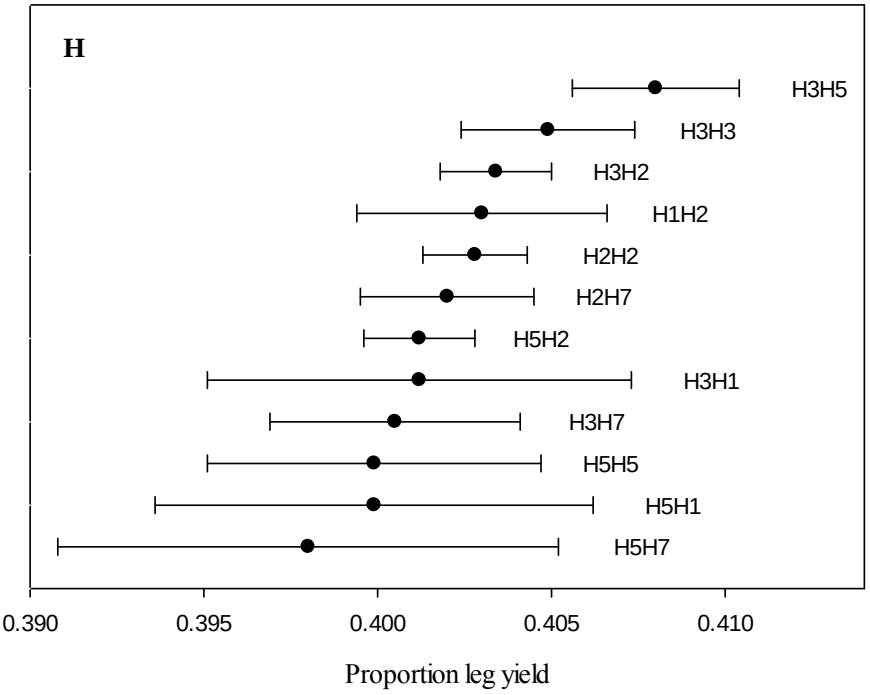


Figure 3.3 The effect of *MSTN* genotype on growth and carcass traits (A) Weaning weight, (B) Growth rate to weaning, (C) Draft age, (D) Loin yield, (E) Leg yield, (F) Total yield, (G) Proportion shoulder yield and (H) Proportion leg yield.

3.3 Discussion

Genetic variation of *MSTN* haplotypes in NZ Romney sheep

Five *MSTN* haplotypes (H1, H2, H3, H5 and H7) were identified in NZ Romney sheep in this chapter. This indicated that greater diversity exists in *MSTN* in NZ Romney sheep than had previously been reported in Chapter 2 where only four haplotypes were detected in Romney sheep. One explanation for this might be the larger number of sheep included in this study. In comparison to the five extended haplotype investigated in *MSTN* in this study, Hickford *et al*. (2010) reported only three haplotypes (A, B and C) in NZ Romney sheep, when a smaller portion of the gene was studied.

In all five haplotypes detected in the NZ Romney sheep in this study, thymine was found at position c.373+101. This is consistent with both the preliminary results described in Chapter 2 and the previous research work by Hickford *et al*. (2010). It also confirms that more variation is present in *MSTN* than previously thought, as the five haplotypes in the current investigation differ from the five haplotypes (named Hap 1-5) identified in Latvian Darkhead sheep (Sjakste *et al.*, 2011). This is presumably due to breed differences.

Haplotypes H4, H6 and H8 were not present in NZ Romney sheep in this study. This result was consistent with the report by Hickford *et al*. (2010) that haplotypes E and D, which were equivalent to haplotypes H4 and H6 in the current study, were not present in NZ Romney sheep. This suggests that haplotypes H4, H6 and H8 do not occur in NZ Romney sheep. One explanation may be that these three *MSTN* genetic variations did not initially exist in the English Romney Marsh sheep, from which NZ Romney sheep were originally developed (Meadows, 2008). Hence, their absence is a founder effect. It is also possible that genetic drift or a genetic bottleneck has in some way removed these haplotypes. Alternatively, these haplotypes may exist in very low frequency and have been bred out of the NZ Romney sheep population because they may not offer any benefit commercially or are a cost to production (McKenzie *et al.*, 2010). Further study on the genetic history and the basis of genetic variation of *MSTN* in both English Romney Marsh and NZ Romney sheep is needed to better understand this result.

The half-sib analyses and pooled-data analyses revealed associations between variation in *MSTN* and various growth and carcass traits. Half-sib analysis is a robust analytical approach as it is assumed that the two progeny groups defined by sire haplotype

segregation are subjected to similar environmental factors that may otherwise affect the phenotype. In effect, a half-sib family is a well-controlled experimental platform for genetic analysis.

In contrast, the pooled-analyses must be interpreted with care, as it is assumed that all variations can be detected and measured, that all the haplotypes are present in each of the sire groups, that all the animals are in similar condition and that the ewes and sires analysed are not highly inbred. All of the above assumptions could not be confirmed as, for example, it was noted that only haplotypes H1, H2, H3, H5 and H7 were identified in the 1206 NZ Romney sheep that were selected for typing. Taken together, the two statistical approaches provide differing types of evidence for the role of genetic variations in *MSTN* in regulating animal growth and carcass traits.

MSTN haplotype H1 carrying c*1232A variation in NZ Romney sheep

This is the first study in which haplotype H1 carrying c*1232A has been observed in NZ Romney sheep and in which its effect on muscle growth and carcass traits was studied. The frequency of the haplotype was 2.79%. This is lower than the frequency of 90% described in Texel sheep (Clop *et al.*, 2006; Kijas *et al.*, 2007), the 30% frequency found in Charollais sheep (Hadjipavlou *et al.*, 2008) and the 10% frequency found in Australian Lincoln, Poll Dorset and White Suffolk sheep (Kijas *et al.*, 2007). The origin of this variation in any given breed remains unclear (Kijas *et al.*, 2007), and it can only be speculated about, given the diverse history of global sheep breeding. These sheep are all European breeds so it is likely that the haplotype had a common origin.

The NZ Romney is derived primarily from the Romney Marsh breed from Southern England (Meadows, 2008) although the introgression of other genetics is possible given the vagueness in the description of development of the breed in NZ. The low haplotype frequency of H1 could be because of genetic drift or, once again, due to the founder effect during the breed development. Alternatively, Kijas *et al*. (2007) suggested that Texel sires have been responsible for transferring this variation into other breeds and humans have bred for it due to its increased effect on muscles. This could also explain the presence of H1 in NZ Romney sheep, because the Texel sheep breed was likely crossed into the Romney during recent breed development in order to increase its muscle production (Meadows, 2008).

H1 associated with heavier birth weight and increased muscle growth

In the Fernvale1106.02 half-sib analyses, there was an increase in birth weight of 0.46 ± 0.28 kg in progeny inheriting H1 compared with those that inherited H5. This contrasts with the previous reports that *MSTN* c.*1232A has no effect on the mean birth weight of Charollais sheep (Hadjipavlou *et al.*, 2008), Norwegian White sheep (Boman *et al.*, 2010), and Australian White Suffolk, Poll Dorset and Lincoln sheep (Kijas *et al.*, 2007). However, the low number of progeny that inherited the variant from their dam within families probably explained the absence of an effect of *MSTN* on birth weight reported by Hadjipavlou *et al*. (2008). H1 was not associated with birth weight in the pooled-data analyses in this study. This might also explain why Hickford *et al*. (2010) did not see the association effect of *MSTN* on birth weight, although five hundred sheep were studied in their work.

Birth weight is the first indicator of the growth potential of a lamb and ultimately reflects the mature body weight potential of sheep (Wardrop, 1968). It is considered a key determinant of animal growth rate, particularly during pre-weaning growth (Farokhad *et al.*, 2011). Increased tailing weights and draft weights were also observed in progeny that inherited H1 compared with those that inherited H5 in the Fernvale 1106.02 half-sib, and tailing weight was also higher in progeny that inherited H1 in the pooled-data analyses. There were positive correlations between birth weight, tailing weight, weaning weight, growth rate to weaning and draft weight in this study, strongly suggesting that selecting for increased birth weight with the presence of H1, would result in improvement in growth in NZ Romney sheep.

In spite of the potential benefits of high birth weights with *MSTN*, there is a case for caution as well. The pleotropic effect of *MSTN* variation has been associated with calving difficulty in cattle (Wiener *et al.*, 2002). Higher mortality rates were found in double-muscled animals with homozygous *MSTN* loss-of-function variation (Wiener *et al.*, 2002), which was reportedly due to the heavy weight of the calves at birth (Esmailizadeh *et al.*, 2008). In *MSTN* knock-out mice, there have also been concerns about the small, brittle and hypo-cellular tendons in females (Mendias *et al.*, 2008), which may have negatively affected birth processes.

In sheep, birth difficulty can be significantly increased with heavier lambs (Dwyer & Bünger, 2012). Lamb mortality is considered a major cause of reproductive loss, especially

in southern regions of NZ where weather conditions are more severe (Gourt *et al.*, 2010). To ensure good lamb survival, lambs should weigh between 4 and 5.5 kg at birth (Gourt *et al.*, 2010). Lambs over 5.5 kg at birth are considered vulnerable to difficult birth or dystocia (Gourt *et al.*, 2010). In view of these reports and the mean birth weight of progeny of 5.31 ± 0.20 kg with H1 in the Fernvale1106.02 sire-line in this study, the effect of H1 on birth weight and the likelihood of heavier birth weight contributing to increased dystocia appear critical, particularly in the context of high fecundity genetics promoted in the NZ sheep industry. Thus, caution may be necessary in further introducing this *MSTN* variation into the NZ Romney sheep breed.

H1 and variation in carcass traits

Haplotype H1 was associated with increased loin yield, leg yield, total yield and proportion loin yield in both half-sib and pooled-data analyses. This increased muscularity was not unexpected. The reason for this is that *MSTN* c.*1232A leads to an inhibition of miRNA-mediated translation of *MSTN* which results in a "double-muscled" phenotype. This was initially identified in Belgian Texel sheep (Clop *et al.*, 2006). The association of *MSTN* c.*1232A with increased muscle mass has also been well documented in Texel flocks in both New Zealand and Australia (Johnson *et al.*, 2009; Kijas *et al.*, 2007). This variant was, subsequently found in other sheep breeds such as the Charollais (Hadjipavlou *et al.*, 2008), Norwegian White sheep (Boman *et al.*, 2010), Norwegian Spælsau sheep (Boman & Våge, 2009); Beltex sheep (Gan *et al.*, 2008) and White Suffolk, Poll Dorset and Lincoln sheep in Australia (Kijas *et al.*, 2007).

Several studies have investigated the effect of *MSTN* c.*1232A on carcass traits. The results of this study were consistent with previous reports that *MSTN* c.*1232A was associated with an increase in *M. longissimus* muscle as reported for NZ Texel sheep (Johnson *et al.*, 2009) and Charollais sheep (Hadjipavlou *et al.*, 2008), and with heavier hindquarters in Belgian Texel sheep (Clop *et al.*, 2006). Meanwhile, the result of increased shoulder yield observed in progeny inheriting haplotype H1 in the Fernvale 1106.02 half-sib was similar to that reported by Kijas *et al*. (2007), who found enhanced forequarters in Australian cross-bred half-sib progeny of Texel sheep with *MSTN* c.*1232A.

Hadjipavlou *et al*. (2008) suggested that the magnitude and the mode of action of the *MSTN* effects on phenotypes was probably affected by the genetic background of the

breeds in which the variation occurred. This would appear to be correct for the NZ Romney, with the breed having higher loin yield, leg yield and total yield with genotype H1H5 when compared with other genotypes. Unfortunately no homozygote H1H1 genotypes were found in this study, and so the expected benefit of being homozygous for c.*1232A could not be assessed.

The muscular effect in progeny inheriting H1 is valuable economically. Progeny inheriting H1 had increased loin, shoulder, leg and total yield by 1.01 ± 0.28, 0.69 ± 0.38, 1.00 ± 0.41 and 2.65 ± 0.84% respectively, compared with progeny inheriting H5 in the Fernvale 1106.02 half-sib analyses. Thus, for an 'average' 18 kg lamb carcass, these yield improvements would equate to an extra 250g of lean meat. While this may seem small, it is never-the-less valuable because of its location on the carcass. For example, an increase in the loin area is of economic value in terms of yielding desirable high priced lamb cuts such as Frenched-racks and boned-out "backstraps". Some Western nations are now suggesting regular daily meat intakes of 65-100g/day of lean meat (Record & Leppard, 2000). Accordingly 250g of lean meat yield is equivalent to at least 2 daily allowances of meat.

Based on the Alliance pay-out scheme for producers in 2011, farmers were paid $15.00/kg loin, $13.80/kg back leg and $7.50/kg shoulder. The average yield of loin, shoulder and leg from an average Alliance lamb is 13.5%, 16.45% and 20.7% respectively, hence, this extra increase in meat yield from inheriting H1 would generate an additional $1.00 per carcass. New Zealand exported 27 million lamb carcasses in 2011 (Beef + Lamb NZ, 2011b). This extra benefit across all carcasses would translate into $27 million more income for the sheep meat industry, assuming that H1 was incorporated into all flocks and assuming all sheep flocks responded similarly to having H1 in the way the NZ Romney lambs studied here did.

The approximately 1% increase in muscling in the loin or leg associated with the presence of H1 appeared lower than reported previously. Hadjipavlou *et al*. (2008) reported a 14% additive genetic variance of muscle depth at the third lumbar vertebra with the presence of *MSTN* c.*1232A in Charollais sheep. At least a 5% muscling increase in the leg and rump was claimed by Pfizer with one or two copies of *MSTN* c.*1232A (Pfizer Animal Health, Dunedin, NZ). One possible reason for the reduced muscling effect with H1 that was observed in this study, might be that only the heterozygous form of *MSTN* c.*1232G>A

was present in NZ Romney sheep, whereas the maximum muscling effect was achieved with the homozygous form of *MSTN* c.*1232A in NZ Texel sheep (Johnson *et al.*, 2009).

Given that the sire-lines in this work were ranked in the top 20% of the SIL-DPO breeding index, an index which is based on measuring genetic merit for a variety of traits of value to the NZ sheep industry, these sire-lines have arguably already been selected for their high productivity. Additionally, a possible explanation for the "small" muscular effect associated with H1 might be that the variation in carcass traits attributable to *MSTN* have been masked by other sheep genes that are located near the markers that are described on murine chromosomes 3, 5,7,11, 16 and X, and which appear to modify the expression of the $MSTN^{Cmpt\text{-}dl1\ Abc}$ phenotype in mice (Varga *et al.*, 2003).

MSTN haplotypes H2 and H5 and lamb growth and carcass traits

In NZ, the weight of a lamb is the most important measurement that determines when to draft and forward lambs for slaughter. The half-sib analyses revealed that a heavier draft weight of 3.63 ± 1.6 kg with an approximately 23-day earlier draft age occurred in the Doughboy 41/06 sire-line with progeny that inherited H2, but not in those inheriting H5. H2 would therefore have major economic benefits due to the lambs achieving their target draft weights earlier. Faster growing lambs gain weight with less accrued feed maintenance cost and can be slaughtered earlier. This is important, particularly in areas where early mating is not possible, as stock may be slaughtered at a time when prices are frequently higher such as prior to Christmas. Having fast growing lambs is also important if pasture growth is low during late spring or early summer, and any feed not used to finish lambs can be used to add condition to the next season's ewes.

In this study, the increase in loin, shoulder and total yield by 0.89 ± 0.36, 0.99 ± 0.43 and 2.75 ± 1.02% respectively in progeny inheriting H2 compared with those inherited H5 in the Doughboy 41/06 half-sib analyses contrasts the results from the pooled-data analyses. In the pooled-data analyses, a decrease in leg yield was observed when H2 was present. This decreased effect is consistent with the results of Hickford *et al*. (2010) who reported that haplotype A (which is the equivalent of H2 in the current study), was associated with a decreased leg, loin and total yield of lean meat. The reduction in loin and total yield reported by Hickford *et al*. (2010) were not found associated with H2.

As discussed in the statistical analyses section of this chapter, the half-sib analysis is a well-controlled analytical approach compared with pooled-data analysis as the effect of compounding factors is balanced across the progeny. However, the pooled-data approach allows more samples to be analysed to represent the population. The apparent discrepancy in the whole effect of haplotype when using the different analytical methods was also reported by Kijas *et al*. (2007). Because the half-sib analyses are better controlled than the pooled-data analyses, the H2 effect in the pooled-data analyses may have been masked or interfered with by other sire effects or other haplotype effects, particularly when it was assumed that all the haplotypes at this locus can be detected and measured, and that all the haplotypes/variants are present in each of the sire groups in the pooled-analyses. Not all these assumptions can be confirmed as robust.

It should be noted that a large proportion of the sires (Of the total 19 sire-lines used in this study: 5 were H2H2, 5 were H2H3, 3 were H2H5, 2 were H2H7, 2 were H3H5, and one was for each of H1H5 and H1H2) and progeny (34.08% were H2H2, 26.87% were H2H3 and 16.34% were H2H5) were homozygous for H2 or heterozygous for H2 in a genotype with H3, H5 and H7. Given that all the sire-lines used in this work have been ranked in the top 20% of the SIL-DPO breeding index, it could be believed that the NZ Romney sheep breeders have been primarily selecting their genetics based on increased productivity. Thus, it could be concluded that H2 not only benefits overall performance (as measured by SIL-DPO), but may also improve selection for increased loin, shoulder and total yield as indicated in the half-sib analyses.

In this study, the apparent discrepancy on the effect of H5 on carcass traits using the two different statistical approaches shows the importance of using both half-sib and the pooled-data analyses. The decreased loin yield, shoulder yield and total yield of progeny inheriting H5 when compared with those inheriting H1 or H2 in Fernvale 1106.02 and Doughboy 41/06 respectively, was identified using half-sib analyses. These results were not confirmed by pooled-data analyses. Indeed there were no significant effects of haplotype H5 on carcass traits using pooled-data analyses in this study. The failure to detect a relationship between H5 and carcass traits was also reported by Hickford *et al*. (2010) using pooled-data analyses. In fact, an adverse effect of the homozygous form of the H5H5 genotype on growth rate to weaning, loin yield, leg yield and total yield compared with other genotypes was actually observed in progeny in the current study. This provides

further support for the negative effect of H5 on meat yields as suggested by the half-sib analyses.

Furthermore, if we were to accept the negative effect of H5 on meat yields, then it is somewhat strange that it is found in NZ Romney sheep. This would suggest that perhaps H5 is associated with and/or beneficial for some other desirable traits selected on the SIL index.

MSTN haplotype H3 and lamb growth and carcass traits

In this study, there were also conflicting results in the associations with H3. In the half-sib analyses, weaning weight and growth rate to weaning for the progeny inheriting haplotype H3 tended to be higher than those inherited H2 in the View Hill 480/04 half-sib. This is in contrast with the Doughboy 167/07 half-sib, where progeny inheriting H3 tended to have a lower mean growth rate to weaning, draft weight and later draft times compared with progeny inheriting H2. The potential increased effects of H2 on muscle growth and carcass traits are discussed in prior discussion. In the pooled-data analyses, H3 had no association with lamb growth with the exception of an association with tailing weight where H3 was associated (P=0.037) with a lower tailing weight (when present with H1, H2, H5 and H7).

H3 was associated with some variation in carcass traits. For example, a higher proportion loin yield was observed in the progeny inheriting H3 compared with those inheriting H2 in the Sudeley 181.06 half-sib. This was consistent with the reports by Hickford *et al*. (2010) and Sjakste *et al*. (2011). In addition, H3 had a positive effect on hot carcass weight in the pooled-data (when present with H1, H2, H5 and H7). The homozygous H3H3 genotype was associated with higher growth rate to weaning, leg yield and total yield, and the heterozygous genotype H3H2 and H3H5 were also associated with higher growth rate to weaning, leg yield and total yield. In view of these results, it is reasonable to suggest that H3 is associated with increased growth rate to weaning during lamb growth, and increased leg yield and total yield of lean meat on carcass traits.

Is *MSTN* haplotype H7 associated with decreased muscularity?

This is the first study showing an association of haplotype H7 carrying the c.101G>A variation with lamb growth and carcass traits. There were conflicting results as regards

associations with H7 in the pooled-data analyses and the effect was only observed in the pooled-data analyses, and not in the half-sib analyses.

MSTN haplotype H7 carrying the c.101G>A variation, results in a Glu/Gly amino acid substitution. This is the only nucleotide substitution identified in the coding region of *MSTN* in NZ Romney sheep (described in Chapter 2) and previously reported by Zhou *et al*. (2008). It may therefore affect the function of the MSTN pro-peptide (Tellgren *et al.*, 2004) and, as a consequence of this, skeletal muscle growth might be disrupted.

In this study, the presence of H7 was associated with increased hot carcass weight but decreased leg yield when other haplotypes (i.e. H2, H3 and H5) were introduced into the model. This suggests that H7 may be having an effect on growth, but possibly at the expense of meat yield. If we were to accept this result, then an argument may be that if historically NZ Romney sheep were selected based on live weight gain, then this might lead to heavier carcass weights as a consequence of increased bone weight and not meat weight. Bone density is 1.47g/cm^3 (Campbell *et al.*, 2003) which is heavier than meat density at 1.05g/cm^3 (Lambe *et al.*, 2011), so H7 conceivably offers more carcass weight, but without any meat yield benefit.

Given the relatively low frequency of H7 in sire-lines and progeny in this study, it could be presumed that this haplotype may have already been bred out of the NZ Romney owing to its negative effect on leg yield. In addition, the failure to detect H7 in both Coopworth and Perendale sheep (Chapter 2), both breeds being based on NZ Romney genetics, suggests H7 is of little value to production in these two breeds.

The suggested negative effect of haplotype H7 on leg yield may explain the non-detection of positive effects for H2, which was the equivalent of the variant A reported by Hickford *et al*. (2010). In the current study, three different *MSTN* haplotypes, H1, H2 and H7, were resolved from Hickford *et al*. (2010) variant A sequence. It could be speculated that the negative effect of variant A associated with leg, loin and total yield suggested by Hickford *et al*. (2010) was therefore due to this variant A being H7. Regardless, more research on H7 is probably required before its effect on growth or carcass traits can be fully understood.

Pleiotropic effect of *MSTN* c.*1232A on gender

This is the first study to report an association between *MSTN* c.*1232 genotype and gender ratio in New Zealand's most common sheep breed, the NZ Romney.

In the initial study with *MSTN*, McPherron *et al*. (1997) suggested that *MSTN* was primarily expressed in skeletal muscle, but it was not restricted to this tissue as low levels of *MSTN* were detected in adipose tissue. Subsequently, *MSTN* has also been detected in other tissues including the heart muscle of mouse and sheep (Sharma *et al.*, 1999), spleen (Helterline *et al.*, 2007) and brain (Ng *et al.*, 2007) of mouse and mammary glands of pigs (Ji *et al.*, 1998). In humans, MSTN is synthesized and released within the placenta, and acts as a paracrine regulator involving maternal/foetal nutrient partitioning (Mitchell *et al.*, 2006). In light of these reports, the potential for other pleiotropic functions of *MSTN* along with frequently reported effect on skeletal muscle growth is implied. Thus, the pleiotropic effect of *MSTN* which may potentially affect other physiological activities during embryonic development offers an explanation for the abnormal gender ratio effect observed in this study. However, the actual mechanism by which this gender effect is initiated is unclear.

In transgenic mice with the muscle-specific "over expression" of MSTN, a decrease in muscle mass was only detected in male progeny and not in females (Reisz-Porszasz *et al.*, 2003). In contrast, an increase in body and muscle mass was associated with a decrease in the expression of processed MSTN in male mice compared with females (McMahon *et al.*, 2003). This suggests that in female transgenic mice, there are gender-specific mechanisms that can possibly override the effects of MSTN on muscle mass. The putative embryonic roles of MSTN may therefore be gender-specific.

According to the lambing records of the progeny from the Fernvale 1106.02 sire, only one lamb died at birth. Thus, the difference in gender ratio associated with the *MSTN* c.*1232 genotype could not be simply explained by lamb mortality.

It is therefore worth studying this gender effect in more detail as *MSTN* c.*1232A is currently being bred into other sheep breeds such as Charollais (Hadjipavlou *et al.*, 2008) and White Suffolk, Poll Dorset and Lincoln sheep in Australia (Kijas *et al.*, 2007) in an effort to increase meat yield. If the presence of *MSTN* c.*1232A affects the viability of the male embryos, then a potential exists for inadvertently selecting for increased male

mortality whilst selecting for increased muscle growth. Thus it is important for the livestock industry to balance the new opportunity of increased muscle growth against potential new threats caused by deleterious effects of *MSTN* c.*1232A. It is accepted that this is a small and preliminary study and that a trial involving more sheep is required to validate these initial observations.

In summary, the five extended *MSTN* haplotypes (H1, H2, H3, H5 and H7) identified in the NZ Romney sheep in this study supports the contention that more extensive investigation of *MSTN* variation in sheep is a valuable exercise. Given the associations revealed between *MSTN* haplotypes and various growth and carcass traits in NZ Romney sheep, and the associations between various genetic variants and muscularity reported in other sheep breeds (Boman *et al.*, 2009; Clop *et al.*, 2006; Hadjipavlou *et al.*, 2008; Han *et al.*, 2010; Hickford *et al.*, 2010; Johnson *et al.*, 2009; Kijas *et al.*, 2007), genetic improvement through marker-assisted selection based on this gene, would be a valid approach to improving lamb production.

In the context of the overwhelming economic importance of the lamb export sales in the NZ sheep meat industry, it is obvious that lamb growth and carcass traits are valuable performance measures. In the context of the research described herein, it would seem advantageous to promote selection for haplotypes H2 and H3 and against haplotypes H5 and H7 in NZ Romney breed. Caution may however be needed in breeding for haplotype H1 in NZ Romney owing to the possibility of undesirable pleiotropic effects associated with this haplotype.

Chapter 4
General summary and future work

The major focus of this thesis was to assess haplotypic diversity of ovine *MSTN* and ascertain whether this genetic variation is associated with lamb growth, carcass traits and reproduction in NZ Romney sheep.

Initially genetic variation across an extended region of *MSTN* was investigated in a wide variety of NZ sheep breeds. This is the first research of this kind undertaken in NZ and is considered important, because characterising the variation of *MSTN* across sheep breeds would be fundamental to establishing how to potentially use this genetic information to improve sheep meat production.

In summary, eighteen sets of PCR primers spanning from c.-1199 to c.*1331 were designed and used to investigate a diverse range of NZ sheep breeds. These included dual-purpose breeds for meat and wool production (Coopdale, Coopworth, Corriedale, Perendale and NZ Romney), meat breeds (Dorper, Dorset Down, Poll Dorset, Suffolk and Texel), a wool breed (Merino) and some other cross-bred sheep. A total of 28 single nucleotide substitutions were found in the *MSTN* region studied using PCR-SSCP and nucleotide sequencing. Ten of the substitutions are reported for the first time in this study.

Based on the genetic variations found, extended haplotypes of *MSTN* were determined. Eight haplotypes (H1-8) of ovine *MSTN* spanning the 5'UTR, coding regions, intronic regions and 3'UTR were resolved. These regions may all affect MSTN function. Two nucleotide substitutions (c.101G>A and c.*83A>G) specifically defined haplotypes H7 and H8 respectively in this study and have not been reported previously.

Of the eight haplotypes, some (H2 and H3) were found with high frequencies whereas others (e.g. H8) were less common. Although the haplotype frequencies may only be representative of the sheep that were sampled for typing in the study, the results still illustrate the extent of the variability within and across breeds. In the future, a study on the genetic history and background of existing sheep breeds is needed although this will be doubtless a time consuming and difficult task.

Of the various sheep breeds studied, the NZ Romney is the most common breed in NZ. Investigation of the association between the *MSTN* haplotypes and variation in lamb growth and various carcass traits in NZ Romney sheep was carried out. Given the role of MSTN in regulating muscularity, adiposity and tendon structure, it is conceivable that variation in *MSTN* potentially affects sheep meat production and thus is of economic value to the NZ sheep industry.

Five extended *MSTN* haplotypes (H1, H2, H3, H5 and H7) were first identified in the NZ Romney sheep studied. Of these, haplotype H1 carrying *MSTN* c.*1232A is reported for the first time in NZ Romney sheep. H1 was associated with an increase in birth weight, tailing weight and draft weight in lambs, and had a significant increase effect on loin yield, leg yield, total yield and proportion loin yield in lamb carcasses. Breeding for increased number of lambs carrying H1 may therefore lead to an improvement in lamb growth and meat yield, and thus underpin increased productivity and economic returns to the industry. However, caution is needed, as the pleiotropic effects of H1 are unknown and it could be speculated that it might increase birthing difficulties and potentially affect gender ratio.

This is the first report of an association between haplotype H1 and increased birth weight in NZ Romney sheep. However birthing difficulties can occur with heavier lambs which have a higher mortality rate at birth (Dawson *et al.*, 2002). Indeed there is an increased need for birthing assistance reported for Texel sheep (Speijers *et al.*, 2010). Dwyer and Bünger (2012) reported a mean birth weight of 4.32kg for Texel lambs in one study which is lighter than the birth weight of 6.21kg for lambs carrying *MSTN* c.*1232A in the NZ Romney sheep reported in this study. It is therefore suggested that birth weight *per se* may not necessarily be the sole factor contributing to birthing difficulties in Texel sheep, a point also noted by Dwyer and Bünger (2012).

The high frequency of *MSTN* c.*1232A and its effect on muscling have been well described in Texel sheep. However small, brittle and hypo-cellular tendons have been reported in *MSTN*-deficient mice (Mendias *et al.*, 2008), which suggests that tendon structure and function may also negatively affect birthing processes, leading to the need for more lambing assistance in Texel sheep. *MSTN* may therefore have other, as yet, unknown pleiotropic effects that contribute to increased birthing difficulties and lamb mortality. Ewes that require more assistance at lambing is a cost to farmers, and is unacceptable in the NZ “easy-care” sheep farming systems. NZ Romney have been bred for and are

recognized as an "easy-care" breed from lambs at birth and through to weaning. The effect of H1 carrying *MSTN* c.*1232A in NZ Romney sheep needs to be investigated further to establish its acceptance as a valuable genetic sequence.

This is also the first report of a potential pleiotropic effect of *MSTN* on gender ratio. The results in Chapter 3 suggest H1 (containing *MSTN* c.*1232A) may potentially affect embryonic physiological activities during its development, and thus lead to the *MSTN* c.*1232AG genotype being more common in female lambs. This may also affect lamb survival, although the actual mechanism by which *MSTN* affects the survival of a particular gender is unclear. Thus, in order to precisely ascertain the potential pleiotropic effects of haplotype H1 on the NZ sheep production system, a large study of Romney sheep carrying *MSTN* c.*1232A is required in the future. It may be a challenge to obtain the benefits from increased birth weight, lamb growth and carcass yields if negative traits are also enhanced.

In NZ Romney breed, it would seem beneficial to promote selection for haplotypes H2 and H3 rather than haplotypes H5 and H7 due to that positive effects of H2 and H3 and negative effects of H5 on various growth and carcass traits were identified in the NZ Romney sheep studied. These conclusions were achieved following consideration of the results of the half-sib and pooled-data analyses. While some of the results conflicted at times, it is believed that the haplotype effects may have been confounded by other sire-line or haplotype effects in pooled-data analyses.

Haplotype H7 carrying c.101G>A which results in a Glu/Gly amino acid substitution in the coding region is considered unique and has been only reported in NZ sheep breeds. The possible effect of H7 on increased hot carcass weight but with decreased leg yield creates a sheep production challenge in NZ. The results suggest that H7 may be simply associated with an increased weight of bone, but not lean meat. The non-detection of H7 in other meat sheep breeds studied may suggest that H7 has not been selected over time for meat production.

Selection of NZ sheep is most frequently based on live weight gain, and payment to farmers is primarily based on the carcass weight with some extra bonus payments for carcass grade. A heavier carcass weight is, therefore, an economic benefit to farmers. In contrast, from the consumer point of view, more meat per unit weight of carcass is desirable. Caution is, however, needed if breeding for H7 in NZ Romney sheep is

promoted due to the need to consider the balance between the economic returns to farmers versus the consumption preferences of consumers. Further work is required to ascertain the existence of haplotype H7 in other sheep breeds within and outside NZ.

The sheep meat industry is an essential and major contributor to the export trade and economy of NZ. A successful and viable sheep industry must produce products that meet the demands of future consumers. Previous studies have shown the effect of variation in *MSTN* on valuable carcass traits (Clop *et al.*, 2006; Kijas *et al.*, 2007; McPherron *et al.*, 1997) without there being any apparent effect on meat tenderness (Johnson *et al.*, 2005; Kijas *et al.*, 2007). *MSTN* also has an effect on carcass fatness (Boman *et al.*, 2010; Hadjipavlou *et al.*, 2008; McPherron *et al.*, 1997) and the percentage of intramuscular fat (Kijas *et al.*, 2007). Despite these apparent positive attributes, a negative effect on eating quality has been reported by Kijas *et al*. (2007) in sheep carrying c.*1232A. It would therefore be important to determine if genetic variation in *MSTN* affects meat quality factors such as tenderness, juiciness, colour and flavour in various NZ sheep breeds, but this would require a future study. Such meat quality factors could influence the consumer preference for meat and therefore the price consumers are prepared to pay. Thus, achieving a high meat yield in animal which produces high quality meat post-mortem remains the ultimate goal for the NZ sheep meat industry.

The work presented in this thesis suggests that using *MSTN* variation as a selection tool for improved carcass traits in NZ Romney may be feasible. Gene-marker assisted breeding offers a great potential for farmers to capitalise on the genetic potential of their animals by increasing the selection accuracy and reducing the generation interval in breeding. Validation of the effect of various haplotypes on sheep meat production and meat quality traits across different sheep breeds are still needed. These studies need to be completed prior to utilising the identified *MSTN* haplotypes for marker-assisted selection, particularly if the advocated genetic selection marker has other negative effects for the breed.

In addition, further investigation is needed into the interaction of *MSTN* with other genes involved in muscle growth such as Myf5, MyoD, MRF4 and MYOG. These are all key genes that are involved in the physiological mechanisms of myoblast proliferation and differentiation during the embryonic development of muscle. Such studies would provide a more accurate understanding of the association between an animal's genotype and its phenotypic performance, and also potentially provide more precision in animal breeding.

Appendices

Appendix A. Variation identified in bovine *MSTN* sequences in different breeds

Variation name[1]	Description of variation[1]	Amino acid changes	Location	Bovine breeds[4]	Reference
nt1-805C>G	nt-805C>G		Promoter	AA	Crisa *et al*., 2003; O'Rourke *et al*., 2009
nt1-371T>A	nt-371T>A		Promoter	AA	Crisa *et al*., 2003; O'Rourke et al., 2009
nt76A>T	nt76A>T	aa 26, asparagine→tyrosine	Exon 1	NE	Grisolia *et al*., 2009
nt111G>T	nt111G>T	silent, no amino acid change	Exon 1	NE	Grisolia *et al*., 2009
nt267A>G	nt267A>G	silent, no amino acid change	Exon 1	AU, BA, SA	Dunner *et al*., 2003
F94L	nt282C>A	aa 94, Phenylalmanine→Leucine	Exon 1	LI	Dunner *et al*., 2003; Esmailizadeh *et al*., 2008; Patent Number 502523
Sl05C	nt314C>G	aa105, Serine→Cysteine	Exon 1	PA	Dunner *et al*., 2003
nt324C>T	nt324C>T	silent, no amino acid change	Exon 1	AU, CH, IN95, MA, SA	Dunner *et al*., 2003
nt374-16(delT)[2]/T>G	nt374-16(delT)/T>G		Intron 1		Grisolia *et al*., 2009
nt374-51T>C	nt374-51T>C		Intron 1	AV, AM, PI, RG, AU, BZ, BA, BR, CH, GA, LI, IN95, MA, NO, PA, SA, AA, AY, BS, DE, GL, HE, HT, LH, SD, BBB mixte, MG	Dunner *et al*., 2003
nt374-50G>A	nt374-50G>A		Intron 1	AV, AM, PI, RG, AU, BA, BR, CH, GA, IN95, MA, PA, SA, AA, AY, DE, GL, HE, LH, SD, TH	Dunner *et al*., 2003
nt374-16del1[2]	nt374-16del 12		Intron 1	AV, AM, PI, RG, AU, BA, BR, CH, GA, IN95, MA, PA, SA, AA, AY, DE, GL, HE, LH, SD, TH	Dunner *et al*., 2003; Patent Number 502523
nt387G>A	nt387G>A	silent, no amino acid change	Exon 2	MA,SA, AY, GL	Dunner *et al*., 2003
nt414C>T	nt414C>T	silent, no amino acid change	Exon 2	AA, AV, AM, PI, RG, AU, BB, BA, BR, CH, CA, GA, IN95, MA, PA, SA, AA, AY, DE, GL, HE, LH, SD, SI, LI, CH, HO, TH	Dunner *et al*., 2003; Smith *et al*., 2000; Patent Number 502523
nt419del7-ins10[3]	nt419del7-ins10	aa140, premature stop codon	Exon 2	MA, PI, IN95, MA, PA	Grobet *et al*., 1998; Dunner *et al*., 2003; Patent Number 502523
nt420T>G	nt420T>G	aa140, Phenylalanine→Leucine	Exon 2	NE	Grisolia *et al*., 2009
nt433A>T	nt433A>T	silent, no amino acid change	Exon 2	NE	Grisolia *et al*., 2009
nt445A>T	nt445A>T	aa149, Asparagine→Tyrosine	Exon 2	NE	Grisolia *et al*., 2009
nt527T>A	nt527T>A	aa176, Leucine→Histidine	Exon 2	NE	Grisolia *et al*., 2009
D182N	nt544G>A	aa182, Aspartic acid→Asparagine	Exon 2	MA	Miranda *et al*., 2002; Dunner *et al*., 2003
Q204X	nt610C>T	aa204, Glutamine→Stop codon	Exon 2	BA, CH, LI, IN95, PA	Grobet *et al*., 1998; Dunner *et al*., 2003; Patent Number 502523
nt641G>A	nt641G>A	aa214, Asparagine→Serine	Exon 2	NE	Grisolia *et al*., 2009
nt694G>A	nt694G>A	aa232, Glutamic acid→Lysine	Exon 2	NE	Grisolia *et al*., 2009
E226X	nt676G>T	aa226, Glutamine→Stop codon	Exon 2	MA, PA	Grobet *et al*., 1998; Dunner *et al*., 2003; Patent Number 502523
nt747+7G>A	nt747+7G>A		Intron 2	DE, LH	Dunner *et al*., 2003
nt747+11A>G	nt747+11A>G		Intron 2	SD	Dunner *et al*., 2003
nt747+116T>G	nt747+116T>G		Intron 2	NE	Grisolia *et al*., 2009
nt747+117C>T	nt747+117C>T		Intron 2	NE	Grisolia *et al*., 2009
nt747+118A>G	nt747+118A>G		Intron 2	NE	Grisolia *et al*., 2009
nt748-78del1[2]	nt748-78del1	Thymine is deleted from sequence	Intron 2	AV, AM, PI, RG, AU, BA, BR, CH, GA, IN95, MA, PA, SA, AA, AY, DE, GL, HE, LH, SD, TH	Dunner *et al*., 2003; Grobet *et al*., 1998; Patent Number 502523

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nt821del11[2]	nt821del11	11bp deletion resulting in a premature stop codon in the bioactive C-terminal region	Exon 3	AM, AU, BB, BA, LI, PA, PI, AV, RG, CH, IN95, NO, DE, LH, SD, BBB MIXTE, BBB	Dunner *et al*., 2003; Grobet *et al*., 1998; Kambadur *et al*., 1997a; McPherron & Lee, 1997;Smith *et al*., 2000; Patent Number 502523
nt840A>G	nt840A>G	silent, no amino acid change	Exon 3	NE	Grisolia *et al*., 2009
E291X	nt874G>T	aa291, Glutamic acid→Stop codon	Exon 3	MG	Dunner *et al*., 2003
nt887A>G	nt887A>G	aa291, Aspartic acid→Glycine	Exon 3	NE	Grisolia *et al*., 2009
C313Y	nt938G>T	aa313, Cysteine→Tyrosine	Exon 3	GA, IN95, PE	Dunner *et al*., 2003; Grobet *et al*., 1998; Kambadur *et al*., 1997a; McPherron & Lee, 1997; Patent Number 502523
nt951T>G	nt951T>G	aa317, Phenylalanine→Lysine	Exon 3	NE	Grisolia *et al*., 2009
nt1083C>T	nt1083C>T	silent, no amino acid change	Exon 3	NE	Grisolia *et al*., 2009
nt1128+27A>C	nt c.*27A>C		3'URT	NE	Grisolia *et al*., 2009
nt1128+151A>C	nt c.*151A>C		3'URT	NE	Grisolia *et al*., 2009
nt1128+159A>T	nt c.*159A>T		3'URT	NE	Grisolia *et al*., 2009
nt1128+180A>C	nt c.*180A>C		3'URT	NE	Grisolia *et al*., 2009
nt1128+201C>A	nt c.*201C>A		3'URT	NE	Grisolia *et al*., 2009
nt1128+263C>T	nt c.*263C>T		3'URT	NE	Grisolia *et al*., 2009
nt1128+273G>A	nt c.*273G>A		3'URT	NE	Grisolia *et al*., 2009
nt1128+377T>C	nt c.*377T>C		3'URT	NE	Grisolia *et al*., 2009
nt1128+381A>C	nt c.*381A>C		3'URT	NE	Grisolia *et al*., 2009
nt1128+401A>C	nt c.*401A>C		3'URT	NE	Grisolia *et al*., 2009
nt1128+421T>A	nt c.*421T>A		3'URT	NE	Grisolia *et al*., 2009
nt1128+492C>A	nt c.*492C>A		3'URT	NE	Grisolia *et al*., 2009
nt1128+598A>C	nt c.*598A>C		3'URT	NE	Grisolia *et al*., 2009
nt1128+687A>G	nt c.*687A>G		3'URT	NE	Grisolia *et al*., 2009
nt1128+702T>C	nt c.*702T>C		3'URT	NE	Grisolia *et al*., 2009
nt1128+742A>T	nt c.*742A>T		3'URT	NE	Grisolia *et al*., 2009
nt1128+761G>C	nt c.*761G>C		3'URT	NE	Grisolia *et al*., 2009
nt1128+773(delT)[2]	nt c.*773delT		3'URT	NE	Grisolia *et al*., 2009
nt1128+779T>C	nt c.*779T>C		3'URT	NE	Grisolia *et al*., 2009
nt1128+801G>C	nt c.*801G>C		3'URT	NE	Grisolia *et al*., 2009
nt1128+836T>C	nt c.*836T>C		3'URT	NE	Grisolia *et al*., 2009
nt1128+888(delT)[2]	nt c.*888delT		3'URT	NE	Grisolia *et al*., 2009
nt1128+900(delA)[2]	nt c.*9000delA		3'URT	NE	Grisolia *et al*., 2009
nt1128+927A>C	nt c.*927A>C		3'URT	NE	Grisolia *et al*., 2009
nt1128+957A>C	nt c.*957A>C		3'URT	NE	Grisolia *et al*., 2009
nt1128+970A>C	nt c.*970A>C		3'URT	NE	Grisolia *et al*., 2009
nt1128+988A>C	nt c.*988A>C		3'URT	NE	Grisolia *et al*., 2009
nt1128+998T>C	nt c.*998T>C		3'URT	NE	Grisolia *et al*., 2009
nt1128+1007A>C	nt c.*1007A>C		3'URT	NE	Grisolia *et al*., 2009
nt1128+1035A>C	nt c.*1035A>C		3'URT	NE	Grisolia *et al*., 2009
nt1128+1039C>A	nt c.*1039C>A		3'URT	NE	Grisolia *et al*., 2009
nt1128+1048T>C	nt c.*1048T>C		3'URT	NE	Grisolia *et al*., 2009
nt1128+1135(delT)[2]	nt c.*1135delT		3'URT	NE	Grisolia *et al*., 2009
nt1128+1197T>C	nt c.*1197T>C		3'URT	NE	Grisolia *et al*., 2009
nt1128+1268C>A	nt c.*1268C>A		3'URT	NE	Grisolia *et al*., 2009
nt1128+1269C>A	nt c.*1269C>A		3'URT	NE	Grisolia *et al*., 2009
nt1128+1277A>T	nt c.*1277A>T		3'URT	NE	Grisolia *et al*., 2009

[1]. The nucleotide (nt) position is based on coding sequence only. Nucleotides in non-coding regions are described by either the additional of nucleotides to the last nucleotide postion in the preceding exon or substracting nucleotides from the first nucleotide position of the next exon;
[2] del = deletion;[3]. ins = insertion; [4] Bovine breeds: Aberdeen Angus (AA), Asturiana de la Montana (AM), Aubrac (AU), Austuriana de los Valles (AV), Ayrshire (AY), Belgian Blue (BB), Bazadaise (BZ), Blanc Bleu Belge culard (BBB), Blanc Bleu Belge Mixte (BBB mixte), Blonde d'Aquitaine (BA), Bretonne Pie Noire (BR), British Shorthorn (BS), Calloway (CA), Charolais E (CH), Devon (DE), Galloway (GL), Gasconne (GA), Hereford (HE), Holstein (HO), Inra95 Sire Line (IN95), Limousine (LI), Lonhorn (LH), Maine-Anjou (MA), Marchigiana (MG), Nellore (NE), Normande (NO), Parthenaise (PA), Piedmontese (PE), Pirenaica (PI), Rubia Gallega (RG), Salers (SA), Simmental (SI), South Devon (SD), Traditional Hereford (TH)

Appendix B. Variation identified in ovine *MSTN* sequences in different breeds

Variation numbering[1]	Description of variation[1]	Amino acid changes	Location	Comments/ original name of variation	Ovine breed[4]	Reference
g.1156G>C	c.-2449G>C		Promoter	g-2449C-G	Texel	Clop *et al*., 2006
g.1226T>C	c.-2379T>C		Promoter	g-2379C-T	Texel	Clop *et al*., 2006
g.2200T>A	c.-1405T>A		Promoter	g-1405A-T	Texel	Clop *et al*., 2006
g.2203A>G	c.-1402G>A		Promoter	g-1402G-A	Texel	Clop *et al*., 2006
g.2391T>C	c.-1214T>C		Promoter	g-1214C-T	Texel	Clop *et al*., 2006
g.2403C>T	c.-1202C>T		Promoter		China Small Tail Han sheep	Du *et al*., 2005
g.2476T>C	c.-1129T>C		Promoter	g-1129T-C	Texel	Clop *et al*., 2006
g.2649T>C	c.-956T>C		Promoter	*-956(T→C)	Huyang; Kazak; Beltex; Dorper; Beltex x Huyang; MPMF; MF	Gan *et al*., 2008
g.2824G>A	c.-781G>A		Promoter	*-781(G→A)	MPMF	Gan *et al*., 2008
g.2841del²A	c.-764del²A		Promoter		China Small Tail Han sheep	Du *et al*., 2005
g.2921G>A	c.-684G>A		Promoter		China Small Tail Han sheep	Du *et al*., 2005
g.3188G>A	c.-417G>A		Promoter		China Small Tail Han sheep	Du *et al*., 2005
g.3564A>C	c.-41A>C		5'UTR	g-41C-A (Clop et al., 2006; Gan et al., 2008; Kijas et al., 2007); c.-40 (Sjakste et al., 2011)	Texel ; Black Suffolk; Coopworth; English Leicester; Lincoln, Merino; Poll Dorset; Polwarth; Romney; Southdown; Tibetan; White Suffolk; Latvian Darkhead sheep	Clop *et al*., 2006; Gan *et al*., 2008; Kijas *et al*., 2007; Sjakste *et al*., 2011
g.3567C>T	c.-38C>T		5'UTR	g-39T-C (Clop et al., 2006); c.-37 (Sjakste et al., 2011)	Texel; Latvian Darkhead sheep	Clop *et al*., 2006; Sjakste *et al*., 2011
g.3574del²T	c.-31del²T		5'UTR		Norwegian White sheep; Norwegian Spælsau sheep; China Small Tail Han sheep; Latvian Blackhead sheep; NZ cross-bred sheep	Boman *et al*., 2009; Boman & Våge, 2009; Du *et al*., 2005; Sjakste *et al*., 2011; Zhou *et al*., 2008;
g.3705G>A	c.101G>A	aa34, Glutamin acid →Clycine	Exon 1	mis-sense veriation	NZ cross-bred sheep;	Zhou *et al*., 2008
g.3724ins³A	c.120ins³A	aa49, premature stop codon	Exon 1	disruptive veriation	Norwegian Spælsau sheep	Boman & Våge, 2009
g.3995T>G	c.373+18T>G		Intron 1	g+391G-T	Texel; NZ Romney; Dorset Down; Merio & Polwarth; Southdown; Beltex sheep; Latvian Blackhead sheep; NZ cross-bred sheep	Clop *et al*., 2006; Hickford *et al*., 2010; Gan *et al*., 2008; Kijas *et al*., 2007; Sjakste *et al*., 2011; Zhou *et al*., 2008
g.4078T>C	c.373+101T>C		Intron 1	g+474C-T	Texel; Latvian Darkhead sheep	Clop *et al*., 2006; Gan *et al*., 2008; Sjakste *et al*., 2011
g.4139A>G	c.373+162A>G		Intron 1	535(A→G)		Gan *et al*., 2008
g.4142A>G	c.373+165A>G		Intron 1	538(A→G)		Gan *et al*., 2008
g.4168T>C	c.373+191T>C		Intron 1	564(T→C)		Gan *et al*., 2008
g.4209C>T	c.373+232C>T		Intron 1	605(C→T)		Gan *et al*., 2008
g.4217C>T	c.373+240C>T		Intron 1	g+613T-C	Texel	Clop *et al*., 2006; Gan *et al*., 2008
g.4218C>T	c.373+241C>T		Intron 1	C.373+241(C-T)	NZ Romney; Dorset Down; Merino & Polwarth; Latvian Darkhead sheep	Hickford *et al*., 2010; Sjakste *et al*., 2011

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g.4220A>G	c.373+243A>G	Intron 1	g+616G-A	NZ Romney; Dorset Down; Texel; Merino & Polwarth; Latvian Darkhead sheep	Clop *et al*., 2006; Gan *et al*., 2008; Hickford *et al*., 2010; Sjakste *et al*., 2011
g.4223T>C	c.373+246T>C	Intron 1	g+619T-C	Dorset Down; Merino & Polwarth; Texel; Latvian Darkhead sheep	Clop *et al*., 2006; Hickford *et al*., 2010; Sjakste *et al*., 2011
g.4226C>T	c.373+249C>T	Intron 1	g+622T-C	Texel; NZ Romney; Dorset Down; Merino& Polwarth; Latvian Darkhead sheep	Clop *et al*., 2006; Hickford *et al*., 2010; Sjakste *et al*., 2011
g.4236T>G	c. 373+259T>G	Intron 1	g+632G-T	Texel; Beltex; NZ Romney; Dorset Down; Merino& Polwarth; Southdown; Latvian Darkhead sheep	Clop *et al*., 2006; Gan *et al*., 2008; Hickford *et al*., 2010; Sjakste *et al*., 2011
g.4237A>G	c. 373+260A>G	Intron 1	633(A→G)		Gan *et al*., 2008
g.4300T>C	c.373+323T>C	Intron 1	g+696C-T	Texel; NZ Romney; Dorset Down; Merino & Polwarth; Latvian Darkhead sheep	Clop *et al*., 2006; Gan *et al*., 2008; Hickford *et al*., 2010; Sjakste *et al*., 2011
g.4370C>A	c.373+393C>A	Intron 1	766(C→A)		Gan *et al*., 2008
g.4371A>T	c.373+394A>T	Intron 1	767(A→T)		Gan *et al*., 2008
g.4374A>T	c.373+397A>T	Intron 1	770(A→T)		Gan *et al*., 2008
g.4412C>A	c.373+435C>A	Intron 1	c.373+435C>A	Latvian Darkhead sheep	Sjakste *et al*., 2011
g.4417G>C	c.373+440G>C	Intron 1	813(G→C)		Gan *et al*., 2008
g.4519A>G	c.373+542A>G	Intron 1	915(A→G)		Gan *et al*., 2008
g.4616C>T	c.373+639C>T	Intron 1	1012(T→C)	Beltex	Gan *et al*., 2008
g.4783T>G	c.373+806T>G	Intron 1	1179(T→G)		Gan *et al*., 2008
g.4829G>A	c.373+852G>A	Intron 1	1225(G→A)		Gan *et al*., 2008
g.4874G>A	c.373+897G>A	Intron 1	1270(A→G)	Beltex	Gan *et al*., 2008
g.4949A>G	c.374-862A>G	Intron 1	1345(A→G)		Gan *et al*., 2008
g.4961T>A	c.374-850T>A	Intron 1	1357(T→A)		Gan *et al*., 2008
g.5026T>A	c.374-785T>A	Intron 1	1422(T→A)		Gan *et al*., 2008
g.5052T>C	c.374-759T>C	Intron 1	1448(T→C)		Gan *et al*., 2008
g.5083G>A	c.374-728G>A	Intron 1	1479(G→A)		Gan *et al*., 2008
g.5087A>T	c.374-724A>T	Intron 1	1483(A→T)		Gan *et al*., 2008
g.5153C>T	c.374-658C>T	Intron 1	1549(C→T)		Gan *et al*., 2008
g.5163A>G	c.374-648A>G	Intron 1	1559(A→G)	Beltex	Gan *et al*., 2008
g.5225T>G	c.374-586T>G	Intron 1	1621(T→G)		Gan *et al*., 2008
g.5280C>T	c.374-531C>T	Intron 1	1676(C→T)		Gan *et al*., 2008
g.5294G>A	c.374-517G>A	Intron 1	1690(G→A)		Gan *et al*., 2008
g.5308G>A	c.374-503G>A	Intron 1	1704(G→A)		Gan *et al*., 2008
g.5313G>A	c.374-498G>A	Intron 1	1709(G→A)		Gan *et al*., 2008
g.5467T>C	c.374-344T>C	Intron 1	1863(T→C)	Beltex	Gan *et al*., 2008
g.5487T>C	c.374-324T>C	Intron 1	1883(T→C)	Beltex	Gan *et al*., 2008
g.5529G>A	c.374-282G>A	Intron 1	1925(G→A)		Gan *et al*., 2008
g.5624A>C	c.374-187A>C	Intron 1	2020(A→C)		Gan *et al*., 2008
g.5668A>G	c.374-143A>G	Intron 1	2064(A→G)		Gan *et al*., 2008
g.5698G>A	c.374-113G>A	Intron 1	2094(G→A)		Gan *et al*., 2008
g.5725A>T	c.374-86A>T	Intron 1	2121(A→T)		Gan *et al*., 2008
g.5758C>T	c.374-53C>T	Intron 1	g+2154C>T		Kijas *et al*., 2007
g.6347G>A	c.747+164G>A	Intron 2	2743(G→A)	Beltex	Gan *et al*., 2008

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g.6492T>A	c.747+308T>A		Intron 2	2888(T→A)	Beltex	Gan *et al*., 2008
g.6739T>C	c.747+555T>C		Intron 2	g+3135C-T	Texel	Clop *et al*., 2006
g.6839T>C	c.747+655T>C		Intron 2	3235(T→C)		Gan *et al*., 2008
g.7019G>T	c.747+835G>T		Intron 2	3415(G→T)		Gan *et al*., 2008
g.7031G>T	c.747+847G>T		Intron 2	3427(G→T)		Gan *et al*., 2008
g.7405T>C	c.748-810T>C		Intron 2		Beltex	Gan *et al*., 2006
g.7420C>T	c.748-795C>T		Intron 2	3816(C→T)	Beltex	Gan *et al*., 2008
g.7640C>A	c.748-575C>A		Intron 2	g+4036A-C	Texel; Black Suffolk; Coopworth; English Leicester; Lincoln; Merino; Poll Dorset; Polwarth; Romney; Southdown; Tibetan; White Suffolk	Clop *et al.*, 2006; Gan *et al*., 2008; Kijas *et al*., 2007
g.7648T>C	c.748-568T>C		Intron 2	g+4044C-T	Texel	Clop *et al*., 2006; Gan *et al*., 2008
g.8427del[2]G	c.960del[2]G	disruption reading frame from aa320 to a premature stop codon at aa359	Exon 3	disruptive veriation	Norwegian White sheep	Boman *et al*., 2009
g.9827G>A	c.*1232G>A		3'UTR	G→A transition creates a target site for miRNA binding which causes the translational inhibition of the myostatin gene in skeletal muscle /named wrongly as g+6723G>A in Clop et al., 2006); c.2360G>A (Boman et al., 2010)	Texel; Norwegian White sheep; Norwegian Spælsau sheep, White Suffolk; Poll Dorset; Lincoln; Charollais; Beltex; Bletex x Huyang	Clop *et al*., 2006; Boman & Våge, 2009; Boman *et al*., 2010; Gan *et al*., 2008; Hadjipavlou *et al*., 2008; Hickford *et al*., 2009; Johnson *et al*., 2005; Kijas *et al*., 2007; Takeda *et al*., 2010;
g.9911A>G	c.*1316A>G		3'UTR		U. S. sheep	Heaton *et al*., 2007
g.9940C>T	c.*1345C>T		3'UTR		U. S. sheep	Heaton *et al*., 2007
g.10188C>T	c.*1593C>T		3'UTR		U. S. sheep	Heaton *et al*., 2007
g.10475G>A	c.*1880G>A		3'UTR	g+6871G>A		Kijas *et al*., 2007

[1]The nucleotide position is based on the level of genomic reference sequence and coding DNA sequence only. Nucleotides in non-coding regions are described by either the addition of uncleotides to the last nucleotide position in the preceding exon or substracting nucleotides from the first nucleotide position of the next exon (http://www.hgvs.org/mutnomen/recs-DNA.html#number); [2]del = deletion; [3]Ins = insertion;

[4]ovine breeds: Meat and Multi-prolific Chinese Merino Fine Wool (MPMF), Meat Chinese Merino Fine Wool (MF).

Appendix C. *MSTN* haplotype comparison between this study and previous works

Haplotype	BM81124[1]	Promoter		5'UTR		Exon 1	Intron 1								Intron 2	3'UTR		BULGE20[1]	Sheep breed[2]
		Nucleotide		Nucleotide		Nucleotide	Nucleotide c.373+								Nucleotide	Nucleotide			
		c.-956	c.-781	c.-41~	c.-38~	c.101	18	101	241	243	246	249	259	323	c.748-575	c.*83	c.*1232		
Current work																			
H1*				A	T	A	G	T	T	G	T	T	G			G	A		TX, NC
H2*				A	T	A	G	T	T	G	T	T	G			G	G		NR, CP, CR, DP, PD, SF, MN, DD, CD, PS, TX, NC
H3[#]				A	T	A	T	T	C	A	T	T	T			G	G		NR, CP, CR, DP, PD, MN, DD, CD, NC
H4[$]				A	T	A	T	T	T	G	T	T	T			G	G		CR, DP, SF, MN, CD, NC
H5[¥]				A	T	A	T	T	T	A	T	C	T			G	G		NR, CP, CR, SF, CD, PS, NC
H6[§]				A	C	A	T	T	T	A	C	T	T			G	G		CR, DP, DD, NC
H7*				C	T	G	G	T	T	G	T	T	G			G	G		NR, CR, MN, NC
H8*				A	T	A	G	T	T	G	T	T	G			A	G		NC
Sjakste *et al*. (2011)																			
Hap 1*				C	T		G	C	T	G	T	T	G	C					LD
Hap 2[#]				C	C		T	C	C	A	T	T	T	C					LD
Hap 3							G	C	C	A	T	T	T	C					LD
Hap 4							T	C	T	G	T	T	G	C					LD
Hap 5[$]				A	C		T	C	T	G	T	T	T	C					LD
Hickford *et al*. (2010)																			
A*							G	T	T	G	T	T	G	C					NR, DD, TX, MP, SD
B[#]							T	T	C	A	T	T	T	C					NR, DD, MP
C[¥]							T	T	T	A	T	C	T	T					NR, DD, MP
D[§]							T	T	T	A	C	T	T	C					DD, MP
E[$]							T	T	T	G	T	T	T	C					MP, SD
Gan *et al*. (2008)																			
Haplo1		C	G	A													G		HY, KZ, BxH, DP, MPMF, MF
Haplo2		C	G	A													A		BT, BxH
Haplo3		C	G	C													G		HY, KZ, DP, MPMF, MF

(Continue on next page)

(Continued)

Haplo4		C	G	C		A	BT, BxH
Haplo5		C	A	A		G	MPMF
Haplo6		C	A	C		G	MPMF
Haplo7		T	G	A		G	HY, KZ, BT, BxH, DP, MPMF, MF
Haplo8		T	G	A		A	BxH
Haplo9		T	G	C		G	HY, KZ, DP, MPMF, MF
Haplo10		T	G	C		A	
Haplo11		T	A	A		G	MPMF
Haplo12		T	A	C		G	MPMF
Kijas *et al*. (2007)							
1	186			C	A	G	139 PS
2	204			C	A	G	135 PS
3	195			C	A	G	149 PS, TX
4	218			C	A	A	141 PS, TX
5	186			A	A	G	141 PS
6	216			C	A	G	151 PS
7	222			C	A	G	149 PS
8	192			C	A	G	135 PS
9	188			A	A	G	149 PS
10	184			C	A	G	139 WS
11	188			A	A	G	151 WS
12	218			C	A	G	135 PS, WS
13	186			A	C	G	137 PS, WS
14	218			C	A	G	137 PS, SF, TX, WS
15	216			A	C	G	139 SF, WS
16	216			C	A	G	137 SF, WS
17	214			A	A	G	149 PS,SF, WS
18	186			C	A	G	137 SF, TX
19	212			C	A	G	149 PS
20	216			A	C	G	143 PS

[1] Microsatellite markers

[2] Sheep breed: Beltex x Huyang (BxH), Beltex (BT), Coopdale (CD), Coopworth (CP), Corriedale (CR), Dorset Down (DD), Dorper (DP), Huyang (HY), Kazak (KZ), Latvian Darkhead (LD), Meat Chinese Merino Fine Wool (MF), Merino (MN), Poll Dorset (PS),Texel (TX), Merino & Polwarth (MP), Meat and Multi-Prolific Chinese Merino Fine Wool (MPMF), NZ cross-bred (NC), NZ Romney (NR), Parendale (PD), Southdown (SD), Suffolk (SF), White Suffolk (WS)

~ nucleotide positions observed in Sjakste *et al*. (2011) were c.-40 and c.-37

Symbols *, #, $, ¥, §mark the haplotypes/variants are identical within nucleotide position from c.373+18 to c.373+259 except c.373+101 in intron 1 region.

Appendix D. Sire and birth rank effects on means of lamb growth

Sire-line	Mean[1] ± SEMs											
	n (male lambs)	Birth weight (kg)	n (male lambs)	Tailing weight (kg)	n (male lambs)	Weaning weight (kg)	n (male lambs)	Growth rate to weaning (g/day)	n (male lambs)	Draft weight (kg)	n (male lambs)	Draft age (days)
Doughboy 45/04	46	5.89 ± 0.13	39	15.39 ± 0.38	39	30.85 ± 0.29	39	278.0 ± 7.1	30	41.71 ± 0.80	30	130.5 ± 4.0
Longridge 626/02	43	5.62 ± 0.13	36	14.42 ± 0.37	37	30.78 ± 0.28	37	275.0 ± 6.8	32	43.06 ± 0.78	32	128.1 ± 3.9
Mana 83/04	38	6.00 ± 0.13	29	15.08 ± 0.36	31	31.03 ± 0.28	31	283.6 ± 6.8	27	41.26 ± 0.82	27	124.4 ± 4.1
Mana 90/01	46	5.75 ± 0.13	37	14.95 ± 0.36	38	30.74 ± 0.27	38	269.8 ± 6.7	35	41.74 ± 0.78	35	131.9 ± 3.9
Offord 414/01	49	5.83 ± 0.13	46	15.08 ± 0.36	45	30.98 ± 0.28	45	269.2 ± 6.7	43	42.81 ± 0.70	43	130.2 ± 3.5
Clifton Downs 497.04	42	5.76 ± 0.13	38	15.66 ± 0.40	39	31.72 ± 0.28	37	304.0 ± 6.7	36	41.60 ± 0.74	36	121.7 ± 3.7
Fernvale 1106.02	40	5.64 ± 0.13	37	15.22 ± 0.37	36	30.88 ± 0.28	36	303.7 ± 6.7	34	41.97 ± 0.77	34	116.7 ± 3.8
Suddeley 181.06	39	5.66 ± 0.13	36	15.46 ± 0.40	36	31.83 ± 0.28	35	301.2 ± 6.6	36	41.31 ± 0.74	36	124.8 ± 3.7
Holly Farm 398.05	28	5.22 ± 0.14	n.d.	n.d.	19	36.87 ± 0.31	19	234.9 ± 8.8	21	40.34 ± 0.99	21	170.4 ± 4.6
Paka-iti 617.04	13	5.04 ± 0.19	n.d.	n.d.	11	36.56 ± 0.43	11	241.2 ± 11.3	12	41.71 ± 1.32	10	166.5 ± 6.3
Waio 408.07	23	5.15 ± 0.16	n.d.	n.d.	18	36.79 ± 0.36	18	236.2 ± 9.8	19	41.22 ± 1.08	18	173.6 ± 5.0
Doughboy 41/06	33	5.78 ± 0.14	n.d.	n.d.	26	31.02 ± 0.32	26	325.7 ± 7.5	27	40.08 ± 0.84	27	113.1 ± 4.2
Fernvale 671/06	33	5.74 ± 0.13	n.d.	n.d.	32	30.60 ± 0.30	32	339.6 ± 7.0	32	42.60 ± 0.79	32	112.4 ± 3.9
View Hill 480/04	35	6.11 ± 0.13	n.d.	n.d.	27	30.94 ± 0.30	27	357.4 ± 6.9	26	41.97 ± 0.83	26	99.1 ± 4.0
Trigg 6448/07	13	5.03 ± 0.18	n.d.	n.d.	13	37.27 ± 0.39	13	232.4 ± 10.9	12	39.54 ± 1.23	12	143.5 ± 6.1
Doughboy 167/07	34	6.43 ± 0.13	32	17.92 ± 0.37	32	32.66 ± 0.29	32	350.1 ± 6.6	32	43.11 ± 0.76	32	100.3 ± 3.7
Gatton Park 114/08	40	6.61 ± 0.13	36	17.56 ± 0.37	35	32.89 ± 0.29	35	332.8 ± 6.7	35	42.24 ± 0.74	35	104.7 ± 3.6
Offord 55/05	32	6.41 ± 0.14	29	18.15 ± 0.40	29	32.66 ± 0.31	29	357.5 ± 7.0	29	44.71 ± 0.78	29	98.2 ± 3.8
Ram Hill 468/06	41	6.35 ± 0.12	34	17.36 ± 0.35	31	33.40 ± 0.29	31	335.4 ± 6.7	29	43.08 ± 0.78	31	102.4 ± 3.8
P value (sire-line)		<0.001		<0.001		<0.001		<0.001		0.002		<0.001
Birth rank:												
Singles	134	6.33 ± 0.08	69	17.65 ± 0.28	118	33.14 ± 0.19	117	318.2 ± 4.4	116	42.58 ± 0.50	115	119.6 ± 2.5
Multiples	534	5.25 ± 0.07	360	14.39 ± 0.22	456	32.17 ± 0.15	454	274.2 ± 3.5	431	41.21 ± 0.41	431	132.2 ± 2.0
P value (birth rank)		<0.001		<0.001		<0.001		<0.001		0.008		0.287

[1]For each lamb growth anaysis: predicted mean values were calculated from REML mixed-model, which included sire-line, birth rank and gender as fixed factors;
n.d. = no data

Appendix E. Sire and birth rank effects on means of carcass traits

	Means[1] ± SEMs									
Sire-line	**n (male lambs)**	**Hot carcass weight (kg)**	**n (male lambs)**	**Loin yield[2]**	**Shoulder yield[2]**	**Leg yield[2]**	**Total yield[3]**	**Proportion loin yield[4]**	**Proportion shoulder yield[4]**	**Proportion leg yield[4]**
Doughboy 45/04	35	17.85 ± 0.38	35	13.70 ± 0.19	16.53 ± 0.18	20.05 ± 0.27	50.54 ± 0.50	0.2726 ± 0.0020	0.3272 ± 0.0023	0.3991 ± 0.0023
Longridge 626/02	32	18.17 ± 0.38	30	14.26 ± 0.18	16.58 ± 0.18	20.82 ± 0.26	51.41 ± 0.51	0.2757 ± 0.0019	0.3225 ± 0.0024	0.4026 ± 0.0023
Mana 83/04	21	17.46 ± 0.44	21	14.24 ± 0.20	16.68 ± 0.21	20.31 ± 0.29	51.09 ± 0.58	0.2777 ± 0.0021	0.3267 ± 0.0027	0.3960 ± 0.0025
Mana 90/01	35	18.14 ± 0.38	33	14.29 ± 0.18	16.90 ± 0.18	20.71 ± 0.26	51.74 ± 0.51	0.2750 ± 0.0019	0.3267 ± 0.0024	0.3987 ± 0.0023
Offord 414/01	40	18.00 ± 0.35	40	14.34 ± 0.16	16.72 ± 0.16	20.56 ± 0.23	51.62 ± 0.46	0.2776 ± 0.0017	0.3241 ± 0.0022	0.3982 ± 0.0020
Clifton Downs 497.04	36	17.48 ± 0.37	32	14.86 ± 0.17	17.15 ± 0.18	21.77 ± 0.25	53.68 ± 0.50	0.2761 ± 0.0018	0.3196 ± 0.0023	0.4045 ± 0.0021
Fernvale 1106.02	34	17.99 ± 0.38	33	14.72 ± 0.18	17.15 ± 0.18	21.62 ± 0.25	53.35 ± 0.51	0.2748 ± 0.0018	0.3216 ± 0.0024	0.4039 ± 0.0022
Suddeley 181.06	36	17.42 ± 0.37	32	14.75 ± 0.18	16.78 ± 0.18	21.24 ± 0.25	52.68 ± 0.50	0.2793 ± 0.0018	0.3185 ± 0.0024	0.4024 ± 0.0022
Holly Farm 398.05	17	19.36 ± 0.48	17	14.34 ± 0.22	17.86 ± 0.23	21.14 ± 0.31	53.37 ± 0.63	0.2688 ± 0.0023	0.3348 ± 0.0030	0.3960 ± 0.0027
Paka-iti 617.04	10	19.07 ± 0.62	10	14.88 ± 0.28	18.23 ± 0.29	21.78 ± 0.40	54.87 ± 0.82	0.2707 ± 0.0030	0.3327 ± 0.0039	0.3965 ± 0.0035
Waio 408.07	15	19.34 ± 0.53	14	14.88 ± 0.25	18.07 ± 0.26	21.63 ± 0.36	54.67 ± 0.72	0.2723 ± 0.0026	0.3309 ± 0.0034	0.3961 ± 0.0031
Doughboy 41/06	25	17.66 ± 0.42	25	15.49 ± 0.19	18.23 ± 0.20	22.19 ± 0.27	55.73 ± 0.55	0.2768 ± 0.0020	0.3274 ± 0.0026	0.3964 ± 0.0024
Fernvale 671/06	32	18.96 ± 0.39	30	15.32 ± 0.18	18.17 ± 0.19	21.51 ± 0.26	54.93 ± 0.52	0.2785 ± 0.0019	0.3311 ± 0.0025	0.3904 ± 0.0023
View Hill 480/04	26	18.22 ± 0.40	26	14.68 ± 0.18	17.98 ± 0.19	21.29 ± 0.26	53.83 ± 0.52	0.2719 ± 0.0019	0.3340 ± 0.0025	0.3943 ± 0.0022
Trigg 6448/07	11	17.30 ± 0.60	11	14.62 ± 0.27	17.07 ± 0.28	22.00 ± 0.39	53.51 ± 0.79	0.2720 ± 0.0029	0.3190 ± 0.0037	0.4094 ± 0.0034
Doughboy 167/07	31	19.05 ± 0.36	31	14.44 ± 0.17	17.29 ± 0.17	20.98 ± 0.24	52.59 ± 0.48	0.2737 ± 0.0018	0.3289 ± 0.0022	0.3980 ± 0.0021
Gatton Park 114/08	35	18.64 ± 0.36	35	14.66 ± 0.17	16.97 ± 0.17	21.33 ± 0.24	52.87 ± 0.48	0.2765 ± 0.0018	0.3211 ± 0.0023	0.4024 ± 0.0021
Offord 55/05	29	18.83 ± 0.37	29	13.72 ± 0.17	16.97 ± 0.17	20.92 ± 0.24	51.64 ± 0.49	0.2654 ± 0.0018	0.3288 ± 0.0023	0.4055 ± 0.0021
Ram Hill 468/06	31	18.47 ± 0.36	30	14.54 ± 0.18	17.26 ± 0.18	21.57 ± 0.25	53.21 ± 0.49	0.2722 ± 0.0018	0.3245 ± 0.0023	0.4037 ± 0.0022
P value (sire-line)		<0.001		<0.001	<0.001	<0.001	<0.001	0.007	<0.001	<0.001
Birth rank:										
Singles	115	18.86 ± 0.24	110	14.54 ± 0.12	17.43 ± 0.12	21.13 ± 0.16	53.04 ± 0.33	0.2737 ± 0.0012	0.3288 ± 0.0015	0.3978 ± 0.0014
Multiples	416	17.71 ± 0.20	404	14.59 ± 0.10	17.16 ± 0.10	21.33 ± 0.14	52.99 ± 0.27	0.2745 ± 0.0010	0.3239 ± 0.0012	0.4016 ± 0.0012
P value (birth rank)		<0.001		0.94	<0.001	0.072	0.413	0.199	<0.001	<0.001

[1] For each carcass trait anaysis: predicted mean values were calculated from REML mixed-model, which included sire-line and birth rank as fixed factors;
[2] Lean meat yield expressed as a percentage of hot carcass weight; [3] Total yield is the sum of the loin, shoulder and leg yield;
[4] The proportion yield of leg, loin or shoulder is the yield of the specific area divided by the total yield.

Appendix F. Numbers of the associated haplotypes of various lamb growth and meat carcass traits in each sire-line

Sire-line	Number of sheep samples for various traits (n)																					
	Tailing weight						Weaning weight		Growth rate to weaning		Draft age				Loin, shoulder, leg and total yield & proportion loin, shoulder and leg yield							
	Haplotype H1		Haplotype H2		Haplotype H3		Haplotype H2		Haplotype H1		Haplotype H2		Haplotype H3		Haplotype H1		Haplotype H2		Haplotype H3		Haplotype H7	
	Absent	Present	Absent	Present	Absent	Present	Absent	Present	Absent	Present	Absent	Present	Absent	Present	Absent	Present	Absent	Present	Absent	Present	Absent	Present
Doughboy 45/04	68			68	57	11		66	66			30	24	6	34			34	26	8	34	
Longridge 626/02	64		22	42	15	49	23	42	65		11	21	7	25	30		10	20	8	22	30	
Mana 83/04	67		12	55	50	15	11	54	65		5	21	22	3	20		3	17	16	3	20	
Mana 90/01	78		8	70	30	47	8	68	76		4	29	14	18	31		4	27	13	17	31	
Offord 414/01	76		13	63	61	15	13	61	74		6	37	34	9	40		6	34	32	8	22	18
Clifton Downs 497.04	76			76	54	21		75	73			40	31	8	36			36	28	7	35	1
Fernvale 1106.02	41	36	22	55	58	19	22	53	40	35	7	26	27	6	19	13	7	25	26	6	32	
Suddeley 181.06	73		11	62	31	42	11	62	72		5	31	16	20	32		4	28	14	18	32	
Holly Farm 398.05							5	48	53		2	18	8	12	17		2	15	7	10	17	
Paka-iti 617.04							4	18	22		2	7	8	1	9		2	7	8	1	9	
Waio 408.07								36	36			16	11	5	12			12	7	5	12	
Doughboy 41/06							8	40	48		5	20	16	9	24		5	19	15	9	24	
Fernvale 671/06								62	62			31	29	2	29			29	27	2	27	2
View Hill 480/04							16	45	61		9	19	11	17	28		9	19	11	17	27	1
Trigg 6448/07							7	23	12	18	4	7	7	4	4	6	3	7	7	3	10	
Doughboy 167/07	76		15	61	30	46	15	58	73		5	34	16	23	38		5	33	15	23	38	
Gatton Park 114/08	76		9	66	53	23	9	61	71		6	36	29	14	42		5	36	28	14	20	22
Offord 55/05	62			62	44	18		62	62			36	26	10	36			36	26	10	36	
Ram Hill 468/06	76		21	55	36	40	18	48	66		10	25	17	18	33		9	24	16	17	33	

References

Acosta, J., Carpio, Y., Borroto, I., Gonzalez, O., & Estrada, M. P. (2005). Myostatin gene silenced by RNAi show a zebrafish giant phenotype. *Journal of Biotechnology, 119*, 324-331.

Amthor, H., Otto, A., Macharia, R., McKinnell, I., & Patel, K. (2006). Myostatin imposes reversible quiescence on embryonic muscle precursors. *Development Dynamics, 235*, 672-680.

Arthur, P. F. (1995). Double muscling in cattle: a review. *Australian Journal of Agricultural Research, 46*, 1493-1515.

Asakura, A., Komaki, M., & Rudnicki, M. (2002). Cellular and molecular mechanisms regulating skeletal muscle development. In: *Mouse Development*. Orlando, FL:Academic. In (pp. 253-278).

Babbitt, C. C., Fedrigo, O., Pfefferle, A. D., Boyle, A. P., Horvath, J. E., Furey, T. S., & Wray, G. A. (2010). Both noncoding and protein-coding RNAs contribute to gene expression evolution in the primate brain. *Genome Biology and Evolution, 2*, 67-79.

Baron, E. E., Wenceslau, A. A., Alvares, L. E., Nones, K., Ruy, D. C., Schmidr, G. S., Zanella, E. L., Continho, L. L., & Ledur, M. C. (2002). High level of polymorphism in the myostatin chicken gene. *Proceedings of the 7th World Congress on Genetics Applied to Livestock Production, Montpellier, France, Session 4*

Beef + Lamb, NZ. (2009). *Overview of the international sheepmeat sector: current trends in demand and supply*. Retrieved. from http://www.meatnz.co.nz/main.cfm?id=31&nid=245.

Beef + Lamb, NZ. (2011a). Compendium of New Zealand farm facts, Beef + Lamb New Zealand Ltd Economic Service 35th Edition. In: Wellington, Publication No. P11005

Beef + Lamb, NZ. (2011b). *Sheep & beef mid season update 2010-11. Economic Service Publication P10051*. Retrieved. from http://www.dannevirke.net.nz/uploads/78215/files/B_LNZ_Mid_Season-_Update_-_Jan_2011.pdf.

Bellinge, R. H., Liberles, D. A., Iaschi, S. P., O'Brien P, A., & Tay, G. K. (2005). Myostatin and its implications on animal breeding: a review. *Animal Genetics, 36*, 1-6.

Bogdanovich, S., Krag, T. O. B., Barton, E. R., Morris, L. D., Whittemore, L. A., Ahima, R. S., & Khurana, T. S. (2002). Functional improvement of dystrophic muscle by myostatin blockade. *Nature, 420*, 418-421.

Boman, I. A., & Våge, D. I. (2009). An insertion in the coding region of the myostatin (*MSTN*) gene affects carcass conformation and fatness in the Norwegian Spælsau (*Ovis aries*). *BMC Research Notes, 2*, 98-102.

Boman, I. A., Klemetsdal, G., Blichfeldt, T., Nafstad, O., & Våge, D. I. (2009). A frameshift mutation in the coding region of the myostatin gene (*MSTN*) affects carcass conformation and fatness in Norwegian White Sheep (Ovis Aries). *Animal Genetics, 40*, 418-422.

Boman, I. A., Klemetsdal, G., Nafstad, O., Blichfeldt, T., & Våge, D. I. (2010). Impact of two myostatin (*MSTN*) mutations on weight gain and lamb carcass classification in Norwegian White Sheep (Ovis aries). *Genetics, Selection, Evolution, 42*, 4.

Browning, S. R. (2008). Missing data imputation and haplotype phase inference for genome-wide association studies. *Human Genetics, 124*, 439-450.

Campbell, A. W., Bain, W. E., McRae, A. F., Broad, T. E., Johnstone, P. D., Dodds, K. G., Veenvliet, B. A., Greer, G. J., Glass, B. C., Beattie, A. E., Jopson, N. B., & McEwan, J. C. (2003). Bone density in sheep: genetic variation and quantitative trait loci localisation. *Bone, 33*, 540-548.

Casas, E., Bennett, G. L., Smith, T. P. L., & Cundiff, L. V. (2004). Association of myostatin on early calf mortality, growth and carcass composition traits in crossbred cattle. *Journal of Animal Science, 82*, 2913-2918.

Cieślak, D., Blicharski, T., Kapelański, W., & Pierzchala, M. (2003). Investigation of polymorphisms in the porcine myostatin (GDF8; MSTN) gene. *Czech Journal of Animal Science, 48*, 69-75.

Clop, A., Marcq, F., Takeda, H., Pirottin, D., Tordoir, X., Bibé, B., Bouix, J., Caiment, F., Elsen, J. M., Eychenne, F., Larzul, C., Laville, E., Meish, F., Milenkovic, D., Tobin, J., Charlier, C., & Georges, M. (2006). A mutation creating a potential illegitimate microRNA target site in the myostatin gene affects muscularity in sheep. *Nature Genetics, 38*, 813-818.

Cockett, N. E., Shay, T. L., & Smit, M. (2001). Analysis of the sheep genome. *Physiological Genomics, 7*, 69-78.

Condit, C. M., Achter, P. J., Lauer, I., & Sefcovic, E. (2002). The changing meanings of "mutation": a contextualized study of public discourse. *Human Mutation, 19*, 69-75.

Cossu, G., & Biressi, S. (2005). Satellite cells, myoblasts and other occasional myogenic progenitors: possible origin, phenotypic features and role in muscle regeneration. *Seminars in Cell & Developmental Biology, 16*, 623-631.

Cotton, R. G. H. (2002). Communicating "mutation:" modern meanings and connotations. *Human Mutation, 19*, 2-3.

Culley, G. (1807). Observations on livestock, 4th ed. London, G. Woodfall.

Cusella-De Angelis, M. G., Molinari, S., Le Donne, A., Colletta, M., Vivarelli, E., Bouche, M., Molinaro, M., Ferrari, S., & Cossu, G. (1994). Differential response of embryonic and fetal myoblasts to TGF beta: a possible regulatory mechanism of skeletal muscle histogenesis. *Development, 120*, 925-933.

Davidson, S. (2000). Research suggests importance of haplotypes over SNPs. *Nature Biotechnology, 18*, 1134-1135.

Dawson, L. E. R., Carson, A. F., & McClinton, L. O. W. (2002). Comparison of the productivity of Texel and Rouge de l'Ouest ewes and their crosses. *Animal Science, 75*, 459-468.

Di Stasio, L., & Rolando, A. (2005). A PCR-RFLP method for genotyping the myostatin locus in Piedmontese cattle. *Animal Genetics, 36*, 511-542.

Dmitriew, D. A., & Rakitov, R. A. (2008). Decoding of superimposed traces produced by direct sequencing of heterozygous indels. *PLoS Computational Biology, 4*, e1000113.

Du, R., An, X., Chen, Y., & Qin, J. (2007a). Functional analysis of the Myostatin gene promoter in sheep. *Science China Life Sciences, 50*, 648-654.

Du, R., An, X. R., Chen, Y. F., & Qin, J. (2007b). Some motifs were important for myostatin transcriptional regulation in sheep (Ovis aries). *Journal of Biochemistry and Molecular Biology, 40*, 547-553.

Du, R., Chen, Y. F., An, X. R., Yang, X. Y., Ma, Y., Zhang, L., Yuan, X. L., Chen, L. M., & Qin, J. (2005). Cloning and sequence analysis of myostatin promoter in sheep. *DNA Sequence, 6*, 412-417.

Dunner, S., Chariler, C., Farnir, F., Brouwers, B., Canon, J., & Georges, M. (1997). Towards interbreed IBD fine mapping of the mh locus: double-muscling in Australia de los Valles breed involves the same locus as in Belgian Blue cattle breed. *Mammalian Genome, 8*, 430-435

Dunner, S., Miranda, M. E., Amigues, Y., Cañón, J., Georges, M., Hanset, R., Williams, J. L., & Ménissier, F. (2003). Haplotype diversity of the *myostatin* gene among beef cattle breeds. *Genetics, Selection, Evolution, 35*, 103-118.

Dwyer, C. M., & Bünger, L. (2012). Factors affecting dystocia and offspring vigour in different sheep genotypes. *Preventive Veterinary Medicine, 103*, 257-264.

Esmailizadeh, A. K., Bottema, C. D. K., Sellick, G. S., Verbyla, A. P., Morris, C. A., Cullen, N. G., & Pitchford, W. S. (2008). Effects of the myostatin F94L substitution on beef traits. *Journal of Animal Science, 86*, 1038-1046.

Fahey, A. J., Brameld, J. M., Parr, T., & Buttery, P. J. (2005). Ontogeny of factors associated with proliferation and differentiation of muscle in the ovine fetus. *Journal of Animal Science, 83*, 2330-2338.

Farokhad, M. L., Roshanfekr, H., Amiri, S., Mohammadi, K., & Mirzadeh, K. (2011). Genetic trends estimation for some of the growth traits in Arman sheep. *Journal of Animal and Veterinary Advances, 10*, 1801-1803.

Galat, A. (2011). Common structural traits for cystine knot domain of the TGFβ superfamily of proteins and three-gingered ectodomin of their cellular receptors. *Cellular and Molecular Life Sciences*. doi:DOI 10.1007/s00018-011-0643-4

Gamer, L. W., Wolfman, N. M., Celeste, A. J., Hattersley, G., Hewick, R., & Rosen, V. (1999). A novel BMP expressed in developing mouse limb, spinal cord, and tail bud is a potent mesoderm inducer in *Xenopus* embryos. *Developmental Biology, 208*, 222-232.

Gan, S., Du, Z., & Li, N. (2006). A new novel mutation in Beltex sheep 3'UTR is strongly associated with the trait muscular hypertrophy (unpublished paper). NCBI number DQ990914.,

Gan, S. Q., Du, Z., Liu, S. R., Yang, Y. L., Shen, M., Wang, X. H., Yin, J. L., Hu, X. X., Fei, J., Fan, J. J., Wang, J. H., He, Q. H., Zhang, Y. S., & Li, N. (2008). Association of SNP haplotypes at the myostatin gene with muscular hypertrophy in sheep. *Asian-Australasian Journal of Animal Sciences, 21*, 928-935.

Gourt, J., Hides, S., & Webb-Ware, J. (2010). Reproductive management. In J. Gourt, S. Hides & J. Webb-Ware (Eds.), *Sheep farming for meat and wool*. Collingwood: CSIRO Publishing.

Grisolia, A. B., Angelo, G. T. D., Porto Neto, L. R., Siqueira, F., & Garcia, J. F. (2009). Myostatin (GDF8) single nucleotide polymorphisms in Nellore cattle. *Genetics and Molecular Research, 8*, 822-830.

Grobet, L., Poncelet, D., Royo, L. J., Brouwers, B., Pirottin, D., Michaux, C., Menissier, F., Zanotti, M., Dunner, S., & Georges, M. (1998). Molecular definition of an allelic series of mutations disrupting the myostatin function and causing double-muscling in cattle. *Mammalian Genome, 9*, 201-213.

Grobet, L., Pirottin, D., Farnir, F., Poncelet, D., Royo, L. J., Brouwers, B., Christians, E., Desmecht, D., F., C., Kahn, R., & Georges, M. (2003). Modulating skeletal muscle mass by postnatal, muscle-specific inactivation of the myostatin gene. *Genesis, 35*, 227-238.

Grobet, L., Martin, L. J. R., Poncelet, D., Pirottin, D., Brouwers, B., Riquet, J., Schoeberlein, A., Dunner, S., Menissier, F., Massabanda, J., Fries, R., Hanset, R., & Georges, M. (1997). A deletion in the bovine myostatin gene causes the double-muscled phenotype in cattle. *Nature Genetics, 17*, 71-74.

Gu, W., Schneider, J. W., Condorelli, G., Kaushal, S., Mahdavi, V., & Nadal-Ginard, B. (1993). Interaction of myogenic factors and the retinoblastoma protein mediates muscle cell commitment and differentiation. *Cell, 72*, 309-324.

Guimaraes, S. E. F., Stahl, C. H., Lonergan, S. M., Geiger, B., & Rothschild, M. F. (2007). Myostatin promoter analysis and expression pattern in pigs. *Livestock Science, 112*, 143-150.

Hadjipavlou, G., Matika, O., Clop, A., & Bishop, S. C. (2008). Two single nucleotide polymorphisms in the myostatin (GDF8) gene have significant association with muscle depth of commercial Charollais sheep. *Animal Genetics, 39*, 346-353.

Hameric, M. W. (2003). Increased bone mineral density in the femora of GDF8 knockout mice. *The anatomical record. Part A, Discoveries in molecular, cellular, and evolutionary biology, 272A*, 388-391.

Han, J., Zhou, H., Forrest, R. H., Sedcole, J. R., Frampton, C. M., & Hickford, J. G. H. (2010). Effect of Myostatin (*MSTN*) g+6223G>A on production and carcass traits in New Zealand Romney sheep. *Asian-Australasian Journal of Animal Sciences, 23*, 863-866.

Hathaway, M. R., Pampusch, M. S., Hembree, J. R., & Dayton, W. R. (1994). Transforming growth factor beta-1 facilitates establishing clonal populations of ovine muscle satellite cells. *Journal of Animal Science, 72*, 20001-22007.

Hayashi, K. (1991). PCR-SSCP: A simple and sensitive method for detection of mutations in the genomic DNA. *Genome Research, 1*, 34-38.

Hayes, B., & Goddard, M. E. (2001). The distribution of the effects of genes affecting quantitative traits in livestock. *Genetic Selection Evolution, 33*, 209-229.

Heaton, M. P., Smith, T. P. L., Freking, B. A., Wray, J. E., & Leymaster, K. A. (2007). Characterization of SNPs in the myostatin 3'UTR region of U. S. sheep (unpublished paper). NCBI number EF182738.

Helterline, D. L., Garikipati, D., Stenkamp, D. L., & Rodgers, B. D. (2007). Embryonic and tissue-specific regulation of myostatin-1, and -2 gene expression in zebrafish. *General and Comparative Endocrinology, 151*, 90-97.

Heslop, L., Beauchamp, J. R., Tajbakhsh, S., Buckingham, M. E., Partridge, T. A., & Zammit, P. S. (2001). Transplanted primary neonatal myoblasts can give rise to functional satellite cells as identified using the Myf5$^{nlacZ/+}$ mouse. *Gene Therapy, 8*, 778-783.

Hickford, J. G. H., Forrest, R. H., & Zhou, H. (2009). Association between a g.+6723G-A SNP in the myostatin gene (*MSTN*) and carcass traits in New Zealand Texel sheep. *Journal of Animal Science, 87*, 1853.

Hickford, J. G. H., Forrest, R. H., Zhou, H., Fang, Q., Han, J., Frampton, C. M., & Horrell, A. L. (2010). Polymorphisms in the ovine myostatin gene (*MSTN*) and their association with growth and carcass traits in New Zealand Romney sheep. *Animal Genetics, 41*, 64-72.

Hill, J. J., Qiu, Y., Hewick, R. M., & Wolfman, N. M. (2003). Regulation of myostatin *in vivo* by growth and differentiation factor-associated serum protein-1: a novel

protein with protease inhibitor and follistatin domains. *Molecular Endocrinology, 17*, 1144-1154.

Hill, J. J., Davies, M. V., Pearson, A. A., Wang, J. H., Hewick, R. M., Wolfman, N. M., & Qiu, Y. (2002). The myostatin propeptide and follistatin-related gene are inhibitory binding proteins of myostatin in normal serum. *The Journal of Biological Chemistry, 277*, 40735-40741.

Hiller, M., Zhang, Z. Y., Backofen, R., & Stamm, S. (2007). Pre-mRNA secondary structures influence exon recognition. *PLoS Genetics, 3*, 2147-2155.

Ji, S., Losinsky, R. L., Cornelius, S. G., Frank, G. R., Willis, G. M., Gerrard, D. E., Depreux, F. F., & Spurlock, M. E. (1998). Myostatin expression in porcine tissues: tissue specificity and developmental and postnatal regulation. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology, 275*, R1265-1273.

Jiang, Y. L., Li, N., Plastow, G., Liu, Z. L., Hu, X. X., & Wu, C. X. (2002). Identification of three SNPs in the porcine myostatin gene (*MSTN*). *Animal Biotechnology, 13*, 173-178.

Johnson, P. L., McEwan, J. C., Dodds, K. G., Purchas, R. W., & Blair, H. T. (2005). A directed search in the region of GDF8 for quantitative trait loci affecting carcass traits in Texel sheep. *Journal of Animal Science, 83*, 1988-2000.

Johnson, P. L., Dodds, K. G., Bain, W. E., Greer, G. J., McLean, N. J., McLaren, R. J., Galloway, S. M., van Stijn, T. C., & McEwan, J. C. (2009). Investigations into the GDF8 g+6273G-A polymorphism in New Zealand Texel sheep. *Journal of Animal Science 87*, 1856-1864.

Joulia-Ekaza, D., & Cabello, G. (2006). Myostatin regulation of muscle development: Molecular basis, natural mutations, physiopathological aspects. *Experimental Cell Research, 312*, 2401-2414.

Joulia-Ekaza, D., & Cabello, G. (2007). The myostatin gene: physiology and pharmacological relevance. *Current Opinion in Pharmacology, 7*, 310-315.

Joulia, D., Bernardi, H., Garandel, V., Robenoelina, F., Vernus, B., & Cabello, G. (2003). Mechanisms involved in the inhibition of myoblast proliferation and differentiation by myostatin. *Experimental Cell Research, 286*, 263-275.

Kambadur, R., Sharma, M., Smith, T. P., & Bass, J. J. (1997). Mutations in myostatin (GDF8) in double-muscled Belgian Blue and Piedmontese cattle. *Genome Research, 7*, 910-916.

Karim, L., Coppieters, W., Grobet, L., Valentini, A., & Georges, M. (2000). Convenient genotyping of six myostatin mutations causing double-muscling in cattle using a multiplex oligo-nucleotide ligation assay. *Animal Genetics, 31*, 396-399.

Kijas, J. W., McCulloch, R., Edwards, J. E., Oddy, V. H., Lee, S. H., & van der Werf, J. (2007). Evidence for multiple alleles effecting muscling and fatness at the Ovine GDF8 locus. *BMC Genetics, 8*, 80-90.

Kijas, J. W., Townley, D., Dalrymple, B. P., Heaton, M. P., Maddox, J. F., McGrath, A., Wilson, P., Ingersoll, R. G., McCulloch, S. M., Tang, D., McEwan, J., Cockett, N. E., Oddy, V. H., Nicholas, F. W., & Raadsma, H. (2009). A genome wide survey of SNP variation reveals the genetic structure of sheep breeds. *PLoS ONE, 4*, e4668.

Kim, H. S., Liang, L., Dean, R. G., Hausman, D. B., Hartzell, D. L., & Baile, C. A. (2001). Inhibition of preadipocyte differentiation by myostatin treatment in 3T3-L1 cultures. *Biochemical and Biophysical Research Communications,* (281), 902-906.

Kirk, S., Oldham, J., Kambadur, R., Sharma, M., Dobbie, P., & Bass, J. (2000). Myostatin regualtion during skeletal muscle regeneration. *Journal of Cellular Physiology, 184*, 356-363.

Kollias, H. D., & McDermott, J. C. (2008). Transforming growth factor-β and myostatin signaling in skeletal muscle. *Journal of Applied Physiology, 104*, 579-587.

Konstantinos, K. V., Panagiotis, P., Antonios, V. T., Agelos, P., & Argiris, N. V. (2008). PCR-SSCP: A method for the molecular analysis of genetic diseases. *Molecular Biotechnology, 38*, 155-163.

Lambe, N. R., Richardson, R. I., Macfarlane, J. M., Nevison, I., Haresign, W., Matika, O., & Büngar, L. (2011). Genotypic effects of the Texel muscling QTL (TM-QTL) on meat quality in purebred Texel lambs. *Meat Science*. doi:10.1016/j.meatsci.2011.03.014

Larzul, C., Lefaucheur, L., Ecolan, P., Gogue, J., Talmant, A., Sellier, P., & Monin, G. (1997). Phenotypic and genetic parameters for Longissimus muscle fibre characteristics in relation to growth, carcass and meat quality traits in Large White pigs. *Journal of Animal Science, 75*, 3126-3137.

Lawrie, R. A., & Ledward, D. A. (2008). In *Lawrie's Meat Science* (6th ed.). Cambridge: Woodhead Publishing Limited.

Lee, S. J. (2004). Regulation of muscle mass by myostatin. *Annual Review of Cell and Developmental Biology, 20*, 61-86.

Lee, S. J. (2007a). Sprinting without myostatin: a genetic determinant of athletic prowess. *Trends in Genetic, 23*, 475-477.

Lee, S. J. (2007b). Quadrupling muscle mass in mice by targeting TGF-beta signaling pathways. *PLoS ONE, 2*, e789.

Lee, S. J., & McPherron, A. C. (2001). Regulation of myostatin activity and muscle growth. *Proceedings of the National Academy of Science of the United States of America, 98*, 9306-9311.

Lee, S. J., Reed, L. A., Davies, M. V., Girgenrath, S., Goad, M. E., Tomkinson, K. N., Wright, J. F., Barker, C., Enrmantraut, G., Holmstrom, J., Trowell, B., Gertz, B., Jiang, M. S., Sebald, S. M., Matzuk, M., Li, E., Liang, L. F., Quattlebaum, E., Stotish, R. L., & Wolfman, N. M. (2005). Regulation of muscle growth by multiple ligands signaling through activin type II receptors. *Proceedings of the National Academy of Science of the United States of America, 102*, 18117-18122.

Li, S. H., Xiong, Y. Z., Zheng, R., Li, A. Y., Deng, C. Y., Jiang, S. W., Lei, M. G., Wen, Y. Q., & Cao, G. C. (2002). Polymorphism of porcine myostatin gene. *Yi Chuan Xue Bao, 29*, 326-331.

Lin, J., Arnold, H. B., Della-Fera, M. A., Azain, M. J., Hartzell, D. L., & Baile, C. A. (2002). Myostatin knockout in mice increases myogenesis and decreases adipogenesis. *Biochemical and Biophysical Research Communications 291*, 701-706.

Lowe, T. M., & Eddy, S. R. (1997). tRNAscan-SE: a program for improved detection of transfer RNA genes in genomic sequence. *Nucleic Acids Research, 25*, 955-964.

Luff, R. A., & Goldspink, G. (1970). Total number of fibers in muscles of several strains of mice. *Journal of Animal Science, 30*, 891-893.

Ma, K., Mallidis, C., Artaza, J., Taylor, W., Conzalez-Cadavid, N., & Bhasin, S. (2001). Characterization of 5'-regulatory region of human myostatin gene: regulation by dexamethasone *in vitro*. *American Journal of Physiology Endocrinology and Metabolism, 281*, E1128-E1136.

Maccatrozzo, L., Bargelloni, L., Radaelli, G., Mascarello, F., & Patarnello, T. (2001). Characterization of the myostatin gene in the gilthead seabream, *Sparus aurata*: sequence, genomic structure, and expression pattern. *Journal of Marine Biotechnology, 3*, 224-230.

Maccatrozzo, L., Bargelloni, L., Patarnello, P., Radaelli, G., Mascarello, F., & Patarnello, T. (2002). Characterization of the myostatin gene and a linked microsatellite marker in shi drum (*Umbrina cirrosa*, Sciaenidae). *Aquaculture, 205*, 49-60.

Marcq, F., Larzul, C., Marot, V., Bouix, J., Eychenne, F., Laville, E., Bibé, B., Leroy, P. L., Georges, M., & Elsen, j. M. (2002). Preliminary results of a whole-genome scan targeting QTL for carcass traits in a Texel X Romanov intercross. *Proceedings of the 7th World Congress on Applied Livestock Production (Montpellier, France)*, 12-14.

Martínez, A., Aldai, N., Celaya, R., & Osoro, K. (2010). Effect of breed body size and the muscular hypertrophy gene in the production and carcass traits of concentrate-finished yearling bulls. *Journal of Animal Science, 88*, 1229-1239.

Mauvais-Jarvis, F., Virkamaki, A., Michael, M. D., Winnay, J. N., Zisman, A., Kulkarni, R. N., & Kahn, C. R. (2000). A model to explore the interaction between muscle insulin resistance and β-cell dysfunction in the development of type 2 diabetes. *Diabetes, 49*, 2126-2134.

McCarthy, J. J., & Esser, K. A. (2007). MicroRNA-1 and microRNA-133a expression are decreased during skeletal muscle hypertrophy. *Journal of Applied Physiology, 102*, 306-313.

McCroskery, S., Thomas, M., Maxwell, L., Sharma, M., & Kambadur, R. (2003). Myostatin negatively regulates satellite cell activation and self-renewal. *The Journal of Cell Biology, 162*, 1135-1147.

McFarland, D. C., Velleman, S. G., Pesall, J. E., & Liu, C. (2007). The role of myostatin in chicken (*Gallus domesticus*) myogenic satellite cell proliferation and differentiation. *General and Comparative Endocrinology, 150*, 351-357.

McKenzie, G. W., Abbott, J., Zhou, H., Fang, Q., Merrick, N., Forrest, R. H., Sedcole, J. R., & Hickford, J. G. H. (2010). Genetic diversity of selected genes that are potentially economically important in feral sheep of New Zealand. *Genetics Selection Evolution, 42*, 43-50.

McMahon, C. D., Popovic, L., Jeanplong, F., Oldham, J. M., Kirk, S. P., Osepchook, C. C., Wong, K. W., Sharma, M., Kambadur, R., & Bass, J. J. (2003). Sexual dimorphism is associated with decreased expression of processed myostatin in males. *American Journal of Physiology - Endocrinology and Metabolism, 284*, E377-E381.

McNally, E. M. (2004). Powerful genes - myostatin regulation of human muscle mass. *The New England Journal of Medicine, 350*, 2642-2644.

McPherron, A. C., & Lee, S. J. (1997). Double muscling in cattle due to mutations in the myostatin gene. *Proceedings of the National Academy of Science of the United States of America, 94*, 12457-12461.

McPherron, A. C., & Lee, S. J. (2002). Suppression of body fat accumulation in myostatin-deficient mice. *The Journal of Clinical Investigation, 109*, 595-601.

McPherron, A. C., Lawler, A. M., & Lee, S. J. (1997). Regulation of skeletal muscle mass in mice by a new TGF-β superfamily member. *Nature, 387*, 83-90.

Meadows, G. (2008). Sheep breeds. In *Pocket guide to sheep breeds of New Zealand* (pp. 97-170). Auckland: New Holland Publishers (NZ) Ltd.

Mendias, C. L., Bakhurin, K. I., & Faulkner, J. A. (2008). Tendons of myostatin-deficient mice are small, brittle, and hypocellular. *Proceedings of the National Academy of Science of the United States of America, 105*, 388-393.

Ménissier, F. (1982a). Present state of knowledge about the genetic determination of muscular hypertrophy or the double muscled trait in cattle. In M. F. King (Ed.), *Current topics in veterinary medicine and animal science* (Vol. 16, pp. 23-53).

Ménissier, F. (1982b). General survey of the effect of double muscling on cattle performance. In J. W. B. King & F. Ménissier (Eds.), *Muscle hypertrophy of genetic origin and its use to improve beef production* (pp. 23-53): Martinus Nijhoff Publishers: The Hague.

MIA. (2010). *2010 Meat industry Association of New Zealand Annual Report. Source: Statistics New Zealand, Overseas Merchandise Trade* Retrieved. from http://www.mia.co.nz/docs/statistics/2010/summary_of_meat_industry_exports_year_ended_june_2010.pdf.

Miranda, M. E., Ménissier, F., Canon, J., Vallejo, M., Boscher, M. Y., & Dunner, S. (2002). Myostatin gene polymorphism and double muscling expression in cattle breeds: preliminary results. *7th World Congress on Genetics Applied to Livestock Production, Montpellier, France,*

Mitchell, M. D., Osepchook, C. C., Leung, K. C., McMahon, C. D., & Bass, J. J. (2006). Myostatin is a human placental product that regulates glucose uptake. *The Journal of Clinical Endocrinology & Metabolism, 91*, 1434-1437.

Molkentin, J. D., & Olson, E. N. (1996). Defining muscling in cattle due to mutations for muscle development. *Current Opinion in Genetics & Development, 6*, 445-453.

Mosher, D. S., Quignon, P., Bustamante, C. D., Sutter, N. B., Mellersh, C. S., Parker, H. G., & Ostrander, E. A. (2007). A mutation in the myostatin gene increase muscle mass and enhances racing performance in heterozygote dogs. *PLoS Genetics, 3*, 779-786.

Newman, S. A. N., McEwan, J. C., & Young, M. J. (2009). A DECADE OF SHEEP IMPROVEMENT LIMITED (SIL). *Proceedings of Association for the Advancement of Animal Breeding and Genetics 18*, 624-627.

Ng, L., Pathak, S. D., Kuan, C., Lau, C., Dong, H., Sodt, A., Dang, C., Avants, B., Yushkevich, P., Gee, J. C., Haynor, D., Lein, E., Jones, A., & Hawrylycz, M. (2007). Neuroinformatics for genome-wide 3D gene expression mapping in the mouse brain. *IEEE/ACM Transactions on Computational Biology and Bioinformatics, 4*, 382-393.

O'Rourke, B. A., Greenwood, P. L., Arthur, P. F., & Goddard, M. E. (2009). Association between myostatin DNA markers and muscularity in Angus cattle. *Association for the Advancement of Animal Breedings and Genetics, 18*, 163-166.

Oldham, J. M., Martyn, J. A. K., Sharma, M., Jeanplong, R., Kambadur, R., & Bass, J. J. (2001). Molecular expression of myostatin and myoD is greater in double-muscled than normal-muscled cattle fetuses. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology, 280*, R1488-R1493.

Olson, E. N. (1992). Interplay between proliferation and differentiation within the myogenic lineage. *Developmental Biology, 154*, 261-272.

Ontell, M., & Kozeka, K. (1984). Organogenesis of the mouse extensor digitorum logus muscle: A quantitative study. *American Journal of Anatomy, 171*, 149-161.

Peter, C., Bruford, M., Perez, T., Dalamitra, S., Hewitt, G., Erhardt, G., & the ECONOGENE Consortium. (2006). Genetic diversity and subdivision of 57 European and Middle-Eastern sheep breeds. *Animal Genetics, 38*, 37-44.

Rebbapragada, A., Benchabane, H., Wrana, J. L., Celeste, A. J., & Attisano, L. (2003). Myostatin signals through a transforming growth factor beta-like signaling pathway to block adipogenesis. *Molecular and Cellular Biology, 20*, 7230-7242.

Record, S. J., & Leppard, P. (2000). Red meat consumption in Australia: intakes, nutrient contribution and changes over time. *Australian Journal of Nutrition and Dietetics, 57*, S1-S36.

Rehfeldt, C., Fiedler, I., Dietl, G., & Ender, K. (2000). Myogenesis and postnatal skeletal muscle cell growth as influenced by selection. *Livestock Production Science, 66*, 177-188.

Rehfeldt, C., Walther, K., Albrecht, E., Nürnberg, G., Renne, U., & Bünger, L. (2002). Intrinsic properties of muscle satellite cells are changed in response to long-term selection of mice for different growth traits. *Cell and Tissue Research, 310*, 339-348.

Reisz-Porszasz, S., Bhasin, S., Artaza, J. N., Shen, R., Sinha-Hikim, I., Hogue, A., Fielder, T. J., & Gonzalez-Cadavid, N. F. (2003). Lower skeletal muscle mass in male transgenic mice with muscle-specific overexpression of myostatin. *American Journal of Physiology - Endocrinology and Metabolism, 285*, E876-E888.

Ríos, R., Carneiro, I., Arce, V. M., & Devesa, J. (2002). Myostatin is an inhibitor of myogenic differentiation. *American Journal of Physiology - Cell Physiology, 282*, C993-999.

Rodgers, B. D., & Garikipati, D. K. (2008). Clinical, agricultural, and evolutionary biology of myostatin: A comparative Review. *Endocrine Reviews, 29*, 513-534.

Romney NZ Inc. (2011). Retrieved from http://www.nzromney.co.nz/organisation/home

Salerno, M. S., Thomas, M., Forbes, D., Watson, T., Kambadur, R., & Sharma, M. (2004). Molecular analysis of fiber type-specific expression of murine myostatin promoter. *American Journal of Physiology - Cell Physiology, 287*, C1031-C1040.

Sanguinetti, C. L., Dias Neto, E., & Simpson, A. J. G. (1994). Rapid silver staining and recovery of PCR products separated on polyacrylamide gels. *BioTechniques, 17*, 915-921.

Schiaffino, S., & Reggiani, C. (1996). Molecular diversity of myofibrillar proteins: gene regulation and functional significance. *Physiological Reviews, 76*, 371-423.

Schuelke, M., Wagner, K. R., Stolz, L. E., Hübner, C., Riebel, T., Kömen, W., Braun, T., Tobin, J. F., & Lee, S. J. (2004). Myostatin mutation associated with gross muscle hypertrophy in a child. *The New England Journal of Medicine, 350*, 2682-2688.

Schultz, E. (1996). Satellite cell proliferative compartments in growing skeletal muscle. *Developmental Biology, 175*, 84-94.

Shahin, K., & Berg, R. T. (1985). Growth patterns of muscle, fat and bone, and carcass composition of double muscled and normal cattle. *Canadian Journal of Animal Science, 65*, 279-294.

Sharma, M., Kambadur, R., Matthews, K. G., Somers, W. G., Devlin, G. P., Conaglen, J. V., Fowke, P. J., & Bass, J. J. (1999). Myostatin, a transforming growth factor-beta superfamily member, is expressed in heart muscle and is upregulated in cardiomyocytes after infarct. *Journal of Cellular Physiology, 180*, 1-9.

Shelton, G. D., & Engvall, E. (2007). Gross muscle hypertrophy in whippet dog is caused by a mutation in the myostatin gene. *Neuromuscular Disorders, 17*, 721-722.

Sjakste, T., Paramonova, N., Grislis, Z., Trapina, I., & Kairisa, D. (2011). Analysis of the single-nucleotide polymorphism in the 5'UTR and part of intron 1 of the sheep *MSTN* gene. *DNA and Cell Biology, 30*, 433-444.

Smet, S. D., Webb, E. C., Claeys, E., Uytterhaegen, L., & Demeyer, D. I. (2000). Effect of dietary energy and protein levels on fatty acid composition of intramuscular fat in double-muscled Belgian Blue bulls. *Meat Science, 56*, 73-79.

Smith, J. A., Lewis, A. M., Wiener, P., & Williams, J. L. (2000). Genetic variation in the bovine myostatin gene in UK beef cattle: Allele frequencies and haplotype analysis in the South Devon. *Animal Genetics, 31*, 306-309.

Smith, T. P. L., Lopez-Corrales, N. L., Kappes, S. M., & Sonstegard, T. S. (1997). Myostatin maps to the interval containing the bovine *mh* locus *Mammalian Genome, 8*, 742-744.

Sonstegard, T. S., Rohrer, G. A., & Smith, T. P. L. (1998). Myostatin maps to Chromosome 15 by linkage and physical analyses. *Animal Genetics, 29*, 19-22.

Speijers, M. H., Carson, A. F., Dawson, L. E., Irwin, D., & Gordon, A. W. (2010). Effects of sire breed on ewe dystocia, lamb survival and weaned lamb output in hill sheep systems. *Animal, 4*, 486-496.

Stinckens, A., Bijttebier, J., Luyten, T., Van Den Maagdenberg, K., Harmegnies, N., De Smet, S., Georges, M., & Buys, N. (2005). Detection of polymorphisms in the myostatin gene in Belgian Pietrain pigs. *Communications in Agricultural and Applied Biological Sciences, 70*, 37-41.

Stinckens, A., Luyten, T., Bijttebier, J., Van den Maagdenberg, K., Dieltiens, D., Janssens, S., De Smet, S., Georges, M., & Buys, N. (2008). Characterization of the complete porcine *MSTN* gene and expression levels in pig breeds differing in muscularity. *Animal Genetics, 39*, 586-596.

Sturm, R. A., Das, G., & Herr, W. (1988). The ubiquitous octomer-binding protein Oct-1 contains a POU domain with a homeo box subdomain. *Genes & Development, 2*, 1582-1599.

Takeda, H., Charlier, C., Farnir, F., & Georges, M. (2010). Demonstrating polymorphic miRNA-mediated gene regulation in vivo: application to the g+6223G>A mutation of Texel sheep. *RNA, 16*, 1854-1863.

Tapio, M., Miceikienė, I., Vilkki, J., & Kantanen, J. (2003). Comparison of microsatellite and blood protein diversity in sheep: inconsistencies in fragmented breeds. *Molecular Ecology, 12*, 2045-2056.

Taylor, W. E., Bhasin, S., Artaza, J., Byhower, F., Azam, M., Willard, D. H., Kull, F. C., & González-Cadavid, N. F. (2001). Myostatin inhibits cell proliferation and protein synthesis in C2C12 cells. *American Journal of Physiology - Endocrinology and Metabolism, 280*, E221-E228.

Tellgren, A., Berglund, A. C., Savolainen, P., Janis, C. M., & Liberles, D. A. (2004). Myostatin rapid sequence evolution in ruminants predates domestication. *Molecular Phylogenetics and Evolution, 33*, 782-790.

Terova, G., Bernardini, G., Binelli, G., Gornati, R., & Saroglia, M. (2006). cDNA encoding sequences for myostatin and FGF6 in sea bass (Dicentrarchus labrax, L.) and the effect of fasting and refeeding on their abundance levels. *Domestic Animal Endocrinology, 30*, 304-319.

Thomas, M., Langley, B., Berry, C., Sharma, M., Kirk, S., Bass, J., & Kambadur, R. (2000). Myostatin, a negative regulator of muscle growth, functions by inhibiting myoblast proliferation. *The Journal of Biological Chemistry, 275*, 40235-40243.

Tobin, J. F., & Celeste, A. J. (2005). Myostatin, a negative regulator of muscle mass: implications for muscle degenerative diseases. *Current Opinion in Pharmacology, 5*, 328-332.

Uytterhaegen, L., Claeys, E., & Demeyer, D. (1994). Effects of exogenous protease effectors on beef tenderness development and myofibrillar degradation and solubility. *Journal of Animal Science, 72*, 1209-1223.

Vali, U., Einarsson, A., Waits, L., & Ellegren, H. (2008). To what extent do microsatellite markers reflect genome-wide genetic diversity in natural populations? *Molecular Ecology, 17*, 3808-3817.

Varga, L., Müller, G., Szabó, G., Pinke, O., Korom, E., Kovács, B., Patthy, L., & Soller, M. (2003). Mapping modifiers affecting muscularity of the myostatin mutant (*Mstn*$^{Cmpt\text{-}dl1Abc}$) compact mouse. *Genetics, 165*, 257-267.

Vianello, S., Brazzoduro, L., Dalla Valle, L., & Belvedere, P. (2003). Myostatin expression during development and chronic stress in zebrafish (*Danio rerio*). *Journal of Endocrinology, 176*, 47-59.

Walling, G. A., Visscher, P. M., Simm, G., & Bishop, S. C. (2001). Confirmed linkage for QTLs affecting muscling in Texel sheep on chromosomes 2 and 18. *Proceedings of 52nd Animal Meeting of the European Association for Animal Production, Budapest, Hungary, 26th-29th August 2001, Paper G5.6*

Wang, Z., & Burge, C. B. (2008). Splicing regulation: from a parts list of regulatory elements to an integrated splicing code. *RNA, 14*, 802-813.

Wardrop, I. D. (1968). Birth weight, liveweight gain in early life, and subsequent gain in sheep and cattle. *Australian Journal of Agricultural Research, 19*, 837-844.

Wegner, J., Albrecht, E., Fiedler, I., Teuscher, F., Papstein, H. J., & Ender, K. (2000). Growth- and breed-related changes of muscle fiber characteristics in cattle. *Journal of Animal Science, 78*, 1485-1496.

Welle, S., Bhatt, K., Pinkert, C. A., Tawil, R., & Thornton, C. A. (2007). Muscle growth after postdevelopmental myostatin gene knockout. *American Journal of Physiology Endocrinology and Metabolism, 292*, E985-E991.

Wiener, P., Smith, J. A., Lewis, A. M., Woolliams, J. A., & Williams, J. L. (2002). Muscle-related traits in cattle: the role of the myostatin gene in the South Devon breed. *Genetics Selection Evolution 34*, 221-232.

Winters, S. J., & Moore, J. P. (2007). Paracrine control of gonadotrophs *Seminars in Reproductive Medicine, 25*, 379-387.

Xu, C., Wu, G., Zohar, Y., & Du, S. J. (2003). Analysis of myostatin gene structure, expression and function in zebrafish. *The Journal of Experimental Biology, 206*, 4067-4079.

Ye, X. H., Brown, S. R., Nones, K., Coutinho, L. L., Dekkers, J. C. M., & Lamont, S. J. (2007). Associations of myostatin gene polymorphisms with performance and mortality traits in broiler chickens. *Genetic Selection Evolution, 39*, 73-89.

Yu, L., Tang, H., Wang, J. H., Wu, Y., Zou, L., Jiang, Y., Wu, C., & Li, N. (2007). Polymorphisms in the 5' regulatory region of myostatin gene are associated with early growth traits in Yorkshire pigs. *Science China Life Sciences, 50*, 642-647.

Zavolan, M., Nimwegen, E. V., & Gaasterland, T. (2002). Splice variation in mouse full-length cDNAs identified by mapping to the mouse genome. *Genome Research, 12*, 1377-1385.

Zhang, G. X., Zhao, X. H., Wang, J. Y., Ding, F. X., & Zhang, L. (2011). Effect of an exon 1 mutation in the *myostatin* gene on the growth traits of the Bian chicken. *Animal Genetics*. doi:doi:10.1111/j.1365-2052.2011.02274.x

Zhou, H., & Hickford, J. G. (2008). Clonal polymerase chain reaction-single-strand conformational polymorphism analysis: An effective approach for identifying cloned sequences. *Analytical Biochemistry, 378*, 111-112.

Zhou, H., Hickford, J. G., & Fang, Q. (2006). A two-step procedure for extracting genomic DNA from dried blood spots on filter paper for polymerase chain reaction amplification. *Analytical Biochemistry, 354*, 159-161.

Zhou, H., Hickford, J. G. H., & Fang, Q. (2008). Variation in the coding region of the myostatin (GDF8) gene in sheep. *Molecular and Cellular Probes, 22*, 67-68.

Zhu, X. Y., Topouzis, S., Liang, L. F., & Stotish, R. L. (2004). Myostatin signaling through Smad2, Smad3 and Smad4 is regulated by the inhibitory Smad7 by a negative feedback mechanism. *Cytokine, 26*, 262-272.

Zubay, G. (1998). The building blocks of proteins: amino acids, peptides, and polypeptides. In R. Worthington (Ed.), *Biochemistry* (Fourth ed., pp. 60-75). Kerper Boulevard, Dubuque, USA: The McGraw-Hill Companies.