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Maternal Feed Intake and Myogenesis in Sheep

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ABSTRACT

Perturbations of the *in utero* environment may influence development of organs and tissues of fetal sheep. Altered myogenesis caused by maternal feed restriction, placental insufficiency and manipulations of the early embryo environment have been reported. However, the underlying mechanisms responsible for altered muscle development have received scant attention in the ovine fetus.

This thesis describes two models developed to investigate the effect of maternal feed intake on muscle cellularity and myogenic gene expression of fetal sheep. In model one, ewes were fed either *ad libitum*, maintenance or restricted amounts of the same diet prior to and throughout pregnancy with fetuses sampled on days 50, 92 and 133 of gestation. Growth-retardation in feed restricted fetuses was evident by late pregnancy (day 133), and was associated with lower fetal plasma glucose, urea and IGF-I and reduced muscle mass, myofibre size, total muscle DNA and protein, and protein concentration.

Messenger RNA expression of candidate genes (MRFs, myostatin, IGFs) occurred in a coordinated manner reflecting their reported roles from *in vitro* studies and other species. Key times during muscle development were identified when gene expression may be altered by nutrition, which may alter myogenesis. Delayed myogenesis in growth-retarded fetuses was associated with repressed GAPDH mRNA expression, or delayed differentiation or reduced satellite cell activity as indicated by elevated myostatin and myf-5 mRNA in growth-restricted fetuses at day 92 and 133, respectively. Whether these later two changes are causative or a result of altered myogenesis remains to be determined. Nevertheless, this study is the first to report the spatial and nutritional regulation of the MRFs, IGFs and myostatin in sheep at various stages of development.

In model two superovulated donor ewes were fed either 1.5 (H) or 0.5 (L) of maintenance requirements for 18 days before and 6 days after ovulation (day 0), embryos were transferred to recipient ewes on day 6, and fetuses were collected on day 75. Although fetal body dimensions, organ development and muscle weights were not altered by maternal intake, total muscle fibre number was greater in the H treatment. The treatment effect resulted from an increased secondary to primary fibre ratio. This new finding suggests that short-term changes in maternal feed intake during

the peri-conception period may influence myogenesis. It is speculated that epigenetic modifications of the embryo and possibly myogenic programming may be involved.

In conclusion, manipulation of maternal feed intake for long or short duration differentially altered myogenesis in fetal sheep. Differential mRNA expression levels of myogenic regulatory genes may be one mechanism that altered myogenesis in response to manipulation of maternal feed intake for a prolonged period. While variable maternal feed intake imposed for a short period during peri-conception also influenced myogenesis, the mechanisms responsible require further investigation.

DECLARATION OF ORIGINALITY

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

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

Simon Quigley

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At the commencement of this candidature, I had little experience in the areas of fetal physiology and muscle biology, with no skills in molecular biology and muscle histology. The past three and a half years have been a learning experience beyond anticipation. The acquisition of technical and statistical skills was an imperative part of the journey. More important was the development of skills to critically assess problems and develop strategies to solve them. The development of these skills and the completion of this work would not have been possible without the expert guidance, assistance and patience of many people.

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PUBLICATIONS ARISING FROM THIS WORK

Scientific Papers

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Conference Proceedings

Quigley, S.P., Walker, S.K., Speck, P.A., Barritt, S.R. and Kleemann, D.O. 2001. Pre-mating nutrition affects the onset and synchrony of oestrus in Merino ewes treated with progesterone CIDR dispensers. *Journal of Animal Science*. **79 (Suppl 1):** 451.

Quigley, S.P., Kleemann, D.O., Owens, J.A., Bawden, C.S., Speck, P.A. and Nattrass, G.S. 2002. Real-time RT-PCR analysis of the ontogeny and nutritional regulation of myostatin mRNA expression in skeletal muscle of fetal sheep. *Australian Society of Animal Production* **24:**343.

Quigley, S.P., Kleemann, D.O., Walker, S.K., Owens, J.A., Hynd, P.I., Nattrass, G.S., Barritt, S.R. and Speck, P.A. 2001. Influence of long-term maternal nutrition on ovine fetal growth and development. *Journal of Animal Science*. **79 (Suppl 1):** 428.

ABBREVIATIONS

ad libitum:	without restraint
ANOVA:	analysis of variance
ATPase:	adenosine triphosphatase
bp:	base pair
BSA:	bovine serum albumin
cDNA:	complementary DNA
CIDR:	controlled intra-vaginal drug release
CORR:	correlation
CSA:	cross-sectional area
CV:	coefficient of variation
°C:	degrees Celsius
d:	day (s)
DM:	dry matter
DNA:	deoxyribose nucleic acid
dNTP:	deoxynucleoside triphosphate
et al.:	*et alia* (and others)
etc.:	*et cetera* (and so on)
g:	gram (s)
g:	relative centrifugal force
GAPDH:	glyceraldehyde 3-phosphate dehydrogenase
GDF:	growth differentiation factor
gDNA:	genomic DNA
GLM:	general linear model
h:	hour (s)
IGF:	insulin-like growth factor
IgG:	immunoglobulin G
kb:	kilobase
kDa:	kiloDalton
kg:	kilogram (s)
L:	litre (s)
ME:	metabolizable energy
M:	molar
μCi:	microcurie (s)

μg:	micorgram (s)
μl:	microlitre (s)
μM:	micromolar
mA:	milliampere
mg:	milligram (s)
min:	minute (s)
mM:	millimolar
mV:	millivolt (s)
MQ water:	milli-Q-filtered water
MRF:	myogenic regulatory factor
mRNA:	messenger RNA
n:	number
NEFA:	non-esterified fatty acid
ng:	nanogram
nm:	nanometres
nM:	nanomolar
OD:	optical density
PCR:	polymerase chain reaction
PVP:	polyvinylpyrrolidone
RNA:	ribose nucleic acid
RO water:	reverse osmosis water
rRNA:	ribosomal RNA
RT:	room temperature
RT:	reverse transcription
SAS:	statistical analysis system
SEM:	standard error of the mean
sec:	second (s)
TGF:	transforming growth factor
U:	units
UV:	ultraviolet
vol:	volumes
v /v:	volume :volume
w /v:	weight :volume

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CHAPTER 1

1 LITERATURE REVIEW

1.1 Introduction

The ability to manipulate muscle growth in domestic species, such as sheep and cattle, has important implications for the red meat industry. Most research effort has focused on factors that may affect lean meat production and efficiency of production in the post-natal period. However, sheep and cattle grown for meat production spend up to 30% of their growing life, from conception, *in utero*. With most tissues and organs actively mature by birth, recently increased emphasis has been placed on the significance of events occurring pre-natally and how they may influence subsequent development and growth.

In general, muscle growth occurs by an increase in muscle fibre number (hyperplasia) and the subsequent growth of individual fibres (hypertrophy). The number of muscle fibres is essentially fixed at birth. The potential to which fibres can grow may also be influenced by the pre-natal period. Therefore, we hypothesised that the main factors impinging on potential muscle growth are inherited genetic factors and the pre-natal environment.

The main variable affecting the pre-natal environment is that of placental restriction; mediated by maternal nutritional status and/ or litter size. Depending on the heritability of muscle fibre characteristics, the pre-natal environment could potentially have a dramatic influence on post-natal growth and carcass attributes.

Like all cellular processes, muscle development, activity and function is controlled by the expression of specific genes. Many genes involved in the regulation of muscle development have been identified and their functions characterised. The myogenic regulatory factors (MRFs) are involved in commitment, proliferation and differentiation of muscle cells; myostatin is involved in muscle cell proliferation and differentiation; the insulin-like growth factors (IGFs) are implicated in the regulation of muscle cell proliferation, differentiation and growth.

The process of muscle development is well understood and many of the genes involved have been investigated. However, little is known as to how and when nutrition regulates the expression of these genes. The implications of these nutrient-gene interactions during the pre-natal period and their consequences for post-natal growth have received limited investigation *in vivo*, particularly in sheep. The following literature review provides an insight into the processes of muscle development and growth and some of the important genes involved in these processes.

1.2 Aspects of muscle structure that influence muscle growth

Muscle tissue contains a diverse range of cell types; fibroblasts, capillary endothelial cells, adipocytes, mononucleate satellite cells and muscle fibres (Harper and Buttery, 1992). Muscle fibres are responsible for the majority of muscle protein and structure and the co-ordinated activity of muscle. The components of typical skeletal muscle structure are displayed in Figure 1.1.

1.2.1 Muscle structural proteins

Muscle cells can be distinguished from other cells by the presence of myofibrils, which give muscle its contractile properties. The myofibril is a contractile thread consisting of 12-14 myofibrillar proteins (Dayton and Hathaway, 1989). The muscle structural proteins contribute as much as 55% to total muscle protein, with myosin and actin being the most abundant (Dayton and Hathaway, 1989). The major myofibrillar proteins are myosin heavy chain, myosin light chain, actin, tropomyosin and troponin. Richter *et al.* (1989) reviewed the regulation of expression of these proteins and outlined the functional differences between isoforms.

1.2.2 Primary and secondary muscle fibres

It is generally accepted that two populations of myofibres exist, primary and secondary, which differ in their timing and method of formation; a third population of tertiary fibres has been described in sheep (Wilson *et al.*, 1992) and pigs (Mascarello *et al.*, 1992). Evans *et al.* (1994) determined that primary and secondary fibres form from a single population of cells. Primary muscle fibres develop first by the rapid fusion of primary myoblasts (Kelly and Zacks, 1969) in the embryo and early fetus. In the pig, this contributes about 10-20% of myofibres present at birth (Swatland, 1973).

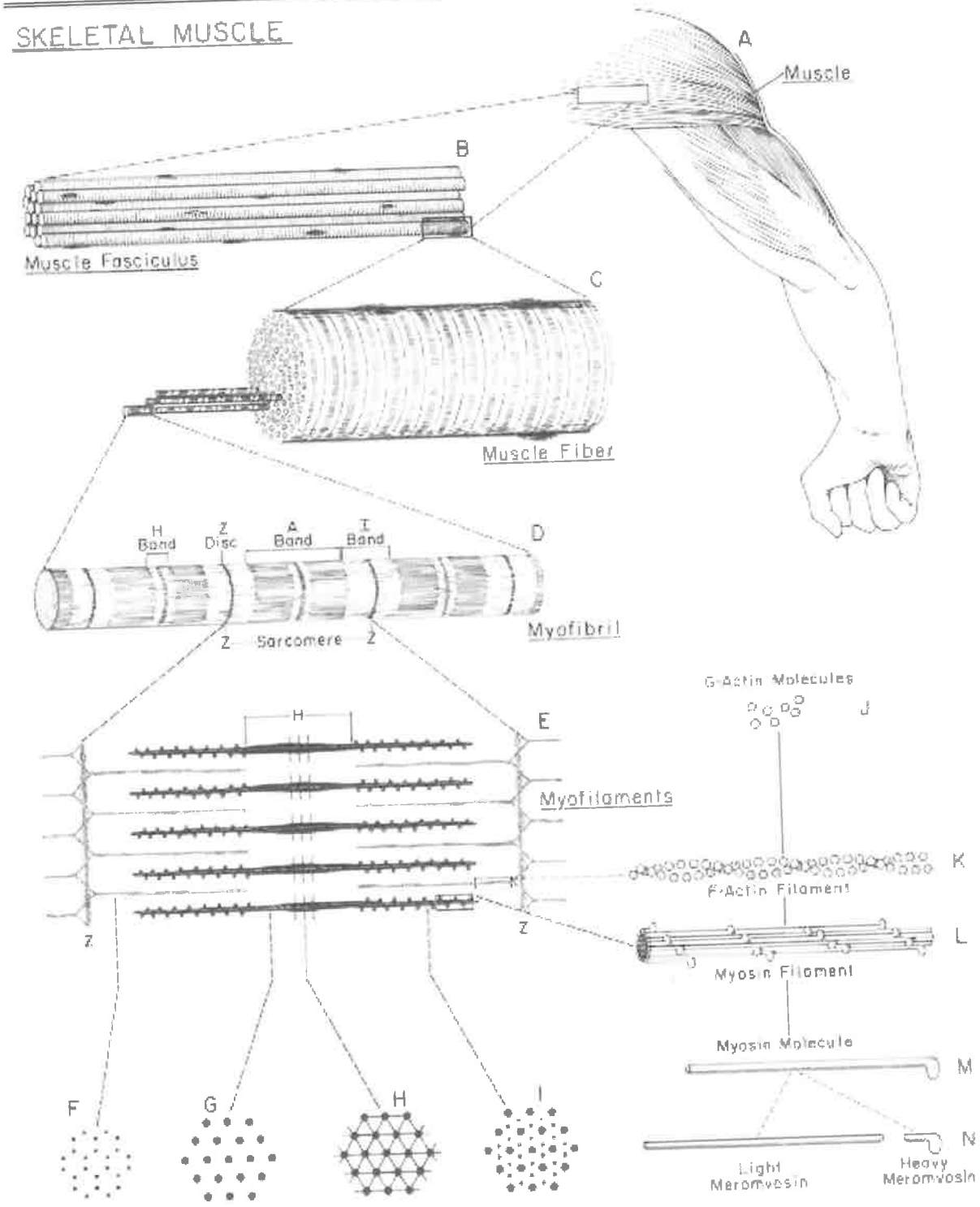


Figure 1.1: The anatomical and fine structure of typical skeletal muscle (from Bloom and Fawcett, 1986).

The primary myofibres act as supporting central structures upon which a slower fusion of a larger population of myoblasts occurs to form the secondary fibres, which contribute 80-90% of myofibres present at birth (Swatland, 1973). Swatland (1973) proposed that the extent of secondary myofibre formation might be influenced by primary myofibres. Hence, the formation of primary myofibres, although relatively small in number, may ultimately influence the number of muscle fibres at birth.

The variation in muscle fibre number is mainly due to responses of secondary muscle fibres to external influences, such as nutrition, while primary myofibre number remains relatively fixed. Evidence of this can be drawn from several studies on pigs, including some on littermates (Wigmore and Stickland, 1983; Handel and Stickland, 1987; Dwyer and Stickland, 1991; Dwyer *et al.*, 1993; Dwyer *et al.*, 1994), rats (Wilson *et al.*, 1988) and guinea pigs (Ward and Stickland, 1991) nutritionally restricted during gestation. This may be explained by the relative demands the fetus places on the mother during the respective phases of fetal development. Primary fibres develop immediately prior to the period of rapid fetal growth, days 30 to 50 in sheep. Secondary muscle fibre development corresponds with a time when rapid fetal development is occurring, placing increasing demands on nutrients from the mother. Alternatively, primary fibre number may be genetic in origin (Swatland and Keifer, 1974; Stickland and Handle, 1986) or a fundamental requirement for survival (i.e. support and mobility).

1.2.3 Muscle fibre number

The number of muscle fibres is essentially fixed at birth. Muscle growth occurs through increased muscle fibre length and width. Muscle fibre number and diameter are both positively related with total muscle size (reviewed by Rehfeldt *et al.*, 2000). There is a strong inverse relationship between muscle fibre number and muscle fibre growth (Rehfeldt *et al.*, 2000); animals that display a high number of muscle fibres exhibit decreased growth of individual muscle fibres.

The growth potential of muscle appears to be determined by muscle fibre number (Rehfeldt *et al.*, 2000). Factors that have been demonstrated to influence overall muscle growth, such as growth hormone, increase fibre growth rate but do not affect maximum fibre size. The increased muscle fibre number witnessed in double-muscled cattle results in a 20% increase in muscle mass. However, these breeds are generally late maturing with lower growth rates than other breeds (see section 1.4.2). This observation further supports the relationships between muscle fibre number and lean

muscle growth. Dwyer *et al.* (1993) found that post-natal growth and the gain to feed ratio was significantly correlated with muscle fibre number in growing pigs. Differences in growth rates, feed conversion efficiency and carcass composition between males and females are widely reported; males generally grow faster and deposit greater protein and less fat than females. Nordby *et al.* (1987) and Shackelford *et al.* (1995) reported male lambs having greater numbers of muscle fibres than females after treatments with beta-agonists and nutrition *in utero*, respectively.

Given the importance of muscle fibre number and size to overall muscle growth, it would be of interest to exploit these factors in selection programs. Rehfeldt *et al.* (2000) suggested that the majority of phenotypic variation in muscle fibre number can be attributed to genetic variation and that the heritability of this can be as high as 0.88 (although it is quite variable, ranging from 0.12 to 0.88, depending on the species and muscle investigated). In contrast, Cameron *et al.* (1998) found low phenotypic and genetic correlations between muscle fibre frequency and carcass characteristics, with similarly low heritability. Therefore, if muscle fibre number is not solely genetic in origin, but is determined before birth, then the pre-natal period must contribute significantly to muscle fibre number development (Rehfeldt *et al.*, 2000). This has been observed to be 17% of the phenotypic variance in the extensor digitorum muscle in mice (Rehfeldt *et al.*, 1988). Therefore, manipulation of the maternal environment and its interactions with fetal development may play a significant role in determining potential muscle growth of the offspring.

1.2.4 Muscle fibre type

Primary and secondary muscle fibres develop distinct characteristics. Muscle fibres can be classified broadly, in relation to their activity and metabolism, as slow oxidative, fast oxidative and fast glycolytic:

- Slow oxidative (SO, type I; β-red): contract slowly, but maintain contraction for a longer period of time; obtain energy from oxidative pathways; mostly found in deeper and postural muscles; small fibre diameter; redder in colour; higher haemoglobin content; higher concentration of mitochondria in muscles.
- Fast oxidative (FO, type IIa; α-red): intermediate between FG and SO; contract rapidly; obtain energy from either oxidative or glycolytic pathways; intermediate in diameter.

- Fast glycolytic (FG, type IIb; α-white): contract rapidly, for a short duration; obtain energy mainly from anaerobic pathways; mostly found in the superficial and limb muscles; large fibre diameter; whiter in colour.

Two trains of thought exist on the development of fibre type. The first thought is that different sub-populations of primary myoblasts form both fast twitch and slow twitch fibres, whereas secondary myoblasts are initially homogenous but form different fibre types depending on their environment. The second, generally accepted, thought is that primary myoblasts form slow twitch fibres, while secondary myoblasts can become either slow or fast twitch fibre types, depending on the muscle. As the fetal/ neonatal animal develops, a transformation occurs that leads to the formation of mature functional muscles. Mature myosin isoforms (I, IIa, IIb and IIx) are enhanced while immature ones (fetal and neonatal) are suppressed. In later life, fibre phenotype tends to be governed by amount and type of muscle activity (Abernethy *et al.*, 1990; Stevens *et al.*, 2000), genotype (Wegner *et al.*, 2000; Maltin *et al.*, 2001), age (Wegner *et al.*, 2000) and nutrition (Vestergaard *et al.*, 2000).

In fetal sheep, it appears that the proportion of each muscle fibre type within a specific muscle does not change between day 90 and 140 of gestation (Javen *et al.*, 1996). Javen *et al.* (1996) determined that the differentiation of muscle fibre type occurs between day 90 and 125 of gestation in sheep. At day 90 slow twitch soleus muscle had similar contractile properties to fast twitch flexor digitorum longus and medial gastrocnemius. However, contraction time increased in slow twitch muscles and decreased in fast twitch muscles with age (Javen *et al.*, 1996).

Fibre composition is continually changing during muscle development. Generally the increase in type IIb fibres, commonly witnessed, is due to a transformation from type IIa to IIb. There is little change in the proportion of type I fibres. Moreover, type I fibres tend to cease growth earlier than type II fibres (Harper, 1999) suggesting early maturing type animals, or muscles, may have a higher proportion of type I fibres. Due to this transient nature of myofibre type, individual myofibres can express more than one myosin isoform (Picard *et al.*, 1998).

The factors that regulate the distribution of fibre types within a muscle could affect muscle size and/ or growth, due to differences in fibre diameter between the muscle fibre types (Dayton and Hathaway, 1989) and growth rate of individual fibres. Type IIb

fibres are thicker and grow faster than other fibres (Kiessling, 1979). Kiessling (1979) proposed the reason for this faster growth might be attributed to differences in metabolic activity. Type II fibres derive their energy from anaerobic metabolism of glucose which is readily available in the form of stored glycogen whereas type I fibres need to diffuse oxygen and substrates into the cell to meet their energy requirements (Kiessling, 1979). Anaerobic metabolism would be a much quicker and efficient way of producing energy than aerobic metabolism.

Slow twitch muscles contain a low ratio of secondary to primary fibres. As primary fibres are generally unaffected by under-nutrition, it has been concluded that slow muscles are not as affected by under-nutrition as fast muscles (Ward and Stickland, 1991).

1.2.5 Satellite cells and muscle fibre hypertrophy

Satellite cells are small spindle shaped mononucleate cells enclosed between the muscle fibre and the basement membranes of muscle tissue (see Figure 1.2). Satellite cells play an important role in post-natal growth and are most abundant in the young animal (Gibson and Schultz, 1983). They remain as a population that can proliferate, differentiate and migrate to form new myonuclei as required for growth or injury repair (Grounds *et al.*, 2002) or can be returned to quiescence. Satellite cells returned to quiescence maintain a constant population per unit length of myofibre (McCroskery *et al.*, 2003) available for future regeneration. However, their frequency declines with increasing maturity and a reduced capacity for proliferation and differentiation has been reported in satellite cells from aged muscle (Chen and Goldhammer, 2003). Satellite cells are thought to be greater in number and as a proportion of all nuclei in slow compared to fast muscles (Zammit *et al.*, 2002).

The origins of satellite cells remain unclear, but three main thoughts exist. Firstly, satellite cells may arise prior to activation of the myogenic lineage and, as such, can be considered true myogenic precursor cells, distinct from myoblasts. Secondly, satellite cells may originate from committed undifferentiated myoblasts, inhibited from undergoing fusion to form muscle fibres, that migrate and reside beneath the basal lamina of muscle fibres (Dayton and Hathaway, 1989). Thirdly, satellite cells may be derived from non-muscle sources, such as neural stem cells and bone-marrow cells, which have been demonstrated to have myogenic properties similar to skeletal muscle satellite cells (reviewed by Zammit and Beauchamp, 2001; Chen and Goldhammer,

2003). However, what is clear is that the formation and activation of satellite cells is under the control of many specific factors (Zammit and Beauchamp, 2001; Chen and Goldhammer, 2003). Of these factors, pax-7 is thought to play a critical role in satellite cell formation, as pax-7 null mice lack satellite cells (Seale *et al.*, 2000). Myostatin and the MRFs are thought to be key regulators of satellite cell proliferation and differentiation.

As discussed earlier, at birth the number of muscle fibres is essentially fixed. Muscle growth occurs by an increase in length and width (hypertrophy) of existing fibres. DNA content of a muscle may increase from 2-100 fold (Young, 1985); protein to DNA ratio also increases (Winick and Noble, 1965). However, myofibre nuclei do not divide. Moss and Leblond (1971) demonstrated that the new nuclei within fibres originated from the proliferation, differentiation and fusion of satellite cells. With satellite cells contributing between 50 to 95% of all myonuclei (Greenwood *et al.* 1999; McCoard *et al.* 2001) in late fetal and young post-natal sheep, their contribution to fetal muscle development may be significant. In rats, severe maternal under-nutrition reduces the satellite cell population in progeny (Beermann *et al.*, 1983). Further, reduced muscle weights due to fetal growth restriction of sheep have been associated with decreased satellite cell frequency (McCoard *et al.*, 2001) and activity (Greenwood *et al.*, 1999).

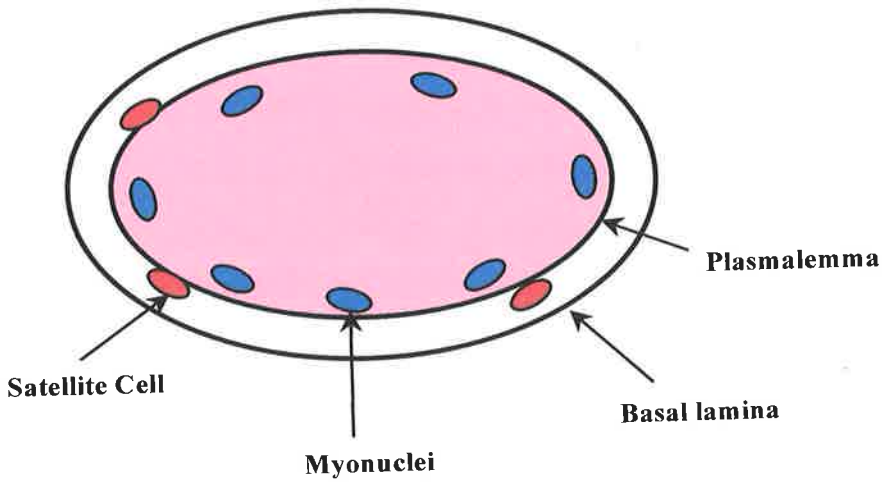


Figure 1.2: Location of satellite cells within the membranes of muscle fibres.

1.3 Myogenesis

1.3.1 Formation of muscle

Formation of specific cells, tissues and organs occurs as a two-step process. The first stage involves commitment of multipotent cells to a distinct lineage. The second step, terminal differentiation, involves the development of the cell, acquiring properties (gene functions) that allow it to become a functioning specialised cell (Hu *et al.*, 1995). In skeletal muscle, this process is referred to as myogenesis, or the sequence of events that results in the formation of functional muscle.

Essentially, myogenesis involves proliferation of single nucleated muscle precursor cells, derived from the myotome of the multi-potent somites, followed by differentiation to form multinucleated myotubes (Figure 1.3). te Pas and Visscher (1994) chronologically classified this process into five steps:

1. Commitment of precursor somite cells to the muscle cell lineage (Determination/ Commitment).
2. Migration of myoblasts to appropriate locations within the embryo.
3. Increase in precursor cell number (Proliferation).
4. Fusion of myoblasts to form myotubes and myofibres and altered programming to allow production of proteins characteristic of muscle cells (Differentiation).
5. Maintenance of the terminally differentiated state (i.e. irreversible departure from the cell cycle).

1.3.1.1 Commitment/ Determination

During gastrulation three embryonic cell layers form; endoderm, ectoderm and mesoderm. It is generally accepted that muscle forms from the myotome of the segmented somites that are found in the mesoderm (Tortora and Anagnostakos, 1990). Therefore, cells present in the myotome are considered irreversibly committed to the myogenic lineage. However, there is evidence that some embryonic cells may commit to a myogenic fate prior to the formation of the somites (George-Weinstein *et al.*, 1997). Depending on the location of presumptive myoblasts within the myotome, they migrate to specific locations within the embryo where they proliferate and differentiate.

1.3.1.2 Proliferation

Allen *et al.* (1979) reviewed the main processes of muscle cell proliferation in both pre- and post-natal (via satellite cells) muscle. Essentially, the process of proliferation increases muscle cell number by the continual propagation of identical daughter cells,

via the cell cycle (see Figure 1.4). Proliferation of precursor cells is completed prior to the onset of differentiation. The increased muscle cell number will subsequently have a role in determining the number of muscle fibres an animal will have. The significance of increased fibre number is demonstrated in double-muscled cattle which display enlarged muscles; the result of increased muscle fibre number (Swatland and Kieffer, 1974). Myostatin, a member of the transforming growth factor-β (TGF-β) family of secreted proteins, has been implicated in the regulation of myoblast proliferation and will be discussed in detail later (section 1.4.2).

1.3.1.3 Differentiation

Differentiation involves:

- Irreversible departure of committed cells from the cell cycle
- Acquisition of the ability to produce proteins characteristic of muscle cells
- Cell alignment and fusion to form myotubes
- Maintenance of the differentiated state and maturation of myofibres.

The generally accepted mechanism of muscle cell differentiation is that put forward by Nadal-Ginard (1978); differentiating cells in G0/G1 either depart the cell cycle and fuse, or continue to proliferate by re-entering the cell cycle based on the time spent in G0/G1 (see Figure 1.4). This is probably regulated by the concentration of mitogens, such as IGF, FGF and TGF-β present at that time (Harper and Buttery, 1992).

Andrés and Walsh (1996) further detailed the sequential events and controlling factors of myogenic differentiation. They reported that myogenin, a member of the myogenic regulatory factor (MRF) family, was expressed first in myoblasts, committing them to differentiation. This was followed by the expression of p21, which inhibits the cell cycle and activates muscle specific genes, such as the myosin heavy chain structural proteins. Finally, differentiated myoblasts fuse with others to form multi-nucleated myotubes. Differentiated myotubes do not divide but mature to form myofibres, while myonuclei within the fibres are also incapable of further division or replication.

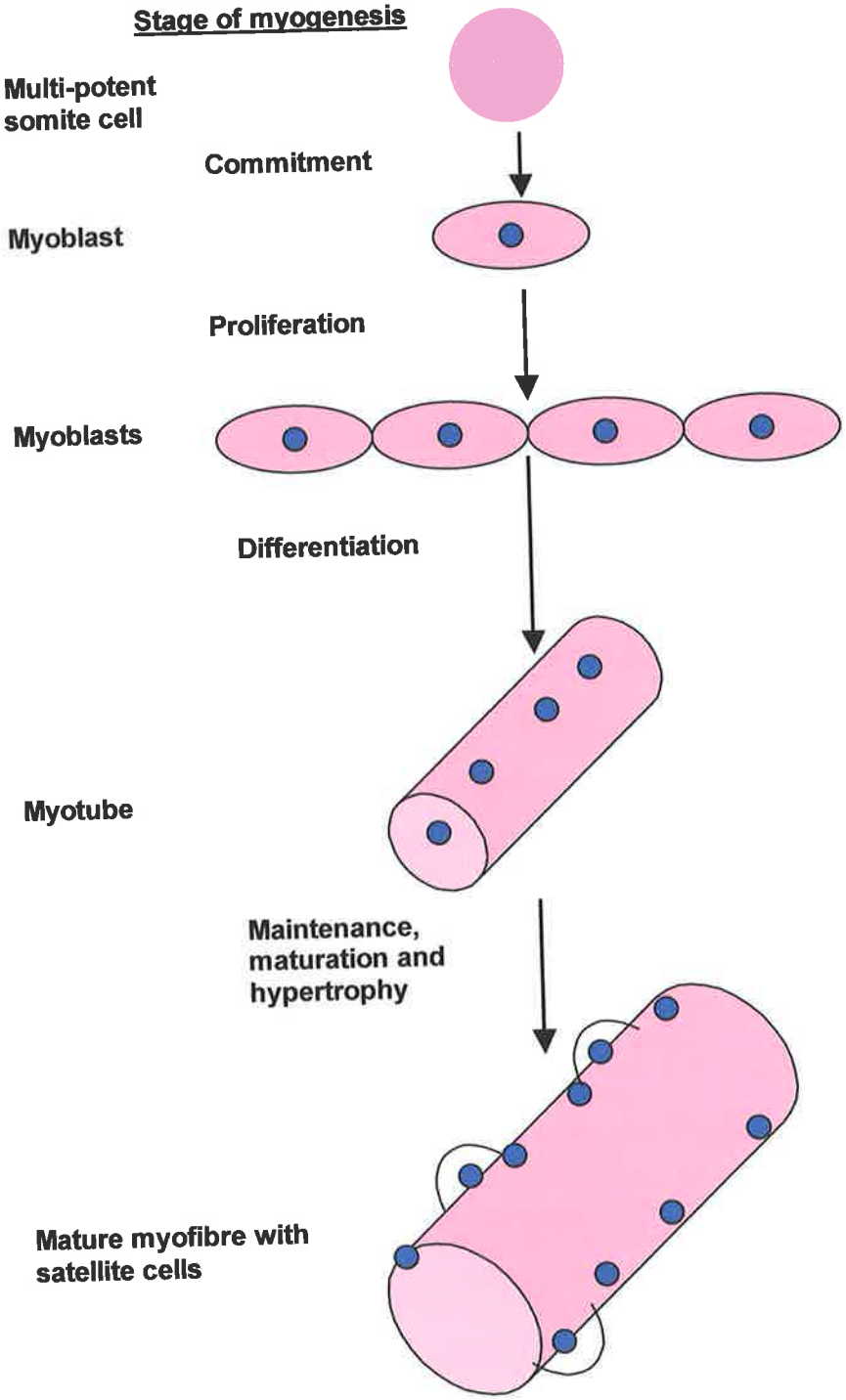


Figure 1.3: The myogenic pathway.

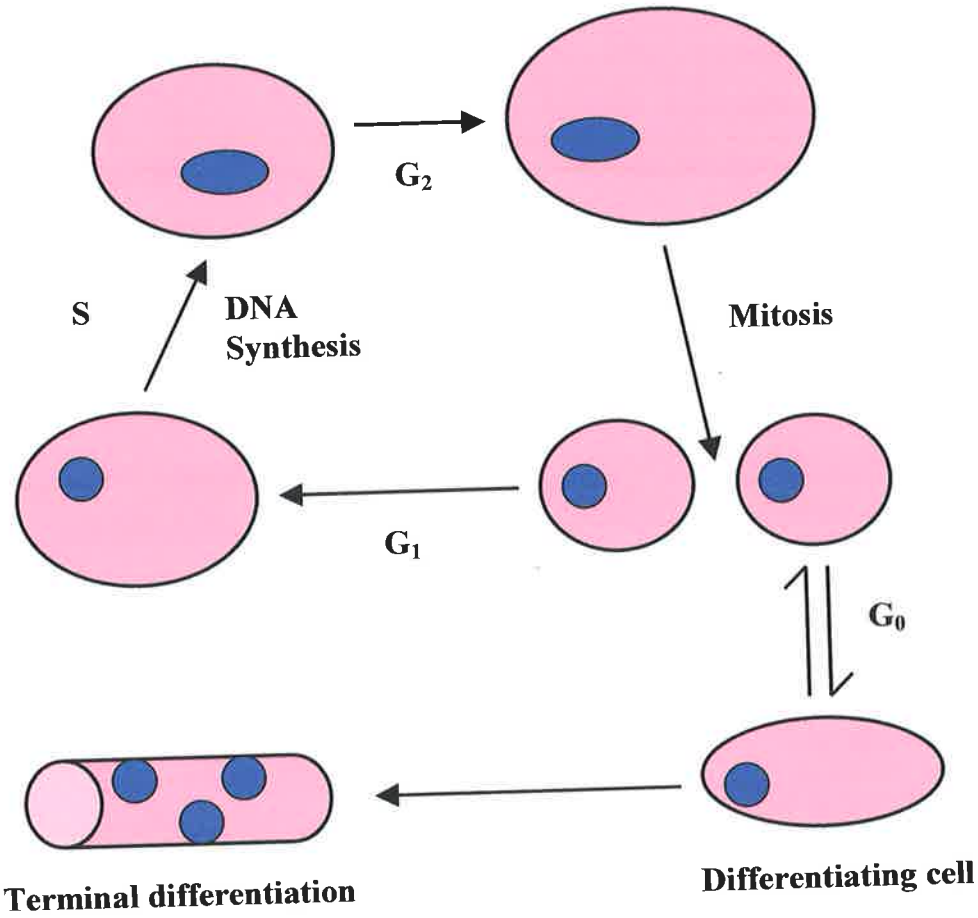


Figure 1.4: The muscle cell cycle (adapted from Harper and Buttery, 1992).

1.3.2 Myogenesis in sheep

There is little information on the events that precede myogenesis (commitment and migration) in sheep and how they may affect muscle development.

Muscle hyperplasia commences during early fetal development in sheep. Primary fibres appear at day 30 (Wilson *et al.*, 1992) with the formation of secondary fibres commencing between days 38 (Wilson *et al.*, 1992) and 50 (Ashmore *et al.*, 1972). Swatland and Cassens (1973) indicated that muscle hyperplasia is insignificant after day 80 of gestation in sheep, supporting the early work of Joubert (1955,1956). However, considerable evidence exists to suggest that hyperplasia continues beyond day 90 (Everitt, 1965) and into the last third of pregnancy, days 100-115 (Maier *et al.*, 1992; Greenwood *et al.*, 1999). Further, McCoard *et al.* (2000a, b) demonstrated that muscle fibre number continued to increase in several muscles up to day 140 of gestation. If the observed increase in apparent myofibre number beyond day 100 is

relatively small, it may be attributed to the growth of intrafascicular terminating myofibres (Swatland and Cassens, 1972). However, the large increases reported by Everitt (1965) and McCoard *et al.* (2000a, b) are unlikely to be the result of increased length of existing fibres alone. Nevertheless, muscle hyperplasia (proliferation and differentiation) is completed pre-natally.

Hypertrophic muscle growth is thought to commence during the final third of pregnancy, as indicated by increasing muscle protein content relative to DNA content (Rattray *et al.*, 1975; Greenwood *et al.*, 1999) and increasing size of individual muscle fibres (Joubert, 1955, 1956; Ashmore *et al.*, 1972) with little increase in muscle fibre number. Total DNA content in fetal muscle continues to increase during late gestation due to the proliferation and incorporation of satellite cells (Greenwood *et al.*, 1999).

Information regarding myogenesis in other animal species used for meat production is provided in a recent review by Picard *et al.* (2002).

1.3.3 Myogenesis and nutrition

The early studies of Everitt (1965) indicated that restriction of maternal nutrient supply throughout pregnancy might reduce muscle fibre number in fetal sheep at days 90 and 140 of gestation. However, in other studies with sheep, myofibre number was relatively resistant to changes due to maternal under-nutrition (Nordby *et al.*, 1987; Krausgrill *et al.*, 1999) and competition between littermates (Greenwood *et al.*, 1999; Greenwood *et al.*, 2000a; McCoard *et al.*, 2000a). In studies where myofibre number is not altered, retarded muscle development has been related to decreased cell cycle activity and DNA and protein accretion (Greenwood *et al.*, 1999), smaller myofibre cross-section area (McCoard *et al.*, 1997, 2000a) and slower longitudinal growth of intrafascicularly terminating myofibres (Swatland and Cassens, 1973). In the porcine species where the number of littermates may be large, runt offspring have smaller muscles and this is believed to be associated with a lower secondary to primary muscle fibre ratio (Wigmore and Stickland, 1983; Dwyer *et al.*, 1994).

The effects of an altered *in utero* environment on muscle development may depend on the stage of gestation at which the perturbation occurs. For example, competition between littermates for nutrients and /or placental insufficiency may alter fetal development at a stage of gestation after commitment of cells to the myogenic lineage and myogenesis. Nevertheless, reduced muscle weights and DNA content are

witnessed in growth-restricted late gestation fetuses and lambs at birth due to intra-uterine competition for nutrients and /or placental insufficiency (Greenwood *et al.*, 1999; McCoard *et al.*, 2000b; Greenwood *et al.*, 2000a). In contrast, Everitt (1965) reduced the number of muscle fibres in fetal sheep by imposing severe nutrient restriction on mothers from an early stage of gestation, when commitment and proliferation may be occurring, suggesting maternal nutrient status during early gestation may program subsequent myogenic events. However, Nordby *et al.* (1987) found that restricting feed intake of ewes from 30 days prior to conception to day-100 of gestation resulted in no difference in muscle fibre number of seven-month-old lambs.

Restricting maternal nutrition during the first 25 days of a 68-day gestation period in Guinea pigs resulted in a significant reduction in myofibre number (Dwyer *et al.* 1995). However, no adverse effect was observed if maternal nutrition was restricted for the first 15 days only. Further, Dwyer *et al.* (1994) and Gatford *et al.* (2003) found that increasing the recommended level of feeding immediately before fibre hyperplasia (days 25 to 50 of gestation) increased the production of secondary myofibres in the semitendinous muscle of pigs. Improvements in growth rate and food conversion efficiency from day 70 post-natal to slaughter at 80kg were evident in progeny from sows that were offered increased feed intake between days 25 and 50 of gestation (Dwyer *et al.*, 1994). Only a small increase in the number of secondary fibres was witnessed if the same high nutritional regime was imposed during fibre hyperplasia (days 50 to 80) (Dwyer *et al.*, 1994). This indicates that level of nutrition prior to hyperplasia may be more significant in secondary fibre formation, than that during hyperplasia. Further, Rehfeldt *et al.*, (2001) demonstrated that the administration of growth hormone to pregnant sows from days 10-27 of gestation, altered muscle development, increasing hyperplastic and hypertrophic cellular growth. It, therefore, appears nutrient supply during the early stages of myogenesis may have a greater, possibly irreversible, impact on subsequent muscle development than in late gestation, despite the increasing demands of the fetus.

1.4 Gene regulation of myogenesis

The process of myogenesis has been outlined above. Each step in the myogenic cascade is under the coordinated control of different factors, which respond to cues to either initiate or inhibit different steps throughout myogenesis. Some of these factors, their role in myogenesis and significance in animal production will be discussed.

1.4.1 Myogenic Regulatory Factors (MRFs)

The discovery of a family of genes involved in committing multipotential somite cells to the muscle cell lineage has contributed significantly to the understanding of myogenesis. The first indications of the importance of the MRFs (myf-5, myoD, myogenin and myf-6) in myogenesis came when it was concluded that myoD could convert non-muscle cells to muscle cells in culture (Davis *et al.*, 1987; Weintraub *et al.*, 1989). Subsequently it was demonstrated that all four factors could induce myogenic properties in non-muscle cells in culture (Braun *et al.*, 1989; Rhodes and Konieczny, 1989; Wright *et al.*, 1989).

1.4.1.1 Structure and activity of the MRFs

The MRFs belong to the basic helix-loop-helix (bHLH) family of DNA-binding proteins. The HLH structure allows the MRFs to form heterodimers with E proteins. The DNA binding site of the HLH is present in the promoters of many skeletal muscle specific genes and mediates gene activation in an MRF dependent manner (Sabourin and Rudnicki, 2000).

a) MyoD and Myf-5

The myoD and myf-5 genes are expressed in the proliferating mononucleate myoblasts, although Ott *et al.* (1991) detected myf-5 in the somite prior to myotome formation, suggesting a possible role in directing precursor cells to the muscle lineage or as a marker of future myotomal cells (Buckingham, 1992). Further studies (Kelley and Mulvaney, 1992; Rudnicki *et al.*, 1993; Braun *et al.*, 1994) have confirmed the expression of myf-5 prior to myoD.

Gene targeting in mice has allowed closer examination of the function of myf-5 and myoD in commitment to muscle lineage and differentiation of muscle cells. Once activated, myf-5 and myoD are co-expressed in the majority of myoblasts and together they probably activate genes that determine the subsequent identity of the myoblast. Gene knockout experiments in mice have demonstrated the following:

- Null mutation of myoD causes no drastic alteration to muscle differentiation, although the expression of myf-5 is elevated and extended (Rudnicki *et al.*, 1992), possibly substituting for the loss of myoD.
- Null mutation of myf-5 similarly results in no drastic change to muscle differentiation, although mice die perinatally due to rib malformation (Braun *et al.*, 1992). In addition, no expression of myogenin or myf-6 occurs at the expected time. In contrast, the expression of myoD is unaffected by the null mutation to myf-5. Myogenin expression occurs immediately after myoD activation (Braun *et al.*, 1994).
- Double mutants, where both myf-5 and myoD were inactivated, fail to develop any skeletal muscle (Rudnicki *et al.*, 1993), lacking both myofibres and myoblasts.

The knockout mice demonstrate the importance of myf-5 and myoD to the normal development of muscle. It is generally considered that myf-5 may be responsible for committing cells to the muscle cell lineage. However, commencement of muscle cell differentiation will proceed in the presence of myoD, expressed independently of myf-5, or with elevated and prolonged myf-5 expression. Kitzmann *et al.* (1998) demonstrated the differential expression of myf-5 and myoD during the cell cycle, where myoD is elevated and myf-5 suppressed during G1, while the converse occurs during G0. As such, myoD is considered a likely marker for actively proliferating satellite cells (Koishi *et al.*, 1995; McCoard *et al.*, 2001). Interestingly, myoD expression is elevated in double-muscled animals, a condition in which increased muscle fibre number exists probably due to a prolonged proliferative phase of the muscle cell cycle (Oldham *et al.*, 2001).

b) Myogenin

Myogenin, first reported by Wright *et al.* (1989), is expressed in all myoblasts from the start of differentiation and continues to be expressed during the fusion of myoblasts into myofibres. The expression of myogenin marks the end of proliferation. Hasty *et al.* (1993) and Nabeshima *et al.* (1993) generated mutations in the murine myogenin gene, which allowed homozygous mice to survive fetal development but die at birth. Mice homozygous for the mutation demonstrated reduced skeletal muscle differentiation, with reduced fibre density and increased mononucleated cells where fibres would normally exist. MyoD levels at an early embryonic stage were comparable to those in normal mice. Taken together, it would appear that myogenin is not required for

commitment of cells to the muscle lineage but it is essential, and irreplaceable by other MRFs, for differentiation.

te Pas *et al.* (1999a) proposed that manipulation of myogenin function or timing of expression could influence the number of muscle fibres that develop during myogenesis. For example, if the expression of myogenin is delayed, myoblast proliferation may continue longer, increasing the number of myoblasts and hence the number of overall muscle fibres. As discussed earlier, increasing muscle fibre number has implications on increased muscle weight at birth initially, but more significantly on growth rate and lean body weight.

c) Myf-6

The myf-6 gene is expressed during early myogenesis (Rhodes and Konieszny, 1989; Miner and Wold, 1990). However, myf-6 is the main MRF family member expressed once differentiation is completed (Kelley and Mulvaney, 1992). Braun and Arnold (1995) inactivated the myf-6 gene in mice and observed mild alterations in skeletal muscle development; an imbalance of contractile protein isoform expression was reported, with embryonic myosin heavy chain expression, in particular, being dramatically reduced in myf-6 knockout mice. This suggests myf-6 has a role during differentiation when muscle fibres acquire muscle-specific proteins and properties (Braun and Arnold, 1996). te Pas and Vischer (1994) proposed that myf-6 was involved in maintaining the terminally differentiated state and possibly in the fusion of satellite cells during hypertrophic growth.

1.4.1.2 MRF ontogeny and tissue specificity

In vertebrates, myf-5, myogenin, myf-6 (also termed MRF4 or herculin) and myoD, are initially expressed in that order (see reviews Olson, 1990; Weintraub *et al.*, 1991; Buckingham, 1992; Sabourin and Rudnicki, 2000). However, levels of myogenin and myf-6 decline, with myf-5 and myoD being most abundant early in myogenesis, while myogenin and myf-6 expression levels increase as myogenesis proceeds. Myf-6 mRNA expression peaks in the post-natal animal.

Myogenin mRNA abundance peaks at day 85 in fetal sheep (Fahey *et al.*, 2003) and day 120 in fetal cattle (Oldham *et al.*, 2001), declining thereafter. Similarly, myoD expression peaks between day 70 and 120 in normal bovine fetuses, declining thereafter (Oldham *et al.*, 2001). The presence of myogenin in young fetuses is likely to

correspond with the differentiation of the smaller population of primary myofibres, increasing with the later differentiation of the larger population of secondary myofibres, then decreasing to a minimum when myofibre differentiation is completed (Picard *et al.*, 1995).

1.4.1.3 Nutritional and hormonal regulation of the MRFs

There is little information describing the direct relationship between nutrition and the MRFs, particularly during myogenesis. Only Jeanplong *et al.* (2003) provides some evidence for an interaction between nutrient intake and MRF mRNA expression by underfeeding and re-feeding eight month old growing sheep. Myf-5, myoD and myogenin all decline significantly in immediate response to underfeeding, myf-5 and myoD tend to increase thereafter. Similarly, a sharp increase in the three factors is evident when sheep are re-fed, indicating a possible involvement in regulating satellite cell activity.

The roles of several specific nutrients on MRF regulation have also been investigated, *in vitro*. Retinoic acid (vitamin A) reduced myf-5 gene transcription in a dose-dependent manner (Carnac *et al.*, 1993) and induced myoD and myogenin *in vitro* (Arnold *et al.*, 1992; Alric *et al.*, 1998), suggesting it may play a role in differentiation of myoblasts. To date no investigations into the role of maternal vitamin A levels on myogenesis *in vivo* have been reported. However, it is known that Vitamin A is critical for normal organogenesis during fetal development (Antipatis *et al.*, 2000). Vitamin A deficiency is a common ailment among pregnant women in developing countries during the last trimester of pregnancy; while over supplementation of Vitamin A during early pregnancy can impair fetal development (World Health Organization, 2003). Similarly, ascorbic acid 2-phosphate (Vitamin C) increased myoblast myogenin expression *in vitro* and promoted muscle differentiation (Mitsumonto *et al.*, 1994), while I-mfa (inhibitor of the MRFs) expression was increased in the presence of Vitamin D3 in osteoblasts (Tsuji *et al.*, 2001). The use of Clenbuterol, a β-agonist known to reduce muscle wasting atrophy in the hind-limbs of rats, was associated with a 360% increase in myogenin expression compared to levels in immobilized hind-limbs of rats not fed Clenbuterol; treatment with Clenbuterol did not alter myoD levels (Delday and Maltin, 1997).

Hormones and growth factors, which are nutritionally regulated, have been reported to interact with the MRFs. Manipulation of the maternal-fetal hormonal/ growth factor axis

by maternal nutrition (or other *in utero* interventions) may indirectly affect MRF expression. Unlike other hormones, IGFs stimulate rather than inhibit myogenic differentiation. This is believed to occur through the induction of myogenin mRNA (Florini *et al.*, 1991b) expression via the IGF-I receptor, IGF-II receptor and IGFBPs (reviewed by Florini *et al.*, 1991a). Treatment of cells *in vitro* with IGF-I results in large increases in myogenin mRNA concentrations (Florini *et al.*, 1991b). Mangiacapra *et al.* (1992) found that IGF-I induced differentiation resulted in progressively decreased myf-5 expression. Thyroid hormones may also be involved in the control of myogenesis by regulating expression of the MRFs. Carnac *et al.* (1992) reported that T_3 increased myoD expression and expedited terminal differentiation, possibly by inducing earlier expression of myogenin (Downes *et al.*, 1993). Therefore, the effect of maternal nutrition on the abundance of circulating factors, namely the IGFs and thyroid hormones, may indirectly regulate the expression of the MRFs.

1.4.1.4 Muscle fibre type and the MRFs

Voytik *et al.* (1993) reported the accumulation of myoD and myogenin mRNAs in fast and slow muscles respectively in normal mice. However, myoD knockout mice did not demonstrate drastic differences in fast muscle phenotype (Rudnicki *et al.*, 1992). Others (Hughes *et al.*, 1993) have witnessed a parallel change in myoD and myogenin expression, when muscle fibre type changes were induced. For example, activation of fast myosin heavy chain in slow muscles (Soleus) by treatment with T_3 and β-agonist was associated with accumulation of myoD. Similarly, over-expression of myogenin induced a transition from glycolytic to oxidative enzymatic properties, although no change in myosin isoforms was reported (Hughes *et al.*, 1999). In contrast, Christensen *et al.* (2000) determined that myf-3 (homologue of myoD) expression was greater in slow twitch fibres of pigs, while there was no association between myogenin content and fibre type.

1.4.1.5 Role of the MRFs in animal production

The main effect of the MRFs on post-natal muscle growth appears to be via their role in regulating myogenesis during the embryonic and fetal periods and, therefore, establishing potential muscle growth. te Pas *et al.* (1999b) found no evidence of an association between the myf-5 gene locus and variation in meat production traits, in pigs. In contrast, Li *et al.* (2004) reported that a single nucleotide polymorphism on intron 2 of the myf-5 gene was significantly associated with average daily weight gain from birth to weaning and weight gain on feed in cattle. te Pas *et al.* (1999a) estimated

the contribution of the myogenin locus, in pigs, to the total phenotypic variance of traits measured, including birth weight (4%), carcass weight (4%), growth rate (4%) and lean weight (5.8%). Coutinho *et al.* (1993), using a hyperplastic quail line selected for heavy body weight, demonstrated that there is a delay in activation of myogenic factors in the hyperplastic line, producing a delay in the start of myoblast differentiation. It can be assumed that this would result in prolonged proliferation of myoblasts, resulting in an increased myofibre number.

te Pas *et al.* (2000) depicts the importance of the myoD genes to lifetime performance, including post-natal growth. In lines of pigs, selected for fast growth (F-line) or against backfat thickness (L-line), they found mRNA expression of myf-5, myoD and myogenin to be generally greater in the F-line than the L-line in some muscles. Conversely, myf-6 expression tended to be higher in the L-line. They also found a negative relationship between myogenin and backfat in the F-line. The fast growth rate exhibited by the F-line probably results from increased satellite cell activity caused by an altered pattern of myf-5, myoD and myogenin expression. MyoD has been used as a marker for detecting actively proliferating satellite cells in fetal and neonatal sheep (McCoard *et al.*, 2001) and in regenerating rat hind-limb muscles (Koishi *et al.*, 1995). te Pas *et al.* (2000) suggested that the increased myf-6 witnessed in the lean line is related to myf-6's function in maintaining the muscle fibres. The higher lean mass exhibited by the L-line may require more myf-6 to maintain the fused state of muscle fibres.

1.4.2 Myostatin

One of the most significant recent findings related to the regulation of muscle development and growth was the discovery of a member of the TGF-β family, myostatin or Growth Differentiating Factor-8 (GDF-8). McPherron *et al.* (1997) generated myostatin null mice, which displayed 30% greater body weights than normal wild type counter-parts. The increased body weight was due to increased musculature. Knockout mice exhibited dramatically (200–300%) increased skeletal muscle mass compared to wild type mice and decreased adiposity but no differences in other tissues (McPherron *et al.*, 1997). The increase in muscle mass of myostatin knockout mice is thought to result from both muscular hyperplasia and hypertrophy (McPherron *et al.*, 1997). The 25-30% increase in muscle mass displayed by another line of mice that expressed a dominant negative myostatin (dnMS) transgene was almost solely from hypertrophy (Zhu *et al.*, 2000). These mice display 23-40% less processed myostatin than normal. The difference in muscle mass between dnMS and null mice may result

from incomplete dominance of dnMS so that dimerization and cleavage of normal myostatin is not fully blocked in dnMS mice (Zhu *et al.*, 2000). This may suggest differential roles for myostatin in regulating hyperplastic and hypertrophic muscle growth. The myostatin gene is highly conserved across species, including cattle, sheep, poultry and pigs (McPherron and Lee, 1997). Mutations are present in the myostatin gene of several European breeds of cattle that display the double-muscled (DM) phenotype (e.g. Belgian Blue, Peidmontese; Kambadur, *et al.*, 1997; McPherron and Lee, 1997). Six disruptive mutations, which result in inactive myostatin, have been found in cattle (Karim *et al.*, 2000).

Evidence of the inhibitory role of myostatin on muscle development has been further demonstrated, with increased myostatin expression observed in the following instances:

- Chronic muscle wasting in HIV-infected men (Gonzalez-Cadavid *et al.*, 1998).
- Loss of muscle of rats exposed to a micro-gravity environment (Lalani *et al.*, 2000), resulting in an average decrease of approximately 21% in muscle mass; a 200-500% increase in myostatin mRNA expression was measured, while myostatin protein also increased.
- Muscle atrophy associated with hind limb unloading for 10 days in rats (Wehling *et al.*, 2000), resulting in a 16% decrease in plantaris mass; a 110% increase in myostatin mRNA expression and a 37% increase in myostatin protein were observed. Interestingly, McMahon *et al.* (2003a) found that loss of muscle mass in response to hind limb unloading was greater in myostatin knockout mice compared with wild type controls.
- Low birth weight runt piglets, 40% the birth weight of normal littermates; synthesize significantly higher levels of myostatin mRNA than normal birth weight piglets (Ji *et al.*, 1998).

A possible role in muscle cell regeneration has also been proposed. Kirk *et al.* (2000) and Zhu *et al.* (2000) reported decreased myostatin expression in regenerating myotubes and in regenerating skeletal muscle from muscular dystrophy mice, respectively. Conversely, Sharma *et al.* (1999) reported that myostatin levels were increased, and maintained at high levels, in cardiomycytes surrounding an area of infarction in sheep heart tissue.

1.4.2.1 Structure and activity of myostatin

The structure and activity of myostatin has been determined by investigating the known properties of other TGF-β family members. Myostatin is a secreted protein synthesised by muscle cells. Unprocessed myostatin (pre-cursor) consists of a N-terminal propeptide (latency associated peptide; LAP) bound to a C-terminal domain. Upon processing, the C-terminal domain is released as the active mature form of (processed) myostatin. The majority of myostatin circulates in a latent complex with its propeptide (Zimmers *et al.*, 2002) that, upon acidification, is dissociated to release mature myostatin (Zimmers *et al.*, 2002), typical of TGF-β (reviewed by Massaugué, 1990).

Thomas *et al.* (2000a, b) demonstrated that myostatin exerts an inhibitory affect on myoblast proliferation, specifically the G1 to S and the G2 to M phases of the cell cycle, in a dose dependent manner. The subsequent removal of myostatin results in cell proliferation continuing at rates similar to those witnessed before myostatin addition (Thomas *et al.*, 2000a, b). It is therefore likely that a mutation in the myostatin gene, as displayed by DM cattle, inactivates these inhibitory effects on cell proliferation. This results in prolonged myoblast proliferation and an increased number of muscle cells undergoing terminal differentiation, which ultimately produces an increased number of muscle fibres of a normal size. Thomas *et al.* (2000a, b) propose that this occurs via interactions with other genes during the myogenic cascade. Specifically, myostatin up-regulates p21, inactivating Rb (retinoblastoma) by down-regulating Cdk2 (cyclin dependent kinase), and arresting myoblasts in the G1 phase of the cell cycle where they undergo differentiation. While Spiller *et al.* (2002) reported that myoD might be responsible for the myostatin up-regulation in muscle cells in culture.

Myostatin will also inhibit differentiation of myoblasts in culture in a concentration dependent manner (Ríos *et al.*, 2002), possibly by down regulating the expression of myoD (Langley *et al.*, 2002) and myogenin (Joulia *et al.*, 2003).

Recently, Lee and McPherron (2001) reported the roles of the propeptide, activin type II receptors and follistatin in the regulation of myostatin signalling in mice. Activin type II receptors play a role in the initial signalling of TGF-βs, while the propeptide and follistatin are both known to inhibit TGF-β activity. Dominant negative activin type II receptor mice display 50 to 125% greater muscle weights than normal mice. Additionally, over-expression of a follistatin transgene resulted in large increases in muscle weights; one individual exhibited muscle weights 194-327% relative to normal

wild type controls (Lee and McPherron, 2001). The increased muscle weights witnessed from dominant negative activin type II receptor or over-expression of the propeptide and follistatin were the result of both increased hyperplasia and hypertrophy (Lee and McPherron, 2001). Further, follistatin knockout mice generated by Matzuk *et al.* (1995), have lower muscle mass at birth than normal mice. The increase in musculature demonstrated in the follistatin transgenic mice is greater than that seen in the myostatin knockout mice (McPherron and Lee, 1997) and suggests that other negative regulators of muscle development exist (Lee and McPherron, 2001). The *in vivo* action of the propeptide and follistatin (or a follistatin-related gene, FLRG) in the circulation of normal humans and mice was confirmed by Hill *et al.* (2002). It was determined that the propeptide and FLRG bind to, and inhibit the actions of, circulating myostatin *in vivo*. Further, it was estimated that 70% of serum myostatin is bound to the propeptide (Hill *et al.*, 2002).

1.4.2.2 Myostatin ontogeny and tissue specificity

Myostatin has been detected in the myotome compartment of the somites and in most muscles throughout development (McPherron *et al.*, 1997). Ji *et al.* (1998) detected myostatin mRNA in pig fetuses aged 21 and 35 days, with the level doubling by day 49 (gestation 114 days). By birth, levels had declined, reaching an absolute minimum at 2 weeks post-natal and then generally increasing with increased live weight, although pre-natal levels were not reached. Kambadur *et al.* (1997) reported a similar pattern of developmental expression in cattle. In poultry, myostatin expression is detected at all stages of embryonic development (Kocamis *et al.*, 1999), with peak expression on days 0-1, with levels declining 80% between days 2-6, and then increasing 3-fold for the remainder of embryonic development (days 7-20). Examination of the ontogeny of myostatin mRNA reveals some concurrence with the development of muscle fibres in the porcine and bovine fetuses. The highest level of expression, (day 49 to 105 in pigs and day 90 in cattle), coincides with the time of high incorporation of cells into muscle fibres (i.e. secondary fibre formation). The decline in myostatin mRNA expression to its lowest level in the neonatal animal would correspond with a time of increased muscle cell activity (i.e. rapid incorporation of satellite cells into muscle fibres during the late pre-natal and early post-natal periods). The gradual increase of myostatin mRNA expression with live weight post-natally may be related to satellite cell activity. Rates of satellite cell activation, proliferation and incorporation decline with animal maturity, corresponding to increased myostatin expression. McCroskery *et al.* (2003) demonstrated muscles from myostatin null mice had increased satellite cell number per

unit length of muscle fibre and an increased proportion of active satellite cells which undergo faster proliferation, which were associated with prolonged expression of myoD and delayed expression of myogenin.

Initial studies detected myostatin expression predominantly in fetal and adult muscle (McPherron *et al.*, 1997). More recently, expression has been detected in several other tissues and organs. Ji *et al.* (1998) detected myostatin expression in the tubuloalveolar secretory lobules of mammary tissue from lactating pigs. Sharma *et al.* (1999) demonstrated the presence of myostatin in fetal and adult heart from sheep and cattle. McPherron *et al.* (1997) detected low levels of myostatin in adipose tissue and reported decreased adiposity of myostatin null mice (McPherron and Lee, 2002). In support of this observation, Kim *et al.* (2001) showed that treatment of 3T3-L1 pre-adipocytes in culture with myostatin inhibited pre-adipocyte differentiation. A decrease in CCAAT/ enhancer binding protein-α, peroxisome proliferator-activated receptor-γ expression and decreased glycerol-3-phosphate dehydrogenase activity (involved in fatty acid synthesis) was associated with the inhibition of pre-adipocyte differentiation (Kim *et al.*, 2001).

1.4.2.3 Nutritional and hormonal regulation of Myostatin

There appears to be a dearth of information regarding the interactions between nutrition and myostatin expression in the regulation of muscle growth in livestock. Ji *et al.* (1998) found no relationship between myostatin expression and food intake (*ad libitum* versus deprived, over 3 days), growth hormone administration (over 9 days) or dietary oil content (over 16 days) in piglets. Similarly, Carlson *et al.* (1999) found no effect of short-term feed intake on skeletal muscle myostatin mRNA in rats. Interestingly, short term fasting elevated myostatin mRNA in tilapia larvae, while prolonged fasting reduced myostatin mRNA; in adult tilapia myostatin mRNA was unaffected regardless of the severity of the fast, suggesting myostatin may be differentially affected by nutrition depending on the stage of development (Rodgers *et al.*, 2003). While in chickens, underfeeding reduced myostatin expression of both hatchlings and adult broilers (Guernec *et al.*, 2004). These findings may further support myostatin acting on muscle metabolism in response to long term rather than acute influences. However, fasting of trout for a long period (10 weeks) resulted in no change in myostatin mRNA (Chauvigné *et al.*, 2003). Somewhat in contrast, Jeanplong *et al.* (2003), using Northern blots, detected a short-term decrease in myostatin mRNA in the semitendinosus muscle of eight month old ewes, after one week of feed restriction

(30% maintenance requirements). After four and twenty-two weeks of underfeeding, transcript levels were similar to and lower than controls, respectively. Subsequent *ad libitum* refeeding of these animals saw myostatin mRNA levels increase rapidly. Changes in myostatin mRNA were associated with differential responses in processed myostatin, LAP and precursor proteins, suggesting altered translational regulation. Processed myostatin initially decreased over the first week of underfeeding and then gradually increased. In contrast, the precursor and LAP increased over the first four weeks and then decreased. After one week of re-feeding, there was a fall in precursor, while LAP and mature myostatin increased (Jeanplong *et al.*, 2003).

A study investigating the relationship between myostatin and complementary growth in pigs (Ji *et al.*, 1999), by altering feed access, found that serum myostatin protein content was lower in pigs consuming *ad libitum* over the last ten days of feeding (i.e. compensatory and unrestricted weight gain groups). In contrast, myostatin mRNA tended to be lower in pigs that had intake restricted for all, or part of, the feeding period.

1.4.2.4 Muscle fibre type and Myostatin

Spatial differences in myostatin expression between muscles have been detected (Kambadur *et al.*, 1997; McPherron *et al.*, 1997; Jeanplong *et al.*, 2001). Mott and Irvine (2002) reported greater myostatin expression in pectoralis (breast) compared with quadriceps (thigh) muscles in poultry. Expression of myostatin mRNA is similar in sexually dimorphic muscles of sheep (Mateescu and Thonney, 2002) and mice (McMahon *et al.*, 2003b), however, myostatin protein was 40-60% higher in female mice (McMahon *et al.*, 2003b) suggesting differences in myostatin translation between males and females.

As mentioned, DM cattle have an increased number of muscle fibres compared with non-DM cattle. Wegner *et al.* (2000) reported no difference in type I fibres between DM and other breeds and determined that the increase in muscle fibre number witnessed in DM animals was due to increases in type IIb and IIa fibres. This suggests that the increased muscle fibre number is derived from increased number of type II fibres. Given that early primary muscle fibre formation results solely in type I fibre production, the period of secondary fibre formation may be the critical time for the increase in muscle fibre number displayed in DM animals. In support of this, Gagnière *et al.* (1997)

and Deveaux *et al.* (2001) reported delayed differentiation of oxidative metabolism and a lower proportion of slow MHC in DM fetuses, respectively.

A strong relationship exists between myosin heavy chain II b isoform, large cross-sectional areas, and myostatin expression in several hind-limb muscles of mice (Carlson *et al.*, 1999), suggesting an association between muscle fibre type and myostatin expression (i.e. myostatin expression is higher in white fast twitch muscle fibres). Ji *et al.* (1999) supported this observation when they found the deep red portion of the semitendinosus muscle of pigs had less myostatin mRNA than superficial white semitendinosus.

1.4.2.5 Myostatin and animal production

To date most myostatin-related work in livestock has been based on the double-muscled (DM) phenotype in cattle. The myostatin gene has been mapped to the *mh* (muscular hypertrophy) locus in DM cattle (eg Belgian Blue and Piedmontese breeds) (Smith *et al.*, 1997), on bovine chromosome 2 (Charlier *et al.*, 1995). Jeanplong *et al.* (2001) recently published the genomic organization of the bovine myostatin gene. These breeds display enlarged muscles (20-25%) and leaner carcases (see review by Arthur, 1995). Wegner *et al.* (2000) reported these breeds having double the number of muscle fibres of other breeds. Myostatin mRNA expression is higher in the skeletal muscle of DM animals (Oldham *et al.*, 2001; Berry *et al.*, 2002), but there is less processed myostatin protein (Berry *et al.*, 2002).

The DM condition in cattle, unlike mice, is more likely the result of hyperplasia, rather than hypertrophy. DM fetuses exhibit increased muscle fibre number during fetal development (Swatland and Keiffer, 1974) and at birth (Ashmore *et al.*, 1974). Picard *et al.* (1995) concluded this might be due to slower myofibre differentiation in DM fetuses, similarly myoblasts of high muscle growth capacity fetuses also underwent elevated proliferation and delayed differentiation (Duris *et al.*, 1999). This supports the reported role myostatin plays in muscle development during the embryonic and fetal periods.

One interesting aspect of the involvement of myostatin in animal production is that, despite high conservation between species, a naturally occurring mutation in the myostatin protein has only appeared in cattle. While the DM phenotype in cattle has been intensively selected for, intensive selection for growth rates in different poultry lines (e.g. layers vs. broilers) has not altered myostatin gene structure, or affected

message or protein abundance (Mott and Ivarie, 2002). Similarly, myostatin mRNA in lines of chicken selected for increased breast muscle yield was not different to controls (Guernec *et al.*, 2003). Further, different breeds of cattle with identifiable mutations in the myostatin protein display different muscling phenotypes dependent on the type of mutation that they possess (e.g. Belgian blue vs. Piedmontese).

Understanding the regulation of myostatin mRNA and protein expression may have significant implications for the efficient production of lean meat from livestock. It appears that substances may exist which block the negative actions of myostatin on muscle growth (Lee and McPherron, 2001; Hill *et al.*, 2002) and these could provide enormous benefit to animal production systems.

1.4.3 Insulin-like Growth Factors (IGFs)

The relationships between the IGF axis and development and growth are well established (for reviews see McGuire *et al.*, 1992; Hossner *et al.*, 1997). The ability of IGFs to promote cellular proliferation and differentiation makes them of particular interest during the pre-natal period, when organogenesis occurs. The IGFs act in endocrine, paracrine and autocrine fashions on many tissues and cell types within the body. Their expression is clearly determined by stage of development, as are the associated receptors and binding proteins. IGF receptors and binding proteins play a significant role in restricting or facilitating the actions of IGF ligands. It is beyond the scope of this review to discuss all aspects of the IGF axis, so all that shall be presented here is information that is directly related to the current understanding of the role of the IGF axis in muscle development and growth.

1.4.3.1 Structure and activity of IGFs

The IGFs (also known as somatomedins) are a class of peptides similar in structure to insulin. They have been defined as polypeptides that are structurally homologous to insulin, and exert insulin-like biological effects in the presence of excess anti-insulin antibodies (Zapf *et al.*, 1984). The primary variants are IGF-I (70 amino acids, 7649 daltons, 43% structural homology with insulin) and IGF-II (67 amino acids, 7471 daltons, 41% structural homology with insulin) (Rinderknecht and Humbel, 1978a,b) although other variants have been isolated that result from alternative exon splicing (Zumstein *et al.*, 1985; Gowan *et al.*, 1987). There is high amino acid identity in the IGF sequence between all mammals (Hossner *et al.*, 1997).

A growing body of evidence suggests that the IGFs may play a vital role in the development of muscle during the early embryonic period and somitogenesis. Pre-implantation embryo manipulations have resulted in altered skeletal muscle IGF-II mRNA expression in early age bovine fetuses (Blondin *et al.*, 2000). Endogenous IGF-II levels are associated with increased mesoderm formation, addition of IGF-II to cells in culture increased the expression of mesoderm markers, while the absence of IGF-II in mouse embryonic cell lines inhibited mesoderm development (Morali *et al.*, 2000). Over-expression of IGF-I in the limb of chicken embryos resulted in an increased myoblast population available for differentiation into myofibres (Mitchell *et al.*, 2002).

The anabolic effects of IGFs on skeletal muscle are widely reported (Florini, 1987; Florini *et al.*, 1996). In brief, IGFs stimulate cell proliferation, differentiation, amino acid and glucose uptake and inhibit protein degradation (Ballard and Francis, 1983). IGF-I and IGF-II are known to stimulate proliferation of L6 myoblasts *in vitro* (Ewton *et al.*, 1987), while over-expression of IGF-I in C_2C_{12} cells (Coleman *et al.*, 1995) and IGF-II in mouse embryonic stem cells (Prelle *et al.*, 2000) promotes differentiation. All that shall be alluded to here is that cells express a temporal biphasic response to IGF-I, where stimulation of proliferation occurs for 24-36 hrs followed by stimulated differentiation (Florini *et al.*, 1996). However, IGFs will stimulate differentiation in the absence of proliferation (Florini *et al.*, 1996).

Myogenic differentiation is probably stimulated through induction of myogenin mediated via IGF-I receptor, IGF-II receptor and IGFBPs (reviewed by Florini *et al.*, 1991a). Inactivation of IGF-I receptor has been shown to delay myoblast differentiation; associated with delayed myogenin expression and prolonged Rb phosphorylation (Cheng *et al.*, 2000). Treatment of cells with IGF-I results in large increases in myogenin mRNA concentrations (Florini *et al.*, 1991b) and progressive decreases in myf-5 expression (Mangiacapra *et al.*, 1992).

1.4.3.2 IGF ontogeny and tissue specificity

IGF-I and -II mRNA has been detected in many tissues, including fetal skeletal muscle (Kind *et al.*, 1995). IGF synthesised at non-hepatic sites is believed to act on the producing cells (autocrine action) or on adjacent cells (paracrine action). IGFs are thought to be involved in all stages of development, while expression of the various components that make up the IGF system will depend on the stage of development.

Expression of IGF-I, IGF-II and the type-I receptor has been detected from the one-cell to blastocyst stage in sheep embryos (Hossner *et al.*, 1997).

Gerrard *et al.* (1998) detected increasing IGF-I mRNA expression throughout fetal development in skeletal muscle of pigs, which peaked shortly after birth. While Fahey *et al.* (2003) reported a gradual increase in skeletal muscle IGF-I mRNA in fetal sheep from day 40, peaking at day 100 and declining thereafter, which is in concurrence with the late gestation decrease reported by Dickson *et al.* (1991).

O'Mahony *et al.* (1991) and Fahey *et al.* (2003) detected a decline in IGF-II mRNA expression from day 80 and 85, respectively in fetal sheep muscle. A similar pattern of expression was detected in porcine fetuses, peaking at day 59 (Gerrard *et al.*, 1998). The peak in IGF-II expression may correspond with increased myogenin and a subsequent increase in myoblast differentiation. This appears reasonable given that day 59 of gestation in pigs would be consistent with the commencement of maximal fetal muscle fibre formation, approximately day 60 (Ashmore *et al.*, 1973). Gerrard and Grant (1994) demonstrated that muscle IGF-II mRNA was greater at a later stage of gestation in DM versus normal fetuses; Listrat *et al.* (1999) reported that this was entirely due to prolonged elevation of the 5.1kb transcript in DM fetuses. However, a more recent report by Bass *et al.* (1999) described no delay in IGF-II expression between DM and normal animals. In mice, myostatin gene knockouts had more IGF-II mRNA in the soleus muscle compared with controls, while no difference in IGF-I expression was detected (Kocamis *et al.*, 2002). Given that IGF-II is proposed to be involved in terminal myogenic differentiation (Florini *et al.*, 1991c), it is appealing to imply that delayed or increased expression of IGF-II may play a major role in determining muscle mass.

1.4.3.3 Nutritional and hormonal regulation of IGFs

It is evident from the literature that nutrition is a key regulator of circulating IGFs, however in most cases it appears to be a reversible interaction. The response of skeletal muscle IGF mRNA production to nutrition is less defined, particularly during the fetal period.

Maternal under-nutrition between 28-80 days of gestation in sheep resulted in lower fetal hepatic IGF-I, higher IGF-II and no change in levels of GHR mRNA (Brameld *et al.*, 2000). Re-feeding nutrient restricted ewes from day 80 resulted in increased fetal

hepatic IGF-I, IGF-II and GHR mRNA at day 140 compared with control fetuses. Maternal under-nutrition had no affect on fetal skeletal muscle IGF-I and GHR mRNA. IGF-II was higher in restricted fetuses, but lower in restricted fetuses after re-feeding (Brameld *et al.*, 2000). In contrast, Kind *et al.* (1995) found that fetal skeletal muscle IGF-I mRNA was decreased in response to placental restriction in sheep and this was correlated with circulating IGF-I.

Postnatal ovine skeletal muscle IGF-I mRNA has been shown to be unresponsive to short-term fasting (24 – 48h) (Oldham *et al.*, 1996). Further, Loughna *et al.* (1992) and Tomita *et al.* (2001) demonstrated no change in muscle IGF-I mRNA of underfed rats, despite reduced levels of circulating IGF-I. Tomita *et al.* (2001) also reported increased IGF-I receptor mRNA in the skeletal muscle of underfed rats at 6 and 16 months of age. In contrast, ovine IGF-I mRNA was decreased in response to a long-term nutrient restriction and was elevated with re-feeding in sheep (Jeanplong *et al.*, 2003).

IGF-I mRNA in skeletal muscle has been demonstrated to respond to other hormones. Administration of growth hormone to rats results in an increase in IGF-I mRNA (Isgaard *et al.*, 1989). Thyroidectomy of fetal sheep results in reduced skeletal muscle mass that is associated with decreased IGF-I mRNA but not IGF-II expression (Forhead *et al.*, 2002). IGF-I mRNA was further reduced in response to cortisol infusions (Forhead *et al.*, 2002). The decrease in IGF-I mRNA is likely to be the result of a T_4 deficiency rather than T_3, as infusion of T_3 alone did not alter expression (Forhead *et al.*, 2002). In contrast, insulin increased muscle protein synthesis independent of any change in IGF-I mRNA in rats (Loughna *et al.*, 1992).

1.4.3.4 Muscle fibre type and IGFs

There appears to be little in the literature regarding the expression of IGFs in different muscles or associations with muscle fibre type.

1.4.3.5 IGFs and animal production

The association of circulating IGFs with growth in some domestic species has generated interest in their use as possible markers for selection. The use of IGF-I as a potential marker for production traits in sheep is varied (Medrano and Bradford, 1991; Speck, 1991). In contrast, IGF-I has been associated with growth rate, voluntary feed intake and gain to feed ratio in weaner pigs (Owens *et al.*, 1999; Hermesch *et al.*, 2001) and is commercially available as a test to aid in selection of animals with

superior growth potential (Primegro IGF-I™ overview). In their studies with cattle, Bishop *et al.* (1989) and Davis *et al.* (1995) established that IGF-I levels provided suitable indicators of feed conversion efficiency and weaning weight, respectively. Further, circulating IGF-I levels in cattle were estimated to have a heritability of 32% (Johnston *et al.*, 2001), suggesting potential use in cattle selection programs. Plasma IGF-II was positively associated with backfat thickness in pigs (Owens *et al.*, 1999) and sheep (Gatford *et al.*, 1996). In general, while circulating IGF levels are associated with growth, feed conversion and possibly carcass characteristics, their use as indicators is unreliable due to high variability with other factors such as nutrition, sex, stage of development and physiological state.

1.5 Summary

The current literature provides an insight into the current understanding of the genetic control of pre-natal muscle development in domestic species. This process and the ontogeny of several genes, described throughout this literature review, during the myogenic cascade is summarised in Figure 1.5. This review indicates that the regulation of several key myogenic genes may alter muscle development and therefore impact on meat yields, however, deficiencies in our understanding of how muscle development is affected by an altered *in utero* environment have been revealed. There is very little information regarding the expression of genes involved in myogenesis in sheep and whether they are differentially regulated by nutrition and muscle type, in any species, and if altered gene expression is related to perturbations in muscle cell development that may occur when pre-natal development is compromised.

The subsequent studies are based on the premise that much of the potential for post-natal muscle development is manifested before birth. Variations in the pre-natal environment may interact with, or provide cues for, gene expression that may contribute to the phenotypic variation in muscle development and growth. It is expected that nutritional manipulation of the pre-natal environment will alter fetal development and may influence myogenesis and gene expression. Determining the consequences of different maternal nutritional treatments on the nutrition-gene regulation of muscle development of progeny may ultimately contribute to our understanding of muscle developmental biology and open up opportunities for further investigation.

Therefore, the specific aims of this work were to:

- Develop a model for investigating the effect of long-term maternal feed intake on myogenesis in sheep (Chapter 3).
- Investigate the effect a long-term pre- and post mating divergence in maternal live weight and condition score has on the cellular development of fetal muscle (Chapter 4).
- Investigate the effect a long-term pre- and post mating divergence in maternal live weight and condition score has on the gene regulation of fetal muscle development (Chapter 5).
- Investigate the effect of short-term peri-conceptional maternal feed intake on the cellular development of fetal muscle at mid-pregnancy (Chapter 6).

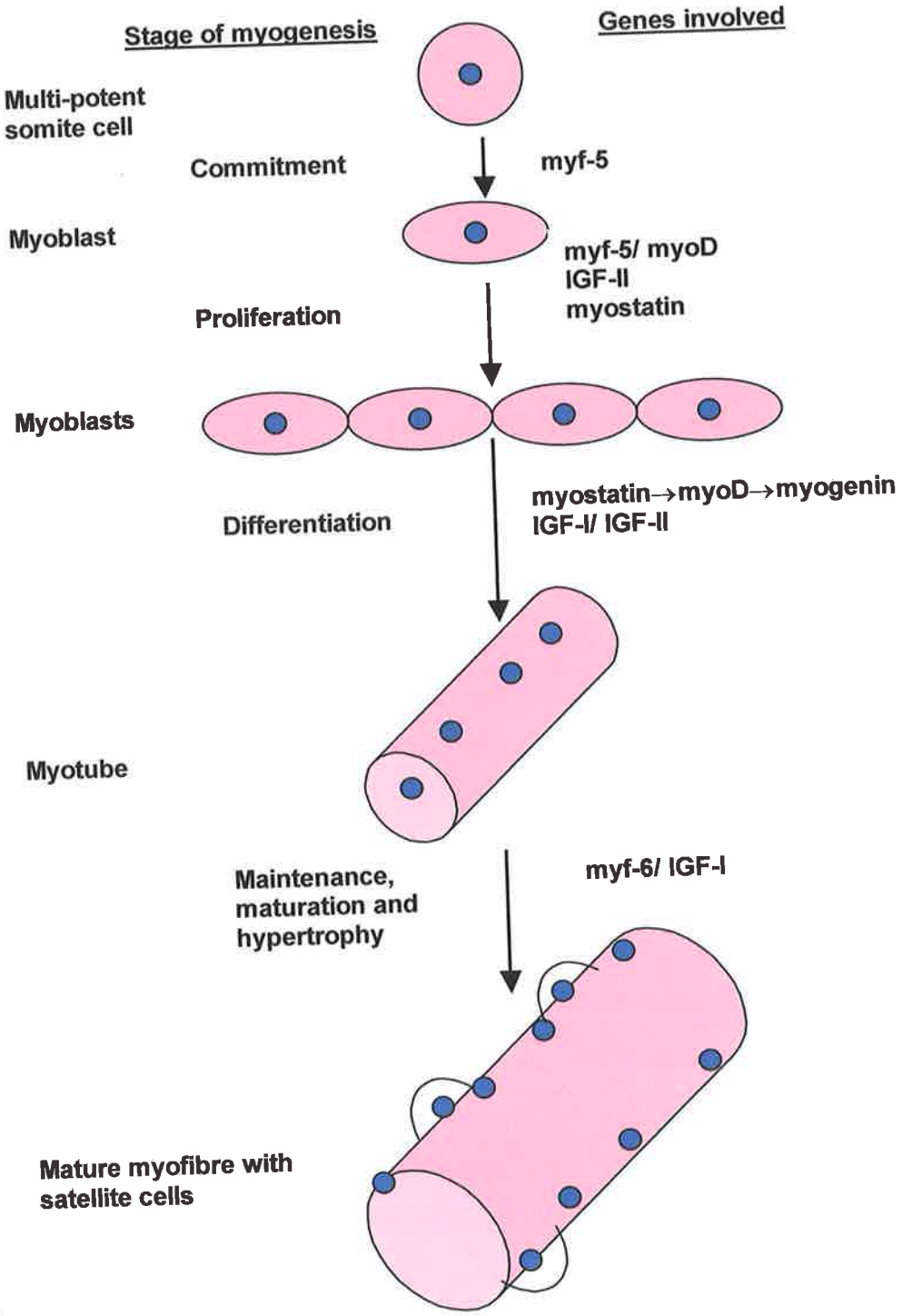


Figure 1.5: The myogenic pathway with gene interactions.

CHAPTER 2

2 MATERIALS AND METHODS

All chemicals and reagents used in the methods described in this thesis were of analytical grade or the highest purity available. The sources of all materials used in the methods are described appropriately throughout the text.

2.1 Plasma metabolite and hormone analysis

2.1.1 Blood collection and preparation

Blood samples (10ml) were taken by jugular venipuncture of pregnant ewes immediately prior to slaughter (described in chapter 3), collected in cooled Lithium Heparin tubes (125 I.U, Disposable Products; Australia) and kept on ice.

Fetal blood was collected from the umbilical artery of individual fetuses at each stage of pregnancy. The umbilical cord was severed and blood collected into 1.5ml Eppendorf (Treff Lab; Switzerland) tubes (day 50) or by needle and syringe into 10ml Lithium Heparin tubes (day 92 and 133) and kept on ice.

Blood samples collected in Lithium Heparin were centrifuged at 2000*g* for 15 min, as soon as possible after collection, and plasma removed on ice. Plasma samples were stored in duplicate aliquots (~1.5ml) at -20°C for later analysis. Blood samples from day 50 fetuses were clotted overnight at 4°C and centrifuged at 1500*g* for 10min and serum was removed on ice and stored at -20°C.

2.1.2 Plasma metabolites and IGF assays

All samples were analysed in duplicate for each assay. Appropriate standards and quality controls were analysed in triplicate. Maternal metabolites were determined on plasma from all pregnant ewes slaughtered on days 50, 92 and 133 of gestation. Fetal metabolite assays were determined on plasma from all fetuses obtained at days 92 and 133 of gestation. Due to the sample volume required and the cost of the assays, the concentration of IGF-I and -II were determined on a subset of fetal plasma samples only (n=19 day 92 and n=19 day 133 of gestation, described in detail in section 3.2.3.7).

2.1.2.1 Plasma Glucose

Maternal and fetal plasma glucose concentrations were determined by measuring the absorbance of the enzymatic production of NADH by spectrophotometry, at 340nm, using the GLUC HK kit (Roche Diagnostic Systems; Germany) on the COBAS MIRA automated analysis system (Roche Diagnostic Systems).

2.1.2.2 Plasma Albumin

Maternal and fetal plasma albumin concentrations were determined by measuring the absorbance of the formation of the albumin-BCG complex by spectrophotometry, at 500nm, using the Albumin Plus (BCG) assay kit (Roche Diagnostic Systems) on the COBAS MIRA automated analysis system.

2.1.2.3 Plasma Urea

Maternal and fetal plasma urea concentrations were determined by measuring the absorbance of the change in NADH concentration by spectrophotometry, at 340nm, using the Urea/ BUN assay kit (Roche Diagnostic Systems) on the COBAS MIRA automated analysis system.

2.1.2.4 Plasma Non-esterified Fatty Acid (NEFA)

Maternal plasma NEFA concentration was determined by an enzymatic colorimetric assay (NEFA-C kit, WAKO Chemicals; Japan), based on the sequential reaction of NEFA with Acyl CoA synthetase and Acyl CoA oxidase, and by measuring the absorbance by spectrophotometry, at 550nm on the COBAS MIRA automated analysis system.

2.1.2.5 Plasma Cholesterol

Maternal plasma cholesterol concentration was determined by measuring the formation of cholestenone from cholesterol ester and measuring the absorbance of peroxidase reactivity by spectrophotometry, at 500nm, using the Cholesterol CHOD-PAP assay kit (Roche Diagnostic Systems) on the COBAS MIRA automated analysis system.

2.1.2.6 Plasma Triglycerides

Maternal plasma triglyceride concentration was determined by measuring the formation of dihydroxyacetone phosphate from glycerol and measuring the absorbance of peroxidase reactivity by spectrophotometry, at 500nm, using the Triglycerides assay kit (Roche Diagnostic Systems) on the COBAS MIRA automated analysis system.

2.1.2.7 Plasma IGF-I and IGF-II

Measurement of IGF-I was conducted by PrimeGro^{LTD} (South Australia). Prior to specific enzyme linked immunosorbent assay (ELISA), plasma IGFs were dissociated from IGFBPs by acid gel chromatography. Plasma samples were acidified by diluting in mobile phase (0.8M acetic acid, 0.2M trimethylamine, pH 2.8 (80% v /v)). Following acidification, samples were mixed with an equal volume of freon to defat plasma, centrifuged at 13 000rpm for 10min at 4°C and the aqueous phase recovered. Samples were then filtered through 0.22μm cellulose micro-filters at 13 000rpm for 5min at 4°C. Filtrate (200μl) was injected into a Protein-Pak 125 column (Waters Corporation; MA, USA.) and high performance liquid chromatography conducted at pH 2.8 at a flow rate of 1ml /min, using a modification of the original procedure (Scott and Baxter, 1986), as described previously (Owens *et al.,* 1990). Fractions were collected at 15sec intervals and neutralised to pH 7.4 with 0.4M Tris.HCl. Concentrations of IGF-I and -II in fetal plasma were then measured by ELISA using the manufacturer's instructions (Diagnostic Systems Laboratories, Inc.; Texas, USA).

2.2 Histochemical techniques

2.2.1 Sectioning and mounting of muscle

Muscles samples, frozen by immersion in isopentane (2-methylbutane, Sigma; New South Wales) chilled over liquid nitrogen and stored at -80°C, were allowed to warm to approximately -20°C and mounted onto specimen chucks with Tissue-Tek® O.C.T. embedding media (Sakura FineTek; CA, USA). 10μM thick entire transverse sections were cut from the medial portion of each muscle using a cryostat (Microtome cryostat HM505E or 2800 Frigocut E, Reichert-Jung; Germany). Muscle orientation and quality of sectioning was verified by DIFFQUIK stain (Bacto Laboratories; New South Wales), serial sections were cut, allowed to air dry on microscope slides, for at least 1h, and stored at -20°C.

2.2.2 Staining procedures

Serial sections (section 2.1.1) were air dried at RT (room temperature). Serial sections analysed in Chapter 4 were reacted for myofibrillar ATPase, oxidative and glycolytic properties and specific proteins. Serial sections analysed in Chapter 6 were stained with hematoxylin and eosin and reacted for myofibrillar ATPase. Each enzymatic reaction was carried out on all samples on the one day. Sections reacted with antibodies were carried out over consecutive days, for each muscle sample four serial sections were reacted with all antibodies on the same day. For each staining experiment, a control section, from the semitendinosus muscle of a 5-month-old lamb, was also reacted to validate the success of the staining protocol.

2.2.2.1 Hematoxylin and Eosin (H and E)

Air-dried sections were stained in 0.5% hematoxylin (Sigma; Appendix 3.1) for 5min, rinsed in 2 washes of Milli-Q filtered water (MQ H_2O) separated by an acid-ethanol rinse (Appendix 3.1), incubated in 0.1% Eosin (Sigma; Appendix 3.1) for 10sec and dehydrated in graded alcohols (70%, 85%, 95% and absolute ethanol, Merck; Victoria), cleared in xylene (Sigma) and mounted using FastMount with colourfast (HistoLabs; New South Wales).

Nuclei were identified by an intense blue stain, while muscle cells stained pink.

2.2.2.2 Myofibrillar ATPase

2.2.2.2.1 Basic pre-incubation at pH 10.3

Slides were pre-incubated in basic pre-incubation buffer (Appendix 3.1) at RT for 10min and rinsed in MQ H_2O, pH 8.5-9.0.

2.2.2.2.2 Acidic pre-incubation at pH 4.3, 4.4 or 4.5

Slides were pre-incubated at pH 4.3, 4.4 or 4.5 (Appendix 3.1) at RT for 5min and rinsed in sodium barbital buffer (Appendix 3.1) and MQ H_2O, pH 8.5-9.0.

2.2.2.2.3 General ATPase incubation at pH 9.4

After pre-incubation at either pH 10.3, 4.3, 4.4 or 4.5 slides were incubated in warmed ATP pH 9.4 incubation solution (Appendix 3.1) at 37°C for 30min. Slides were rinsed in MQ H_2O, pH 8.5-9.0, followed by incubation in 1% $CaCl_2$ (Sigma) for 3min, 2% $CoCl_2$ (Sigma) for 3min and 1% ammonium sulphide (Sigma) for 1min, separated by three rinses in MQ H_2O, pH 8.5-9.0. Sections were dehydrated in graded alcohols (70%, 85%, 95% and absolute), cleared in xylene and mounted using FastMount with colourfast.

Fibres that reacted positively (stained intensely) for acidic pre-incubated ATPase activity (acid stable) were classified as slow twitch, while less intensively stained fibres (acid lable) were identified as fast twitch. The reverse classification was used for basic pre-incubated sections. In Chapter 6, primary fibres were identified based on the presence of a large vacuole and a less intense reaction to ATPase at a basic pH; secondary fibres were smaller, with no vacuole and reacted intensely to ATPase at a basic pH.

2.2.2.3 Nicotinamide adenine dinucleotide (NADH)

The NADH medium (Appendix 3.1) was warmed for 5min at 37°C and air dried slides were incubated for 30min in a 37°C water bath. Slides were rinsed in MQ H_2O, soaked in 50% acetone for 1min and mounted with glycerogel (Sigma).

Fibres that reacted positively (stained intensely) were classified as oxidative, while less intensely stained fibres were identified as less oxidative. However, little differential staining was witnessed for NADH in fetal muscle samples.

2.2.2.4 α-glycerophosphate dehydrogenase (α-GPD)

Slides were air dried and incubated for 60min in a mendione-linked α-GPD medium (Appendix 3.1) in a 37°C water bath. Slides were rinsed in MQ H_2O, soaked in 50% acetone for 1min and mounted with glycerogel.

Fibres that reacted positively (stained intensely) were classified as glycolytic, while less intensely stained fibres were classified as oxidative.

2.2.2.5 Immunohistochemical reactions

The presence of specific proteins was detected by incubation with monoclonal antibodies (mAb) and a streptavidin-biotin amplification detection system (Histostain SP Kit, ZYMED; California, USA). The mAb used, and their working dilutions, were (anti-) slow (1:100; Novo castra Laboratories), (anti-) fast (1:1200; Sigma), (anti-) 1,2b (1:1000; Greenwood) and (anti-) laminin (1:500; Sigma).

Four serial sections from each muscle were air dried and fixed in acetone for 10min. Each section was encircled by a hydrophobic ring, using a PAP pen (ZYMED) and rinsed in 0.01M phosphate-buffered saline (PBS), pH 7.4, to stop fixation. Sections were incubated in 10% non-immune goat serum (ZYMED) for 10min, to block non-specific binding, and then incubated with primary mAbs for 1hr in a 37°C humidity chamber. Sections were washed in PBS for 5-10min and incubated with biotinylated goat anti- (mouse, rabbit, guinea pig, rat) IgG second antibody (ZYMED) for 10min at RT. Following a 10min wash in PBS, sections were labelled with Streptavidin peroxidase conjugate (ZYMED) for 10min at RT, followed by a further 10min PBS wash and MQ H_2O rinse. The colour reaction was carried out by adding diaminobenzidine peroxidase substrate (DAB) (ZYMED) in MQ H_2O to sections at RT for 10min. Sections were then rinsed in MQ H_2O, dehydrated in graded alcohols cleared with xylene and mounted using FastMount with colourfast.

Due to poor resolution of cell outlines, it was decided to counterstain two serial sections for the fast and slow antibodies, respectively, with antibodies for Laminin on the same sections. The protocol outlined above was followed, with the following exception. The antibodies were made at twice the concentration of the normal working dilutions in half the required volume. A cocktail of equal volumes of the fast and laminin antibodies and the slow and laminin antibodies were made, giving the normal working dilution

concentration for each antibody used in the cocktail. Sections were then incubated with respective cocktails as outlined above.

2.2.3 Muscle fibre analysis

2.2.3.1 Cross-sectional area of whole muscle (MCSA)

The mean cross-section area of a muscle (MCSA) was determined by measuring the cross-sectional area of 8 to 10 serial sections of the entire muscle. A low magnification montage of the whole section was projected onto a computer monitor, a reference grid was overlaid and the outline of the image traced with a mouse attached to the computer and the area determined using the Video Pro 32 image analysis software (Leading Edge; South Australia).

2.2.3.2 Estimation of muscle fibre number

Due to the large MCSA of the semitendinosus (ST) muscle, it was decided to count a proportion of the MCSA as opposed to the entire section. Approximately 5% of the total MCSA was counted; the error associated with counting this proportion of total area has been validated by Greenwood *et al.* (2000a). A random sampling protocol, essentially as described by Greenwood *et al.* (2000a), was employed to estimate the apparent number of muscle fibres in an entire muscle cross section. For the purpose of estimation of myofibre number, a basic pre-incubated ATPase stained section was used.

Briefly, it was determined that optimal resolution of fibres would be at 200x magnification. The area of an individual field, at that magnification, was established and the number of fields required to account for 5% of the total MCSA was calculated.

The location of fields to be counted was determined using the reference grid laid over the entire cross section. The total number of grids required to count the total section area and, therefore, the appropriate number of fields to count 5% of MCSA was determined. The location of fields to be counted was determined by random sampling of grid references from the total grid population. The total number of myofibres and the number of individual fibre types within a field were counted (Gundersen *et al.*, 1988) and averaged across the number of fields counted. The estimated number of fibres in a single cross-section was determined by

(muscle cross-section area/ area of single field) x mean fibre number per field

2.2.3.3 Classification of myofibres

An initial objective of this work was to conduct a detailed classification of myofibre populations throughout fetal development with the use of specific antibodies and enzymes. However, due to financial and time constraints, it was unfeasible to optimise all staining protocols for fetal muscle; the success of the different staining protocols employed is mentioned in Chapter 4. The reactivity of muscle fibres to ATPase after pre-incubations at different pH was optimised for fetal muscle (as described in section 2.2.2.2). Identical fields were randomly selected, located in series, photographed and printed. The basic pre-incubated ATPase stained section provided a template for classification. Fibres were classified as described in section 2.2.2.2.3

2.2.3.4 Estimation of myofibre size (FCSA)

Fields that consisted of the mean muscle fibre number and proportion of fibre types were selected (determined in sections 2.2.3.2 and 2.2.3.3), so that CSA of between 400-500 fibres were measured. FCSA of individual muscle fibres, of each fibre type, were determined by tracing the perimeters of all individual fibres within a field using the mouse attached to the computer and the area determined using the Video Pro 32 image analysis software.

2.3 Nucleic acid, protein and dry matter content of muscle tissue

Total muscle RNA, DNA and protein were extracted in triplicate from homogenates of pulverised frozen ST and SS muscles from fetal lambs. RNA and DNA were extracted using the acid-alkali precipitation method based on the original procedure of Schmidt and Thannhauser (1945) and quantified using Baker's yeast RNA type III (Sigma) and calf thymus DNA type I (Sigma) as RNA and DNA standards, respectively. Muscle RNA and DNA concentration were measured using the Orcinol-ribose reaction (Dische and Schwartz, 1937) and the Diphenylamine-deoxyribose reaction (Burton, 1956), respectively. Muscle protein concentration was determined using the protein-dye binding method of Bradford (1976) with bovine serum albumin (BSA, Amresco; Ohio USA) as the standard.

2.3.1 Preparation of DNA and RNA stock standards

50mg of Baker's yeast RNA and Calf thymus DNA were dissolved in 25ml nuclease free water (NFW; Ambion; Geneworks, South Australia) by heating at 37°C for 1h or 15h, respectively, in an orbital mixing incubator (Ratek OM11; Victoria, Australia), with periodic vortexing. Standard stock solutions were then stored in aliquots at -80°C.

2.3.2 Preparation of DNA and RNA standard curves

For each extraction run (section 2.3.4) the amount of RNA and DNA stock solutions required for a standard curve were thawed and concentration determined by spectrophotometry at 260nm using the GeneQuant RNA/ DNA calculator (Pharmacia Biotech; Sweden). The extinction coefficients (ε) used for the RNA and DNA concentration were Absorbance $(A)_{260}$ = 1 = 40μg /ml and 50μg /ml, respectively. Therefore, nucleic acid concentration was determined

$$\text{Concentration} = A_{260} \times \varepsilon \times \text{dilution factor.}$$

Working standards were then prepared using the RNA and DNA stock diluted in 0.3M NaOH (Merck; Appendix 3.2) and 0.4M Perchloric acid (PCA; Asia Pacific Speciality Chemicals; Australia; Appendix 3.2), respectively. Final concentrations of RNA and DNA standards ranged from 0 to 240μg /ml and 0 to 60 μg /ml, respectively.

The working standards were lightly vortexed and stored overnight at 4°C. Standards were heated with unknown samples at 100°C for 20min the following day and then stored with samples until assayed. Therefore, each extraction run was associated with its own standard curve.

2.3.3 Control pool homogenate

A control homogenate was created by pooling 10 samples of ST muscle from day 92 (n=5) and day 133 (n=5) fetuses. The muscles were pooled and homogenised in 1:8 PBS-EDTA buffer, as outlined in section 2.3.4. Therefore, each extraction run (section 2.3.4) included a control pool homogenate extracted in triplicate.

2.3.4 Homogenisation and extraction of RNA and DNA from muscle tissue

Approximately 200mg of powdered frozen muscle was weighed and homogenised, in 8 volumes (vol) of cold PBS-EDTA buffer (Appendix 3.2), pH 7.2, at 20 000rpm using a 5mm rotor attached to an Ultra-Turrax (Janke & Kunkel; Germany).

In triplicate, equal volumes (~100mg) of homogenate were diluted 1 in 5 with ice-cold PBS-EDTA buffer and vortexed. Ice-cold 2.3M PCA (Appendix 3.2) was added and mixed and tubes were then incubated on ice for 15min. Samples were centrifuged at 3000*g* for 12min at 4°C, after which the supernatant was discarded and the pellet resuspended in ice-cold 0.22M PCA (Appendix 3.2) and stored overnight at 4°C.

The samples were then centrifuged at 3000*g* for 12min at 4°C and the supernatant discarded. The pellet was then washed in 0.22M PCA, resuspended in 0.3M NaOH (Merck) and heated at 100°C for 20min. After the alkali digest, samples were cooled on ice and precipitated with ice-cold 2.3M PCA. The pellet formed after sitting on ice for 15min and centrifugation at 3000*g* for 12min at 4°C. The acid-soluble supernatant fraction was removed and stored for RNA determination. The acid-insoluble fraction was resuspended in 0.4M PCA by heating at 100°C for 20min. Samples were cooled on ice and centrifuged at 3000*g* for 12min at 4°C. The supernatant was saved for DNA determination.

2.3.5 Diphenylamine-deoxyribose reaction for muscle DNA determination

DNA samples, including control pool, and associated standards were thawed and incubated with 2 vol Diphenylamine reagent (Appendix 3.2) in the dark at 37°C for 15h. Reactions were cooled on ice for 5min, mixed and warmed to RT in the dark. Absorbance was measured at 600nm (Smart Spec 3000, BioRad; USA).

2.3.6 Orcinol-ribose reaction for muscle RNA determination

RNA samples, including control pool and associated standards were incubated with 2 vol Orcinol reagent (Appendix 3.2) at 100°C for 20min and cooled on ice for 10min.

Samples and standards were warmed to RT and absorbance measured at 600nm (Smart Spec 3000) within 2h.

2.3.7 Muscle protein content determination (Bradford)

Protein standards were prepared by determining concentration of BSA and diluting to a stock concentration of 0.5mg /ml with MQ water. Working standards were then prepared using the BSA stock, diluted with 0.15M NaCl (Merck), over a final concentration range of 0 to 10μg.

Approximately 50mg of homogenate (section 2.3.4) was diluted 1 in 5 with ice-cold PBS-EDTA. 5μL of 1:5 homogenate was added to 95μL 0.15M NaCl and mixed. Standards and unknowns were mixed with 1ml Bradford Reagent (Appendix 3.2) and reacted at RT for 3min. Absorbance was measured within 15min at 595nm.

2.3.8 Muscle dry matter content

Duplicate samples of pulverised muscle tissue were heated at 70°C for 48h. A preliminary study showed that fetal muscle samples of this nature were completely dried after 36h at this temperature, with no further losses beyond this time. A control sample was dried with each determination.

2.4 Molecular techniques

2.4.1 RNA extraction

Total RNA was isolated from muscle samples using TRIzol® Reagent (Invitrogen Life Technologies; Victoria), according to the manufacturer's instructions. Briefly, approximately 100mg of pulverised frozen tissue sample was homogenised in a 5ml polypropylene tube containing 1ml of TRIzol® Reagent. Homogenised samples were incubated at RT for 5min and were then transferred to 2ml screw-capped tubes. Chloroform (200μl; Merck), was added to each sample and the tubes gently inverted several times. Samples were incubated at RT for a further 3min and total RNA was recovered from the aqueous phase after centrifugation at 10 000*g* for 15min at 4°C. Two equal volumes of RNA were transferred to 1.5ml Eppendorf tubes and precipitated at 10000*g* for 15min at 4°C in 1 vol isopropanol (Merck). One pellet was washed in 70% ethanol and resuspended in NFW. The other pellet was resuspended in Solution D (Appendix 3.3); both RNA samples were snap frozen and stored at -80°C.

Quality and quantity of RNA obtained from muscle were assessed by gel electrophoresis and ethidium staining (section 2.4.8.1). The integrity and intensity of the 28S and 18S ribosomal RNA bands were examined under UV light to verify the RNA quality of each sample. The RNA concentration was determined by measuring the absorbance of RNA by spectrophotometery at 260nm, using the GeneQuant RNA/ DNA calculator, as outlined in section 2.3.2. A further indicator of RNA quality that was also used was the ratio of light absorbance at 260nm and 280nm (i.e. A_{260} /A_{280} = 1.7 – 2.0, for good quality RNA), as contaminating proteins and residual organic compounds from the extraction procedure will also absorb light at 280nm.

2.4.2 RNA purification

Contaminating genomic DNA, protein and any possible solution D carryover were removed from RNA using the RNAqueous™-4PCR Kit for Isolation of DNA-free RNA (Ambion). RNA was precipitated from the solution D stored aliquot (section 2.4.1) with an equal vol of isopropanol and resuspended in 100μl of NFW. RNA was then diluted with 250μl lysis solution and 350μl 64% ethanol and transferred to an RNAqueous column. Columns were centrifuged at 8000*g* for 1min and the filtrate discarded; the RNA was maintained on the silica-gel membrane of the column. RNA was then washed once with 700μl Wash solution 1 and twice with 500μl Wash solution 2; each wash included column centrifugation at 8000*g* for 1min. RNA was eluted from the column

with a total of 60μl elution buffer heated to 95°C; elution was done in two steps, as 40μl and 20μl elutions.

The RNAqueous column was used after comparison with the RNeasy column system (Qiagen; Germany). Fetal muscle RNA yields (i.e. (RNA concentration after column / RNA concentration before column) x100) were in the range of 10-25% using the RNeasy columns; postnatal muscle and skin RNA yields ranged from 50-70% through the same batch of columns. A Proteinase K (Qiagen) digest conducted on fetal muscle RNA did not improve yields using the RNeasy columns. Therefore, fetal muscle RNA purification through the Qiagen RNeasy column would provide insufficient RNA material for reverse transcription with our preferred reverse transcriptase. Fetal muscle RNA yields were greater with the RNAqueous columns, ranging from 35-60% (mean approximately 50%). As the exact chemistrys of the two systems of RNA purification are unknown, it is difficult to speculate as to why low yields resulted with the Qiagen system. However, it does appear to be related specifically to the properties of fetal muscle RNA, as postnatal muscle and skin RNA were extracted and treated identically to fetal muscle but resulted in greater RNA yields.

2.4.3 DNase treatment of RNA

Following "on-column" RNA purification (section 2.4.2), DNase treatment was conducted to remove any contaminating genomic DNA from RNA samples using the DNA-*free*™ kit (Ambion). Briefly, 0.1vol of 10xDNase buffer and 2U of DNase I were added to 60μl RNA, mixed and incubated at 37°C for 30min. DNase I inactivation reagent (0.1 vol) was added and the samples mixed thoroughly, incubated at RT for 2min and centrifuged at 10 000*g* for 1min. Treated RNA was then removed and transferred to a new tube. Quality and quantity of DNase I treated RNA were assessed by gel electrophoresis and spectrophotometry at 260nm, respectively, as described earlier (2.4.1).

The effect of "on-column" purification and DNase I treatment of RNA is presented in Table 2.1 and Figure 2.1. Column purification of TRIzol® extracted RNA removed much of the 5S tRNA from RNA samples while DNase I treatment further reduced yields. However, both column purification and DNase I treatment improved RNA purity, as indicated by the greater 260:280 ratio.

Table 2.1: The effect of RNAqueous column purification and DNase treatment on the yield and quality of fetal muscle RNA.

		RNA sample 1	RNA sample 2
Before column purification	RNA yield (μg /ml)	795.2	771.4
	260:280	1.474	1.496
After column purification	RNA yield (μg /ml)	364	446.6
	260:280	1.722	1.749
After DNase treatment	RNA yield (μg /ml)	277.2	327.6
	260:280	1.761	1.784

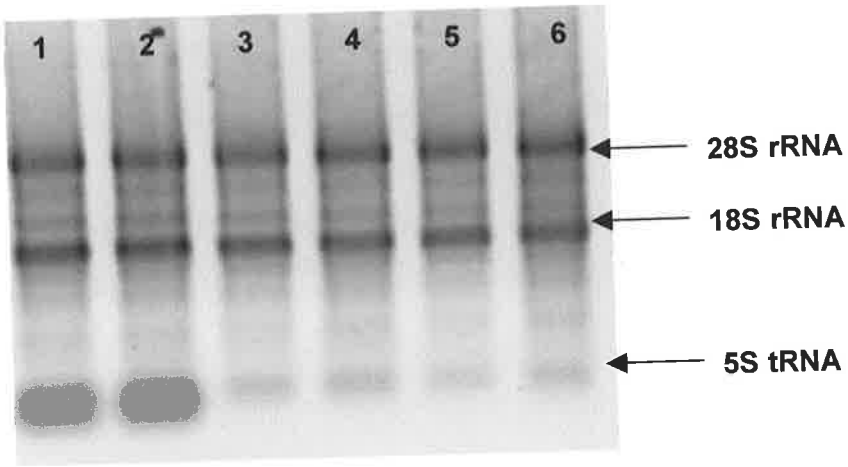


Figure 2.1: The effect of RNAqueous column purification and DNase treatment on the yield and quality of fetal muscle RNA fractionated through a 1% agarose gel. Lanes 1, 3 and 5 and lanes 2, 4 and 6 correspond to RNA samples 1 and 2 from Table 2.1, respectively. Lanes 1 and 2, lanes 3 and 4 and lanes 5 and 6 correspond to RNA before column purification, RNA after column purification and RNA after a subsequent DNase I treatment, respectively.

2.4.4 Quantitation of mRNA expression

Two approaches to quantitate mRNA expression by real-time PCR were employed in this study. The first approach, referred to as the preliminary study, used a random hexamer primed reverse transcription reaction (SuperscriptII, Invitrogen) with mRNA abundance measured on the GeneAmp 5700 system (Applied Biosystems; USA) with a SYBR green I reagent purchased from the same company. Unfortunately, due to the prohibitive cost of this reagent, only a subset of samples across two levels of nutrition and three stages of gestation were examined in the preliminary study. Moreover, only one batch of cDNA was prepared per sample and quantitative real-time PCR (qPCR) measurements were performed in duplicate on only one occasion for each gene. While the overall trends (data not presented) were reflected in our second analysis (primary experiment), this initial experiment lacked the repetition required for accurate quantitation of mRNA expression levels in the limited selection of fetal muscle samples examined.

The second approach, referred to as the primary experiment from which the results presented and discussed in Chapter 5 are derived, involved an anchored olidodT (5'-dT_{14}VN-3') primed reverse transcription reaction (Omniscript, Qiagen). The mRNA abundance in the fetal muscle samples was measured using a homemade SYBR green I reagent on the RotorGene 2000 system (Corbett Research; Sydney). This more comprehensive assessment of fetal muscle mRNA expression was made possible by the development of a low cost SYBR green I reagent and through the use of a liquid handling robotics station (CAS 1200; Corbett Robotics, Brisbane) which facilitated high precision pipetting and provided the level of throughput required to undertake the primary qPCR experiment. Quantitation of mRNA abundance was conducted on two muscles (semitendinosus and supraspinatus) of sheep exposed to three levels of nutrition (Low, Maintenance and High) at four stages of development (50, 92 and 133 day old fetuses and adult ewes, slaughtered in late pregnancy).

2.4.4.1 Reverse Transcription (RT) reactions

Total RNA from each muscle sample was reverse transcribed in oligodT primed cDNA reactions conducted in duplicate using the OmniScript™ First-Strand Synthesis System for RT-PCR (Qiagen). Briefly, 1.5μg of DNase free RNA (section 2.4.3) was incubated at 37°C for 2h in a 25μl reaction mix containing 1xRT buffer, 0.5mM dNTPs and 3.6U Omniscript. The reactions were primed with a combination of dTVN (4μM) and gene-specific primers for 18S rRNA (40nM) and IGF1 receptor (4nM); the final

concentrations of these primers are shown in brackets. Control RNA (50ng /μl), which is contained in the SuperscriptII 1st Strand cDNA Synthesis Kit (Invitrogen; USA), was included in each cDNA synthesis reaction as an exogenous RNA reference to provide an estimate of intra- and inter-assay reverse transcriptase efficiencies. The reactions were terminated by incubation at 70°C for 20min. An identical volume of each duplicate cDNA reaction was pooled and these aliquots stored at -80°C until required for qPCR. For all qPCR studies involving genes other than 18S rRNA, the pooled cDNA was diluted 1:25 in 10mM Tris (pH8.0) as required and stored at -20°C. The abundance of 18S rRNA in total RNA preparations meant that all measurements for this gene were conducted on a further 1:100 dilution of the pooled cDNA. The setting up of cDNA synthesis reactions, pooling of duplicate cDNAs and dilutions (1:25 and 1:100) in Tris buffer were performed with the CAS1200 liquid handling robot (Corbett Robotics; Brisbane).

2.4.4.2 Real-time PCR

2.4.4.2.1 Terminology/ Definitions:

Threshold Cycle (Ct): first cycle where the fluorescence level exceeds a threshold setting that is significantly different to the background fluorescence.

Unknown: samples generated from experimental treatment (e.g. nutrition, stage of development, muscle type), with unknown abundance of mRNA expression.

Relative Abundance: input amount of unknown mRNA relative to a standard curve.

Normalised Abundance: relative abundance of mRNA of each gene of an unknown adjusted to account for RNA loading and /or reverse transcription efficiencies. Normalisation can be to an endogenous gene, accurate quantity of input RNA determined by spectrophotometry, the exogenous control RNA of each unknown or a combination of all these approaches.

Treatment mean: mean of all values within a treatment group.

Calibrator: the treatment mean, after normalisation, to which all other treatment means, after normalisation, are expressed relative to.

2.4.4.2.2 Design of oligonucleotide primers for qPCR

The limited availability of ovine DNA sequence information meant that the majority of the oligonucleotide primers used in qPCR were designed against bovine expressed sequence tags (EST) and cDNA sequences. The exon-intron structure for most of these bovine sequences was not known so highly conserved regions of DNA in these genes were aligned against the human genome sequence to position putative exon-intron boundaries in the bovine cDNA sequences. The alignment of sheep, bovine and human cDNA sequences was performed with the Lasergene (DNAstar Inc.; USA) suite of programs while DNA alignments to the human genome sequence were conducted with internet-based applications at ANGIS (www.angis.org.au) and ENTREZ (www.ncbi.nlm.nih.gov/entrez). Oligonucleotide primers were designed with PrimerExpress (Applied Biosystems), Oligo4.05 (National Biosciences; USA) and PrimerSelect (DNAstar Inc.). The main aims were to design oligonucleotides that:

- spanned at least one intron
- were in highly conserved regions of gene sequence
- were ovine and bovine specific
- produced DNA amplicons between 70 – 200bp in length
- would anneal to target sequences at 60°C and generate a single, specific PCR product

All primers were purified by reversed phase chromatography and supplied at a concentration of 100μM by Proligo (New South Wales). The forward and reverse primer sequences, the expected sizes of amplified cDNA fragments and the Gen Bank accession numbers are presented in Table 2.2.

Table 2.2: Size and melting points of amplified PCR products, and the accession numbers of the gene sequence from which the forward and reverse oligonucleotide primers were designed.

Gene	Amplicon Size (bp)	Amplicon Melt Point (Tm; °C)	Forward Primer	Reverse Primer	GenBank Accession Number
18S rRNA	103	86	5'-cggtcggcgtcccccaactt-3'	5'-gcgtgcagccccggacatctaa-3'	M10098*
Acidic Riboprotein	189	88	5'-caaccctgaagtgcttgacat-3'	5'-gcaagtgggaaggtgtaatca-3'	AF013214
β-actin	191	91	5'-cagcacaatgaagatcaagatcatc-3'	5'-aaagggtgtaacgcagctaacagt-3'	AY141970
Internal Control	104	87	5'-tgcccgcctgatgaatgctc-3'	5'-gctcatggaaaagggtgtaacaa-3'	**
Cyclophilin A	72	85	5'-caaatgctggccccaacaca-3'	5'-tgccatccaaccactcagtc-3'	AB099010
GAPDH	104	86	5'-gccatcaatgacccctttcat-3'	5'-ttcccgttctctgccttgac-3'	U85042
Myf-5	90	86	5'-aacagccctatctggtccagaa-3'	5'-ggagcttttatccgtggcatat-3'	M95684
MyoD	128	93	5'-aactgttccgacggcatgat-3'	5'-gacaccgcagcgctcttc-3'	X62102
Myogenin	52	85	5'-ggcagcgcactggagttt-3'	5'-ccgctgggagcagatgat-3'	AF09174
Myf-6	85	86	5'-ggccaagtgtttcggatcatt-3'	5'-ctactcgaggccgatgaatca-3'	AB110601
Myostatin	101	82	5'-accttcccagaaccaggagaa-3'	5'-tcacaatcaagcccaaaatctct-3'	AF320998
IGF-I	73	80	5'-cattcattcagcaggcttgt-3'	5'-cagcctgtgttgcgtagaa-3'	M30653
IGF-II	84	90	5'-ggcggggagctggtggaca-3'	5'-tcggtttatgcggctggatggt-3'	M89788
IGF-I receptor	115	89	5'-tggcagtacaaccccaagat-3'	5'-gcttgttctcctcgctgtag-3'	X54980

* matched with bovine ESTs

** 900bp control RNA supplied with Superscript First-Strand Synthesis System for RT-PCR (Invitrogen, Life Technologies)

2.4.4.2.3 Standards and reference samples

For each gene, a standard curve was constructed from a series of 10 fold dilutions of a previously amplified, gel purified PCR product (QIAquick® kit, Qiagen). Concentrations of the DNA standards were estimated using the DNA LabChip 1000 kit on a Bioanalyser (Agilent Technologies; Victoria) according to the manufacturers instructions.

In addition to the DNA standards, three cDNA reference samples were included in these qPCR studies to account for the inter-run variation that exists when DNA standards are used alone. The cDNA reference samples were prepared from two fetal and one ewe muscle sample. Essentially, cDNA from each reference sample was synthesised in quintuplicate and pooled (as described in section 2.4.4.1). The three reference samples were treated the same as unknown samples, except they were measured in triplicate in every qPCR run conducted for a particular gene.

2.4.4.2.4 Polymerase chain reaction (PCR)

PCR master mix and reactions were prepared and dispensed into 0.1ml strip tubes by the CAS 1200 liquid handling robotics system (Corbert Research). Each 20μL qPCR contained 5μL of 1:25 cDNA (equivalent to 1μL of 1:5 cDNA) or 5μl of 1:2500 cDNA for the 18S rRNA measurements. Each reaction contained a final concentration of 1xPCR Gold buffer (Applied Biosystems), 3mM $MgCl_2$ (Applied Biosystems), 0.2mM dNTPs (Invitrogen), 400nM (80nM for 18S) forward and reverse primers (Proligo), 0.3xSYBR® Green (Roche) in 10mM Tris, pH 7.5, 0.01% Triton X-100 (Sigma) and 0.5U AmpliTaq Gold (Applied Biosystems). A final concentration of 1M Betaine (Sigma) was included in qPCRs on gene sequences that were GC-rich (i.e. myogenin and myoD). All qPCRs were conducted on the Rotorgene 2000 (Corbert Research) using the 72 well rotor. qPCR cycling parameters for all genes included an initial 10min denaturation step at 95°C, followed by 40 cycles at 95°C for 15 sec (denaturation), 58°C for 20 sec (anneal, after a 5°C touchdown over the first 5 cycles) and 72°C for 15sec (extension). The temperature at which fluorescence data were acquired depended on the melting temperature (Tm) of each DNA amplicon. In general, the acquisition of qPCR data occurred at 4°C below the Tm of the DNA amplicon, and is shown in Table 2.3. At the completion of 40 cycles, a dissociation analysis was conducted to confirm the Tm and specificity of the product.

Table 2.3: Acquisition temperature for fluorescence data of qPCR products.

Acquisition Temperature (°C)	Amplicons measured
75	IGF-I
78	Myostatin
80	ARP, Control, Cyclophilin A, GAPDH, Myf-5, Myogenin
82	Myf-6
84	18S rRNA, IGF-I receptor
86	β-actin, IGF-II, MyoD

In total, 105 pooled cDNA samples were examined in the primary qPCR experiment. Given that a 72 well rotor can measure 24 DNA samples in triplicate per run, a total of five qPCR runs in a row was required to measure the expression of each gene in this study. For each gene, this process was conducted twice; giving two replicate measures of gene expression carried out in triplicate on pooled cDNA reactions that were initially synthesised as separate duplicates.

2.4.4.2.5 Quantitation

The relative standard curve method was used to quantify the relative abundance of mRNA for the genes of interest in this study. From the qPCR data, an amplification plot was constructed (e.g. Figure 2.2). A standard curve was then created by plotting the Log input concentration vs. the C_t of the amplified standards (e.g. Figure 2.3). The C_t of the unknowns is determined from the amplification plot (e.g. Figure 2.4) and the amount of mRNA present in the unknowns is then determined from the equation of the standard curve. Therefore, regardless of absolute standard input amounts, the amount of mRNA in unknowns is determined relative to the standard curve and, therefore, relative to other unknowns (e.g. Figure 2.5; Table 2.4).

The main advantage of the relative standard curve method over other methods of quantitation (i.e. comparative C_t and absolute standard curve) is less optimisation is required to validate the performance of each assay. The accurate quantitation of input amounts of DNA standards is not essential (input amounts in unknowns are expressed relative to each other in arbitrary units) and it is not imperative that different primer sets amplify with identical reaction efficiencies, which is essential when the comparative C_t method is used. For each gene, treatment means (least-squares means) and standard

errors of the means were expressed relative to (i.e. as a percentage of) the calibrator treatment mean. The main reason for this was that exact copy numbers of standards were not known. Therefore, the data were relative rather than absolute and the units were arbitrary, meaning the most consistent way of expressing data was relative to a calibrator.

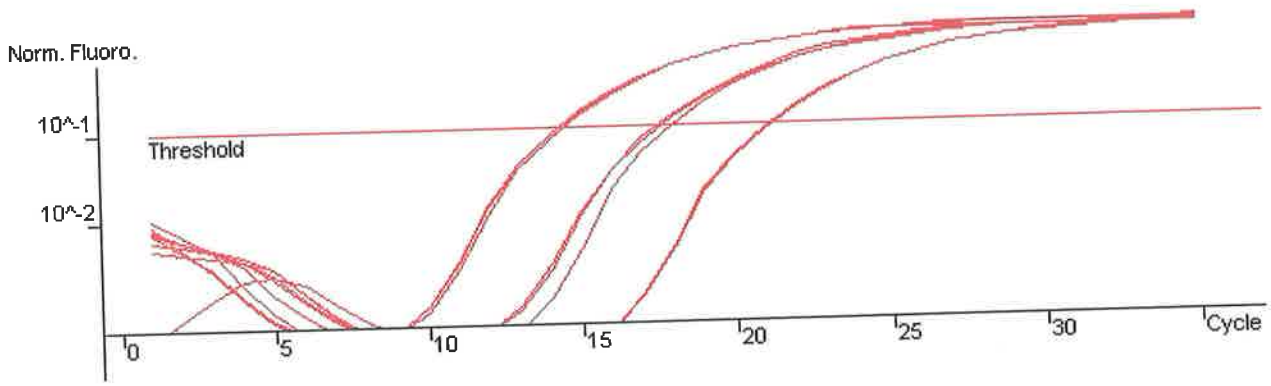


Figure 2.2: Amplification of a 10-fold serial dilution of the β-actin standard converted to the log scale; each dilution is measured in triplicate. Fluorescence is recorded at the end of each cycle and the C_t is the cycle when fluorescence is first detected above the background and occurs in the exponential phase.

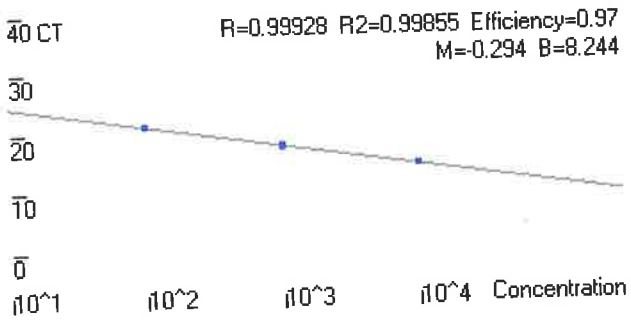


Figure 2.3: β-actin standard curve generated by plotting C_t against the log of the concentration of the standard. The equation of the line (C_t = -0.294 x concentration + 8.244) is then used to determine the concentration of β-actin in unknowns. The efficiency of the standard curve (E=0.97) is a measure of the amplification efficiency of a particular oligonucleotide pair across the dilution series.

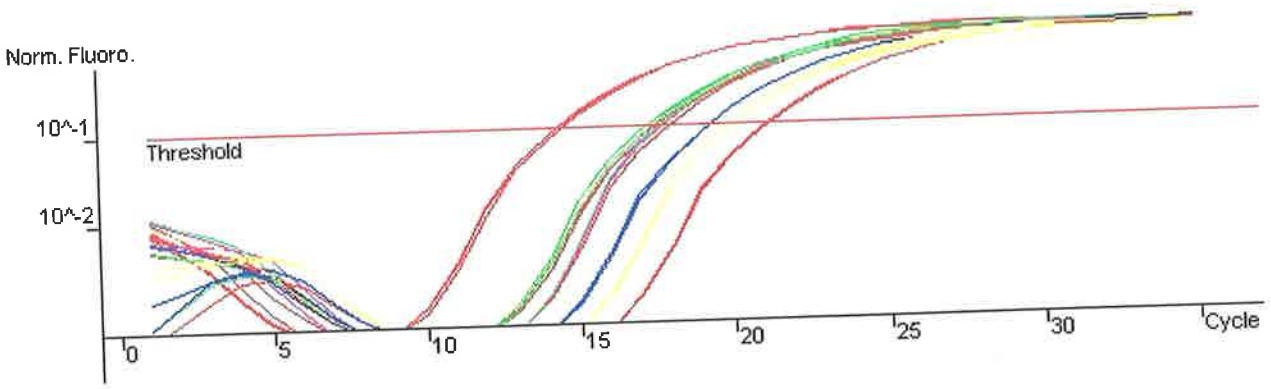


Figure 2.4: Amplification of β-actin standards (red lines, described in Figure 2.2) and unknowns converted to the log scale. The unknowns represent one muscle sample cDNA measured in triplicate from each stage of development (i.e. pink = day 50; green = day 92; blue = day 133; yellow = adult ewe). It appears that day 50 and 92 C_ts are earlier, followed by day 133 and adult ewe, suggesting β-actin is most abundant at day 50 and 92 of fetal development and least abundant in the adult ewe.

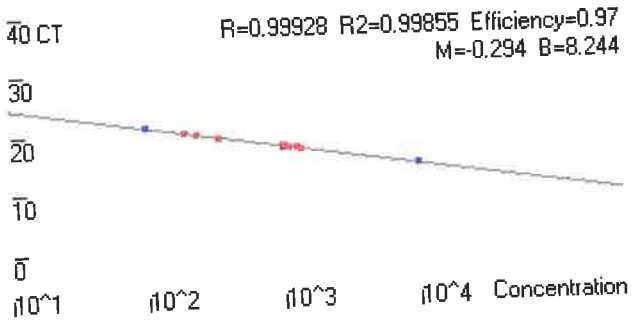


Figure 2.5: The relative concentration of β-actin mRNA in unknown samples. The unknowns (red dots) fall within the linear range of the standard curve (blue dots) and their concentration can be determined from the equation of the line.

Table 2.4: Calculation of relative concentration of β-actin mRNA in muscle samples from fetal and adult sheep.

Name	Type	Ct	Given Concentration (arbitrary units)	Calculated Concentration (arbitrary units)	Mean Concentration (arbitrary units)	Relative Expression (% of day 50)
Ovine β-actin 1	Standard	14.39	10,000.00	10,225.20	9,844.00	
Ovine β-actin 1	Standard	14.46	10,000.00	9,751.50		
Ovine β-actin 1	Standard	14.49	10,000.00	9,555.30		
Ovine β-actin 2	Standard	17.6	1,000.00	1,161.60	1,032.27	
Ovine β-actin 2	Standard	17.75	1,000.00	1,049.40		
Ovine β-actin 2	Standard	18	1,000.00	885.8		
Ovine β-actin 3	Standard	21.2	100	101.3	99.10	
Ovine β-actin 3	Standard	21.3	100	94.7		
Ovine β-actin 3	Standard	21.2	100	101.3		
cDNA Fetal day 50	Sample	17.73		1,063.70	1,065.03	100
cDNA Fetal day 50	Sample	17.82		1,000.80		
cDNA Fetal day 50	Sample	17.64		1,130.60		
cDNA Fetal day 92	Sample	17.33		1,394.80	1,232.87	116
cDNA Fetal day 92	Sample	17.47		1,268.60		
cDNA Fetal day 92	Sample	17.77		1,035.20		
cDNA Fetal day 133	Sample	19.41		340.8	341.57	32
cDNA Fetal day 133	Sample	19.4		343.1		
cDNA Fetal day 133	Sample	19.41		340.8		
cDNA Adult ewe	Sample	19.95		236.3	203.47	19
cDNA Adult ewe	Sample	20.29		187.7		
cDNA Adult ewe	Sample	20.3		186.4		

2.4.5 Verification of amplified cDNA and gDNA

Specificity of the amplified product was confirmed in three ways.

2.4.5.1 Melt curve analysis (for qPCR products only)

On completion of the cycling parameters, an incremental increasing temperature protocol was employed, where temperature increased 1°C every 5 sec and fluorescence was recorded. Product specificity was displayed as a single peak at the products melting temperature (Tm) as shown in Figure 2.6. Non-specific products and primer dimers were identified by a separate melting point peak and a small shoulder on melting point peak, respectively.

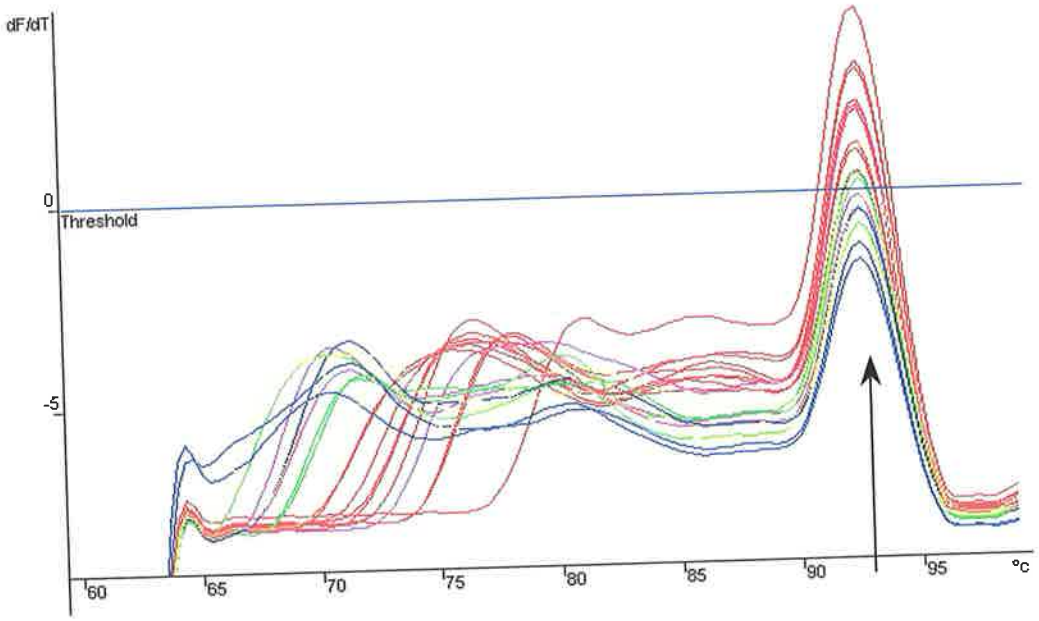


Figure 2.6: Melt curve analysis of the β-actin PCR products amplified and quantitated in the example given in section 2.4.6.1. The red lines are melt curves of the standards; the pink, green, blue and yellow lines are the melt curves for the fetal day 50, 92 and 133 and adult ewe samples, respectively. The arrow indicates a single specific melting temperature of 92.5°C.

2.4.5.2 Size of amplified cDNA and genomic DNA products

Amplified PCR products were subjected to gel electrophoresis (section 2.4.8.1) to examine specific or non-specific amplification in both positive (+RT) and negative (-RT and NTC) cDNA and in gDNA. Further, the size of the amplified product was verified by comparison against either *Hpa*II-restricted pUC19 DNA or *Eco*RI-digested SPP1 DNA (Geneworks) molecular weight markers, depending on the size of the fragment (section 2.4.8).

2.4.5.3 Cloning and sequencing of amplified cDNA and genomic DNA products

2.4.5.3.1 End-filling of DNA fragments amplified by PCR

Double-stranded DNA fragments were "polished" for cloning with Klenow DNA polymerase (Promega Corporation; WI, USA). Fragments were incubated at 37°C for 15 mins in 1 x Klenow buffer (Appendix 3.3) containing 5U Klenow polymerase and 50μM dNTPs to fill in the 5' T-overhang that is present on DNA fragments amplified by Taq polymerase.

2.4.5.3.2 DNA ligation into pBluescript and transformation of *E. coli*

An aliquot of the pBluescript I KS+ plasmid vector that had been linearised with the restriction endonuclease, EcoRV (Promega), and dephosphorylated with Calf Intestinal Phosphatase (New England Biolabs; USA) was provided by Dr. Greg Nattrass. The end-filled cDNA and gDNA fragments were blunt end ligated into the EcoRV site of pBluescript using Cycle Restriction Ligation (CRL; Technical Tips Online; www.biomednet.com/db/tto). The DNA ligation reactions were extracted with phenol /chloroform and purified by ethanol precipitation (Sambrook *et al.*, 1989). Electro-competent *E. coli* (strain DH5α) were transformed in 0.1cm cuvettes using standard bacterial electroporation procedures (Gene Pulser II, BioRad; USA). The transformed cells were plated onto Luria Broth (LB; Appendix 3.3) agar plates containing ampicillin (100μg /ml), X-gal (2.5mg; 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside; Progen; Australia) and IPTG (2.5mg), and incubated overnight at 37°C or longer if the blue /white colouring process of individual colonies was not complete after overnight incubation.

2.4.5.3.3 Identification of recombinant *E. coli* and preparation of plasmid DNA

Individual white colonies were picked off the LB plates with a pipette tip, a portion spotted onto a LB master plate and the remainder of the colony transferred to a PCR tube containing: 1x AmpliTaq Gold reaction buffer, 2mM $MgCl_2$, 0.2mM dNTPs, 200nM

reverse sequencing primers (RSP) and T7 primers and 0.25U AmpliTaq Gold polymerase (Applied Biosystems). The PCR cycling conditions were: 95°C, 55°C and 72°C for 30 seconds each, for 40 cycles. The PCRs were fractionated on 1-2% agarose gels, stained with ethidium bromide and the DNA visualised under UV illumination (section 2.4.8.1). PCR products from pBluescript clones with a DNA insert were larger than PCR products containing pBluescript alone. The clones with DNA inserts in pBluescript were re-picked with a pipette tip from the LB master plate, transferred to 2ml LB with ampicillin (100μg /ml) and mixed at 37°C in an orbital mixing incubator (Ratek) overnight. Plasmid DNA was prepared using the alkaline lysis miniprep protocol (Birnboim and Doly, 1979).

2.4.5.3.4 Restriction endonuclease digestion of DNA

Restriction endonuclease digestion of plasmid DNA was performed in 1x Super Dooper Buffer (SDB) (Appendix 3.3) with 2U of HindIII and PvuI per μg of DNA at 37°C for 2h.

2.4.5.3.5 DNA sequencing

DNA sequencing was carried out by the dideoxy chain termination method using the fmol® DNA Cycle Sequencing System (Promega). For each gene to be sequenced, reactions were carried out with 2μL of each dNTP (A, C, G, T), 1x fmol® Sequencing Buffer, 400nmol T7 or universal sequencing primer (USP) and 1.25U Taq polymerase. Reactions, labelled with α^{33}P dATP, were carried out at 95°C for 3min, and 40 cycles of 95°C, 50°C and 70°C for 40 sec each, followed by denaturation at 75°C for 3min after addition of 3μL of fmol® Sequencing Stop Solution. 3μL of each sequencing reaction was then fractionated through a 5% denaturing acrylamide gel (SequaGel® XR, National Diagnostics; Georgia, USA) by electrophoresis at 120W and 50°C for approximately 2h. The sequencing gels were washed with 12% acetic acid and 20% ethanol before being dried (Gel Dryer Model 583, BioRad) and exposed to X-ray film (Fuji Rx; Japan) in an autoradiography cassette with an intensifying screen (DuPont; USA) for 12-24h at -80°C. Exposed X-ray film was developed (Phenisol X-ray developer 1:4, Ilford; Australia) for 3min, rinsed in RO water and fixed (Hypam fixer 1:4 and Hypam hardener 1:40, Ilford) for 3min in a darkroom under safelights. The DNA sequences were read off the film and compared with the published sequences.

2.4.6 Western transfer

2.4.6.1 Protein quantitation, electophoresis and transfer

Protein was extracted from approximately 100mg of pulverised muscle in 10 vol PBS with 0.05% Nonidet P-40, pH 7.2 with Complete Protease Inhibitor Cocktail (Roche) (Appendix 3.3) by brief homogenisation followed by sonication (duty cycle 50%, output control 3; Sonifier 450, Branson; CT, USA). Protein concentration was determined using the 2D-protein quantitation kit (Amersham Biosciences; New South Wales), according to the manufacturers instructions, with BSA (Amersham Biosciences) as the standard.

Protein (10μg) was diluted in an equal volume of 2x protein loading buffer (Appendix 3.3) and denatured at 95°C for 5min. A positive control peptide, designed from either the C-terminus or N-terminus (section 2.4.8.2.3), was loaded on each gel to confirm primary antibody reactivity. SDS-PAGE was conducted with 72x83x0.75mm polyacrylamide gels (4% stacking-12.5% separating), in the Mini-PROTEAN® 3 Cell system (BioRad). Electrophorosis was carried out in Tris-Glycine-Saline (TGS) running buffer (Appendix 3.3) for 35min at 200V.

The Mini-PROTEAN® 3 Cell system allows two gels to be run simultaneously, therefore each gel was run in duplicate. A pre-stained protein ladder (Benchmark™; Invitrogen Life Technologies) was loaded on each gel for orientation and sizing of proteins. One gel was used to verify equal protein loading by staining with Coomassie blue (Appendix 3.3) for 1h at RT and then destaining in a container of boiling MQ H_2O, with an equal size piece of woollen fabric to absorb excess Coomassie stain, in a microwave. The other gel, to be used for immunoblotting, was prepared for electrophoretic transfer by equilibrating in 3x 10min changes of Towbin-20% methanol (Merck) transfer buffer (Appendix 3.3).

Proteins were transferred to equilibrated ECL Hybond membrane (Amersham-Pharmacia; UK) in Towbin-20% methanol transfer buffer using the Trans-Blot® SD Semi-Dry Electrophoretic Transfer Cell (BioRad) at 15V for 20min. Efficiency of transfer was verified by staining gels in Coomassie Blue after transfer, as described above.

2.4.6.2 Antibodies and positive controls

Several primary and secondary antibodies were evaluated under a range of dilutions and blocking conditions.

2.4.6.2.1 Primary antibodies

GDF-8 (C-20) goat anti-mouse IgG (sc-6884). 200μg of affinity purified polyclonal antibody in 1ml PBS containing 0.1% sodium azide and 0.2% gelatin (Santa Cruz Biotechnology; California, USA). The antibody was raised against a peptide mapped to the C-terminus of mouse myostatin, therefore it is common to processed myostatin and its precursor.

MyoB rabbit anti-human IgG (BL891). 1mg of affinity purified polyclonal antibody in 1ml Tris-citrate /phosphate, pH 7 to 8, containing 0.1% sodium azide (Bethyl Laboratories; Texas, USA). The synthetic peptide used to raise the antibody represents a portion of human myostatin encoded within exon 3 and is located in the C-terminus, therefore it is common to processed myostatin and its precursor.

In-house Protein A purified rabbit anti-bovine IgG. Polyclonal antibodies were raised in two rabbits by Dr. Greg Nattrass to a portion of the bovine myostatin protein that spanned amino acid residues 198–376 (see Appendix 4). The protein preparation used for the immunisations was expressed in *E. coli* from a pQE70 vector construct (Qiagen) that contained a C-terminal 6x-histidine tag. The myostatin His-tagged protein was purified with a nickel-nitrilotriacetic acid (Ni-NTA) metal-affinity chromatography resin, dialysed against PBS and the protein concentration determined by Bradford analysis.

Immune sera (1ml) from the terminal bleeds were filtered through a 0.45μm disposable filter (Schleicher and Schuell; Germany) and washed through with 1ml of 20mM sodium phosphate buffer, pH 7 (Buffer A). The filtrate was applied to a HiTrap Protein A HP column (Amersham Biosciences) attached to a FPLC (Pharmacia Fine Chemicals; Sweden) and washed through with Buffer A at a rate of 1ml /min. Non-IgG serum proteins were collected in fractions 3-10. After the collection of fraction 12, Buffer A was replaced with 0.1M citric acid, pH 3 (Buffer B) to elute the bound IgG. Approximately 85% of the IgG was collected in fraction 16. The pH of the IgG fractions was adjusted to 7, with 350μl of 1M Tris.HCl (pH 9) and stored at 4°C.

2.4.6.2.2 Secondary antibodies

Upon arrival, all secondary anti-bodies were stored in 40μl aliquots at -20°C to avoid freeze thawing.

Rabbit anti-goat IgG (A 5420; Sigma). 7.5mg of whole molecule, affinity purified, horseradish peroxidase-conjugated polyclonal antibody in 1ml 0.01M PBS, pH 7.4 containing 0.01% Thimerosal. Tests conducted in the lab on this antibody showed that it to cross-reacted strongly with ovine IgG in muscle and plasma samples.

Goat anti-rabbit IgG (A 0545; Sigma). 7.5mg of whole molecule, affinity purified, horseradish peroxidase-conjugated polyclonal antibody in 1ml 0.01M PBS, pH 7.4 containing 0.01% Thimerosal.

Monoclonal anti-rabbit IgG (A 2074; Sigma). The affinity purified, horseradish peroxidase-conjugated monoclonal antibody is lypholysed from 0.01M PBS, pH 7.4, containing 1% BSA and 0.01% Thimerosal.

2.4.6.2.3 Positive controls

Two positive controls derived from the bacterial expression of bovine myostatin were used in these studies to test the specificity of the different myostatin antibodies in the immunoblotting procedures. The positive controls included the peptide that was used for the rabbit immunisations (C-terminal region) and another peptide that encoded the N-terminal region of bovine myostatin (amino acid residues 23-262; see Appendix 4).

2.4.6.3 Optimisation of blocking conditions.

The following products were all assessed for their suitability as blocking agents over a range of concentrations and in different combinations: BSA, non-fat dried milk (NFDM; Sigma), skim milk powder (SMP, Country Grove; Australia) and polyvinylpyrrolidone (PVP-40; Sigma). Conducting the blocking step and incubating antibodies with BSA generally resulted in higher background and non-specific binding of the secondary antibody, regardless of concentration. Use of SMP and NFDM to block the membranes was effective at removing all background binding, however more primary antibody was required for specific binding. There was little difference between SMP and NFDM, with the exception that SMP was cheaper and more readily available. The use of PVP in combination with BSA resulted in a lighter block allowing a greater antibody dilution to be used; however, non-specific binding of the secondary antibody was still evident.

Therefore, in combination with the above primary (2.4.6.2.1) and secondary (2.4.6.2.2) antibodies, the optimum blocking and incubating conditions to minimise background and non-specific binding and to maximise antibody titre are described in section 2.4.6.4.

2.4.6.4 Antibody detection

Transferred proteins were blocked overnight at 4°C in 1% PVP-40 /0.3% BSA in 25ml TBS-T (Tris buffered saline with Tween, Appendix 3.3). Membranes were briefly rinsed in TBS-T and then incubated with the in-house Protein A purified rabbit anti-bovine myostatin (1:1000) or pre-immune sera (1:1000) in 0.1% PVP-40 /0.3% BSA in 25ml TBS-T for 2h at RT. Membranes were then rinsed in TBS-T four times for 5min each and incubated with goat anti-rabbit IgG, horseradish peroxidase-conjugated secondary antibody (1:10000) in 5% SMP in TBS-T for 1h at RT. The membranes were again rinsed four times for 5min each in TBS-T at RT and immunoreactive myostatin proteins detected using enhanced chemiluminescence (ECL, Amersham Biosciences) according to the manufacturers instructions. Membranes were then exposed to Hyperfilm ECL (Amersham Biosciences) for 1min. Exposed film was developed (Phenisol X-ray developer 1:4, Ilford) for 3min, rinsed in RO water and fixed (Hypam fixer 1:4 and Hypam hardener 1:40, Ilford) for 3min in a darkroom under safelights.

2.4.7 Gel electrophoresis

2.4.7.1 Agarose gels

Small-scale analytical and preparative gels were electrophoresed using a horizontal (submarine) mini gel apparatus. Gels were prepared on 50x75mm glass slides and contained approximately 10ml of 1-2% agarose dissolved in 1xTAE buffer. Agarose gels loaded with DNA (mixed with 0.1vol agarose load buffer, 10xALB, Appendix 3.3), and RNA (mixed with 1vol formamide loading buffer 2xFLB, Appendix 3.3), were electrophoresed at 100V in 1xTAE buffer (Appendix 3.3). Electrophoresed DNA and RNA were stained in ethidium bromide (10μg /ml), viewed under ultraviolet light and photographed using a Gel Doc 1000 imaging system (BioRad). DNA fragments larger than 500bp and total RNA preparations were resolved on 1% agarose (GIBCO BRL) gels. Smaller DNA fragments (i.e. real-time PCR products) were resolved on 3% low melting point agarose gels (Progen Industries; Queensland) that were poured in a cool room (4°C) to facilitate setting. In general, SPPI DNA markers were used on 1% agarose gels while pUC19 DNA markers were used on 3% LMP gels.

2.4.7.2 Polyacrylamide gels

Polyacrylamide gels used in section 2.4.6.1 were prepared using a 30% acrylamide stock (38:1, acrylamide, Sigma: N,N`-methylene-bis-acrylamide, Biorad), and included 375mM Tris, pH 8.8, 0.4% SDS (Sigma), 0.1% ammonium persulfate (APS; AJAX Chemicals; Australia) and 0.01% Temed (N,N,N`N`-tetra-methylaethylenediamine, Biorad). The separating gel (12.5%) was prepared, poured and set with an overlay of H_2O-saturated butanol (Merck), for approximately 1h. The butanol was rinsed away and the stacking gel (4%) poured and set. Electrophoresis conditions were described earlier (section 2.4.6.1).

2.4.8 Molecular weight markers

The expected sizes of the fragments of the DNA (SPPI and pUC19) and protein markers are shown in the Tables 2.5, 2.6 and 2.7, respectively.

Table 2.5: The expected size and approximate concentration of DNA in fragments in SPPI-EcoRI DNA markers.

Fragment Number	Size (kb)	Amount (ng)
1	8.51	96
2	7.35	83
3	6.11	69
4	4.84	55
5	3.59	40
6	2.81	32
7	1.95	22
8	1.86	21
9	1.51	17
10	1.39	16
11	1.16	13
12	0.98	11
13	0.72	8
14	0.48	5
15	0.36	4

Table 2.6: The expected size and approximate concentration of DNA in fragments of pUC19-HpaII DNA markers.

Fragment Number	Size (basepairs)	Amount (ng)
1	501	93
2	489	91
3	404	75
4	331	62
5	242	45
6	190	36
7	147	27
8	111	20
9	110	20
10	67	12
11	34	6
12	34	6
13	26	5

Table 2.7: The expected size of protein fragments in the protein markers (Benchmark™ Pre-Stained Protein Ladder; Invitrogen Life Technologies).

Fragment Number	Molecular weight (kDa)
1	177.6
2	113.9
3	81.2
4	**60.7***
5	47.4
6	36.1
7	25.3
8	19
9	14.7
10	6.1

*Pink band for orientation

CHAPTER 3.

3 THE EFFECTS OF LONG-TERM MATERNAL FEED INTAKE ON PLACENTAL AND FETAL DEVELOPMENT

3.1 Introduction

Current meat production practices for domestic animals are designed to generate the desired finished product as rapidly as possible. Consequently, an increasing proportion of an animal's total life (i.e. conception to slaughter) is spent *in utero*. Perturbations of the pre-natal environment can affect the development of the fetus and its organs, subsequently impacting on the post-natal viability, growth rate and ultimate size and composition of an individual.

The effects of intrauterine growth retardation (IUGR) via artificial methods (eg. carunclectomy; Owens *et al.*, 1989), maternal under-nutrition (Everitt 1965, Mellor and Murray, 1982), maternal nutrient partitioning (eg. adolescent ewe model; Wallace *et al.*, 2000; Wallace *et al.*, 2001) and litter size (eg. prolific ewe model; Greenwood *et al.*, 1998) on ovine fetal development are widely reported. Severe restrictions of substrate supply may result in maternal, placental and fetal adaptations to the altered pre-natal environment, which allows offspring survival, often at the expense of normal fetal development.

Perturbed fetal development may be apparent as altered fetal/ birth weight, length, girth and disproportionate tissue growth. There is a tendency for substrates to be directed to maintain essential tissues and organs, such as the brain, at the expense of less vital ones. As a result, size at birth, muscle development and growth (Greenwood *et al.*, 1999, Greenwood *et al.*, 2000a), wool follicle formation (Schinkel and Short, 1961), adiposity (Greenwood *et al.*, 1998; Budge *et al.*, 2004) and gastrointestinal tract development (Trahair *et al*, 1997) are often altered in growth-restricted fetuses and newborns. Such perturbations in development may affect neonatal survival or impair post-natal productivity.

Adaptations of the fetus to placental insufficiency not only alter post-natal productivity but are also considered likely precursors to disease in adulthood humans (Barker, 1992). These fetal adaptations are often associated with fetal hypoglycaemia,

hypoxaemia and modifications to the insulin-like growth factor and hypothalmo-pituitary-adrenal axis (reviewed by McMillen *et al.*, 2001; Symonds *et al.*, 2001).

Fetal weight is generally considered to be unaffected by moderate changes in maternal nutrition during early gestation. However, Everitt (1965) reported a 10% reduction in fetal weight when ewes lost 12% of their body weight over the first 90 days of gestation. Mid-gestation maternal nutrient restriction has resulted in small (Mellor, 1983) or large (Russel *et al.*, 1981) fetuses; these differences may be attributed to the weight of the ewe at mating (Russel *et al.*, 1981; De Barro *et al.*, 1993). While restricting maternal feed intake during late gestation can severely reduce fetal growth rate (Mellor and Murray, 1981; Mellor, 1983).

Placental characteristics play a significant role in the development of the fetus, as indicated by the strong correlation that exists between placental weight and fetal and birth weight in sheep (Greenwood *et al.*, 2000b). The effects of maternal nutrition during pregnancy on the development and activity of the placenta are widely reported. However, these appear highly variable, being influenced by age, live weight and condition score of the ewe, stage of pregnancy (treatment and measurement), litter size and severity of restriction imposed (Kelly, 1992; Robinson *et al.*, 1994).

There appear to be no studies documenting the effects of long-term pre- and post-conception nutrition on the development of the placenta in sheep. Everitt (1965) demonstrated that severe maternal feed restriction post-mating resulted in decreased placentome weight at day 90 and a greater decline in placentome weight by late gestation. Similarly, Osgerby *et al.* (2002) report that under-nutrition throughout pregnancy results in a greater reduction in placental mass over late pregnancy. While De Barro *et al.* (1992) reported that light weight ewes under-fed between 30 and 90 days of gestation, had heavier placentas at day 92 than similarly underfed heavy ewes.

The following experiment examined the hypothesis that long-term pre- and post-conception feed intake of the ewe will induce adaptations in placental characteristics and affect fetal development. Therefore, the primary aims of this study were to,

- Investigate the feto-maternal adaptations to a nutritional challenge imposed long-term prior to and throughout pregnancy.
- Develop a model for producing fetuses divergent in growth to assess the nutritional regulation of myogenesis in sheep.

3.2 Experimental

The experimental protocol was reviewed and approved by the University of Adelaide and PIRSA Animal Ethics Committees in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (6th Edition, 1997).

3.2.1 Location

The experiment was conducted at Turretfield Research Centre (34° 33`S, 138° 50`E), South Australia. Ewes were penned in treatment groups in an open-sided covered shed (n=10 /pen).

3.2.2 Animals, diet and experimental design

Freshly shorn, mature age multiparous, South Australian Merino ewes (n=149) were ranked and stratified on live weight and randomly allocated to three feed intake treatment groups. The feed intake treatments imposed were *ad libitum*/ High (H; 1.8 x maintenance, n=60), Maintenance (M; 1.2 x maintenance, n=30) and restricted/ Low (L; 0.6 x maintenance, n=59). Maintenance levels were calculated to be 1.1kg DM /head /day from MAFF (1976),

$$\text{Maintenance (g /day)} = [1.4 + (0.15 \times \text{live weight})] \times 100$$

The ewes were fed a pelleted roughage/ grain ration daily (Johnsons Stockfeed, Kapunda) with *ad libitum* access to water. The nutritional composition of the diet, as determined by Near Infrared Reflectance (NIR) (FEEDTEST®, Agriculture Victoria, Hamilton), is shown in Table 3.1. Ewes were acclimatized to pens and gradually introduced to diet and treatment levels over a 10-day period. Feed intake treatments were then maintained for twelve weeks immediately prior to mating and subsequently throughout pregnancy. Feed refusals, if any, were collected and weighed daily throughout the experiment. Ewe live weight and condition score (Russell *et al.*, 1969) was monitored at weekly intervals.

Table 3.1: Nutritive composition of diet offered to ewes throughout the experimental period (all parameters calculated on a dry matter basis).

Dry Matter (%)	**90.7**
Energy (MJ ME /kg)	**9.5**
Crude Protein (%)	**12**
Digestibility (%)	**65.8**
Acid Detergent Fibre (%)	**23.9**
Ash (%)	**10.5**

Ewe live weight and condition score differed between treatment groups after six weeks of feeding ($p<0.05$). Ewes were fed their allocated ration for a further six weeks, until live weights and condition scores had stabilised, at which time they were mated (day 0), and throughout pregnancy. The model for creating, and maintaining, divergent live weight and condition score prior to mating and throughout gestation is shown in Figures 3.1 and 3.2, respectively. Throughout the experiment, calculation of maintenance requirements was based on initial weight. While the nutritive requirements of an animal vary based on its physiological state, it was considered appropriate not to adjust requirements throughout the experiment. The two reasons for this were:

- H ewes were considered to be consuming *ad libitum* throughout the experiment. Intake of H ewes never exceeded 1.7 x initial dry ewe maintenance requirements. So although live weight and condition of these ewes did increase throughout the experimental feeding period, intake remained relatively fixed, never exceeding 1.8 x maintenance requirements.
- L ewes lost live weight and condition over the pre-mating period, which then remained relatively constant throughout gestation. It was thought that decreasing feed offered in line with loss of live weight during the pre-mating period would result in difficulties in establishing and maintaining pregnancy.

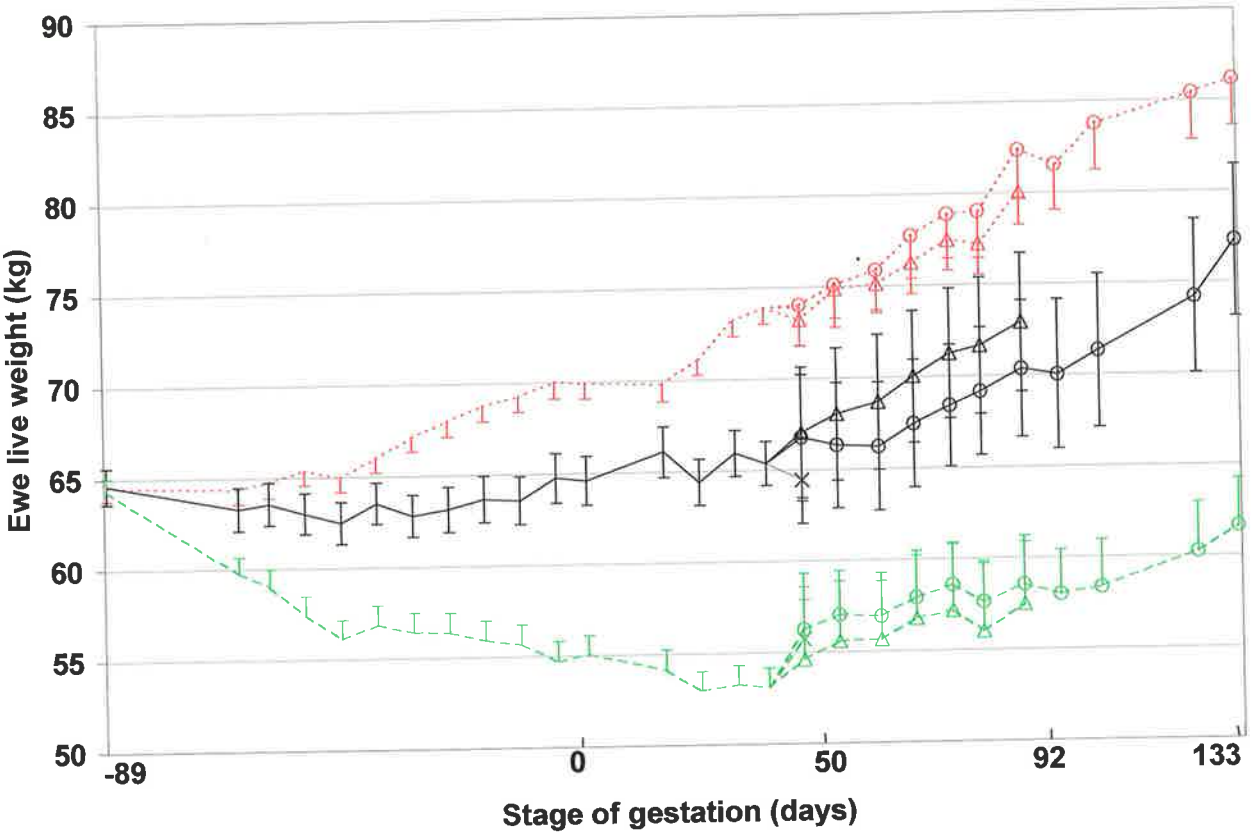


Figure 3.1: Mean (± SEM) live weight of ewes allocated to High (…), Maintenance (¯) and Low (---) levels of nutrition, prior to pregnancy diagnosis (day –89 to 40), and of pregnant ewes within nutritional treatments slaughtered at either day 50 (*), 92 (Δ) or 133 (o) of gestation. See Table 3.2 for sample cohort populations.

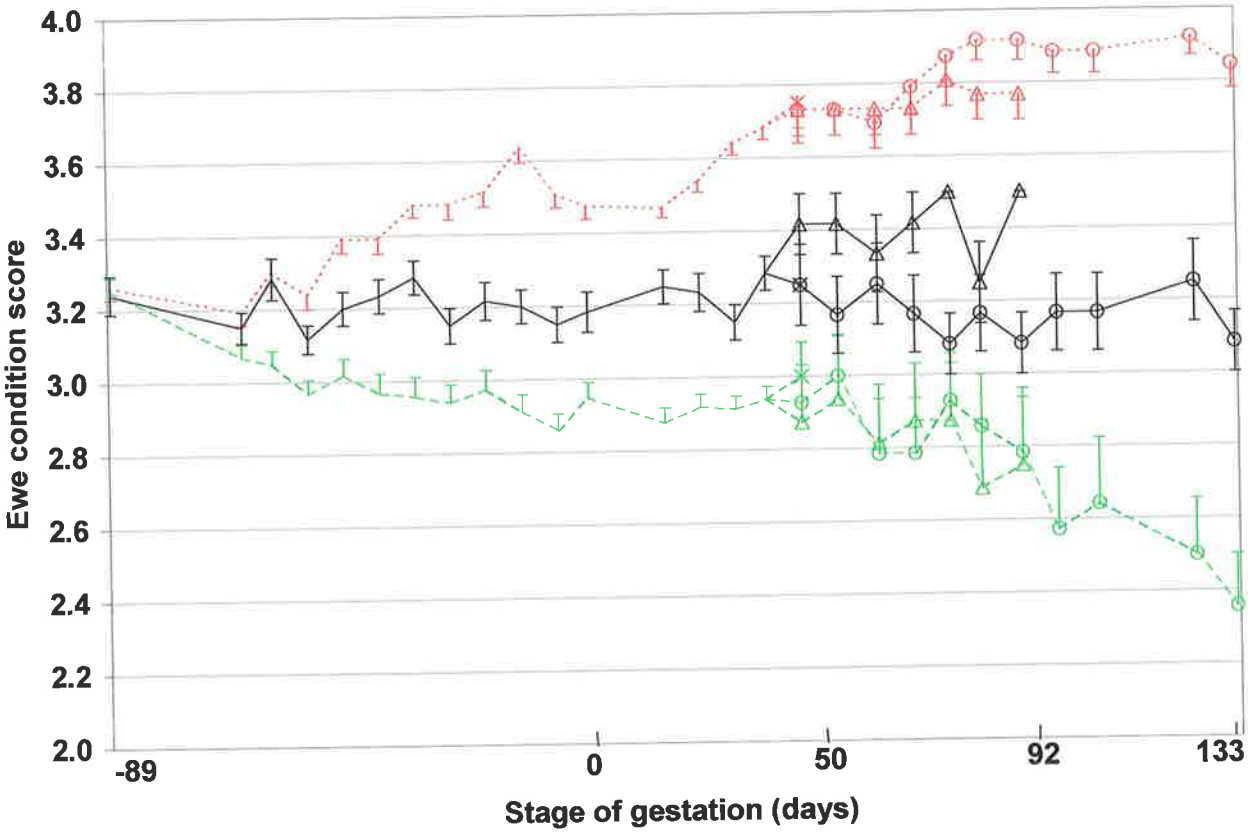


Figure 3.2: Mean (± SEM) condition score of ewes allocated to High (...), Maintenance (‾) and Low (---) levels of nutrition, prior to pregnancy diagnosis (day –89 to 40), and of pregnant ewes within nutritional treatments slaughtered at either day 50 (*), 92 (Δ) or 133 (o) of gestation. See Table 3.2 for sample cohort populations.

To determine the effect of long-term maternal nutrition on placental and fetal development and muscle characteristics, fetuses were obtained at three gestational ages, days 50 (early), 92 (mid) and 133 (late) of pregnancy. Therefore, the overall experimental design was a 3x3 factorial (i.e. three levels of nutrition and three stages of fetal development).

3.2.3 Procedures

3.2.3.1 Joining

One hundred and forty nine ewes were treated with controlled intra-vaginal drug release devices (EAZI-BREED™ CIDR® Sheep and Goat Device, Active Constituent: 0.3g progesterone, in inert silicon elastomer. Pharmica and Upjohn; New South Wales) for twelve days. Ewes were exposed to vasectomized rams, fitted with harnesses and crayons, immediately after CIDR removal. Oestrus was detected at 7:00h, 12:00h and 17:00h over three consecutive days, commencing 24h following CIDR withdrawal, by recording crayon marked ewes.

All ewes detected in oestrus were inseminated via laparoscopy (day 0) with fresh semen collected from a single Poll Dorset ram (Ram identification: ALG 17). In total, 126 ewes were detected in oestrus and inseminated. The remaining ewes were exposed to the same ram (ALG 17) in the paddock for a further 24h.

3.2.3.2 Pregnancy and litter size determination

Pregnancy and litter size was determined by ultrasonography (Echo Camera SSD-500, 3.5MHz probe; Aloka Co Ltd, Tokyo, Japan) on day 44 of gestation. Non-pregnant ewes were subsequently removed from the experiment. Single and multiple bearing ewes were maintained on treatment levels, with live weights and condition scores monitored weekly and feed refusals collected and weighed daily.

3.2.3.3 Sampling schedule

Pregnant ewes were stratified within treatments on litter size and live weight and randomly allocated to three sample cohorts (Table 3.2).

Table 3.2: Actual number of pregnant ewes from three levels of feed intake allocated to the three sample cohorts (day 50, 92 and 133) during gestation (original allocation in parentheses).

	Maternal Feed Intake					
	Low		Medium		High	
Stage (days)	Single	Multiple	Single	Multiple	Single	Multiple
50	6 (7)	2 (2)	6 (5)	0 (1)	4 (10)	10 (4)
92	4 (7)	4 (4)	4 (5)	2 (1)	7 (8)	6 (5)
133	4 (7)	3 (4)	6 (5)	0 (1)	7 (8)	6 (5)

3.2.3.4 Euthanasia

After blood was sampled from the jugular vein (section 2.1.1), ewes were slaughtered by captive-bolt pistol and exsanguination on their allocated sample day. The intact reproductive tract was immediately recovered from sacrificed ewes. After blood was collected from the umbilical artery (section 2.1.1), the exposed fetus was euthanased with a sodium pentobarbitone overdose.

3.2.3.5 Fetal dissection

3.2.3.5.1 Day 50

Fetal weight, crown rump length, abdominal girth, thoracic girth, skull width, skull length, litter size and sex were recorded. The liver, kidneys, heart and lungs were removed, weighed and rapidly frozen at -160°C in liquid nitrogen. Individual muscles could not be identified at day 50, so a composite muscle sample, which incorporated the approximate location of the Semitendinosus and Semimembranosus muscles, was taken from both hind limbs. One sample, frozen by immersion in isopentane, cooled over liquid nitrogen, was wrapped in foil. The other sample was frozen in liquid nitrogen. Both samples were transferred to dry ice (-78.5°C) for not more than 8h and then stored at -80°C.

3.2.3.5.2 Day 92 and 133

Measurements and samples taken from fetuses on days 92 and 133 of gestation were the same as those recorded on day 50 with the following exceptions. Semitendinosus (ST), Longissimus dorsi (LD) and Supraspinatus (SS) muscle were dissected from the left and right side of the fetus. Muscles from the left side were frozen by immersion in

isopentane, cooled in liquid nitrogen, wrapped in labelled foil and stored at -80°C. Only day 133 muscles were weighed before freezing. Muscles from the right side were wrapped in foil and frozen directly in liquid nitrogen. Muscle samples were transferred to dry ice for the remainder of the day and then stored at -80°C.

3.2.3.6 Placental measurements

Individual placentomes were located and trimmed from the placental membranes. The total number of placentomes, weight of individual placentomes, and the number and weight of placentomes per fetus (in the case of multiple litters) were recorded. Placental weight, when mentioned throughout the text, refers to the aggregate weight of individual placentomes. The morphology of individual placentomes was characterised according to their level of eversion (Vatnick *et al.*, 1991) at day 92 and 133. Placentomes were classified as type A (inverted), B, C or D (increasing levels of eversion, respectively).

3.2.3.7 Maternal and fetal metabolic and hormone profiles

Plasma concentrations of glucose, albumin and urea (maternal and fetal) and NEFA, cholesterol and triglycerides (maternal only) were measured as indicators of nutritive status (section 2.1.2). The intra- and inter-assay coefficients of variation of all assays are presented in Table 3.3.

Plasma concentrations of fetal IGF-I and -II were determined (section 2.1.2.7) on a sub-population of fetuses, whose number, litter size, sex and weight are described in Tables 3.4 and 3.5.

Table 3.3: Coefficient of variation (CV%) of plasma metabolite and hormone assays.

	Assay CV (%)	
	Intra	Inter
Glucose	1.8	2.7
Albumin	7.3	2.8
Urea	6.2	4.9
NEFA	3.1	*
Cholesterol	2.9	*
Triglycerides	4.2	*
IGF-I	3.2	4.5
IGF-II	8.8	*

* one assay only

Table 3.4: Fetal population used for plasma IGF analysis.

	Maternal Feed Intake				
	Low		High		Total
Stage of gestation (days)	**1[a]**	**2[b]**	**1**	**2**	
92	4	5	3	7	19
133	4	6	5	4	19
Total	8	11	8	11	**38**
	M[c]	**F[d]**	**M**	**F**	**Total**
92	6	3	5	5	19
133	3	7	5	4	19
Total	9	10	10	9	**38**

[a]singleton fetus [b]twin fetus [c]male fetus [d]female fetus

Table 3.5: Weight of fetuses used for plasma IGF analysis. *all values are presented as least-squares means ± SEM.

Stage of gestation (days)	Maternal Feed Intake		Probability
	Low	High	p
92	649 ± 17* (n=9)	747 ± 17 (n=10)	0.0007
133	3576 ± 128 (n=10)	4445 ± 129 (n=9)	0.0002

3.2.4 Statistical analysis

Analysis of variance procedures (GLM procedure in SAS; Spector *et al.*, 1985) were used to interpret the significance of main effects and their first order interactions on all variables. Variables analysed included maternal live weight and condition score, placental characteristics, fetal dimensions, and plasma metabolite and IGF concentrations. Interactions that tended toward significance ($p<0.1$) and all main effects were retained in the final overall model. Descriptions of the final overall models are presented in Appendix 1. The main effects included maternal feed intake, stage of gestation, litter size and fetal sex. Non-significant interactions ($p>0.1$) were progressively removed from the model in order of least significance.

Data that were not normally distributed or exhibited uneven variances were log transformed before analysis. In some cases, data were segregated into three distinct populations based on stage of gestation. Further, due to unbalanced data amongst twins, specifically no maintenance twins at day 50 and 133, feed intake treatments were analysed independently for single and twin pregnancies at each stage of gestation. Proportional placentome morphology data were arcsin transformed before analysis. Different alphabetical superscripts in Tables and Figures indicate significant (lower case, $p<0.05$) or tendency to significant (upper case, $p<0.1$) differences between the main effects for the mean of the parameter compared.

Relationship coefficients (r) and associated probabilities (p) between maternal, placental and fetal parameters were established using Pearson product-moment correlations (CORR procedure in SAS; Delong and Yuan, 1985). Correlations between parameters were determined within each gestational stage.

3.3 Results

Details of the final experimental population available for analysis are presented in Table 3.6.

Table 3.6: Experimental population of ewes and fetuses at three stages of gestation. Ewes were offered different amounts of feed prior to and throughout pregnancy.

		Maternal Feed Intake			
	Stage	**Low**	**Medium**	**High**	**Overall**
Ewes (n)	**50**	8	6	14	
	92	8	6	13	
	133	7	6	13	
					81
Fetuses (n)	**50**	10	6	24	
	92	12	8	19	
	133	10	6	19	
					114
Litter Size	**50**	1.3 ± 0.2	1.0 ± 0.2	1.7 ± 0.1	
	92	1.5 ± 0.2	1.3 ± 0.2	1.5 ± 0.1	
	133	1.4 ± 0.2	1.0 ± 0.2	1.5 ± 0.1	
					1.41
Females (n) : Males (n)	**50**	2:8	4:2	14:10	
	92	4:8	2:6	9:10	
	133	7:3	3:3	10:9	
					55:59
Fetal Age (days)	**50**	49.6 ± 0.2	50.0 ± 0.2	50.0 ± 0.1	
	92	92.3 ± 0.2	92.5 ± 0.2	92.4 ± 0.1	
	133	133.1 ± 0.2	133.3 ± 0.2	133.6 ± 0.1	

3.3.1 Maternal live weight and condition score

Live weights and condition scores of H and L ewes at mating and on allocated day of slaughter differed (Table 3.7). Weight and condition scores of M ewes were intermediate with the L and H ewes, but were not always significantly different to these (Table 3.7). High ewes had gained 8% of their live weight by mating, while Low ewes had lost 14% of their weight; at mating, the weight of Low ewes was 79% of High ewes. Ewe live weight increased significantly between days 92 and 133 of gestation in all treatment groups, even though allowable intake remained fixed at initial treatment levels, and condition scores of treatment groups continued to diverge. Nevertheless, H ewes were nearly 40% heavier than their L counterparts at late gestation.

Ewe live weight at mating correlated positively with ewe condition score at mating ($r=0.78$; $p<0.001$) and ewe live weight ($r=0.86$; $p<0.001$) and condition score ($r=0.73$; $p<0.001$) on day of slaughter.

Table 3.7: Live weight and condition scores of ewes allocated to nutritional treatments (day -89), detected in oestrus and mated (day 0) and pregnant ewes slaughtered on allocated day of sampling (day 50, 92 or 133 cohorts). *values are least-squares means ± SEM. Different superscripts within rows indicate significant differences ($p<0.05$).

		Feed Intake		
	Stage	**Low**	**Medium**	**High**
Ewes (*n*)	**Allocated**	59	30	60
	Mated	47	27	55
	50	8	6	14
	92	8	6	13
	133	7	6	13
Live weight (kg)	**Allocated**	64.4 ± 0.7*	64.6 ± 1.0	64.5 ± 0.7
	Mated	55.4 ± 1.1^{a}	65.5 ± 1.5^{b}	69.8 ± 1.1^{c}
	50	55.7 ± 2.7^{a}	64.4 ± 3.2^{b}	73.9 ± 2.1^{c}
	92	57.5 ± 3.0^{a}	72.9 ± 3.5^{b}	80.1 ± 2.4^{b}
	133	61.6 ± 3.5^{a}	77.3 ± 3.7^{b}	86.1 ± 2.5^{b}
Condition score	**Allocated**	3.3 ± 0.1	3.2 ± 0.1	3.3 ± 0.1
	Mated	2.9 ± 0.1^{a}	3.2 ± 0.1^{b}	3.5 ± 0.1^{c}
	50	3.0 ± 0.1^{a}	3.3 ± 0.1^{a}	3.8 ± 0.1^{b}
	92	2.8 ± 0.1^{a}	3.5 ± 0.2^{b}	3.8 ± 0.1^{b}
	133	2.4 ± 0.1^{a}	3.1 ± 0.1^{b}	3.9 ± 0.1^{c}

3.3.2 Maternal metabolic profile

The effect of maternal feed intake and stage of gestation, derived from the overall model (section 3.2.4.4), on the concentration of metabolites in the maternal circulation are presented in Table 3.8. Maternal plasma glucose and albumin concentration were reduced in nutrient restricted ewes (Table 3.8). Litter size had no affect on maternal plasma glucose, albumin, urea, cholesterol and triglyceride concentration, however NEFA concentration was elevated in twin bearing ewes (Appendix 1). The effect of feed intake on maternal metabolites at each gestational age is presented in Figures 3.3-3.8.

Table 3.8: The effect of feed intake and stage of gestation on the plasma metabolite concentration of pregnant ewes. *values are marginal least-squares means ± SEM. Different superscripts within rows and main effects indicate significant differences ($p<0.05$).

	Feed Intake			Stage of gestation (day)		
	Low	Medium	High	50	92	133
Glucose (mmol /L)	$4.2 \pm 0.3^{a*}$	5.2 ± 0.3^{b}	5.2 ± 0.2^{b}	5.9 ± 0.2^{a}	5.2 ± 0.2^{a}	3.5 ± 0.3^{b}
Albumin (g /L)	35.6 ± 0.8^{a}	39.5 ± 0.9^{b}	40.7 ± 0.6^{b}	38.3 ± 0.7	38.9 ± 0.7	38.5 ± 0.8
Urea (mmol /L)	4.4 ± 0.2	4.6 ± 0.3	4.5 ± 0.2	4.0 ± 0.2^{a}	4.3 ± 0.2^{a}	5.3 ± 0.2^{b}
NEFA (meq /L)	1.1 ± 0.1^{a}	0.6 ± 0.2^{b}	1.1 ± 0.1^{a}	0.7 ± 0.1^{a}	0.9 ± 0.1^{a}	1.2 ± 0.1^{b}
Cholesterol (mmol /L)	1.8 ± 0.1^{ab}	1.6 ± 0.1^{a}	1.9 ± 0.1^{b}	1.5 ± 0.1^{a}	1.9 ± 0.1^{b}	1.8 ± 0.1^{b}
Triglycerides (mmol /L)	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0

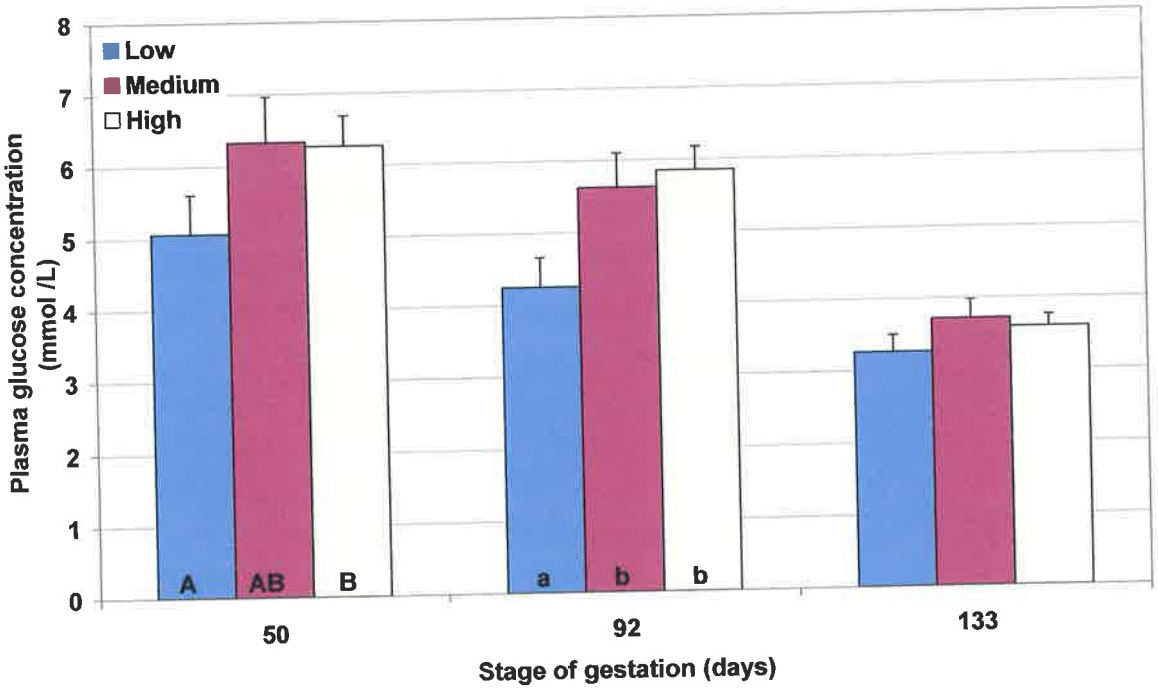


Figure 3.3: The effect of feed intake on maternal plasma glucose concentration at three stages of pregnancy. Different letters at each stage of gestation indicate significant differences between maternal feed intake treatments (lowercase, $p<0.05$; uppercase $p<0.1$).

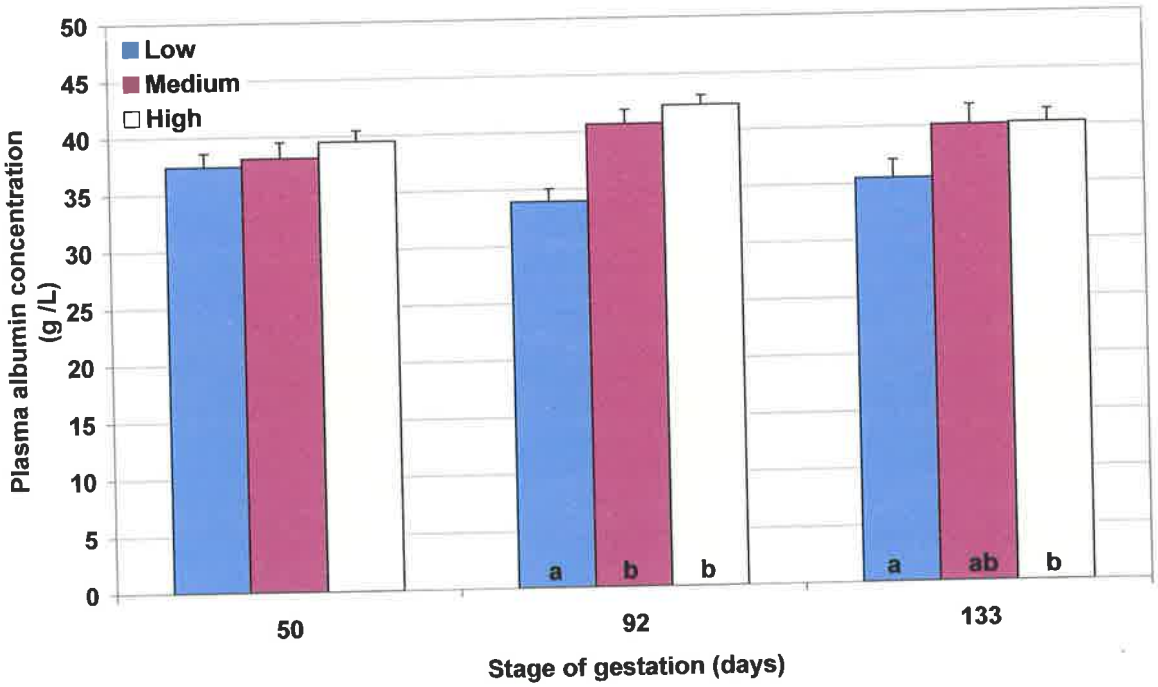


Figure 3.4: The effect of feed intake on maternal plasma albumin concentration at three stages of pregnancy. Different letters at each stage of gestation indicate significant differences between maternal feed intake treatments ($p<0.05$).

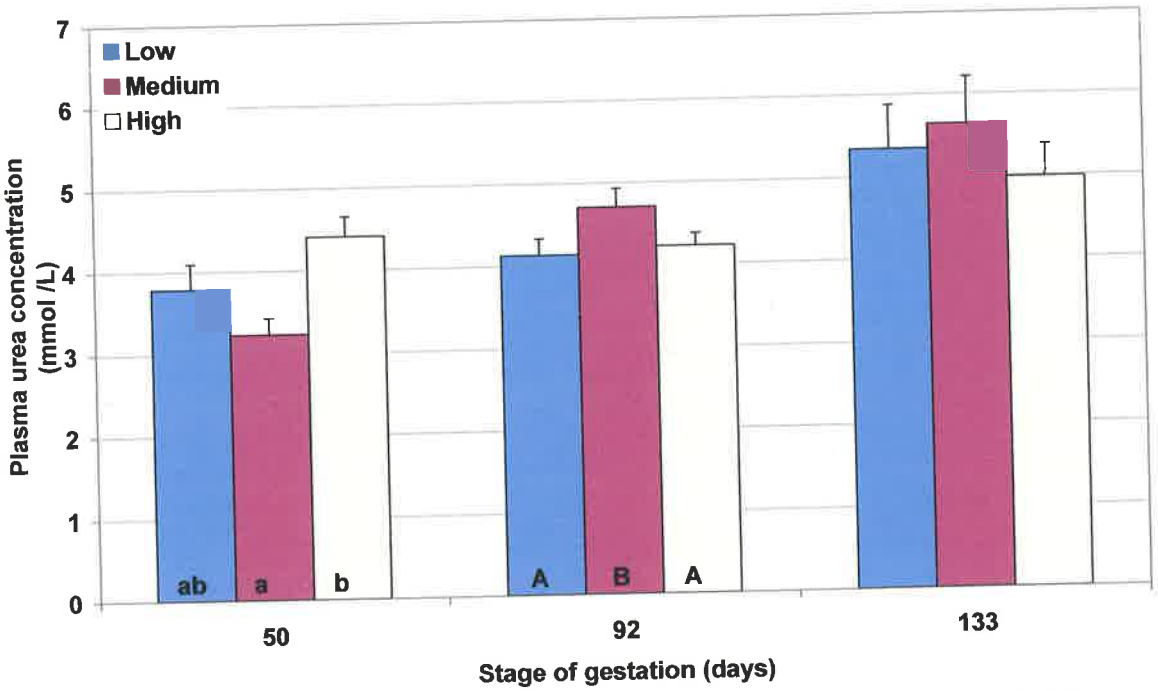


Figure 3.5: The effect of feed intake on maternal plasma urea concentration at three stages of pregnancy. Different letters at each stage of gestation indicate significant differences between maternal feed intake treatments (lowercase, $p<0.05$; uppercase $p<0.1$).

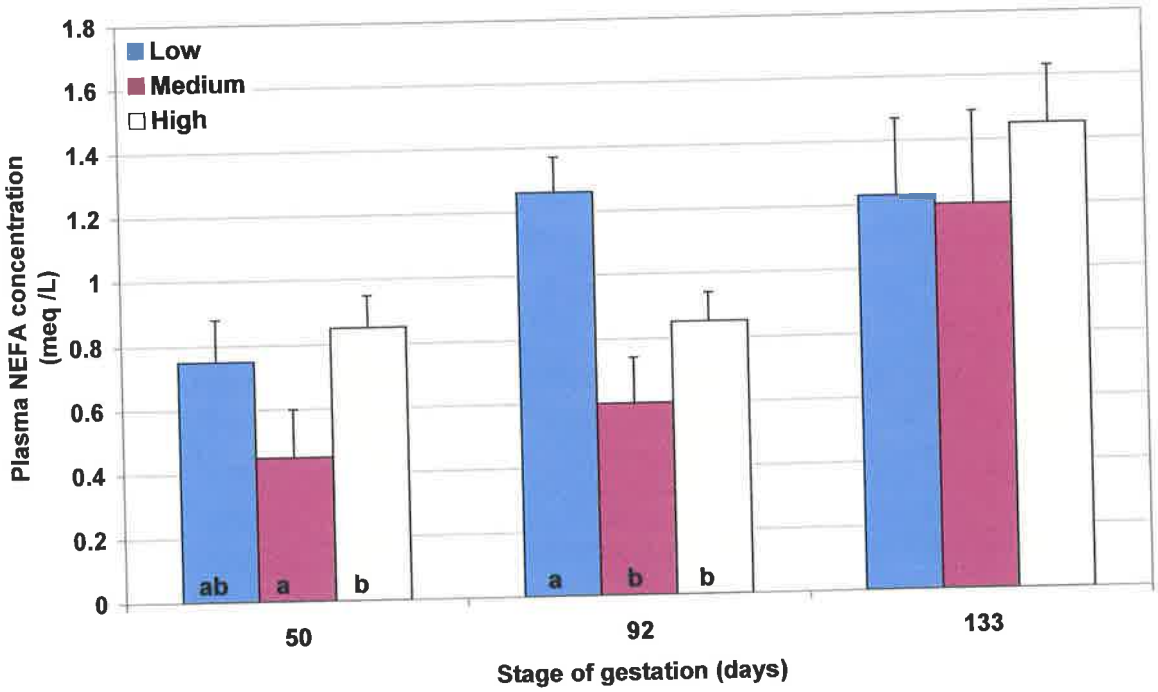


Figure 3.6: The effect of feed intake on maternal plasma NEFA concentration at three stages of pregnancy. Different letters at each stage of gestation indicate significant differences between maternal feed intake treatments ($p<0.05$).

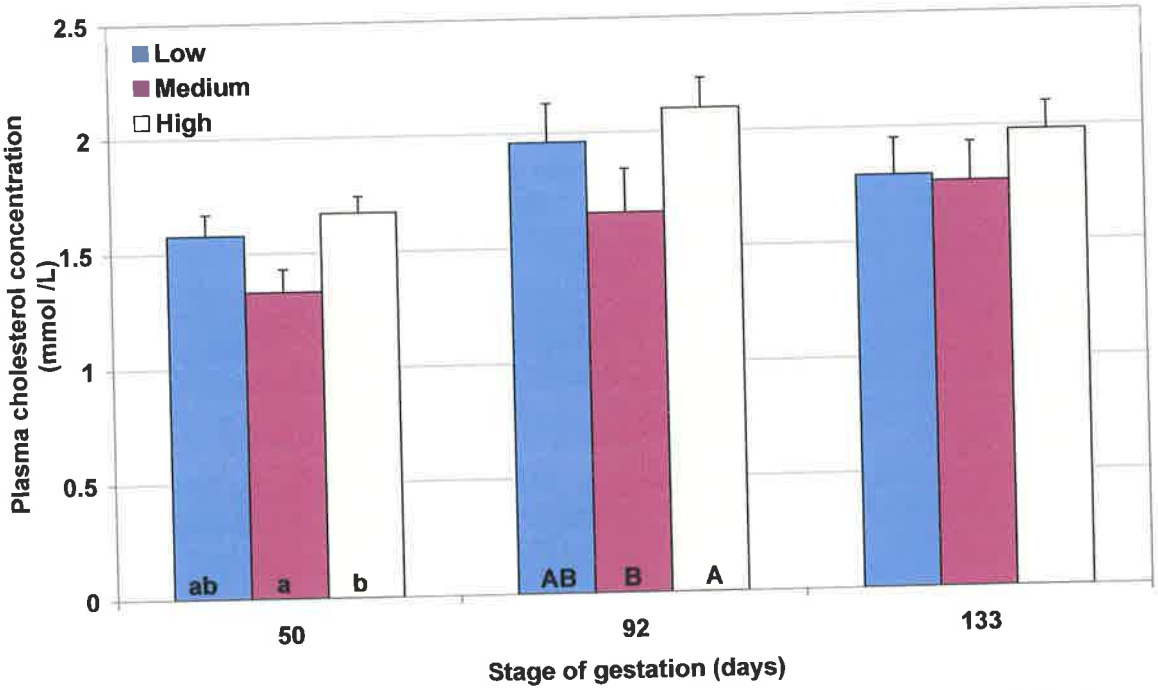


Figure 3.7: The effect of feed intake on maternal plasma cholesterol concentration at three stages of pregnancy. Different letters at each stage of gestation indicate significant differences between maternal feed intake treatments (lowercase, $p<0.05$; uppercase $p<0.1$).

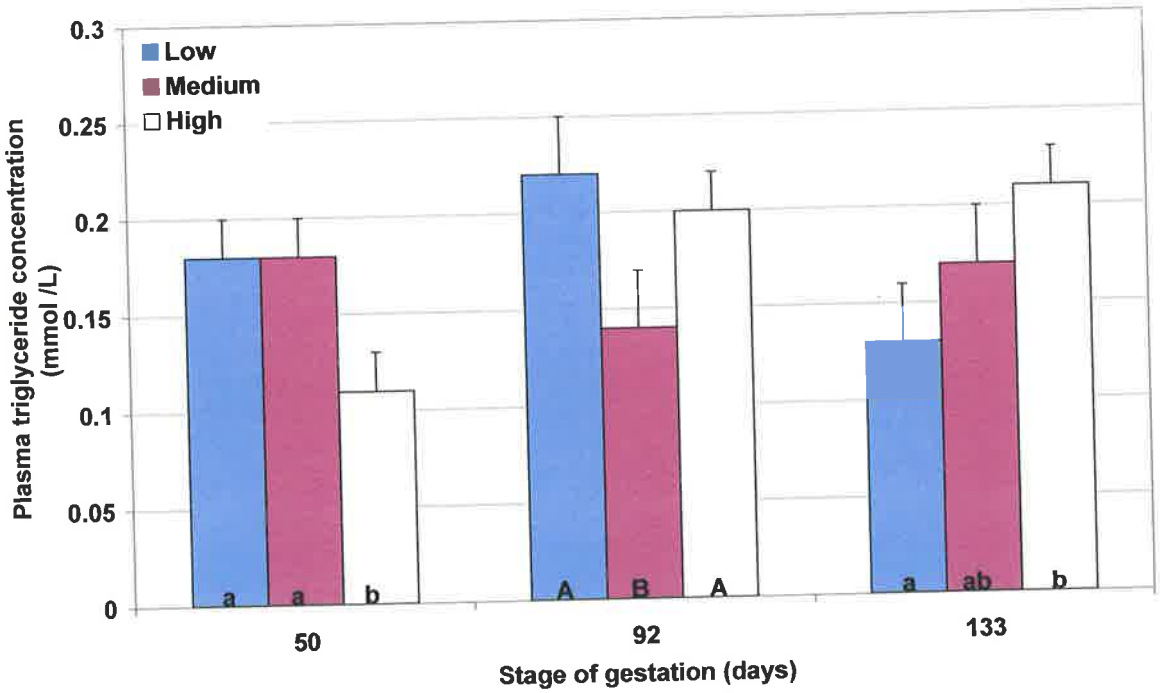


Figure 3.8: The effect of feed intake on maternal plasma triglyceride concentration at three stages of pregnancy. Different letters at each stage of gestation indicate significant differences between maternal feed intake treatments (lowercase, $p<0.05$; uppercase $p<0.1$).

3.3.3 Placental characteristics

Total placentome weight and mean weight of individual placentomes were greatest at day 92, while the number of placentomes remained relatively constant at the different stages of pregnancy (Appendix 1). The combined placental weights from twin bearing pregnancies were significantly heavier than singleton bearing pregnancies at day 50 ($p<0.001$), 92 ($p<0.05$) and 133 ($p<0.001$) of gestation. This resulted from heavier individual placentomes at day 50 ($p<0.001$) and a greater number of individual placentomes at day 92 and 133 ($p<0.001$ and $p<0.05$, respectively).

Given the significant effect of litter size on placental characteristics, the data were re-analysed to interpret the effect of maternal feed intake on placental characteristics within litter size groups. Maternal feed intake had little influence on placentome number, placentome weight (Figures 3.9 and 3.10), individual placentome weight and fetal:placental weight ratio for both single and twin bearing ewes at each stage of gestation (Appendix 1). Further, intake had no influence on placentome morphology of single bearing ewes (Appendix 1). However, a greater proportion of placentomes were everted in twin bearing ewes offered a restricted intake ($p<0.05$) compared with H ewes (Appendix 1).

Total placental weight at day 50 tended to correlate with ewe live weight at mating ($p<0.1$) and at day 50 ($p=0.052$) but at later stages of gestation placental weight was not related to either ewe weight at mating or slaughter. Mean weight of individual placentomes at day 50 and 133, but not day 92, correlated with ewe live weight at mating ($p<0.05$).

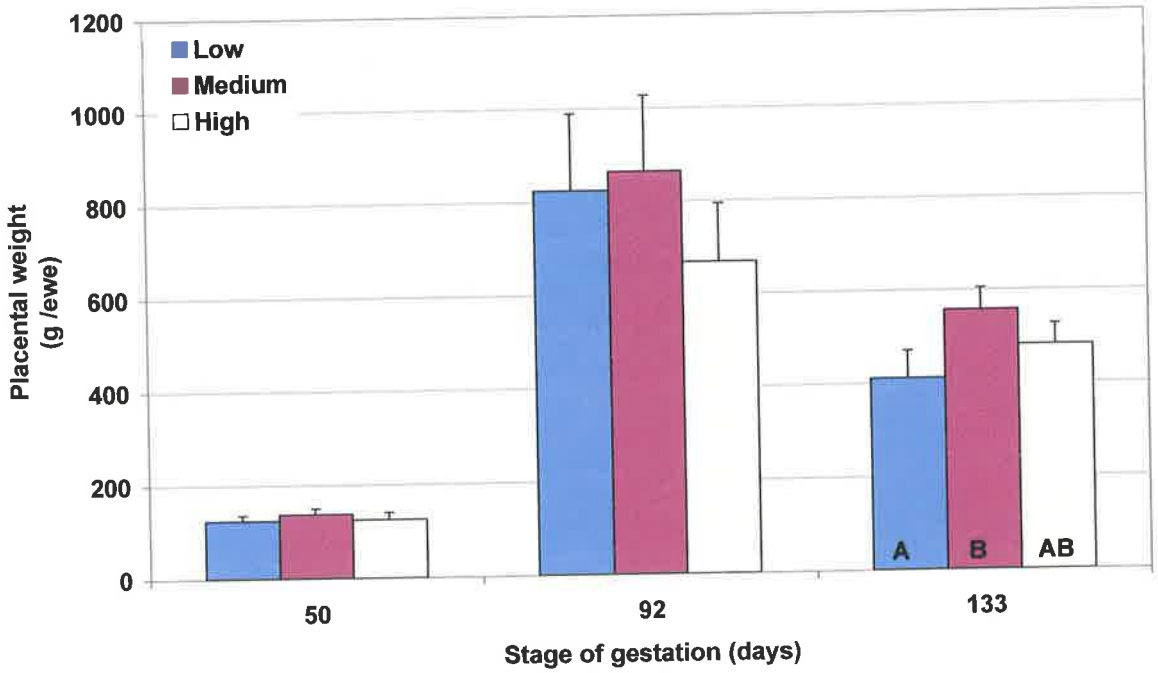


Figure 3.9: Total placentome weight in ewes with singleton pregnancies at three stages of gestation. Different letters at each stage of gestation indicate tendency toward significant differences between maternal feed intake treatments ($p<0.1$).

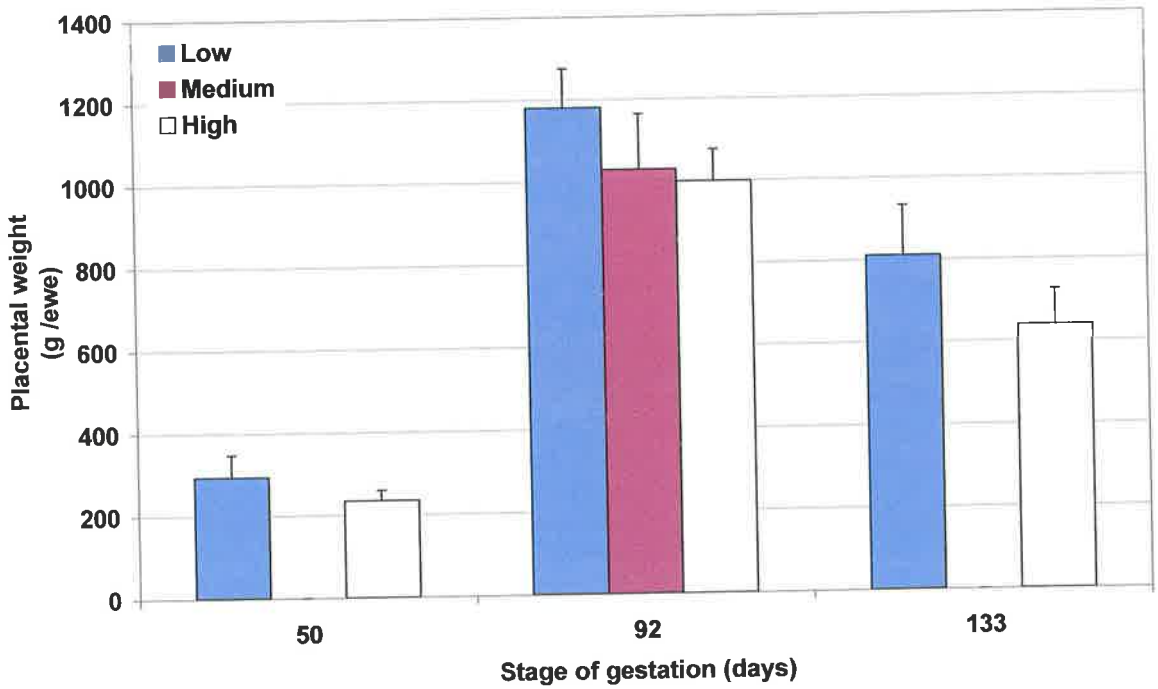


Figure 3.10: Total placentome weight in ewes with twin pregnancies at three stages of gestation.

3.3.4 Fetal body dimensions

Weight of singleton fetuses from High feed intake ewes were greater than singleton fetuses from Low feed intake ewes at each stage of gestation, although only significantly at day 133 ($p<0.05$, Table 3.9). By day 133 of gestation it appeared that Low fetuses were lighter and thinner (smaller abdominal and thoracic girths) but of a similar length to High fetuses (Table 3.9). High feed intake tended to increase weight of twin fetuses, compared to those from restricted ewes at day 92 and 133 of gestation ($p<0.1$; Table 3.10). Fetal sex had no affect on most parameters measured, with the exception of fetal weight and thoracic girth of singleton fetuses at 133 days of gestation, which were greater for males than females ($p=0.04$ and $p=0.05$, respectively).

Fetal weight was correlated positively with total placentome weight (Figure 3.11) and average placentome weight ($r=0.51$, $p<0.01$) but not with placentome number, at day 50 of gestation. Fetal and placental parameters were unrelated at day 92 of gestation (Figure 3.11). Fetal weight correlated positively with both the number of placentomes ($r=0.43$, $p<0.01$) and total placentome weight (Figure 3.11) at day 133 of gestation.

3.3.5 Development of fetal organs

The absolute and relative weights of lungs of Low singleton fetuses were significantly less than High singleton fetuses (Table 3.11) at day 50 of gestation. There was no evidence that restricted maternal feed intake affected the weights of organs of singleton fetuses at day 92 of gestation. However, absolute and relative liver weights of Low twin fetuses were increased compared with High twin fetuses (Table 3.12). At day 133 absolute liver and lung weights of singleton fetuses were reduced in response to restricted maternal feed intake, while the brain was disproportionately larger, compared with High singleton fetuses (Table 3.11). Low twin fetuses had proportionately larger livers and kidneys than High twin fetuses (Table 3.12). Fetal liver and brain weights, as a proportion of fetal weight, correlated positively and negatively with placental weight at day 133 of gestation, respectively (Figure 3.12), indicative of asymmetric growth retardation.

Table 3.9: The effect of maternal feed intake on body dimensions of singleton fetuses at three stages of gestation. * values are least-squares means ± SEM. Different superscripts within rows indicate significant differences (lowercase, $p<0.05$; uppercase, $p<0.1$).

		Maternal Feed Intake		
	Stage	Low	Medium	High
Fetuses (*n*)	**50**	6	6	4
	92	4	4	7
	133	4	6	7
Fetal weight (g)	**50**	16.0 ± 0.7*	17.3 ± 0.7	18.0 ± 1.0
	92	680.9 ± 26.5	695.8 ± 26.5	685.3 ± 20.0
	133	3716.9 ± 259.9^{a}	4333.0 ± 205.8^{ab}	4565.4 ± 191.4^{b}
Crown-rump length (mm)	**50**	97.1 ± 2.9	101.9 ± 2.9	104.5 ± 3.6
	92	332.0 ± 14.4	334.8 ± 14.4	336.9 ± 10.9
	133	513.0 ± 12.5^{a}	552.7 ± 10.2^{b}	536.1 ± 9.5^{ab}
Abdominal girth (mm)	**50**	56.7 ± 2.1	56.0 ± 2.1	58.5 ± 2.6
	92	202.0 ± 6.0	188.5 ± 6.0	198.7 ± 4.6
	133	319.3 ± 11.3^{a}	343.0 ± 9.3^{ab}	349.9 ± 8.6^{b}
Thoracic girth (mm)	**50**	54.3 ± 1.2	55.8 ± 1.2	55.3 ± 1.4
	92	187.8 ± 3.2	194.3 ± 3.2	191.9 ± 2.4
	133	331.7 ± 10.5^{a}	345.8 ± 8.3^{ab}	367.4 ± 7.7^{b}
Skull width (mm)	**50**	14.7 ± 0.4^{A}	15.7 ± 0.4^{AB}	15.9 ± 0.5^{B}
	92	43.8 ± 0.7	44.0 ± 0.8	45.2 ± 0.5
	133	61.8 ± 1.2	62.9 ± 1.0	63.6 ± 0.9
Skull length (mm)	**50**	24.5 ± 0.3^{a}	25.2 ± 0.3^{a}	26.4 ± 0.4^{b}
	92	78.4 ± 1.1^{a}	81.5 ± 1.3^{ab}	81.5 ± 0.8^{b}
	133	117.6 ± 2.1^{A}	123.3 ± 1.7^{B}	121.5 ± 1.6^{AB}

Table 3.10: The effect of maternal feed intake on body dimensions of twin fetuses at three stages of gestation. * values are least-squares means ± SEM. Different superscripts within rows indicate significant differences (lowercase, $p<0.5$; uppercase, $p<0.1$).

		Maternal Feed Intake		
	Stage	Low	Medium	High
Fetuses (n)	**50**	4	.	20
	92	8	4	12
	133	6	.	12
Fetal weight (g)	**50**	18.7 ± 1.0*	.	18.1 ± 0.5
	92	660.6 ± 21.8^{A}	688.8 ± 30.8^{AB}	712.7 ± 17.6^{B}
	133	3385.7 ± 126.4^{A}	.	3676.2 ± 89.4^{B}
Crown-rump length (mm)	**50**	103.3 ± 3.0	.	105.5 ± 1.3
	92	345.0 ± 11.6	317.3 ± 16.3	320.2 ± 9.4
	133	514.7 ± 8.5	.	525.3 ± 6.3
Abdominal girth (mm)	**50**	61.0 ± 2.3	.	58.7 ± 1.0
	92	194.9 ± 5.0^{ab}	182.3 ± 7.1^{a}	201.7 ± 4.1^{b}
	133	309.2 ± 7.4	.	314.9 ± 5.2
Thoracic girth (mm)	**50**	56.9 ± 1.6	.	59.1 ± 0.7
	92	187.8 ± 2.0^{a}	185.8 ± 2.8^{a}	196.0 ± 1.6^{b}
	133	331.2 ± 3.5	.	334.3 ± 2.5
Skull width (mm)	**50**	15.3 ± 0.6	.	15.3 ± 0.3
	92	44.3 ± 0.5^{AB}	43.4 ± 0.8^{A}	44.9 ± 0.4^{B}
	133	59.4 ± 0.7	.	60.5 ± 0.5
Skull length (mm)	**50**	25.3 ± 0.6	.	25.5 ± 0.3
	92	80.7 ± 1.0^{AB}	78.6 ± 1.5^{A}	82.0 ± 0.8^{B}
	133	115.7 ± 1.5^{A}	.	118.9 ± 1.1^{B}

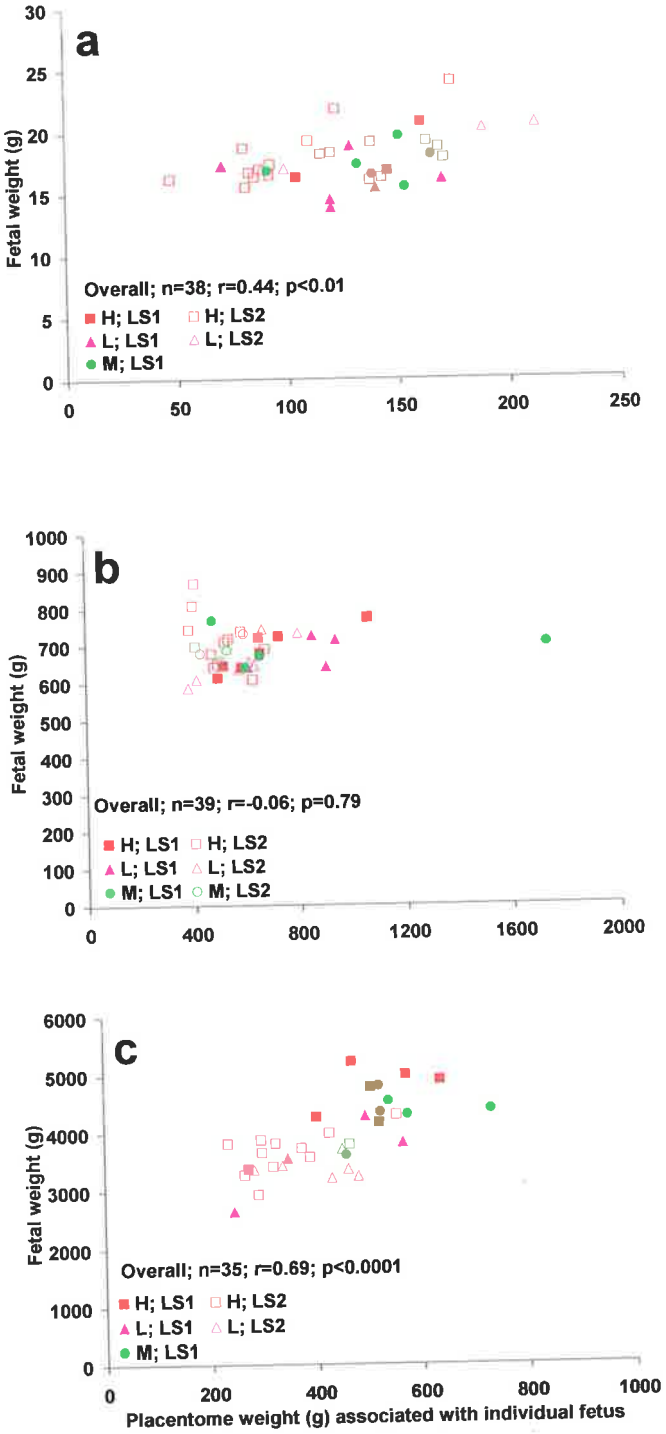


Figure 3.11: Relationship between placental weight and fetal weight of sheep at day 50 (a), 92 (b) and 133 (c) of gestation. High (H), Maintenance (M) and Low (L) maternal feed intake. Singleton (LS1) and twin (LS2) fetuses.

Table 3.11: The effect of maternal feed intake on absolute and relative organ weights of singleton fetuses at three stages of gestation. * values are least-squares means ± SEM. Different superscripts within rows indicate significant differences (lowercase, $p<0.05$; uppercase, $p<0.1$).

	Stage	Maternal Feed Intake			Maternal Feed Intake		
		Low	Medium	High	Low	Medium	High
Fetuses (n)	50	6	6	4	6	6	4
	92	4	4	7	4	4	7
	133	4	6	7	4	6	7
		Absolute Weight (g)			Relative Weight (g /kg fetal weight)		
Liver	50	1.4 ± 0.1*	1.5 ± 0.1	1.3 ± 0.1	90.7 ± 5.6	88.3 ± 5.6	75.3 ± 7.9
	92	43.4 ± 2.5	41.6 ± 2.5	42.9 ± 1.9	64.9 ± 3.4	60.6 ± 3.4	61.7 ± 2.6
	133	108.0 ± 9.3^{a}	143.4 ± 7.4^{b}	133.9 ± 6.9^{b}	27.9 ± 1.7^{a}	33.3 ± 1.4^{b}	28.8 ± 1.3^{a}
Heart	50	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	10.5 ± 0.5	9.4 ± 0.5	10.4 ± 0.7
	92	6.8 ± 0.6	6.2 ± 0.6	6.7 ± 0.5	10.5 ± 1.0	9.4 ± 1.0	9.4 ± 0.8
	133	30.2 ± 3.2	33.7 ± 2.5	35.1 ± 2.3	8.0 ± 0.5	7.8 ± 0.4	7.6 ± 0.4
Lung	50	0.6 ± 0.1^{a}	0.6 ± 0.1^{a}	0.8 ± 0.1^{b}	35.4 ± 2.2^{a}	35.0 ± 2.2^{a}	47.3 ± 2.6^{b}
	92	33.4 ± 1.4	34.9 ± 1.4	34.0 ± 1.0	49.2 ± 1.4	50.2 ± 1.4	49.6 ± 1.0
	133	127.2 ± 12.3^{a}	161.1 ± 10.1^{ab}	164.4 ± 9.3^{b}	36.2 ± 3.4	37.3 ± 2.8	36.9 ± 2.6
Kidney	50	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	11.5 ± 0.8	11.5 ± 0.8	11.0 ± 1.2
	92	9.9 ± 0.6	9.8 ± 0.6	9.8 ± 0.5	14.6 ± 0.7	14.1 ± 0.7	14.3 ± 0.5
	133	19.2 ± 1.8^{A}	23.3 ± 1.4^{B}	22.2 ± 1.3^{AB}	5.1 ± 0.4	5.4 ± 0.4	4.8 ± 0.3
Brain	50	.	.	.	.	.	.
	92	15.8 ± 0.5^{AB}	16.3 ± 0.5^{A}	14.9 ± 0.5^{B}	23.7 ± 1.4	23.7 ± 1.4	20.9 ± 1.3
	133	56.6 ± 2.1	56.0 ± 1.5	55.2 ± 1.4	15.1 ± 0.9^{a}	13.0 ± 0.7^{ab}	12.3 ± 0.7^{b}
Brain:Liver	50	.	.	.	.	.	.
	92	0.4 ± 0.0	0.4 ± 0.0	0.3 ± 0.0	.	.	.
	133	0.5 ± 0.1^{a}	0.4 ± 0.0^{b}	0.4 ± 0.0^{ab}	.	.	.

Table 3.12: The effect of maternal feed intake on absolute and relative organ weights of twin fetuses at three stages of gestation. * values are least-squares means ± SEM. Different superscripts within rows indicate significant differences (lowercase, $p<0.05$; uppercase, $p<0.1$).

		Maternal Feed Intake			Maternal Feed Intake		
	Stage	Low	Medium	High	Low	Medium	High
Fetuses (n)	50	4	.	20	4	.	20
	92	8	4	12	8	4	12
	133	6	.	12	6	.	12
		Absolute Weight (g)			Relative Weight (g /kg fetal weight)		
Liver	50	1.6 ± 0.1*	.	1.4 ± 0.1	83.2 ± 3.7	.	78.6 ± 1.7
	92	46.3 ± 1.7^{a}	42.1 ± 2.4^{ab}	41.6 ± 1.4^{b}	70.7 ± 2.0^{a}	61.0 ± 2.9^{b}	58.3 ± 1.6^{b}
	133	95.3 ± 4.9	.	90.0 ± 3.5	28.2 ± 0.8^{a}	.	24.4 ± 0.6^{b}
Heart	50	0.2 ± 0.0	.	0.2 ± 0.0	10.7 ± 0.8	.	10.0 ± 0.4
	92	6.3 ± 0.3	5.7 ± 0.4	6.5 ± 0.2	9.7 ± 0.4	8.4 ± 0.6	9.2 ± 0.3
	133	28.8 ± 1.7	.	28.7 ± 1.2	8.5 ± 0.4	.	7.8 ± 0.3
Lung	50	0.7 ± 0.1	.	0.7 ± 0.1	35.3 ± 4.4	.	39.3 ± 2.2
	92	31.3 ± 1.4	33.1 ± 1.9	34.2 ± 1.1	47.3 ± 1.4	48.0 ± 2.0	48.0 ± 1.1
	133	135.5 ± 7.7	.	131.2 ± 5.5	40.2 ± 2.2	.	35.8 ± 1.6
Kidney	50	0.2 ± 0.0	.	0.2 ± 0.0	10.1 ± 0.9	.	11.6 ± 0.5
	92	9.8 ± 0.3	9.8 ± 0.5	10.1 ± 0.3	14.9 ± 0.4	14.2 ± 0.5	14.1 ± 0.3
	133	18.5 ± 0.7	.	17.4 ± 0.5	5.5 ± 0.3^{a}	.	4.8 ± 0.2^{b}
Brain	50	.	.	.	.	.	.
	92	15.6 ± 0.7	16.0 ± 1.0	16.8 ± 0.6	23.7 ± 1.1	23.4 ± 1.5	23.7 ± 0.9
	133	50.3 ± 1.6	.	53.2 ± 1.1	14.9 ± 0.5	.	14.5 ± 0.3
Brian: Liver	50	.	.	.	.	.	.
	92	0.3 ± 0.0^{a}	0.4 ± 0.0^{ab}	0.4 ± 0.0^{b}	.	.	.
	133	0.5 ± 0.0^{a}	.	0.6 ± 0.0^{b}	.	.	.

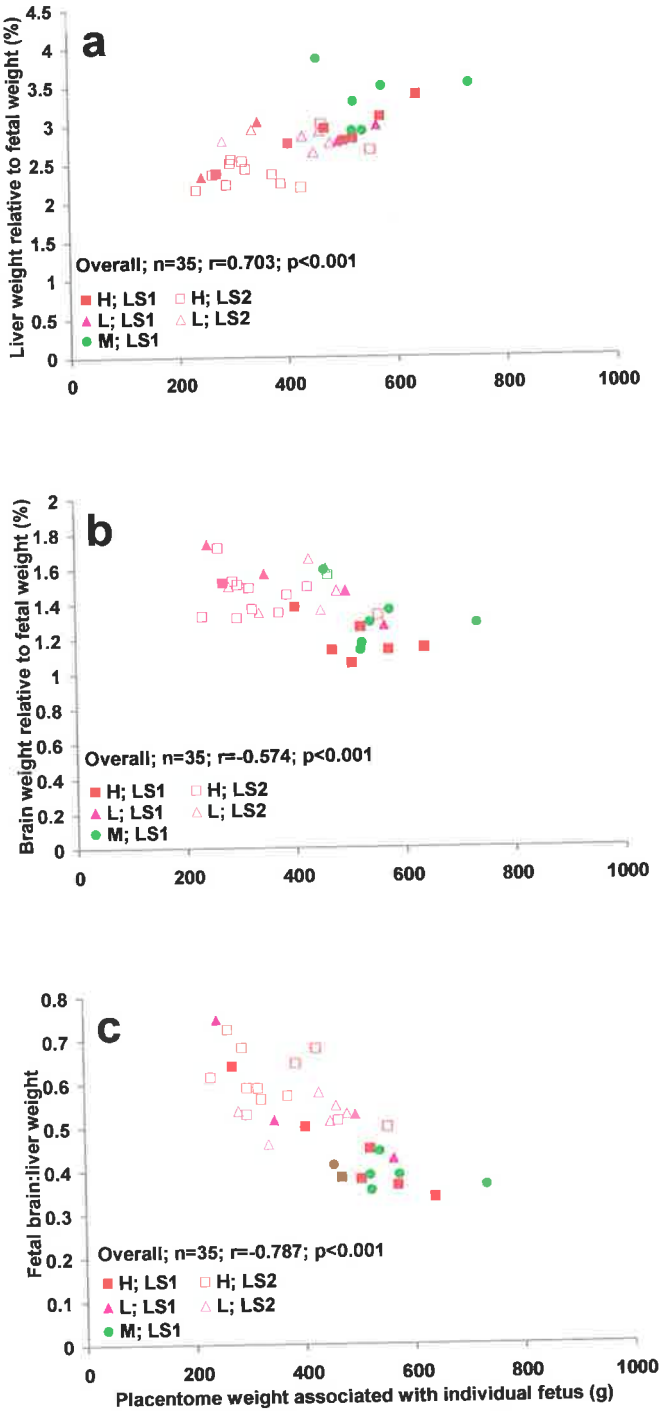


Figure 3.12: Relationship between placental weight and fractional weight of fetal liver (a) and brain (b) and fetal brain: liver ratio (c) at day 133 of gestation. High (H), Maintenance (M) and Low (L) maternal feed intake. Singleton (LS1) and twin (LS2) fetuses.

3.3.6 Fetal metabolic profile

The effect of maternal feed intake and stage of gestation, derived from the overall model (section 3.2.4.4), on the metabolites in the fetal circulation are presented in Table 3.13. Fetal plasma glucose ($p<0.05$), urea ($p<0.05$) and albumin ($p<0.1$) concentrations were all lower in fetuses whose mothers feed intake was restricted (Table 3.13). Fetal plasma glucose concentration decreased ($p<0.05$) and fetal plasma albumin concentration increased ($p<0.05$) as stage of gestation progressed (Table 3.13). Litter size had no affect on plasma albumin and urea concentration, however plasma glucose concentration was significantly lower in twin fetuses (Appendix 1). The effect of feed intake on fetal plasma metabolites at each stage of gestation is presented in Figures 3.13 – 3.15. In general, fetal plasma concentrations of glucose, albumin and urea were not correlated with fetal body dimensions and tissue growth at days 92 and 133 of gestation, the only exception was that fetal plasma glucose correlated positively with fetal weight at day 133 ($r=0.34$, $p<0.05$). Fetal plasma glucose concentration also correlated positively with the number of placentomes ($r=0.36$, $p<0.05$) and placentome weight ($r=0.36$, $p<0.05$) at day 133.

3.3.7 Fetal plasma IGF-I and IGF-II

Fetal plasma IGF-I ($p<0.01$) and IGF-II ($p<0.1$) concentrations increased between day 92 and day 133 of gestation. This was due to the significantly elevated IGF-I in High fetuses compared with Low fetuses at late gestation ($p<0.01$). Plasma IGF-I was not significantly different between High and Low fetuses at day 92 (Figure 3.16). Singleton fetuses had higher plasma IGF-I than twin fetuses ($p<0.05$) but there was no difference in plasma IGF-I between male and female fetuses. Fetal plasma IGF-II concentration was unaffected by maternal nutrition (Figure 3.17), litter size and fetal sex.

There were no relationships between fetal plasma IGF-I and IGF-II and fetal weight, placental weight, liver weight and plasma glucose at day 92 of gestation. However, by late gestation (day 133) fetal weight, placental weight, liver weight, brain weight and plasma glucose were all correlated positively with fetal plasma IGF-I (Figure 3.18). No significant relationships existed between fetal plasma IGF-II and fetal or placental parameters at late gestation.

Table 3.13: The effect of maternal feed intake and stage of gestation on fetal plasma metabolite concentration. *values are marginal least-squares means ± SEM. Different superscripts within rows and main effects indicate significant differences (lowercase, $p<0.05$; uppercase, $p<0.1$).

	Maternal Feed Intake			Stage of gestation (days)	
	Low	Medium	High	92	133
Glucose (mmol /L)	$0.7 \pm 0.1^{a*}$	0.7 ± 0.2^{ab}	1.1 ± 0.1^{b}	1.3 ± 0.1^{a}	0.4 ± 0.1^{b}
Albumin (g /L)	24.2 ± 0.8^{A}	25.2 ± 1.1^{AB}	26.0 ± 0.7^{B}	20.3 ± 0.7^{a}	30.0 ± 0.7^{b}
Urea (mmol /L)	5.4 ± 0.3^{a}	5.8 ± 0.3^{ab}	6.0 ± 0.2^{b}	5.5 ± 0.2	6.0 ± 0.2

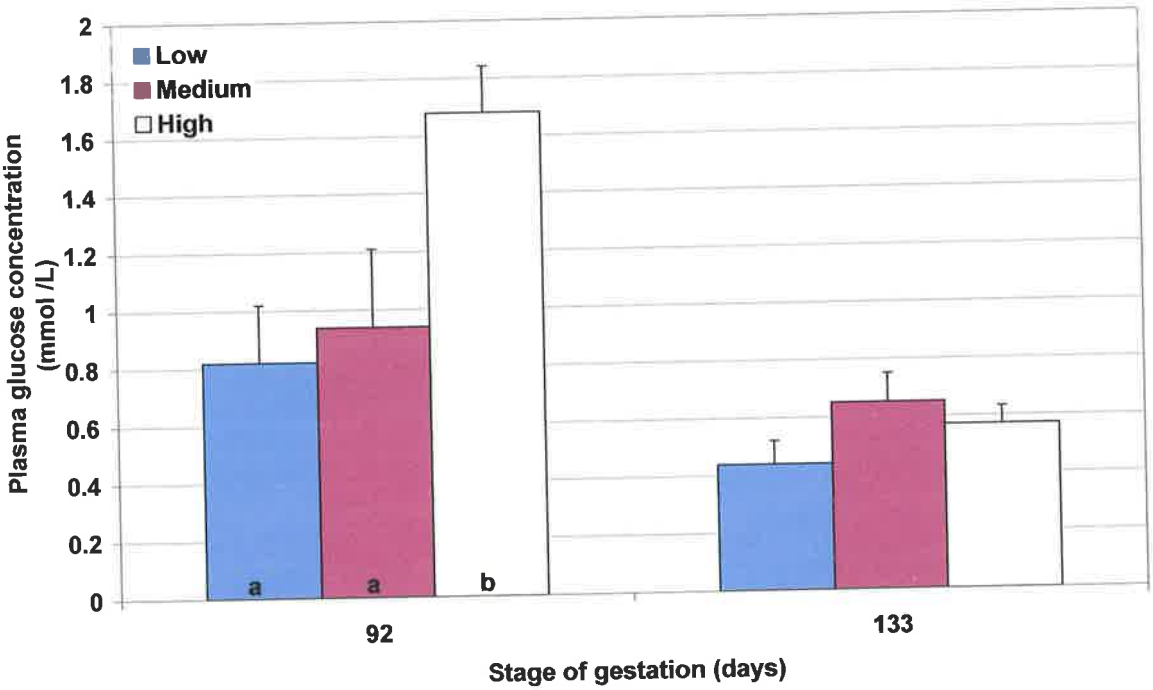


Figure 3.13: The effect of maternal feed intake on plasma glucose concentration in the fetal circulation at mid- and late gestation. Different letters at each stage of gestation indicate significant differences between maternal feed intake treatments ($p<0.05$).

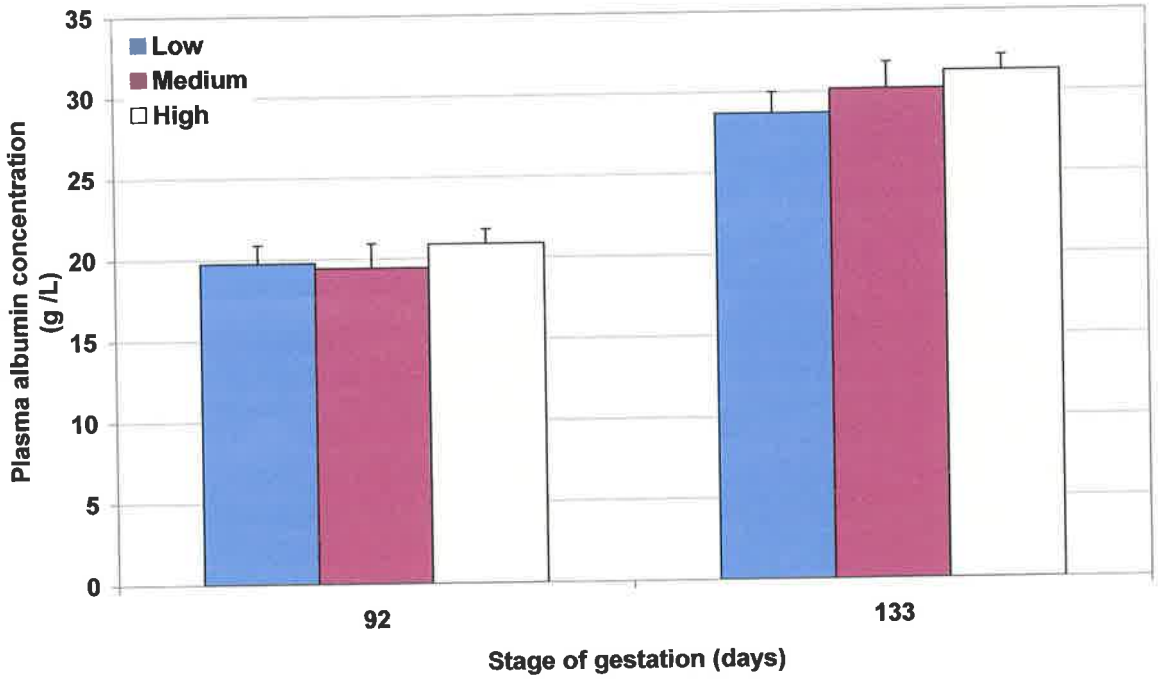


Figure 3.14: The effect of maternal feed intake on plasma albumin concentration in the fetal circulation at mid- and late gestation.

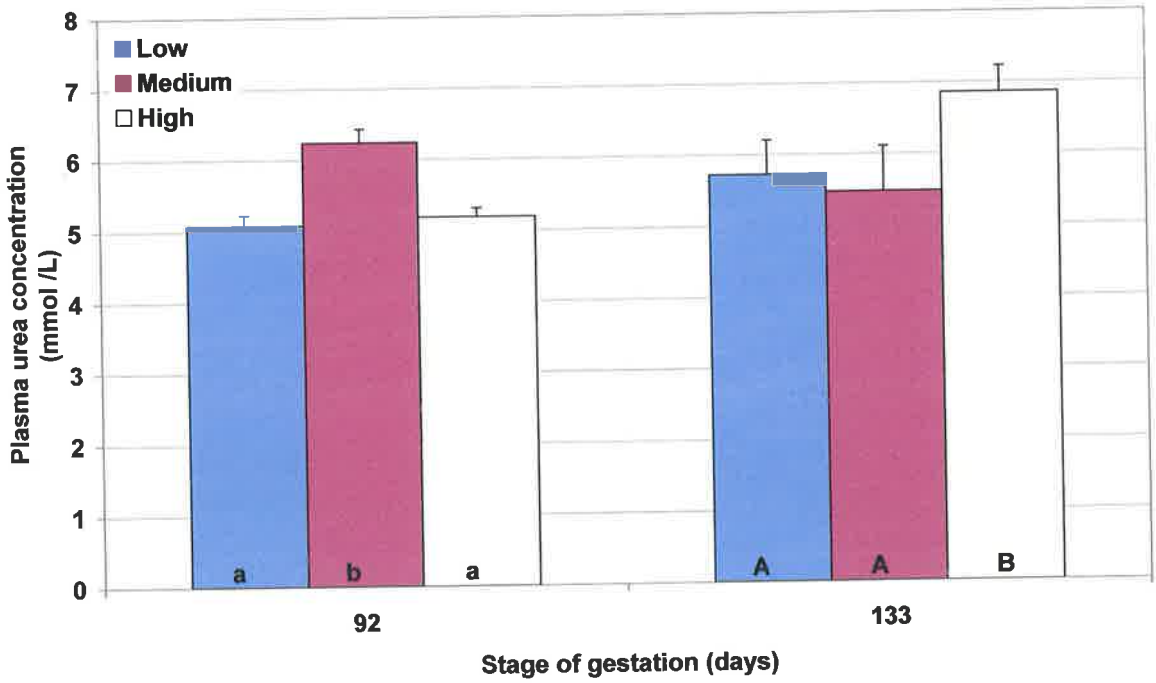


Figure 3.15: The effect of maternal feed intake on plasma urea concentration in the fetal circulation at mid- and late gestation. Different letters at each stage of gestation indicate significant differences between maternal feed intake treatments (lowercase $p<0.05$; uppercase $p<0.1$).

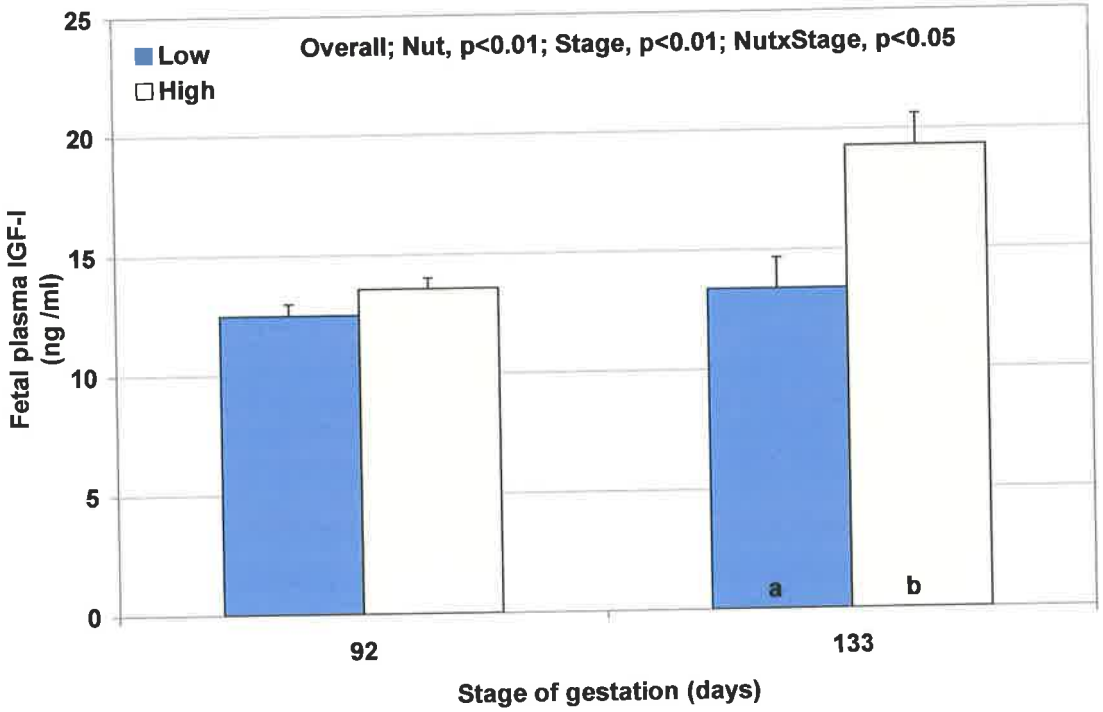


Figure 3.16: The effect of maternal feed intake on the plasma concentration of IGF-I in the fetal circulation at mid- and late gestation. Different letters at each stage of gestation indicate significant differences ($p<0.05$).

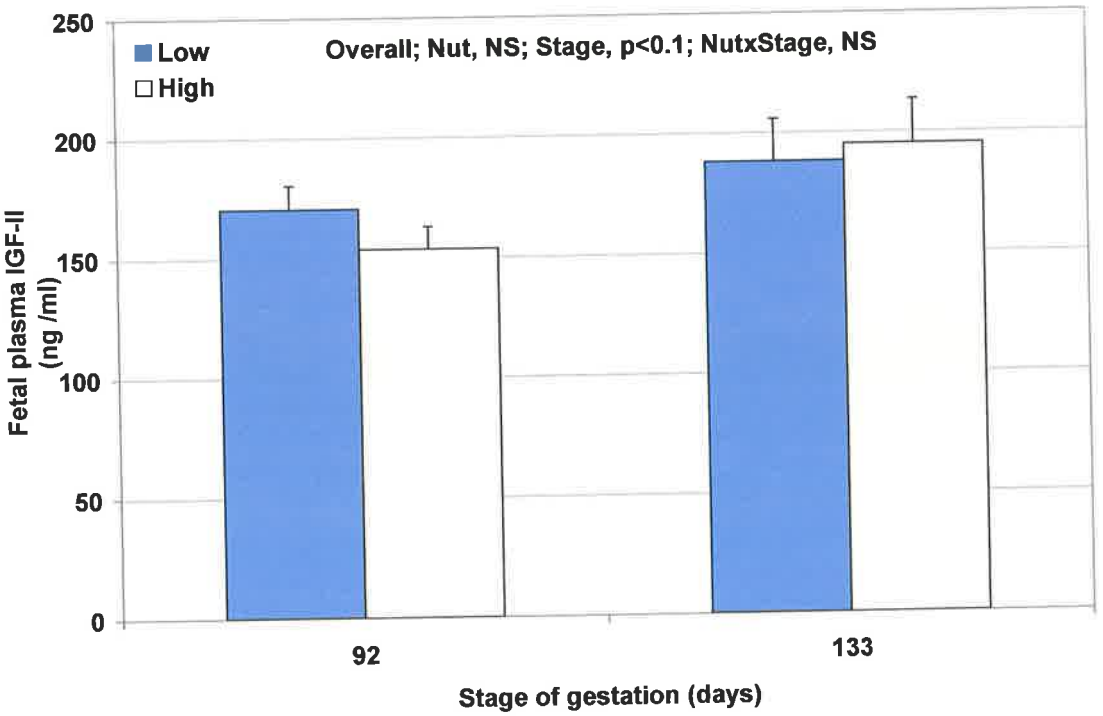


Figure 3.17: The effect of maternal feed intake on the plasma concentration of IGF-II in the fetal circulation at mid- and late gestation.

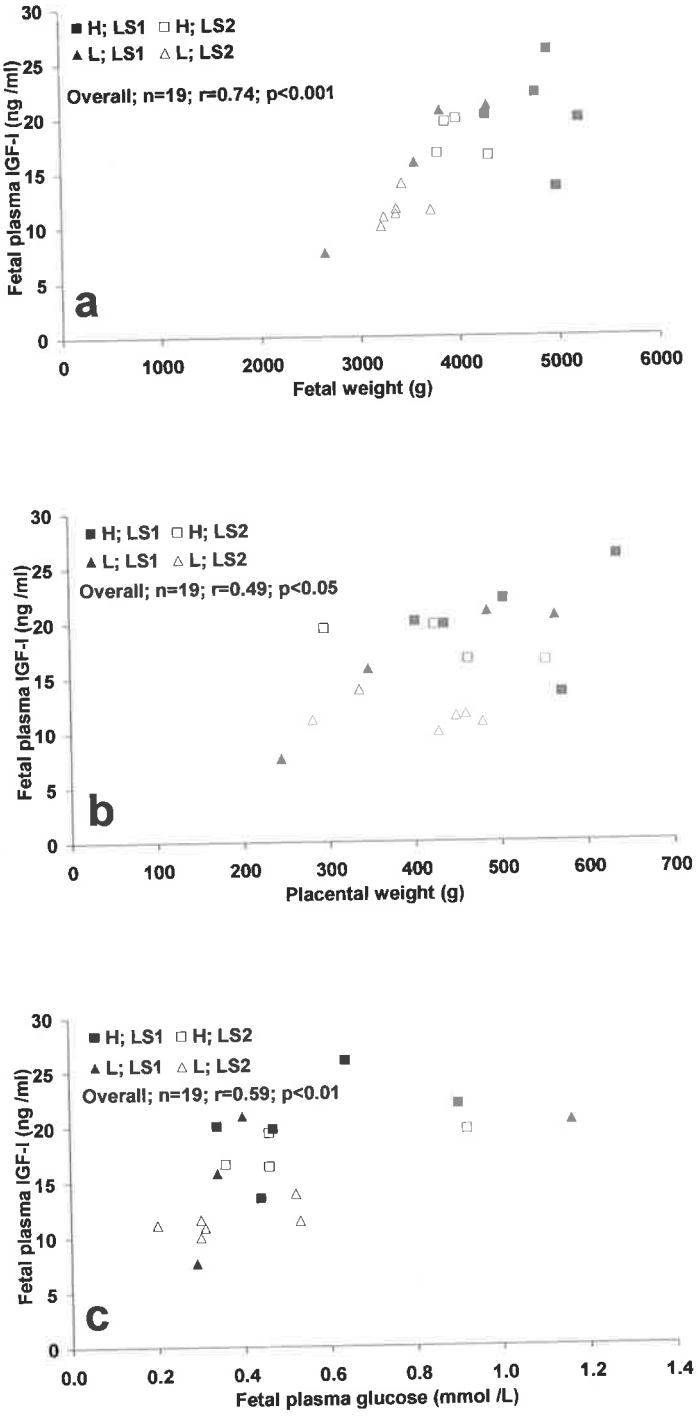


Figure 3.18: Relationship between fetal plasma IGF-I concentration and fetal weight (a), placental weight (b) and fetal plasma glucose concentration (c) at day 133 of gestation. High (H) and Low (L) maternal feed intake. Singleton (LS1) and twin (LS2) fetuses.

3.4 Discussion

Maternal, placental and fetal responses to variable long-term feed intake of sheep were investigated. Maternal under-nutrition resulted in decreased live weight and condition score of ewes, which together with lowered maternal plasma glucose and albumin concentrations, suggests protein and energy deficiencies and the mobilization of protein and fat stores. Fetal growth-restriction was evident by late gestation in mothers that had feed intake restricted, while placental weight was a major influence on fetal weight in late gestation, as reported by Greenwood *et al.* (2000b). Interestingly, some placental restraint of fetal growth was also evident in early gestation, at day 50 of pregnancy. Fetuses whose growth was retarded due to restricted maternal feed intake were lighter and thinner than normal counterparts and were associated with decreased glucose and IGF-I concentrations in the fetal circulation at late gestation. These findings are consistent with those reported by Osgerby *et al.* (2002) and indicate differential maternal feed intakes imposed for a prolonged period before and after conception, coupled with variable litter size, resulted in compensatory adaptations of the maternal, placental and fetal environments, which enabled fetal survival at the cost of growth.

Different amounts of feed offered to ewes prior to and throughout pregnancy, successfully manipulated maternal energy stores and metabolism, as indicated by diverging live weight and condition score prior to and throughout pregnancy and variable plasma metabolite concentrations. The level of divergence in ewe live weight and condition score is similar to that observed by Everitt (1965), which appears to be the only comparable study in terms of duration and severity of treatment.

In the present study, maternal plasma glucose and albumin concentrations were reduced in ewes with restricted intakes, indicative of energy and long-term protein deficiencies, respectively. Maternal NEFA tended to be higher in nutrient restricted ewes in early and mid-gestation, which may suggest these ewes were mobilizing energy reserves at a greater rate than adequately fed counterparts, reflecting the observed changes in condition score. The adaptive response of the underfed mother is to make glucose available for diffusion across the placenta, while the mobilisation of NEFA from her fat reserves provides a source of energy for maternal activity; NEFA do not readily cross the ovine placenta (Pethick *et al.*, 1983) and make an insignificant contribution to energy metabolism in the fetal sheep (Elphick *et al.*, 1979).

Placental weight and the number of placentomes were, for the most part, unresponsive to variations in long-term maternal feed intake in both single and twin pregnancies; the limited number of twins and the high variability in placental weights observed here may have contributed to this. This is in contrast to other studies that showed increased (Faichney and White, 1987; McCrabb *et al.*, 1991) or decreased (Clarke *et al.*, 1998) responses in placental weight to maternal feed restriction. Although the present study is unique in the timing and duration of the restriction imposed, as it is the only study in which placental characteristics were quantified at three stages of gestation when ewes were significantly divergent in condition at mating. In the current experiment, the decline in placental weight between days 92 and 133 of pregnancy apeared greater in Low (50.1%) compared to High feed intake ewes (27.9%). The greater decline in placental mass in Low ewes may explain some of the fetal growth-retardation evident at late gestation, as the capacity for nutrient supply may have been reduced. Therefore, reduced availability of nutrients and reduced transport capacity of the placenta may both have contributed to fetal growth restriction at late gestation.

Despite the insensitivity of placental characteristics to long-term maternal feed intake in this study fetal weight and organ development in early and late gestation were related to placental weight, suggesting that placental weight may have acted on fetal growth, independently of maternal nutritive status. Other factors may have influenced variability in placenta development. All ewes were sourced from the one flock and were assumed to be of similar genetics and backgrounding throughout life. Further, all ewes were mature aged (greater than four years) and were multiparous, reducing a parity affect on placental development. The use of a single sire in this experiment would have eliminated any influence of paternal programming on early placental development. Godfrey *et al.* (1997) reported, small women, of a low birth weight themselves, who are undernourished tend to have small placentas and produce thin babies, suggesting IUGR maybe passed through subsequent generations (although in humans this may be due to societal influences and demographics). Birth weights of the ewes used in the current study were not available, but it is possible that ewes with small placentas had undergone IUGR themselves, adding to the variability in placental mass of the selected population.

Substantial and prolonged nutritional challenge did not vary fetal weight largely, suggesting that considerable feto-placental-maternal adaptations took place, which were sufficient to maintain relatively normal body growth. Differences in late gestational

fetal weight between H and L intake treatments were 19% for singletons and 8% for twins. These values are comparable to nutritionally induced differences reported elsewhere (34% Schinkel and Short, 1961; 22% Mellor and Murray, 1982; 15% McCoard *et al.*, 1996; 11.6% Osgerby *et al.*, 2002) but less than that reported by Everitt (1965; 46%) and much less than that reported for placental restriction through carunclectomy (50% Owens *et al.*, 1994), high litter size (50% Greenwood *et al.*, 1999) and adolescent ewe nutrition (37%, Wallace *et al.*, 2000). A greater divergence in fetal development, between L and H fetuses, may have been evident had pregnancies gone to term. We also found that fetal weight was not increased when pregnant ewes were fed in excess of that required for maintenance, which is in contrast to the finding of Cooper *et al.* (1998).

The relative absence of retarded fetal growth in response to restricted maternal feed intake at early (d 50) and mid-gestation (d 92) suggests that the adaptive mechanisms of the ewe and the placenta were able to satisfactorily compensate at these early stages of gestation, when the conceptus has much lower nutrient requirements for growth compared to near-term. However, in late gestation, when fetal growth accelerates rapidly relative to placental growth and size, maternal and placental adaptations may have been unable to maintain adequate nutrient supply, resulting in compromised fetal growth in response to restricted maternal feed supply.

Different aspects of fetal growth were variably affected by changes in maternal feed intake. Fetal crown-rump length tended to be unaffected by nutrition, while generally fetal weight and girth were less in fetuses from ewes offered restricted nutrition, producing thin light weight fetuses of normal length. The observation that some fetal organs displayed disproportionate growth in response to variations in maternal nutrient supply suggests that low feed intake restricted fetal growth asymmetrically, where growth of the brain was maintained at the expense of less vital organs, such as muscle and lungs (Greenwood *et al.*, 2000a; Harding *et al.*, 2000; McMillen *et al.*, 2001).

Maternal under-nutrition resulted in relative fetal hypoglycaemia at day 92 of gestation. There was no affect of variations in maternal feed intake on circulating fetal albumin at either stage of gestation, despite a large increase in concentration between day 92 and 133, suggesting that circulating protein levels were unaffected by maternal feed intake. The slight increase in fetal urea concentration between day 92 and 133, which

corresponds with decreased fetal glucose, may indicate replacement of fetal glucose with amino acids as a possible source of energy (Faichney and White, 1987).

Restricted nutrient supply to the mother may have directly and indirectly reduced the supply of substrates available for growth of the fetus and altered the production and circulating concentrations of IGF-I in the fetus. IGF-I regulates cell proliferation and differentiation and may be involved in controlling the utilisation of available substrates for metabolism as well as growth. It was apparent by late gestation that fetal plasma IGF-I was positively associated with fetal weight, placental weight and fetal glucose concentration. Fetal plasma IGF-I was lower in fetuses whose mothers received restricted nutriment, consistent with circulating IGF-I regulating fetal growth during late gestation, as demonstrated by Lok *et al.* (1996). In support, fetal circulating IGF-I is decreased with maternal under-nutrition (Bauer *et al.*, 1995; Osgerby *et al.*, 2002), carrunclectomy (Owens *et al.*, 1994) and hypoxaemia (Iwamoto *et al.*, 1992) in the sheep in other studies. Further, IGF-I knockout mice have impaired growth (Liu *et al.*, 1993), while maternal under-nutrition decreases the expression of IGF-I mRNA expression in the liver of fetal sheep (Brameld *et al.*, 2000). Placental restriction in sheep reduced the expression of IGF-I mRNA in the lungs, kidneys and skeletal muscle of fetal sheep, which were correlated with fetal plasma IGF-I concentrations (Kind *et al.*, 1995). Circulating IGF-I was also positively correlated with plasma glucose concentration and the blood oxygen supply in fetal sheep (Owens *et al.*, 1994). It is, therefore, likely that restricted glucose (nutrient) supply to the developing fetus reduced the expression of IGF-I in tissues and the concentration of IGF-I in the circulation; Oliver *et al.* (1993) demonstrated that provision of external glucose, in the presence of under-nutrition, elevated fetal IGF-I levels. Low IGF-I abundance may have limited protein accretion in specific tissues and the fetuses as a whole, however in other studies, skeletal muscle weights (Lok *et al.*, 1996) and protein metabolism (Boyle *et al.*, 1998) were unaffected by circulating IGF-I levels. In contrast, when amino acid and glucose concentrations were maintained at constant levels, IGF-I infusion increased protein synthesis in fetal skeletal muscle (Shen *et al.*, 2003).

It appears that restricted nutrient supply may have inhibited IGF-I production mainly in late gestation. At day 92 fetal plasma IGF-I levels were similar in High and Low fetuses, but by day 133 circulating IGF-I was approximately 45% greater in High fetuses. Therefore, reduced abundance and action of IGF-I may partly explain the reduced fetal growth observed in restricted compared to *ad libitum* feed intake mothers.

Despite evidence that IGF-II is also involved in fetal development, the present study revealed no such associations. This finding is in contrast to Jones *et al.* (1988) and Owens *et al.* (1994) who reported positive and negative associations between fetal weight and IGF-II in fetal sheep, respectively. However, these associations emerged in late gestation after the pre-partum cortisol surge, which inhibits IGF-II production by fetal tissues (Li *et al.*, 1993). This cortisol-induced reduction in fetal IGF-II is excessive in placentally restricted sheep and it is possible a decrease in IGF-II may occur after day 133 of gestation and contribute to fetal growth restriction in feed intake restricted ewes (Owens, Personal Communication).

The present study reveals relatively higher levels of circulating IGF-II than IGF-I in the fetal plasma. Further, it demonstrates an insensitivity of IGF-II in the fetal circulation to maternal feed intake. While circulating IGF-I has been implicated with fetal growth regulation during late gestation, in this and other studies in sheep, this occurs when transitions in the IGF-axis are occurring. During late gestation, IGF-I regulation of fetal tissue growth shifts from local autocrine/ paracrine control to endocrine control. IGF-I mRNA levels in tissues, such as skeletal muscle decline, while liver mRNA and circulating IGF-I increase in response to the actions of growth hormone on the liver during the peri-natal period (reviewed by Fowden, 2003). The significance of both local and circulating IGF-II to post-natal growth is also thought to decline during late gestation.

Circulating IGF-I concentrations reported here (8-26ng /ml) are less than values reported elsewhere (50-200ng /ml) (Owens *et al.*, 1994; Osgerby *et al.*, 2002). In addition, IGF-II values (114-323ng /ml) are considerably lower than those reported by others (200-1200ng /ml; Owens *et al.*, 1994; Carr *et al.*, 1995). The relatively low IGF-II values reprted here may be related to the antibodies used. The antibodies used in the present study were designed in the C-region of human IGF-II, which may have only 10-20% cross reactivity with ovine IGF-II (S. Humphrys, personal communication).

One limitation of the present study, which may account for some of the variation within treatments, was that ewes within treatments were fed as a group. Ideally, if resources were available, ewes would have been individually penned and fed according to their individual maintenance requirements. Other problems, which may have contributed to the variation, include

- The lower than anticipated population of restrictively fed ewes that were able to establish and maintain pregnancy. This resulted in larger standard errors associated with treatment means and hence limited the power of the study to detect statistical significant treatment differences. It is difficult to arrive at an alternate method than the one employed. Embryo transfer is one option, although this introduces other variables, which may affect fetal development independent of maternal nutrition (Barnes *et al.*, 2000). The best solution would be to increase the number of ewes offered restricted nutrition.
- The difficulty in accurately determining litter size before day 45 of gestation, which resulted in an uneven distribution of litter size across the three sample cohorts.

A further limitation of the present study was the blood sampling protocols. Sampling of fetal blood after slaughter may have resulted in asphyxia and affected fetal blood composition. Further, a one off collection of ewe blood by venipuncture may have resulted in blood composition being altered due to stress, rather than experimental treatment influences alone (eg. unphysiologically high NEFA values (Bell, Personal Communication). Nevertheless, given that all blood sampling procedures were, to the best of ability, identical between ewes and fetuses, it was considered that differences in relative values would be due to treatment effects.

3.5 Summary

The impact of long established planes of variable maternal nutrition in the sheep was investigated in this study. Low maternal feed intake in this model resulted in asymmetric IUGR by late gestation, compared to maintenance or *ad libitum* maternal feed intake. In addition, fetal growth appeared to be regulated by placental weight, independent of maternal nutritional status, even during early gestation. However, maternal, placental and fetal adaptations to restricted maternal feed intake adequately maintained normal fetal growth during early and mid-gestation. Restricted fetal growth during late gestation was characterised by hypoglycaemia and reduced circulating IGF-I concentrations. Overall, substantial reductions in maternal nutrient availability resulted in fetal growth-restriction that only became evident late in gestation. This resistance of fetal growth to nutritional limitations in the sheep resembles that seen in humans in the Dutch Famine and contrasts with the impact of placental restriction in sheep and humans. The impact of the fetal growth-restriction evident in this study on post-natal growth rates and carcass composition of livestock remains to be determined. This study provided a source of fetuses, divergent in growth, for further investigations into the nutritional regulation of muscle development *in utero*, described in the next two chapters.

CHAPTER 4.

4 THE EFFECTS OF LONG-TERM MATERNAL FEED INTAKE ON SKELETAL MUSCLE GROWTH AND CELLULARITY IN FETAL SHEEP

4.1 Introduction

In sheep, potential for muscle growth may be determined before birth. The number of myofibres is believed to be established *in utero* (hyperplasia). Subsequent increases in muscle mass results from the growth of individual muscle fibres (hypertrophy), which involves the incorporation of new myonuclei (derived from replication of satellite cells) into the muscle fibre, and the accretion of protein. The number and size of muscle fibres are related to muscle mass and growth rates (Dwyer *et al.*, 1993; Rehfeldt *et al.*, 2000).

It appears that myogenesis may be environmentally regulated, as reduced muscle weights and muscle growth resulting from restricted pre-natal nutrition (Everitt, 1965) and placental insufficiency (Greenwood *et al.*, 2000a) have been reported. However, there is conflicting evidence on how the resulting changes in muscle development are manifested and whether these changes transiently or permanently influence subsequent muscle growth.

Genetic (Staun, 1963; Stickland and Handel, 1986; Wegner *et al.*, 2000) and maternal environmental (Everitt, 1965; Swatland and Cassens, 1973; Wigmore and Stickland, 1983; Dwyer *et al.*, 1994; Greenwood *et al.*, 1999, 2000a) factors influence myogenesis. Given that muscle fibre number is believed to be fixed after birth, then the *in utero* environment may have a significant impact on myogenesis and muscle fibre number, size and type during fetal development.

In Chapter 3, different long-term maternal nutritional regimes provided a population of fetuses from which individuals divergent in growth were obtained at three stages of gestation. The fetus's adaptation to reduced nutrient supply may involve the partitioning of energy substrates to vital organs at the expense of other tissues, such as muscle, or a shift in the use of amino acids from anabolism and muscle protein accretion to catabolism for energy; both of which would reduce muscle growth. Altered fetal growth,

mediated by nutrient supply, described in Chapter 3 provides a model to investigate myogenesis in fetuses on different growth trajectories during early, mid- and late gestation. Therefore, the aim of this study was to investigate the effects that nutritionally mediated fetal growth-retardation might have on myogenesis, and more specifically on muscle cellularity.

4.2 Experimental

4.2.1 Animals

Fetuses divergent in growth were collected at three stages of gestation, as described in Chapter 3. The semitendinosus (ST), longissimus dorsi (LD) and supraspinatus (SS) muscles were dissected from fetuses and prepared as described earlier (section 2.2.1). Individual muscles could not be distinguished at day 50. Distinct muscles were identified at day 92, however muscle weights were not recorded; entire muscle weights were recorded for all fetuses at day 133.

The ST, LD and SS muscles were chosen as they represent muscles that are diverse in fibre type proportions in sheep (fast glycolytic, mixed and slow oxidative, respectively; Briand *et al.*, 1981; Carpenter *et al.*, 1996; Greenwood *et al.*, 2000a) and cattle (Vestergaard *et al.*, 2000), mature differently (ST early maturing, SS late maturing; Vestergaard *et al.*, 2000) and they were readily accessible for removal of the entire muscle.

Analysis of myogenic indices was conducted on two sub-populations of fetuses, based on fetal weight at days 92 and 133 of gestation. Fetuses were considered as either Small (growth-restricted fetuses derived from feed intake restricted ewes) or Large (large fetuses derived from *ad libitum* feed intake ewes).

4.2.2 Muscle fibre analysis

A description of the population of fetuses used for muscle fibre analysis is presented in Table 4.1.

Table 4.1: Weight (g) and number of fetuses (n) selected for determination of the apparent number, size and type of muscle fibres in the semitendinosus muscle. *values are least-squares means ± SEM.

Stage of gestation (days)	Fetal size		Probability
	Small	Large	p
92	653.9 ± 24.5* (n=8)	752.8 ± 21.9 (n=10)	0.0083
133	3488.4 ± 179.7 (n=9)	4217.6 ± 170.5 (n=10)	0.0091

The estimated total apparent muscle fibre number, the proportion of fibre types and the cross-sectional area (CSA) of myofibres were determined on sections reacted for ATPase activity after basic pre-incubation at the mid-belly of the semitendinosus (ST) muscle as described in sections 2.2.2 and 2.2.3. Myofibres were counted in 36.6 ± 13.6 (mean ± standard deviation) and 79.7 ± 29 fields (x200 magnification) at days 92 and 133, respectively, which accounted for approximately 5% of the total muscle mid-belly CSA, as validated by Greenwood *et al.* (2000a) and discussed in Chapter 2.

Muscle fibre analysis was conducted on the ST muscle only. The size of the LD samples taken was too large and they did not freeze properly; muscle fracturing and cellular artefacts were common. Two problems arose with the SS sampling, firstly it was not possible to maintain the integrity of the whole muscle sections; secondly the muscles were not orientated before freezing. The importance of the orientation became significant when it was noted that large areas of the SS muscle were solely type I fibres while other areas were type II fibres, meaning myofibre types were not evenly distributed across the whole cross-section. Suzuki (1995) reported that the SS muscle had greater concentration of type I myofibres in the caudal and deep regions of the muscle, suggesting a postural role for the caudal and deep regions of the muscle and locomotory role for the rest of the muscle.

4.2.3 Muscle DNA, RNA, protein and dry matter content

Muscle nucleic acid, protein and dry matter content were determined in the ST and SS muscles of fetuses described in Table 4.2. The amount of DNA and RNA were determined using the Diphenylanine and Orcinol reactions following perchloric acid extraction, as described in sections 2.3.5 and 2.3.6, respectively. Muscle protein content was determined using the method of Bradford (section 2.3.7). Muscle dry matter content was determined as described earlier (section 2.3.8). The intra- and inter-assay coefficients of variation for each procedure are presented in Table 4.3.

Table 4.2: Weight (g) and number of fetuses (n) selected for determination of DNA, RNA, protein and dry matter content of muscles. *values are least-squares means ± SEM.

Stage of gestation (days)	Fetal size		Probability Value
	Small	Large	
92	623.6 ± 12.5* (n=5)	785.6 ± 12.5 (n=5)	<0.0001
133	3206.8 ± 98.1 (n=5)	4832.0 ± 98.1 (n=5)	<0.0001

Table 4.3: Coefficient of variation (CV%) of DNA, RNA, protein and dry matter content assays of fetal muscle.

	Assay CV (%)	
	Intra	Inter
DNA	9.32	10.41
RNA	6.61	15.35
Protein	8.69	4.75
Dry matter	3.78	2.07

4.2.4 Statistical analysis

All statistical analyses were conducted using the GLM and CORR procedures in SAS. The model for muscle weights of late gestation fetuses included the main effects of nutrition, litter size and sex and their first order interactions. The models for muscle fibre number, size and type included the main effects of fetal size (small and large), stage of gestation (day 92 and 133), sex, and their interactions. The initial model (Appendix 1) included the main effects of fetal size, stage of gestation, muscle (SS and ST) and their interactions. On those occasions when non-normal distribution was noted, data were log transformed prior to analysis. Only those interactions that were significant or tended toward significance ($p<0.1$) were retained in the final models. Descriptions of the final overall models are presented in Appendix 1. Different alphabetical superscripts in Tables and Figures indicate a significant (lower case, $p<0.05$) or tendency to significant (upper case, $p<0.1$) difference between main effect means. Within some charts, level of significance between large and small fetuses is indicated by # $p<0.1$, * $p<0.05$ and ** $p<0.01$.

4.3 Results

4.3.1 Weight of muscles from late gestation fetuses

Overall, maternal feed intake affected absolute weights of the ST ($p=0.06$), LD ($p<0.01$) and SS ($p=0.07$) muscles of late gestation fetuses. Singleton fetuses had significantly ($P<0.01$) heavier LD (40.9 ± 1.4 and 33.4 ± 1.7g) and SS (10.7 ± 0.5 and 8.5 ± 0.6g) muscles than twin fetuses. Fetal sex had no significant affect on muscle weights (Appendix 1). However, litter size elicited a differential response in males and females (Table 4.4), where muscles from singleton males were heavier than those of singleton females but muscles from twins did not vary with sex. The relative weight of the ST, LD and SS muscles (g /kg fetal body weight) were unaffected by maternal feed intake, litter size and fetal sex (Appendix 1).

Table 4.4: The effect of litter size on muscle weights of late gestation male and female fetuses. *values are least-squares means ± SEM. Different superscripts within the rows indicate values are significantly different (lowercase, $p<0.05$; uppercase, $p<0.1$).

	Singleton		Twin	
	Female	Male	Female	Male
Fetuses (n)	10	7	10	8
L. dorsi (g)	$37.45 \pm 1.77^{a*}$	44.26 ± 2.16^{b}	35.17 ± 2.09^{a}	31.56 ± 2.34^{a}
Semitendinosus (g)	7.24 ± 0.63^{A}	9.08 ± 0.77^{B}	7.20 ± 0.72^{A}	6.87 ± 0.80^{A}
Supraspinatus (g)	9.18 ± 0.61^{a}	12.29 ± 0.74^{b}	9.01 ± 0.69^{a}	8.02 ± 0.77^{a}

4.3.1.1 Singleton fetuses

The weights of the LD, ST and SS muscles were reduced by 19%, 23.5% and 21.3% respectively in Low singleton fetuses, although this only tended to significance (Table 4.5). Reductions in muscle weights were proportionate to changes in fetal weight; 83%, 53% and 52% (r^2) of the variation in LD, ST and SS muscle weights were accounted for by fetal weight ($p<0.01$), respectively.

Table 4.5: The effect of maternal nutrition on muscle weights of late gestation singleton fetuses. *values are least-squares means ± SEM. Different superscripts within the rows indicate values are tending toward a significant difference ($p<0.1$).

	Maternal Feed Intake		
	Low	Medium	High
Fetuses (n)	4	6	7
L. dorsi (g)	$35.36 \pm 3.25^{A*}$	43.30 ± 2.58^{B}	43.63 ± 2.39^{B}
Semitendinosus (g)	6.79 ± 0.85^{A}	8.77 ± 0.67^{B}	8.87 ± 0.63^{B}
Supraspinatus (g)	9.46 ± 1.17^{A}	10.48 ± 0.93^{AB}	12.02 ± 0.86^{B}

4.3.1.2 Twin fetuses

LD, ST and SS muscle weights were reduced by 17.4%, 23.4% and 12.4% respectively in Low twin fetuses, although the effect was only significant ($p<0.05$) for LD (Table 4.6). Reductions in fetal muscle weights were proportionate to changes in fetal weight; 60%, 48% and 70% (r^2) of the variation in LD, ST and SS muscle weights were accounted for by fetal weight ($p<0.01$), respectively.

Table 4.6: The effect of maternal nutrition on muscle weights of late gestation twin fetuses. *values are least-squares means ± SEM. Different superscripts within the rows indicate values are significantly different ($p<0.05$).

	Maternal Feed Intake	
	Low	High
Fetuses (n)	6	12
L. dorsi (g)	$29.42 \pm 2.09^{a*}$	35.61 ± 1.62^{b}
Semitendinosus (g)	5.87 ± 0.91	7.66 ± 0.65
Supraspinatus (g)	8.25 ± 0.64	9.36 ± 0.45

4.3.2 Muscle fibre development

4.3.2.1 Muscle fibre arrangement

4.3.2.1.1 Day 50 fetal muscle (early myogenesis)

In the composite muscle taken from the hind limb of day 50 fetuses the muscle fibres appeared as a relatively homogenous population. The majority of fibres were identified as primary myotubes based on their morphology, as described by Ashmore (1972). Fibres classified as primary were circular in appearance with a large centrally located vacuole, identified with ATPase stain after basic pre-incubation (Figure 4.1), these fibres all contained a centrally located nuclei, identified by H and E stain (Figure 4.1) and all appeared to be of a similar size. There was no differential staining between primary myotubes and a small population of small cells, which were assumed to be the early development of secondary myofibres. There appeared to be no organization of myofibres into bundles at this stage of myogenesis (Figure 4.1). Unfortunately, due to the much-hydrated nature of muscle at this stage, and the inability to accurately identify and dissect individual anatomical muscles with confidence, no quantitative data were obtained.

4.3.2.1.2 Day 92 fetal muscle (late myogenesis)

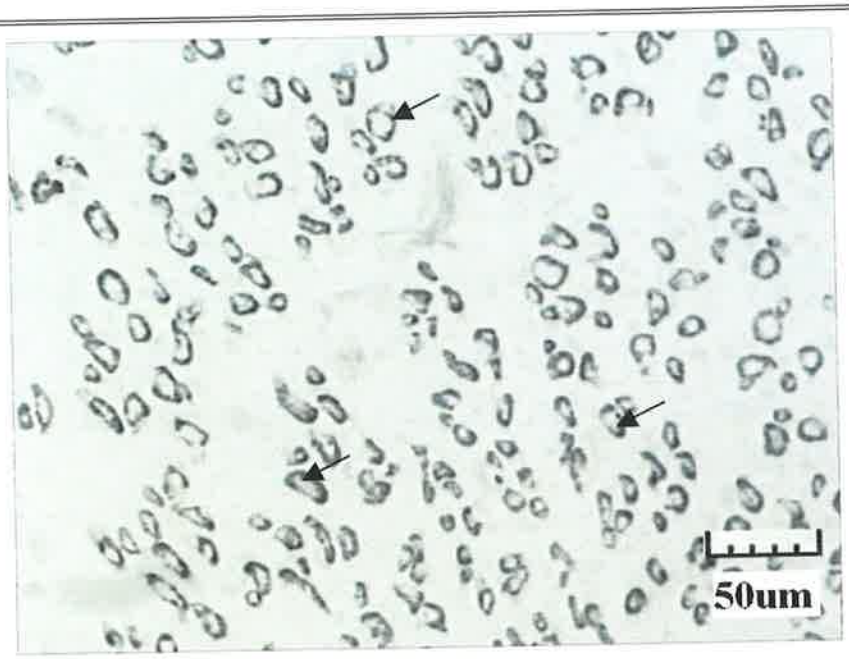
In the semitendinosus muscle from 92-day-old fetuses there appeared to be a significant increase in the frequency and size of myofibres, compared with day 50 muscle. The increase in number was due to the appearance of a large population of smaller fibres, presumed to be secondary fibres, surrounding the larger myofibres (Figure 4.2). Myotubes, indicated by centrally located nuclei, were rare. Differential staining of slow and fast twitch myofibres based on their reactivity with ATPase was apparent in some of the muscles sectioned at this time point, suggesting events occurring around day 92 may initiate the metabolic properties of muscle fibres. Slow fibres were identified based on their ATPase reactivity, and sometimes also contained a central vacuole (nuclei) and were generally located at the centre of a bundle. Fasciculi organization was well advanced with fibres arranged in bundles.

All fibres stained intensely for NADH and GPD at this time point, despite differential staining evident in the post-natal control. In contrast, there was little success in the immuno-staining protocols employed on the fetal muscles, while reactivity was detected with the post-natal control. However, it cannot be ruled out that different conditions may have been required for the optimisation of enzymatic and immuno-staining of fetal muscle at this time point.

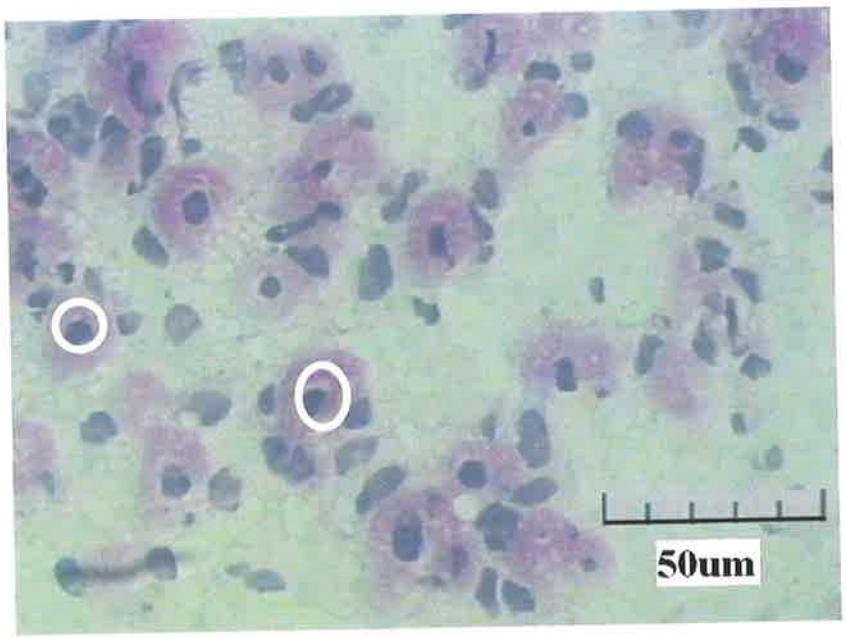
4.3.2.1.3 Day 133 fetal muscle (post-myogenesis)

Muscle fibres in the semitendinosus muscle from 133-day-old fetuses increased significantly in size from day 92 and were polygonal in appearance, with little space between fibres (Figure 4.3). Nuclei were always located peripherally (Figure 4.3); no attempt was made to distinguish myonuclei from satellite cells. Differential reactivity to ATPase was evident in all samples at acidic and basic pH. Fibres were classified as fast or slow according to their reactivity as described in section 2.2.3.

All fibres stained intensely for NADH, while differential GPD activity was apparent, although this was not consistent across all samples. Fibres demonstrated reactivity to anti-slow and anti-fast immuno-staining, which validated ATPase staining at basic pH.

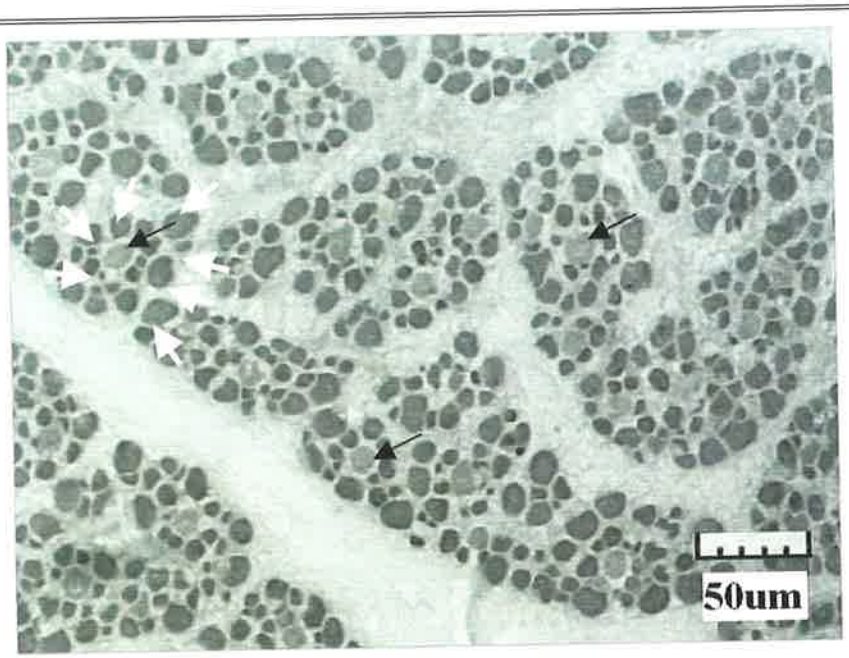


a.

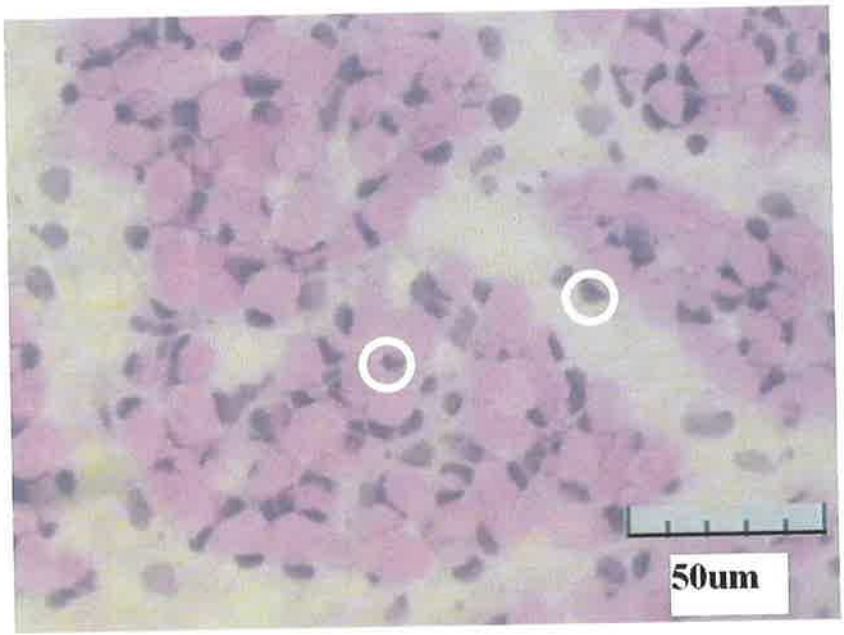


b.

Figure 4.1: **Cross-section of mixed hind-limb muscle of day-50 fetuses, reacted with ATPase after basic pre-incubation (x200) (a.) and H and E (x400) (b.).** Black arrows indicate primary fibres and white circles indicate myonuclei.

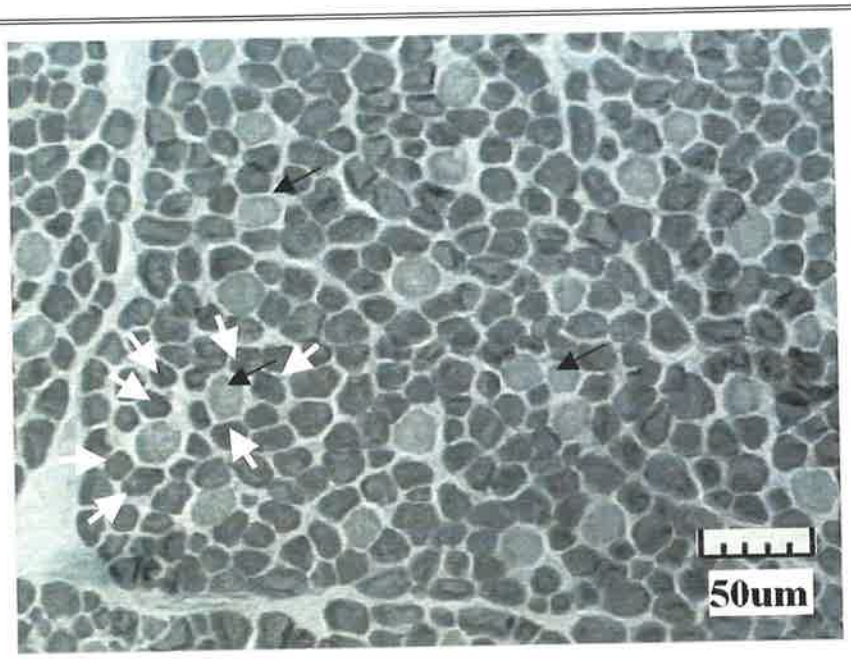


a.

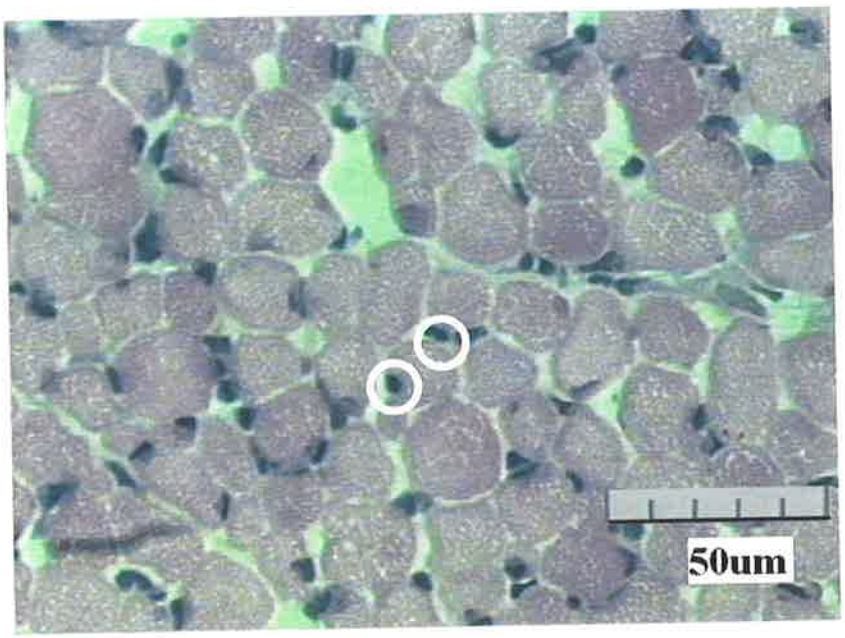


b.

Figure 4.2: Cross-section of the semitendinosus muscle of day-92 fetuses, reacted with ATPase after basic pre-incubation (x200) (a.) and H and E (x400) (b.). Black arrows indicate slow twitch fibres, white arrows indicate fast twitch fibres and white circles indicate myonuclei.



a.



b.

Figure 4.3: Cross-section of the semitendinosus muscle of day-133 fetuses, reacted with ATPase after basic pre-incubation (x200) (a.) and H and E (x400) (b.). Black arrows indicate slow twitch fibres, white arrows indicate fast twitch fibres and white circles indicate myonuclei.

4.3.2.2 Muscle fibre number and type

The estimated apparent total number of muscle fibres at the mid-belly of the ST muscle increased between mid- and late gestation and tended to be greater in small fetuses at day 92 of gestation ($p<0.1$), however there was no difference between small and large fetuses at day 133 (Figure 4.4). The number of fast twitch fibres per slow twitch fibre (S:P ratio) was similar between small and large fetuses at day 92 and 133 of gestation (Figure 4.5). The density of fibres per unit area (mm^2) decreased significantly between mid- and late gestation and was greater in the ST muscle of small fetuses at both stages of gestation, although this was only significant by late gestation (Figure 4.6).

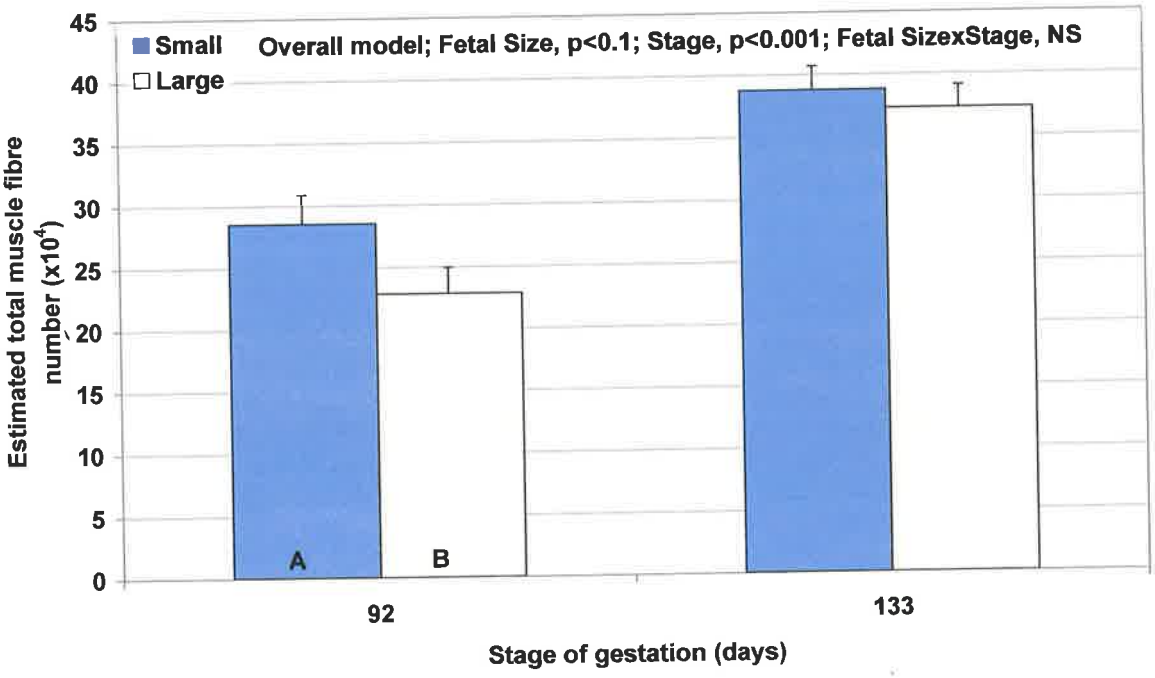


Figure 4.4: Estimated total number of muscle fibres at the mid-belly of the semitendinosus muscle of small and large fetuses at mid- and late gestation. Different uppercase letters within a stage of gestation indicates values are tending toward significance ($p<0.1$). Values are least-squares means ± SEM.

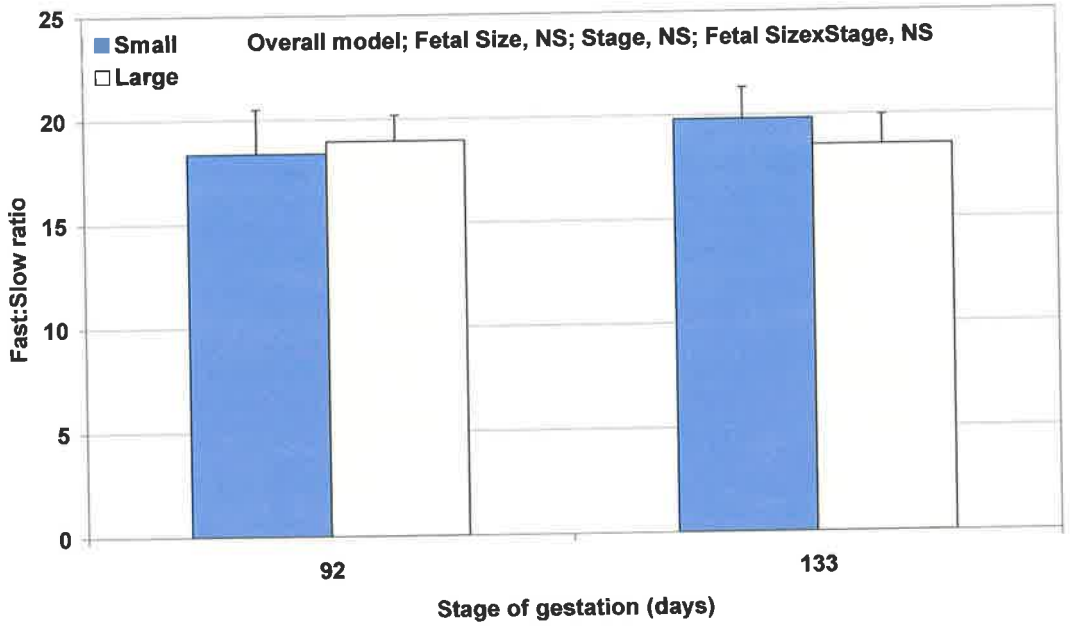


Figure 4.5: Ratio of fast to slow twitch fibres at the mid-belly of the semitendinosus muscle of small and large fetuses at mid- and late gestation, respectively. Values are least-squares means ± SEM.

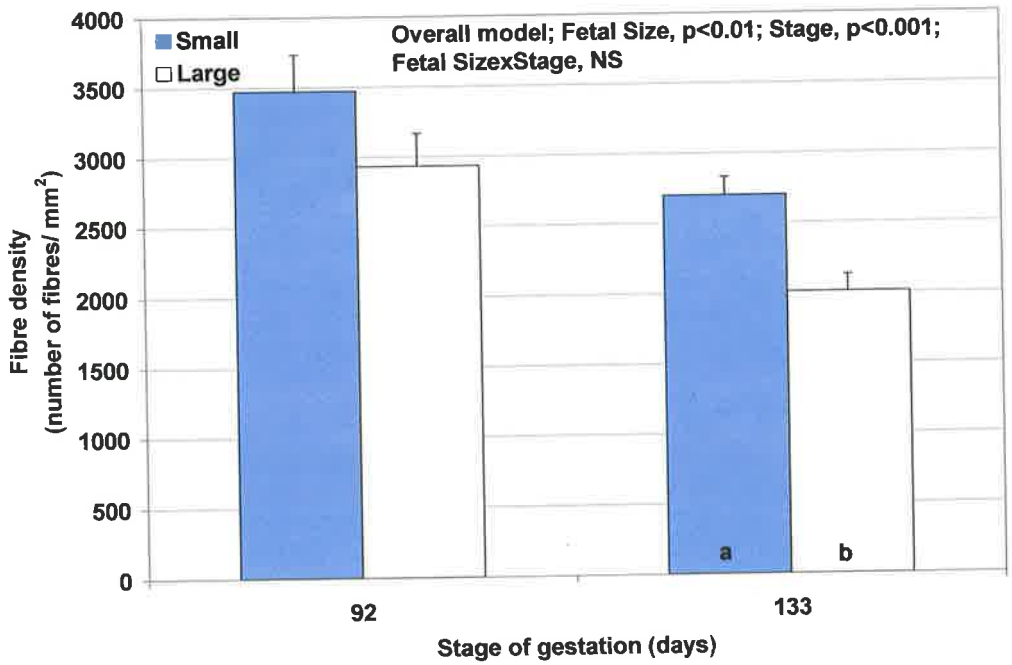


Figure 4.6: Density of muscle fibres at the mid-belly of the semitendinosus muscle of small and large fetuses at mid- and late gestation. Different lowercase letters within a stage of gestation indicates a significance difference ($p<0.05$). Values are least-squares means ± SEM.

4.3.2.3 Muscle fibre size

Slow twitch myofibres had greater cross-sectional area than fast myofibres, at both stages of gestation ($p<0.05$). At both stages of gestation the CSA of all myofibres was greater in large fetuses ($p<0.01$), this was mostly attributed to greater CSA of fast myofibres at both stages ($p<0.01$); the CSA of slow myofibres tended to be similar between small and large fetuses at both time points.

The difference in CSA of myofibres can be attributed to a greater proportion of smaller myofibres in small fetuses and larger myofibres in large fetuses at day 92 (Figure 4.8) and 133 (Figure 4.9). At late gestation the difference in distribution of fibre CSA can be attributed to changes in the distribution of fast myofibre CSA (Figure 4.10); no difference in slow myofibre CSA distribution was evident (Figure 4.11).

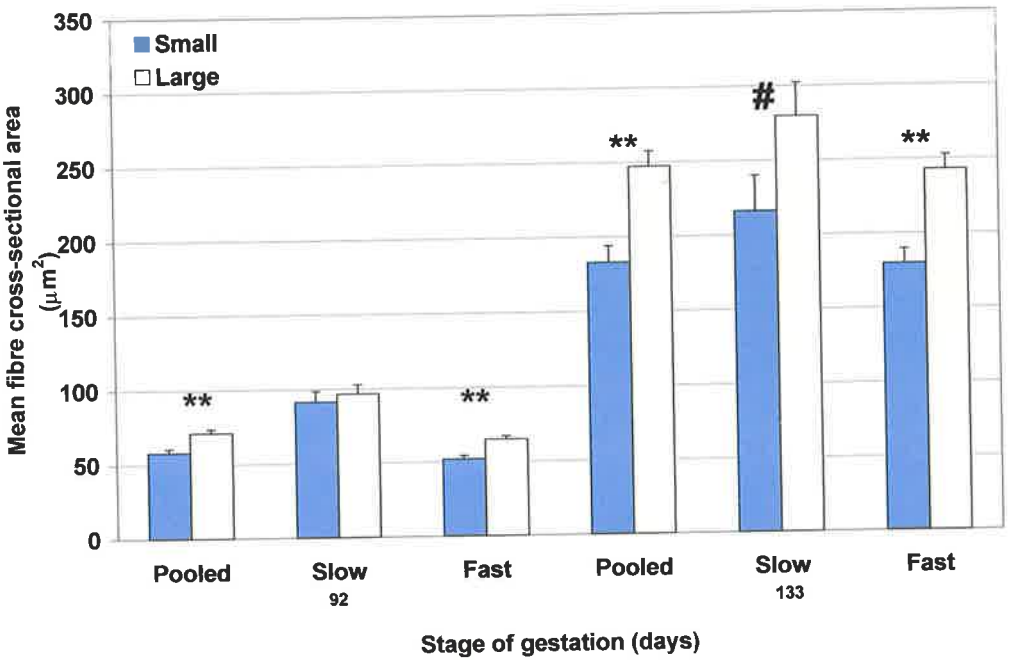


Figure 4.7: Mean myofibre cross-sectional area of all myofibres combined and myofibres classified as either fast or slow twitch at the mid-belly of the semitendinosus muscle of small and large fetuses at mid- and late gestation. Symbols indicate the significance for each fibre type (#, $p<0.1$; **$p<0.01$). Values are least-squares means ± SEM.

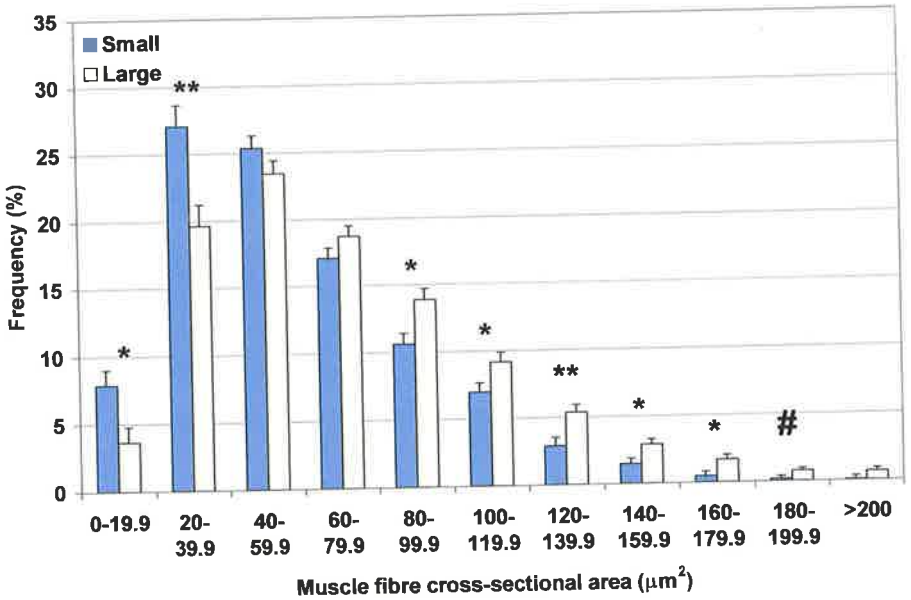


Figure 4.8: Distribution of myofibre cross-sectional area at the mid-belly of the semitendinosus muscle of small and large fetuses at mid-gestation. Symbols indicate level of significance on the frequency of fibres within each 20μm^2 range of cross-sectional area (#, p<0.1; *p<0.05; **p<0.01). Values are least-squares means ± SEM.

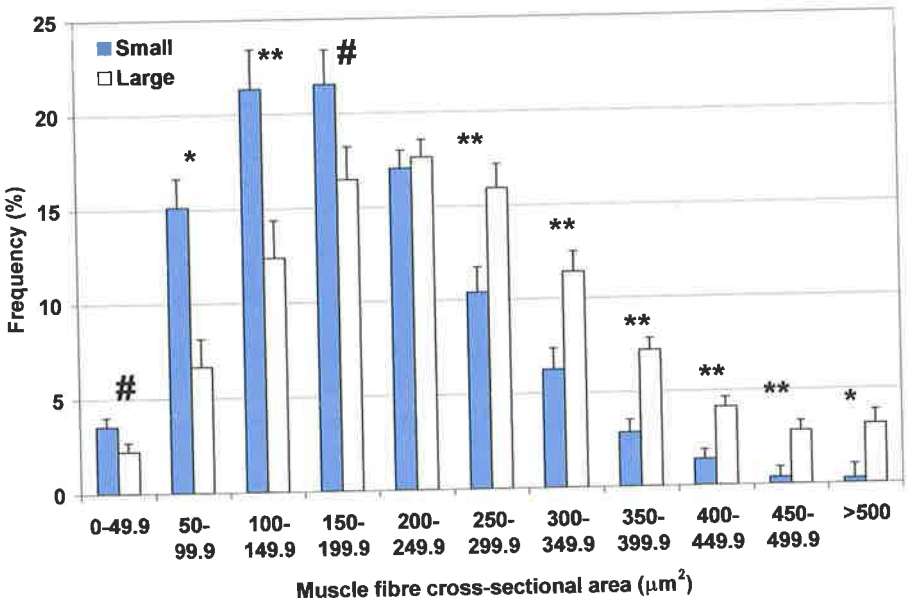


Figure 4.9: Distribution of myofibre cross-sectional area at the mid-belly of the semitendinosus muscle of small and large fetuses at late gestation. Symbols indicate level of significance on the frequency of fibres within each 20μm^2 range of cross-sectional area (#, p<0.1; *p<0.05; **p<0.01). Values are least-squares means ± SEM.

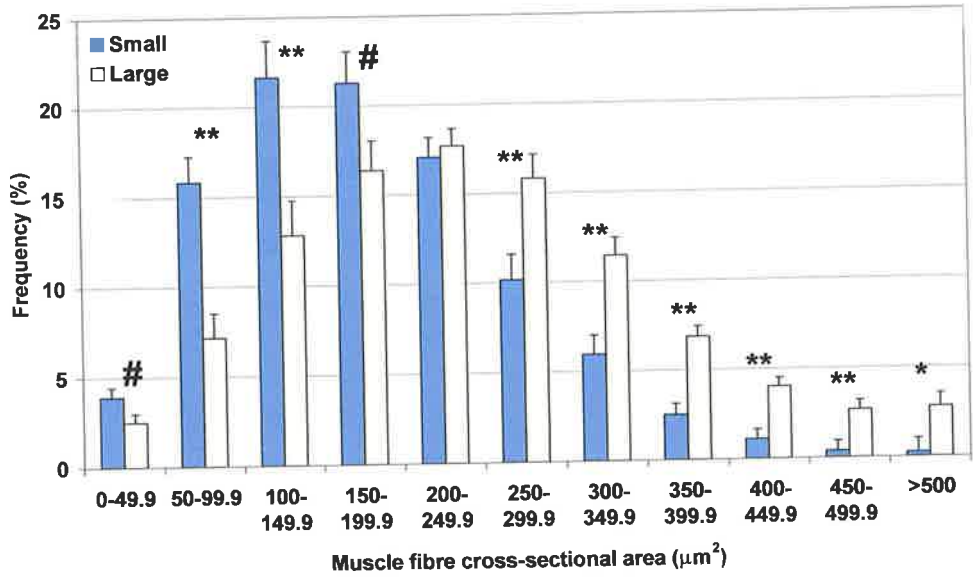


Figure 4.10: Distribution of myofibre cross-sectional area of fast twitch myofibres at the mid-belly of the semitendinosus muscle of small and large late gestation fetuses. Symbols indicate level of significance on the frequency of fibres within each 20μm^2 range of cross-sectional area (#, p<0.1; *p<0.05; **p<0.01). Values are least-squares means ± SEM.

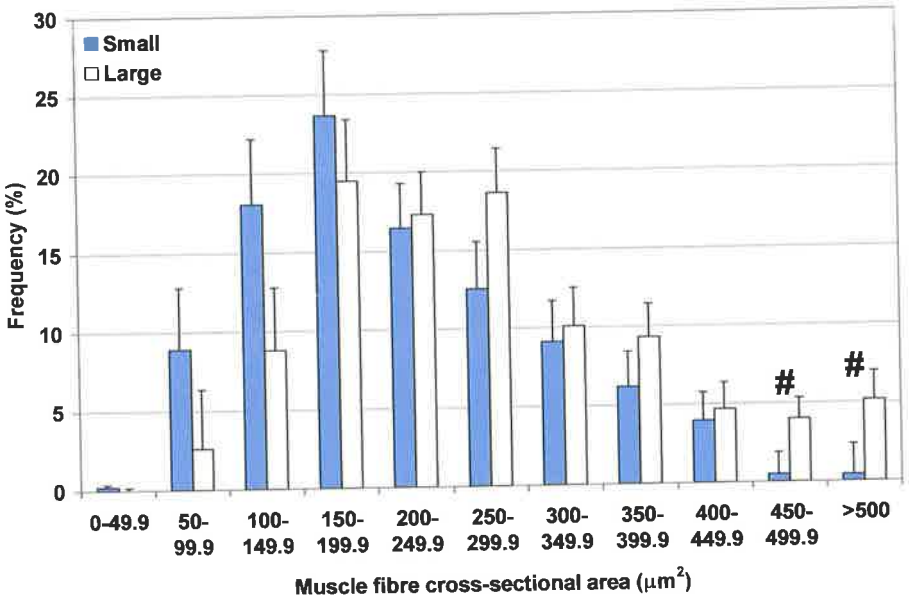


Figure 4.11: Distribution of myofibre cross-sectional area of slow twitch muscle fibres at the mid-belly of the semitendinosus muscle of small and large late gestation fetuses. Symbols indicate level of significance on the frequency of fibres within each 20μm^2 range of cross-sectional area (#, p<0.1; *p<0.05; **p<0.01). Values are least-squares means ± SEM.

4.3.3 Dry matter, DNA, RNA and protein content of fetal muscle

Dry matter content of fetal muscle increased with stage of gestation and tended to be greater in large fetuses at late gestation (Figure 4.12). DNA concentration declined significantly between days 92 and 133 of gestation; the rate of decline was greater in large fetuses, as indicated by the interaction between fetal size and stage of gestation (Figure 4.13). RNA concentration declined between days 92 and 133 of gestation, but only tended to be influenced by fetal size (Figure 4.14). Protein content increased significantly with stage of gestation and was elevated in large fetuses by late gestation (Figure 4.15).

The amount of RNA per unit of DNA and the amount of protein per unit of DNA and RNA increased with stage of gestation (Figures 4.16-4.18), while the amount of protein per unit of DNA and RNA was also elevated in large fetuses at late gestation ($p<0.05$).

The total amount of DNA, RNA and protein were greater in muscles of large fetuses at late gestation (Table 4.7), although this was mostly accounted for by the weight of the muscle (r^2=0.51, 0.85 and 0.92, respectively). There was no difference in the total amount of DNA, RNA and protein between the ST and SS muscles.

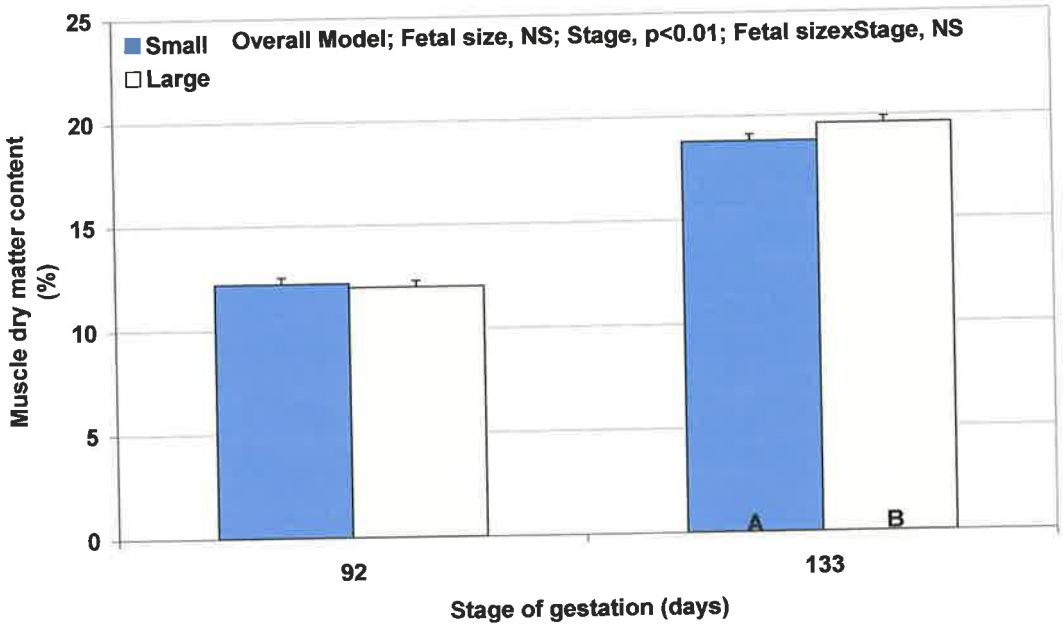


Figure 4.12: Dry matter content (%) of muscle from small and large fetuses at mid- and late gestation. Different uppercase letters within a stage of gestation indicate a tendency towards significance ($p<0.1$). Values are least-squares means ± SEM.

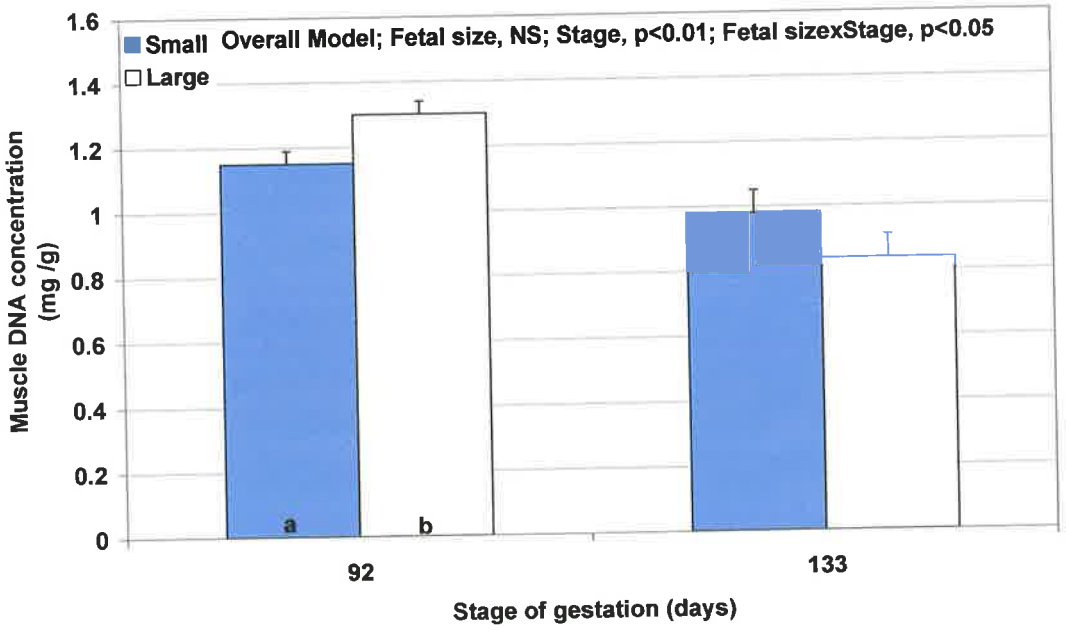


Figure 4.13: Concentration of DNA in muscle of small and large fetuses at mid- and late gestation. Different letters within a stage of gestation indicate a significant difference ($p<0.05$). Values are least-squares means ± SEM.

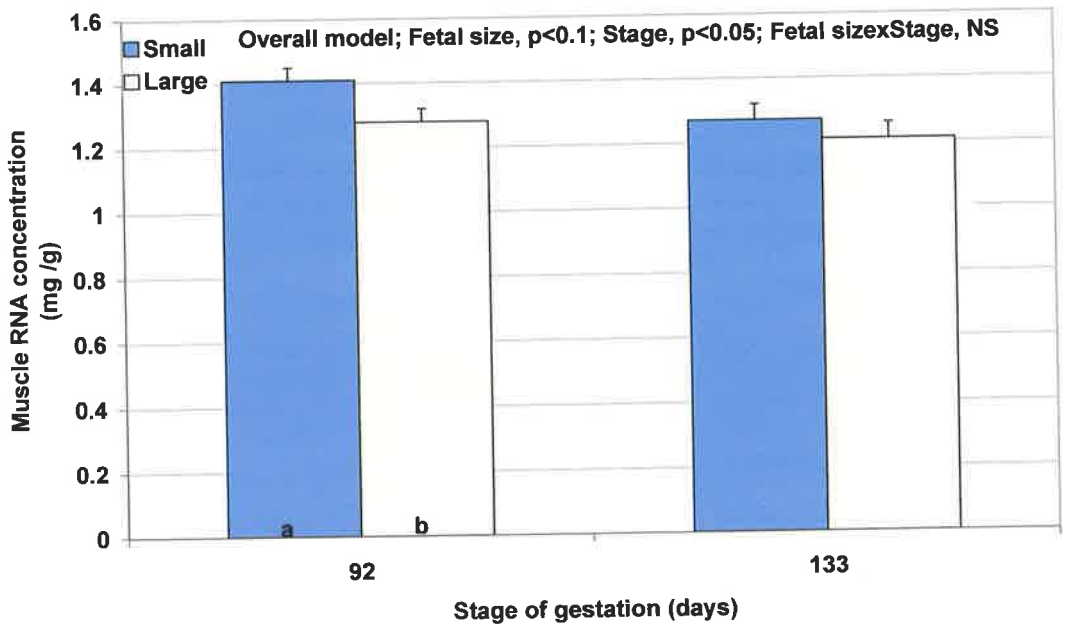


Figure 4.14: Concentration of RNA in muscle of small and large fetuses at mid- and late gestation. Different letters within a stage of gestation indicate a significant difference ($p<0.05$). Values are least-squares means ± SEM.

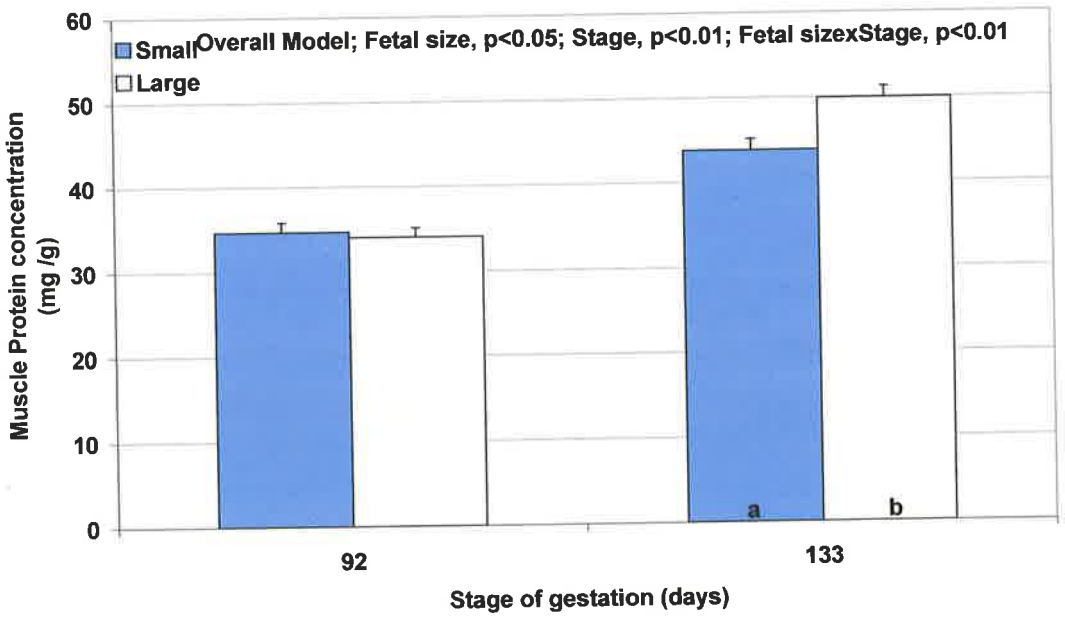


Figure 4.15: Concentration of Protein in muscle of small and large fetuses at mid- and late gestation. Different letters within a stage of gestation indicate a significant difference ($p<0.05$). Values are least-squares means ± SEM.

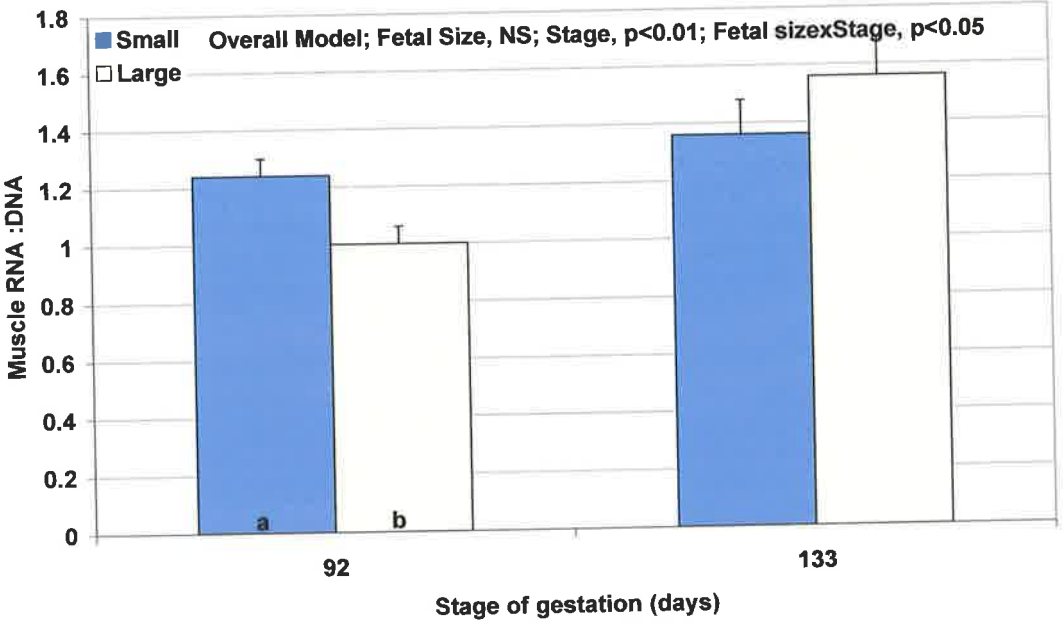


Figure 4.16: Ratio of RNA to DNA in muscle of small and large fetuses at mid- and late gestation. Different letters within a stage of gestation indicate a significant difference ($p<0.05$). Values are least-squares means ± SEM.

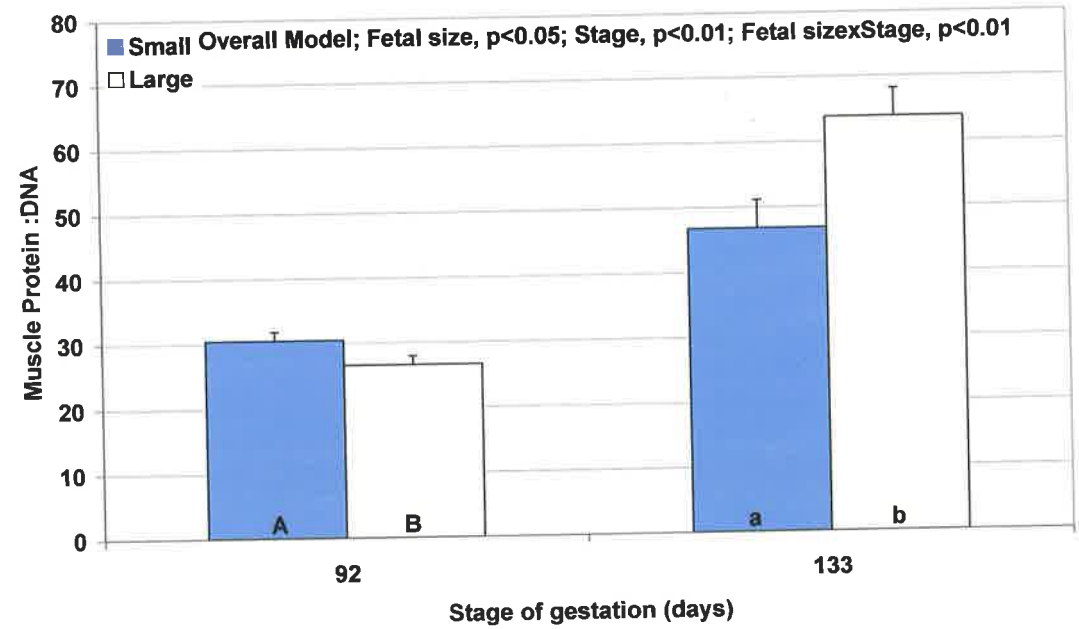


Figure 4.17: Ratio of Protein to DNA in muscle of small and large fetuses at mid- and late gestation. Different letters within a stage of gestation indicate a significant difference (uppercase, $p<0.1$; lowercase, $p<0.05$). Values are least-squares means ± SEM.

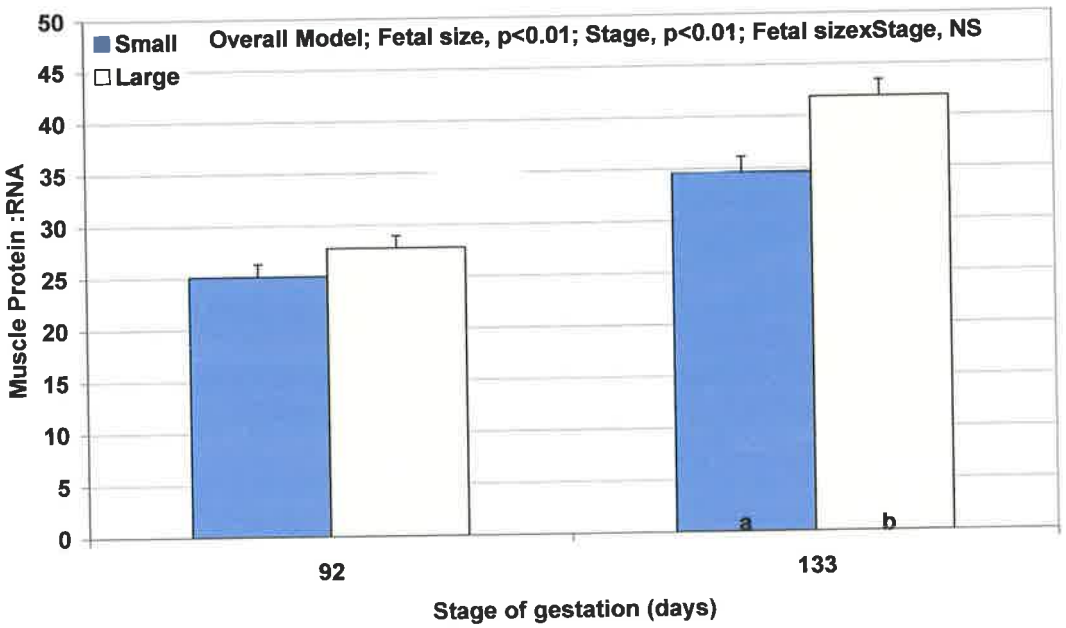


Figure 4.18: Ratio of Protein to RNA in muscle of small and large fetuses at mid- and late gestation. Different letters within a stage of gestation indicate a significant difference ($p<0.05$). Values are least-squares means ± SEM.

Table 4.7: Total DNA, RNA and Protein content of late gestation fetal muscles. *values are least-squares means ± SEM.

	Fetal size		Probability
	Small	Large	p
DNA (mg)	6.2 ± 0.9*	9.9 ± 0.9	0.0075
RNA (mg)	8.2 ± 0.8	14.3 ± 0.9	0.0001
Protein (mg)	281.7 ± 35.4	588.8 ± 35.4	<0.0001

4.4 Discussion

The present study demonstrates that a nutritionally mediated alteration of fetal development (Chapter 3) is associated with modified myogenesis. The results indicate that fetal growth-restriction, due to a severe pre- and post-mating restriction of maternal nutrient supply, may result in delayed or inhibited hypertrophic growth of muscle fibres and, hence, retarded muscle development. The number and types of muscle fibres within the semitendinosus muscle were not affected by fetal size, however growth-restricted fetuses had smaller individual fibres. Muscles of large fetuses had a greater concentration of DNA and protein (mg /g tissue) at mid- and late gestation, respectively, and a greater total DNA and protein content at late gestation, although this was directly related to muscle weight, which was also affected by maternal feed intake in late gestation fetuses.

Decreased muscle mass resulting from maternal under-nutrition and increasing litter size was evident in late gestation fetuses, but was proportionate to decreased fetal weight. Nevertheless, reductions in muscle mass in the range of 12 to 28%, across a range of muscles, due to nutrition and/ or litter size may have a significant impact on post-natal muscle growth and yield, as demonstrated by Greenwood *et al.* (2000a). Feeding ewes above maintenance requirements did not enhance the muscle weights of late gestation fetuses, suggesting little potential to enhance fetal muscle development by maternal over-nutrition.

Estimated muscle fibre number in the late gestation semitendinosus muscle ($380x10^3$) was similar to that reported elsewhere (~$540x10^3$, Nordby *et al.*, 1987; 330-$480x10^3$, McCoard *et al.*, 1997; ~$440x10^3$, Greenwood *et al.*, 2000a; ~404-$426x10^3$, McCoard *et al.*, 2000a;), but more than that reported by Everitt (1965) (60-$85x10^3$). The relatively low apparent myofibre number estimated by Everitt (1965) may suggest problems with the methodology employed by this worker, raising concerns about the validity of the conclusions. There was little difference in estimated muscle fibre number between large and small fetuses, although there tended to be more fibres present in small fetuses at day 92. This finding of increased hyperplasia in small fetuses at day 92 is inconsistent with other studies, which report similar (Greenwood *et al.*, 1999; Greenwood *et al.*, 2000a; McCoard *et al.*, 2000a) or fewer muscle fibre numbers in growth-restricted sheep fetuses (Everitt, 1965) and runt piglets (Wigmore and Stickland, 1983) compared to their well-grown counterparts. This may be due to errors associated with counting myofibres in only 5% of the total muscle area when myofibres

were much smaller than those used in the initial validation of the methodology used, by Greenwood *et al.* (2000a).

Muscle fibre number increased significantly between days 92 and 133 of gestation, regardless of maternal feed intake, suggesting hyperplasia continued into the last third of gestation, as demonstrated by Maier *et al.* (1992) and Greenwood *et al.* (1999). These authors report completion of hyperplasia at approximately 115 days of gestation. In the present study, it was not possible to determine the exact time when or if, hyperplasia ceased, or if it continued beyond day 133, as suggested by McCoard *et al.* (2000a). It would appear that such a significant increase in fibre number between day 92 and 133 is likely to be greater than that due to the increasing length of intrafascicularly terminating fibres alone, as demonstrated by Swatland and Cassens (1972).

There was no difference in the ratio of fast to slow fibres at days 92 or 133 between large and small fetuses in the semitendinosus muscle. This is consistent with Greenwood *et al.* (2000a) and McCoard *et al.* (2000a), but in contrast to Greenwood *et al.* (1999) who found a greater proportion of fast fibres in the peroneus longus muscle of small fetuses at day 130 of gestation. Therefore, it appears that fetal growth-retardation, due to long-term maternal feed restriction, does not impact on the phenotype of muscle fibres in the semitendinosus muscle of fetal sheep.

The greater cross-sectional area of muscle fibres in large fetuses indicates enhanced muscle fibre growth and may be responsible for increased muscle weights. Conversely, the smaller muscles of runt piglets (Wigmore and Stickland, 1983) and twin fetuses (McCoard *et al.*, 2000a) display smaller fibre cross sectional areas than normal size littermates or singleton counterparts, respectively. Further, Swatland and Cassens (1973) reported that muscle fibre diameter was related to the dry weight of the longissimus dorsi. Increased cell growth in this study, as indicated by increased myofibre CSA of fetuses whose mothers feed intake was not restricted, may be attributed to greater fibre hypertophy; the result of greater protein accretion.

In the present study, day 92 muscles of large fetuses had greater DNA concentration (mg /g of muscle), consistent with the work of Greenwood *et al.* (1999) and McCoard *et al.* (2001); this may indicate a greater incorporation of myonuclei within individual fibres or increased satellite cell activation. The amount of myonuclei or DNA in the muscle

fibre has been proposed, and suggested from several studies (Beermann, 1983; Greenwood *et al.*, 1999; 2000a), to be of more importance to muscle growth capacity than muscle fibre number. Greenwood *et al.* (1999) reported that as early as day 85 of gestation approximately 50% of the nuclei associated with myofibres are satellite cells and that reduced DNA content of muscles of growth-restricted fetal sheep was related to declining cell cycle activity, resulting in slower proliferation and incorporation of nuclei into muscle fibres. In rats, severe maternal under-nutrition reduces the satellite cell population in progeny (Beermann *et al.*, 1983). Further, McCoard *et al.* (2001) demonstrated that reduced muscle fibre hypertrophy and muscle growth, due to litter size, was associated with the number of myoD positive cells. A positive reaction for myoD was assumed evidence of an active proliferating satellite cell, with between 75 and 95% of myoD positive nuclei determined to be satellite cells across a range of muscles.

Increasing DNA content is indicative of hyperplasia, with mononucleate myoblasts and satellite cells being incorporated into muscle fibres, suggesting that the rate of myoblast and satellite cell proliferation and differentiation was enhanced in large fetuses. The greater DNA content of individual fibres would subsequently allow greater synthesis of proteins, evident by increased protein content and muscle fibre size at late gestation. Increasing protein concentration, relative to DNA content, is suggestive of true hypertrophic growth of muscle. The higher protein to DNA ratio in large fetuses in late gestation is indicative of greater hypertrophic growth, suggesting delayed hypertrophy, or a decreased rate or efficiency of translation in growth-restricted fetuses. This is further supported by the higher DNA concentration of muscles (mg /g muscle) in small fetuses at late gestation, which indicates that there is less protein, and hence muscle mass, per myonuclei.

The exact mechanisms that determine the rate of cell cycle activity, potential DNA content and regulation of the satellite cell population in fetal muscle remain to be elucidated. The possibility that nutrition interacts with local factors to stimulate or inhibit muscle cell proliferation and differentiation is one that has received limited investigation, particularly in the sheep fetus. The known roles of the MRFs, IGFs and myostatin in regulating proliferation and differentiation of myoblasts makes them ideal candidates to investigate whether specific local nutrition-gene interactions are responsible for altering myogenesis. The effect of nutrition on systemic factors (e.g.

circulating IGFs) has been the focus of more attention, however, too often little attention is given to impacts specific to skeletal muscle.

Two main limitations of this study were evident. Firstly, the protocols employed for muscle sample collection and storage were unsatisfactory. The samples of LD and SS muscle taken were too large for freezing which resulted in poor quality samples available for analysis. Secondly, time and resource constraints meant optimisation of all enzymatic and immuno-histochemical staining protocols in fetal muscle was not possible. If these staining protocols could have been optimised, along with immuno staining with antibodies specific to fetal and neonatal myosin chain isoforms, a better understanding of myofibre transitions as muscle develops and myofibres mature may have resulted.

4.5 Summary

Long-term pre- and post-conception maternal feed intake alters fetal development and myogenesis in sheep. It is likely that maternal under-nutrition results in less myonuclei, due to reduced satellite cell activity, limiting the number of satellite cells available for incorporation into muscle fibres, potentially delaying or decreasing the rate of subsequent hypertrophic growth of myofibres of growth-restricted fetuses. This results in larger muscle fibres and greater muscle mass of fetuses whose growth is uninhibited compared to those that are growth-restricted. Investigation of specific nutrition-gene interactions on muscle development may provide some insight into myogenic regulation in growth-restricted fetuses. The implications of a severe pre-natal nutritional restriction mediated reduction in muscle hypertrophy, such as reported here, on post-natal muscle growth and meat yield and quality across different genotypes and post-natal nutritional regimes are worthy of future study.

CHAPTER 5.

5 THE EFFECTS OF LONG-TERM MATERNAL FEED INTAKE ON THE EXPRESSION PROFILE OF GENES INVOLVED IN MYOGENESIS.

5.1 Introduction

Adverse conditions within the ovine uterine environment can alter normal fetal muscle development and have implications for post-natal muscle growth (Greenwood *et al.*, 1999, 2000a). In chapter 4, it was demonstrated that restricting maternal feed intake before and throughout gestation inhibited the development of muscle in fetal sheep. At late gestation, growth-restricted fetuses had lighter muscles, which were associated with smaller muscle fibres and lower DNA and protein content, indicative of a prolonged proliferative phase or a delayed or slower rate of differentiation, resulting in less hypertrophic development of muscle fibres. It was hypothesised that fetal growth-retardation, caused by a restriction in maternal feed intake, resulted in slower development of muscle fibres.

The function of the MRFs, IGFs and myostatin in regulating muscle cell proliferation and differentiation *in vitro* is well documented (Chapter 1). These genes were identified as candidates likely to regulate myogenesis *in vivo*. However, little is known as to the role they may play when perturbed myogenesis occurs *in vivo*, particularly in fetal sheep. In addition, there is limited information on the role these genes play in the skeletal muscle of mature sheep and if expression is influenced by feed intake at this stage of development. Further, no studies report whether nutrient supply differentially affects the expression of these genes during (fetal) and after (adult) myogenesis in the sheep.

Reasons for a lack of information on gene expression profiles in fetal sheep (and livestock in general), aside from the large expense involved in establishing animal experiments, are that techniques previously used to quantify mRNA abundance (e.g. Northern transfer and Ribonuclease Protection Assays) are laborious and require a large amount of starting material. In recent years, technologies have been developed that require significantly less starting material, can be automated (thereby reducing operator errors) and allow higher throughput. Real-time or quantitative PCR (qPCR) is

one of these technologies and was used in the present study to measure the mRNA abundance of several myogenic regulatory genes in the muscles of fetal and adult sheep.

It was hypothesised that changes in the mRNA expression of the candidate genes known to be involved in myogenesis, would reflect the delays in myogenic differentiation and reduced hypertrophic growth of muscles of growth-restricted fetuses witnessed in Chapter 4. Further, it was hypothesised that mRNA expression of candidate genes would be regulated in a co-ordinated manner fitting with their known roles in myogenesis, derived from other species and *in vitro* studies.

The specific aims of this study were to:

- Develop a method for the rapid and accurate quantification of gene transcripts in fetal and adult muscle samples.
- Assess the usefulness of several reported "housekeeping" genes (GAPDH, β-actin, Cyclophilin A, Acidic Ribosomal Phosphoprotein (ARP) and 18S ribosomal RNA) in experiments that examine developmental, spatial and nutritional aspects of myogenesis.
- Determine the ontogeny, spatial and nutritional regulation of the MRFs, IGFs and myostatin in skeletal muscle of sheep.
- Determine if any of the candidate genes are implicated in the delayed muscle development that occurs in growth-restricted fetuses, witnessed in Chapter 4.

5.2 Experimental

5.2.1 Animals

Fetuses divergent in growth were collected at three stages of gestation, as described in Chapter 3. The semitendinosus (ST) and supraspinatus (SS) muscles were dissected from fetuses and treated as described earlier (section 2.2.1). Individual muscles could not be distinguished at day 50. Distinct muscles were identified at days 92 and 133 of gestation.

Analysis of mRNA abundance was conducted on muscles of three sub-populations of fetuses, based on fetal weights at days 50, 92 and 133 of gestation. Fetuses were classified as Low (L; growth-restricted fetuses derived from feed intake restricted ewes), Medium (M; fetuses derived from maintenance feed intake ewes) or High (H; large fetuses derived from *ad libitum* feed intake ewes) at each of the three stages of gestation. Further, mRNA abundance was measured in the muscles of mature age pregnant ewes slaughtered at day 133 of gestation, after 222 days of restricted, maintenance or *ad libitum* feed intake. Messenger RNA abundance was measured in the composite hind limb muscle of day 50 fetuses and the ST and SS muscles of day 92 and 133 fetuses and mature age ewes. The overall experimental population used for determining mRNA expression profiles is shown in Table 5.1. The weights of fetuses and ewes used in the analysis are presented in Table 5.2.

Table 5.1: Experimental population used for analysis of mRNA abundance of myogenic and housekeeping genes in the semitendinosus (ST) and supraspinatus (SS) muscles of fetuses aged 50, 92 and 133 days and mature ewes slaughtered on day 133 of gestation. *composite hind-limb muscle at day 50, not individual ST.

	Maternal Feed Intake					
Stage of	Low		Maintenance		High	
Development	ST (n)	SS (n)	ST (n)	SS (n)	ST (n)	SS (n)
Fetal day 50	5*		5*		5*	
Fetal day 92	5	5	5	5	5	5
Fetal day 133	5	5	5	5	5	5
Mature ewe	5	5	5	5	5	5

Table 5.2: Weights of fetuses aged 50, 92 and 133 days and mature ewes slaughtered on day 133 of gestation and change in weight of ewes, used for analysis of mRNA abundance of myogenic and housekeeping genes in the semitendinosus and supraspinatus muscles. Values are least-squares means ± SEM; ** change in weight from start of experiment (day -89) to late gestation slaughter (day 133) i.e. 222 days of treatment feeding. Different superscripts within rows indicate significant differences ($p<0.05$).

Stage of Development		Maternal Feed Intake Low	Maintenance	High
Fetal day 50	**(g)**	15.2 ± 0.7*[a] (n=5)	16.9 ± 0.7[a] (n=5)	20.4 ± 0.7[b] (n=5)
Fetal day 92	**(g)**	623.6 ± 19.9[a] (n=5)	689.3 ± 19.9[b] (n=5)	785.3 ± 19.9[c] (n=5)
Fetal day 133	**(g)**	3206.8 ± 171.5[a] (n=5)	4291.6 ± 171.5[b] (n=5)	4805.6 ± 171.5[b] (n=5)
Mature ewe	**(kg)**	56.3 ± 3.2[a] (n=5)	75.9 ± 3.2[b] (n=5)	85.1 ± 3.2[b] (n=5)
Ewe weight change**	**(kg)**	-5.9 ± 1.6[a]	12.9 ± 1.6[b]	20.6 ± 1.6[c]

5.2.2 RNA extraction and reverse transcription

RNA was extracted from fetal and ewe muscle samples as described in section 2.4.1. RNA was column purified (section 2.4.2) and DNase treated (section 2.4.3). RNA concentration and integrity were assessed as described in section 2.4.1. In duplicate, 1.5μg of RNA was reverse transcribed using an anchored oligodT primer with Omniscript™ reverse transcriptase, as described in section 2.4.4.1. Aliquots of neat cDNA duplicates were pooled and then diluted 1:25 with 10mM Tris, pH 8.0, as required. Due to the large number of samples, reverse transcription reactions were prepared in three batches (batch 1, n=33; batch 2, n=47; batch 3, n=25).

5.2.3 Real-time PCR

Real-time PCR was conducted on the 105 pooled cDNA samples as described in section 2.4.4.2.4. For each gene of interest, triplicate PCRs were set-up on the 72-well rotor, allowing 24 triplicate reactions /assay, and conducted on two different occasions (i.e. in duplicate). The set up for analysis of one gene would involve five consecutive assays on the one day (replicate 1), which would be repeated later (replicate 2). Three reference samples were used across all assays and were made by making a large amount of cDNA from three unknown samples from the experimental population (section 2.4.4.2.3). One standard curve was prepared and used for all assays on a day (section 2.4.4.2.3). Preliminary work in our laboratory showed that the stability and reproducibility of cDNA samples was better than that of diluted standards. Further, reference sample cDNA better reflected what was actually going into the PCR of our unknowns than the diluted standards. A typical assay set-up for the experiment described here is outlined in Table 5.3.

Table 5.3: Real-time PCR assay set-up.

Assay Number	Standard Curve*	Reference Samples*	Unknown Samples*	Total
1	3	3	18	24
2		3	21	24
3		3	21	24
4		3	21	24
5		3	21	24

* each measured in triplicate

5.2.3.1 Calculations

A standard curve was constructed by plotting the Log Input Amount vs. the C_t of the amplified standards. The amount of mRNA present in unknowns was then determined from the equation of the standard curve. The relative concentrations of the three reference samples in each of the assays were determined by importing the standard curve from assay 1. As most values are relative, the ratios of the three reference samples (1:2, 1:3 and 2:3) were compared between assays for the one gene, reflecting the reproducibility of the assays (Table 5.4). The Rotorgene 2000 software (Corbert Research) then allows each assay to be adjusted to the most reproducible reference sample to account for C_t variation in the actual values between assays. Data were analysed both with no normalisation to an endogenous or exogenous gene or normalised to 18S rRNA. The data presented in this chapter is normalised to 18S rRNA; data not normalised is presented in Appendix 2. Several reasons the non-normalisation approach was also used included:

- Accurate quantification of total RNA by spectrophotometry meant equal amounts of RNA were added to all cDNA reactions (input RNA).
- The efficiency of the reverse transcription reactions was similar (determined from the abundance of an exogenous control contained in each cDNA sample) within batches of cDNA, but varied between batches.
- The abundance of several commonly used endogenous housekeeping genes was regulated by either stage of development, muscle type or maternal feed intake.

5.2.4 Western transfer

Skeletal muscle proteins (10μg) were separated by SDS-PAGE and transferred to ECL Hybond membrane using the Semi-Dry Electrophoretic Transfer Cell, as described in section 2.4.6.1. Membranes were blocked overnight at 4°C in 1% PVP /0.3% BSA in TBS-T (section 2.4.6.4). Membranes were then rinsed in 2 washes of TBS-T, incubated with 1:1000 primary antibody for 2h at RT and washed in TBS-T 4 times for 5min each (section 2.4.6.4). The HRP-conjugated secondary antibody was then exposed to the membranes at 1:10 000 for 1h at RT, followed by four washes in TBS-T, for 5min each, and 1 to 5min exposure to film (section 2.4.6.4).

Table 5.4: Coefficient of variation (CV%) of qPCR assays measuring the mRNA expression of genes in the skeletal muscle of fetal and mature sheep. CV% was based on repeatability of the three references measured in triplicate in each run across 10 runs per gene.

Gene	Assay CV% Intra	Inter
GAPDH	10.9	14.4
β-actin	12.6	14.4
Cyclophilin A	11.6	18.7
ARP	13.2	8.5
18S rRNA	13	16.5
Exogenous Control	9.6	9.3
Myf-5	10.2	11.9
MyoD	8.1	7.2
Myogenin	12.5	20.3
Myf-6	12.2	16.2
Myostatin	10.3	10.9
IGF-I	12.3	18.4
IGF-II	12.2	24
IGF-I Receptor	9.9	13.5

5.2.5 Statistical analysis

The significance of main effects and their first order interactions on all variables were determined by analysis of variance procedures using the generalized linear model (GLM procedure in SAS). The main effects examined included maternal feed intake (Low, Medium, High), stage of development (fetal day 50, 92 and 133 and Ewe) and muscle type (SS, ST). Reverse transcription batch was included in the model for exogenous control RNA. Interactions that tended to significance ($p<0.1$) and all main effects were retained in the final overall model (Descriptions of the overall models are presented in Appendix 1). Data that were not normally distributed or exhibited uneven variances, due to stage of gestation, was log transformed before analysis. Non-significant interactions were progressively removed from the model in order of least significance.

The data were further reduced with analysis of variance being conducted at each stage of gestation, independent of other stages, and for each muscle at each stage, respectively.

Relationship coefficients (r) and associated probabilities (p) between relative mRNA abundance for each gene with the abundance of other genes, fetal and ewe live weights and fetal muscle weights (where available) were established using Pearson product-moment correlations (CORR procedure in SAS). Correlations between parameters were determined within each gestational stage.

5.3 Results

5.3.1 Real-time PCR quantitation of mRNA

5.3.1.1 Validation of amplification of specific mRNA

5.3.1.1.1 Validation of amplified cDNA and gDNA products

Amplified cDNA fragments were of expected size for each of the genes analysed. The size of the amplified myf-5, myoD, myogenin, myf-6, myostatin and IGF-II fragments in gDNA reflected the spanning of introns, of expected size. Problems encountered with amplicons in the 5` end of the IGF-I and IGF-I receptor genes necessitated the design of qPCR primers in the 3` untranslated regions of both these genes. Therefore, IGF-I, IGF-I receptor and 18S rRNA (intron-less gene) did not span introns so the gDNA fragments were of similar size to cDNA fragments. GAPDH, β-actin, Cyclophilin A and ARP amplicons all spanned introns, however the gDNA fragment amplified from these genes was of a similar size to the amplified cDNA fragment and was thought to be derived from a pseudogene in each case. The size of the fragments amplified using the Robocycler in ovine cDNA and gDNA are shown in Figures 5.1 to 5.5.

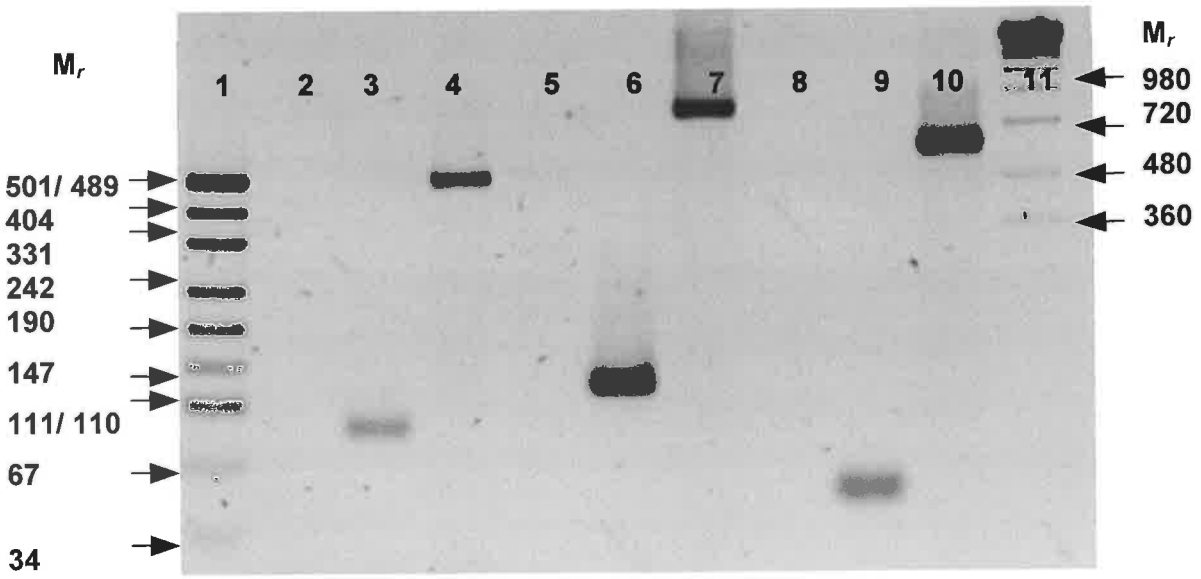


Figure 5.1: Amplified myf-5, myoD and myogenin fragments in ovine cDNA and gDNA, fractionated through 3% low melting point agarose/ TAE gels. Lane 1 pUC19 marker, lanes 2, 5 and 8 no template control, lanes 3, 6 and 9 amplified myf-5, myoD and myogenin cDNA respectively, lanes 4, 7 and 10 amplified myf-5, myoD and myogenin gDNA respectively and lane 11 SPP1 marker.

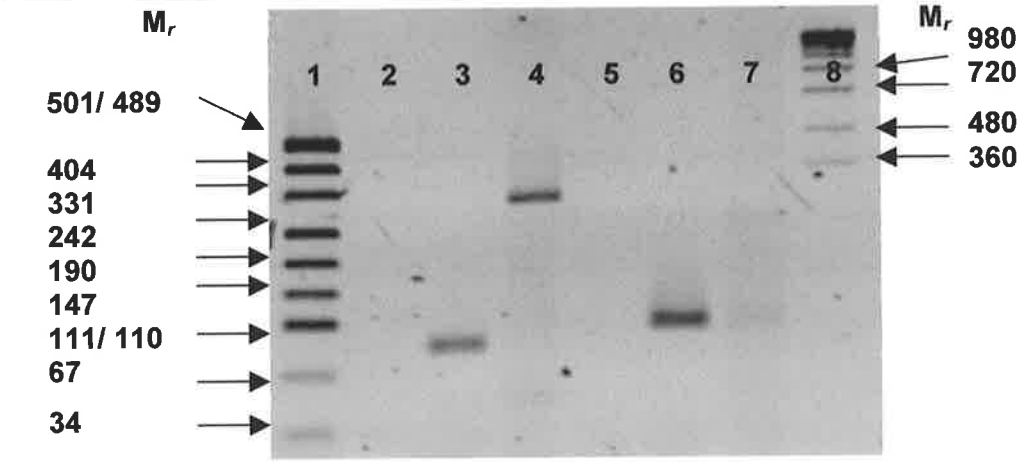


Figure 5.2: Amplified myf-6 and myostatin fragments in ovine cDNA and gDNA, fractionated through 3% low melting point agarose/ TAE gels. Lane 1 pUC19 marker, lanes 2 and 5 no template control, lanes 3 and 6 amplified myf-6 and myostatin cDNA respectively, lanes 4 and 7 amplified myf-6 and myostatin gDNA respectively and lane 8 SPP1 marker.

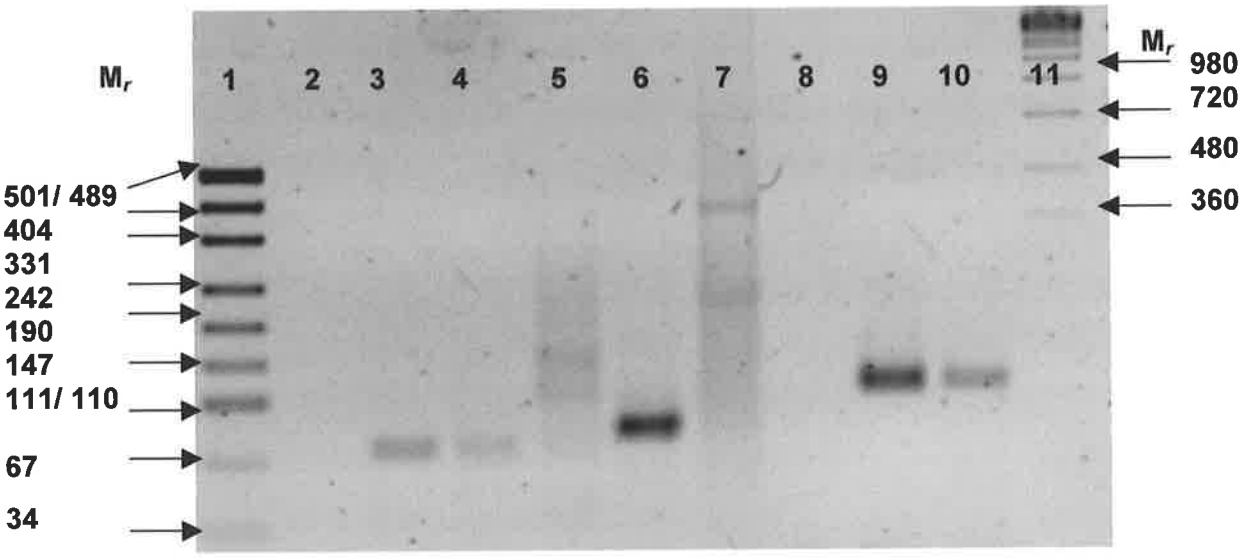


Figure 5.3: Amplified IGF-I, IGF-II and IGF-I receptor fragments in ovine cDNA and gDNA, fractionated through 3% low melting point agarose/ TAE gels. Lane 1 pUC19 marker, lanes 2, 5 and 8 no template control, lanes 3, 6 and 9 amplified IGF-I, IGF-II and IGF-I receptor cDNA respectively, lanes 4, 7 and 10 amplified IGF-I, IGF-II and IGF-I receptor gDNA respectively and lane 11 SPP1 marker.

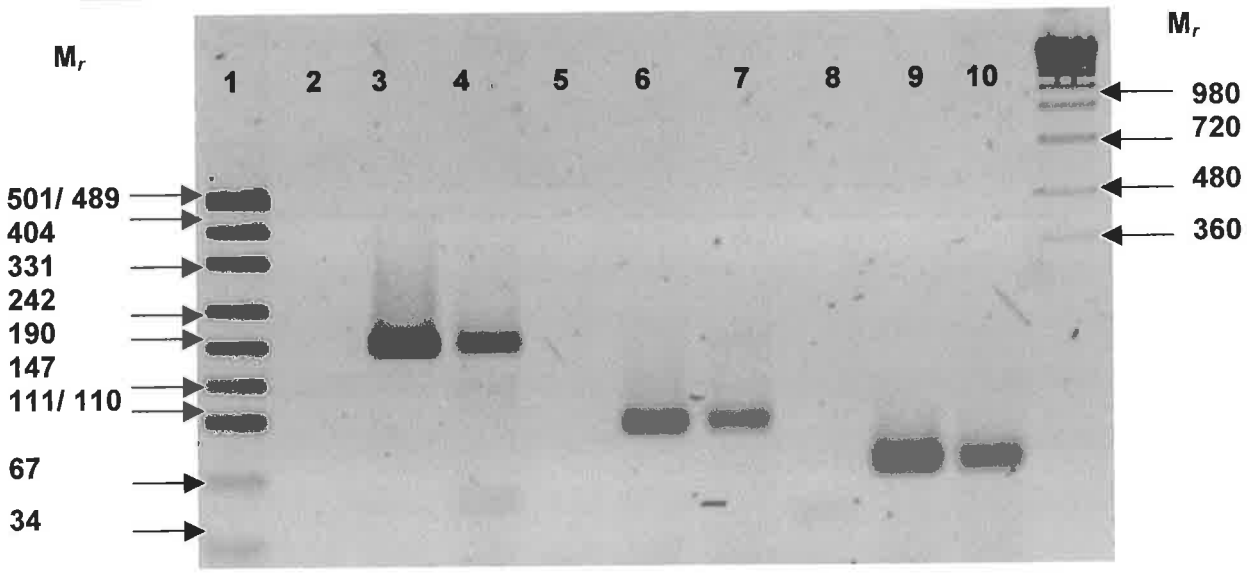


Figure 5.4: Amplified β-actin, GAPDH and Cyclophilin A fragments in ovine cDNA and gDNA, fractionated through 3% low melting point agarose/ TAE gels. Lane 1 pUC19 marker, lanes 2, 5 and 8 no template control, lanes 3, 6 and 9 amplified β-actin, GAPDH and Cyclophilin A cDNA respectively, lanes 4, 7 and 10 amplified β-actin, GAPDH and Cyclophilin A gDNA respectively and lane 11 SPP1 marker.

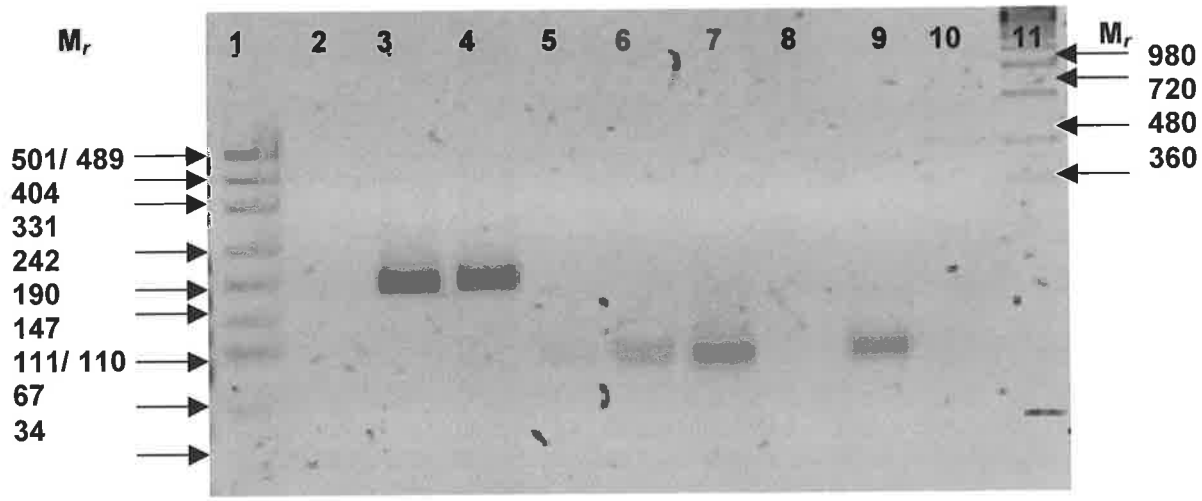


Figure 5.5: Amplified ARP, 18S and exogenous control fragments in ovine cDNA and gDNA, fractionated through 3% low melting point agarose/ TAE gels. Lane 1 pUC19 marker, lanes 2, 5 and 8 no template control, lanes 3, 6 and 9 amplified ARP, 18S and control cDNA respectively, lanes 4, 7 and 10 amplified ARP, 18S and control gDNA respectively and lane 11 SPP1 marker.

5.3.1.1.2 Sequencing of amplified cDNA products

The nucleotide sequence of all amplified products matched the expected published sequences.

5.3.1.1.3 Melt curve analysis

Melt curve analysis was conducted at the end of each experiment on every PCR performed. All products were specific, as indicated by a single melt peak (see Figure 2.6, for example); this was further confirmed by gel electrophoresis of a random selection of cDNA samples amplified in the qPCR studies (data not presented).

5.3.1.2 Quantitative results

5.3.1.2.1 Reverse transcription efficiency

The abundance of exogenous control mRNA measured by real-time qPCR differed between cDNA batches ($p<0.01$) and stage of development ($p<0.05$). However, these two were linked as batch 1 contained samples of day 50 and 92 cDNA, batch 2 contained samples of day 133 and ewe cDNA and batch 3 contained mostly ewe cDNA. Control abundance within a batch was generally unaffected by stage ($p>0.05$). Therefore, the difference in control cDNA witnessed across all stages of development is probably actually due to batch rather than stage. Feed intake and muscle had no affect on the amount of control measured. The overall analysis of variance model is presented in Appendix 1.

5.3.1.2.2 Abundance of housekeeping genes (18S rRNA, GAPDH, β-actin, Cyclophilin A and ARP) mRNA in the skeletal muscle of ovine fetuses and ewes.

The overall analysis of variance models are presented in Appendix 1.

5.3.1.2.2.1 Stage of development

In qPCR using total RNA, there was a higher proportion of 18S rRNA measured in the muscles of early and mid-gestation fetuses compared with late gestation fetuses and mature ewes (Table 5.5). GAPDH mRNA levels increased significantly ($p<0.05$) with fetal age and was lower in ewe muscles compared with late gestation fetal muscles (Table 5.5). In contrast, β-actin and Cyclophilin A mRNA levels both decreased significantly ($p<0.05$) with progressing fetal development and decreased further in ewe muscles (Table 5.5). ARP mRNA levels were significantly ($p<0.05$) greater in muscles

of day 50 fetuses, with little difference in abundance between the other stages of development (Table 5.5).

5.3.1.2.2.2 Muscle type

GAPDH mRNA levels increased ($p<0.1$ or <0.05) in the ST muscle at each stage of development and appeared to increase relative to SS as development increased (Table 5.6). The level of β-actin and ARP mRNA was similar between the SS and ST muscle at all stages of development (Table 5.6). Cyclophilin A mRNA and 18S rRNA were higher ($p<0.05$) in the ST muscle of day 133 fetuses and mature ewes, respectively (Table 5.6).

5.3.1.2.2.3 Feed intake

GAPDH mRNA levels tended to be greater in muscles of day 133 fetuses whose mothers received high feed intakes ($p<0.1$; Appendix 2). Mature aged ewes offered a restricted feed intake had lower GAPDH mRNA in skeletal muscles ($p<0.1$; Appendix 2). Feed intake significantly ($p<0.05$) affected GAPDH mRNA abundance in the ST muscle (see Figure 5.7). Skeletal muscle β-actin mRNA tended to be greater in H fetuses at day 133 of gestation ($p<0.1$; Appendix 2) and lower in High intake mature aged ewes ($p<0.1$; Appendix 2). Mature aged ewes offered *ad libitum* or maintenance requirements had lower skeletal muscle Cyclophilin A mRNA than those offered restricted feed intake ($p<0.05$; Appendix 2). The level of ARP mRNA in fetal skeletal muscle was unaffected by maternal feed intake at mid- and late gestation (Appendix 2), however, ARP mRNA was elevated in mature aged ewes offered restricted feed intake ($p<0.05$; Appendix 2). The results of the analysis of the effect of feed intake on "housekeeping" gene mRNA abundance in each muscle type at each stage of development are presented in Figures 5.6-5.10.

Table 5.5: The abundance of 18S rRNA, GAPDH, β-actin, Cyclophilin A and ARP mRNA in skeletal muscles (pooled averages at each stage) of fetuses aged 50, 92 and 133 days and mature ewes slaughtered on day 133 of gestation, expressed relative to abundance at day 50 (100%). *values are means ± SEM converted to percentages; data were log transformed. Different superscripts within rows indicate significantly different relative mRNA levels between stages ($p<0.05$).

	Stage of Development			
	Fetal day 50	Fetal day 92	Fetal day 133	Mature ewe
18S rRNA	$100 \pm 8.6^{*a}$	102.1 ± 8.3^{a}	55.9 ± 7.9^{b}	40.8 ± 7.1^{b}
GAPDH	100.0 ± 338.0^{a}	739.7 ± 273.1^{b}	3812.2 ± 273.1^{c}	2561.2 ± 273.1^{d}
β-actin	100 ± 4.4^{a}	39.9 ± 2.9^{b}	20.5 ± 2.9^{c}	9.1 ± 2.9^{d}
Cyclophilin A	100.0 ± 5.3^{a}	59.4 ± 3.4^{b}	28.8 ± 3.4^{c}	13.8 ± 3.4^{d}
Acidic RiboProtein	100.0 ± 7.4^{a}	55.6 ± 4.9^{b}	74.4 ± 4.9^{c}	70.6 ± 4.9^{c}

Table 5.6: The abundance of 18S rRNA, GAPDH, β-actin, Cyclophilin A and ARP mRNA in the semitendinosus (ST) muscle expressed as a percentage of mRNA in the supraspinatus (SS) muscle of mid- and late gestation fetuses and mature ewes slaughtered on day 133 of gestation. *values are least-squares means ± SEM converted to percentages. Different superscripts within rows indicate significantly different mRNA levels between muscles at each stage (lowercase, $p<0.05$; uppercase $p<0.1$).

	Fetal day 92		Fetal day 133		Mature ewe	
	SS	ST	SS	ST	SS	ST
18S rRNA	$100.0 \pm 7.8^{*}$	98.5 ± 7.8	100.0 ± 11.7	116.7 ± 11.7	100.0 ± 15.7^{a}	149.0 ± 15.7^{b}
GAPDH	100.0 ± 9.9^{A}	126.3 ± 9.9^{B}	100.0 ± 22.4^{a}	172.3 ± 22.4^{b}	100.0 ± 15.5^{a}	161.7 ± 15.5^{b}
β-actin	100.0 ± 10.1	121.0 ± 10.1	100.0 ± 12.3	106.7 ± 12.3	100.0 ± 12.1	71.8 ± 12.1
Cyclophilin A	100.0 ± 9.4	107.4 ± 9.4	100.0 ± 23.7^{a}	173.8 ± 23.7^{b}	100.0 ± 11.4	89.0 ± 11.4
Acidic RiboProtein	100.0 ± 9.1	96.3 ± 9.1	100.0 ± 9.6	84.4 ± 9.6	100.0 ± 9.9	110.3 ± 9.9

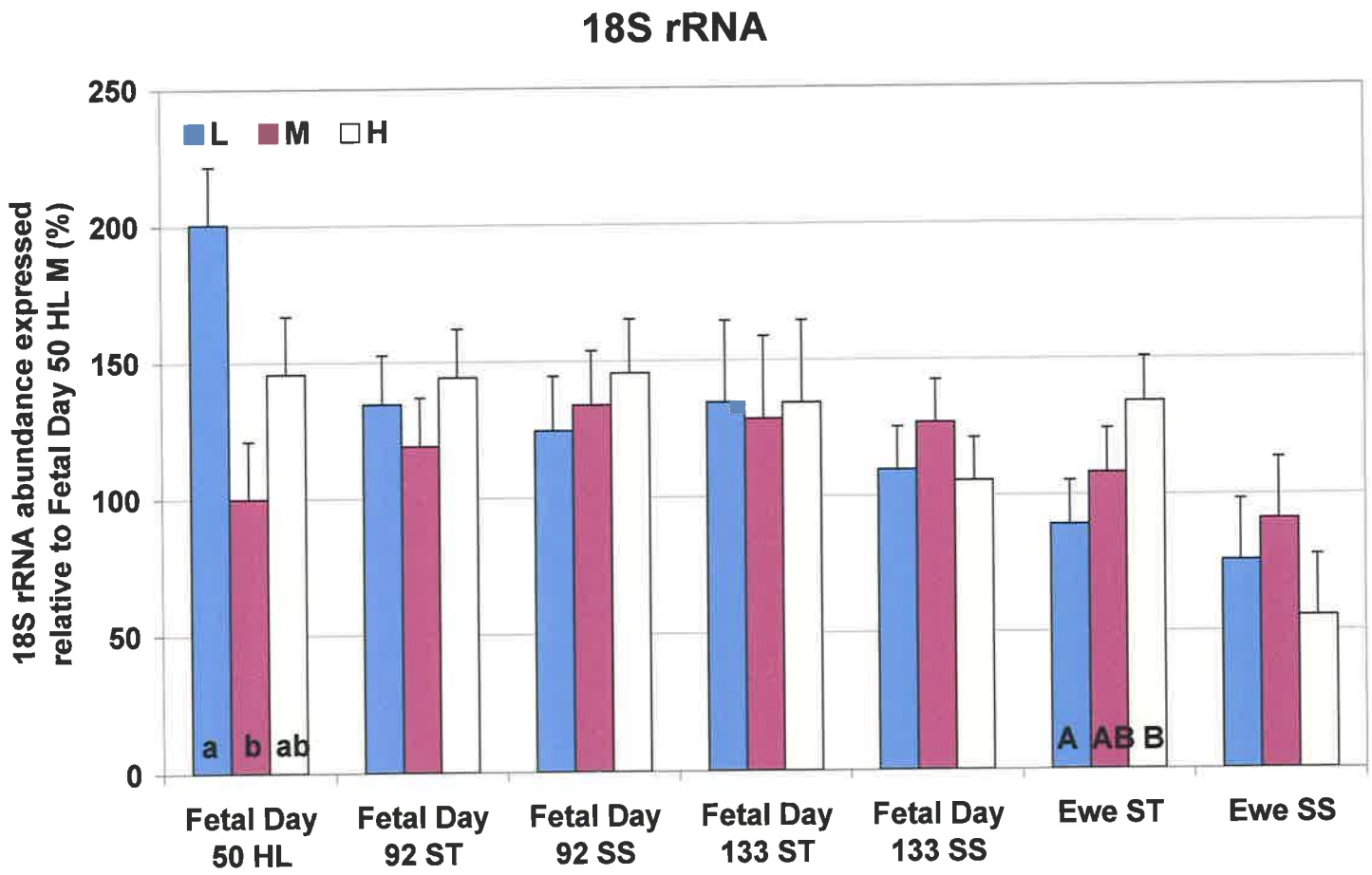


Figure 5.6: The effect of feed intake (L, M, H) on the abundance of 18S rRNA in the semitendinosus (ST) and supraspinatus (SS) muscles of fetuses aged 50, 92 and 133 days and mature ewes slaughtered on day 133 of gestation. All values are expressed relative to rRNA abundance in the hind-limb (HL) of maintenance fetal sheep at day 50 (100%). Different letters within each muscle at each stage of development indicate a significant difference (lowercase, $p<0.05$; uppercase $p<0.1$).

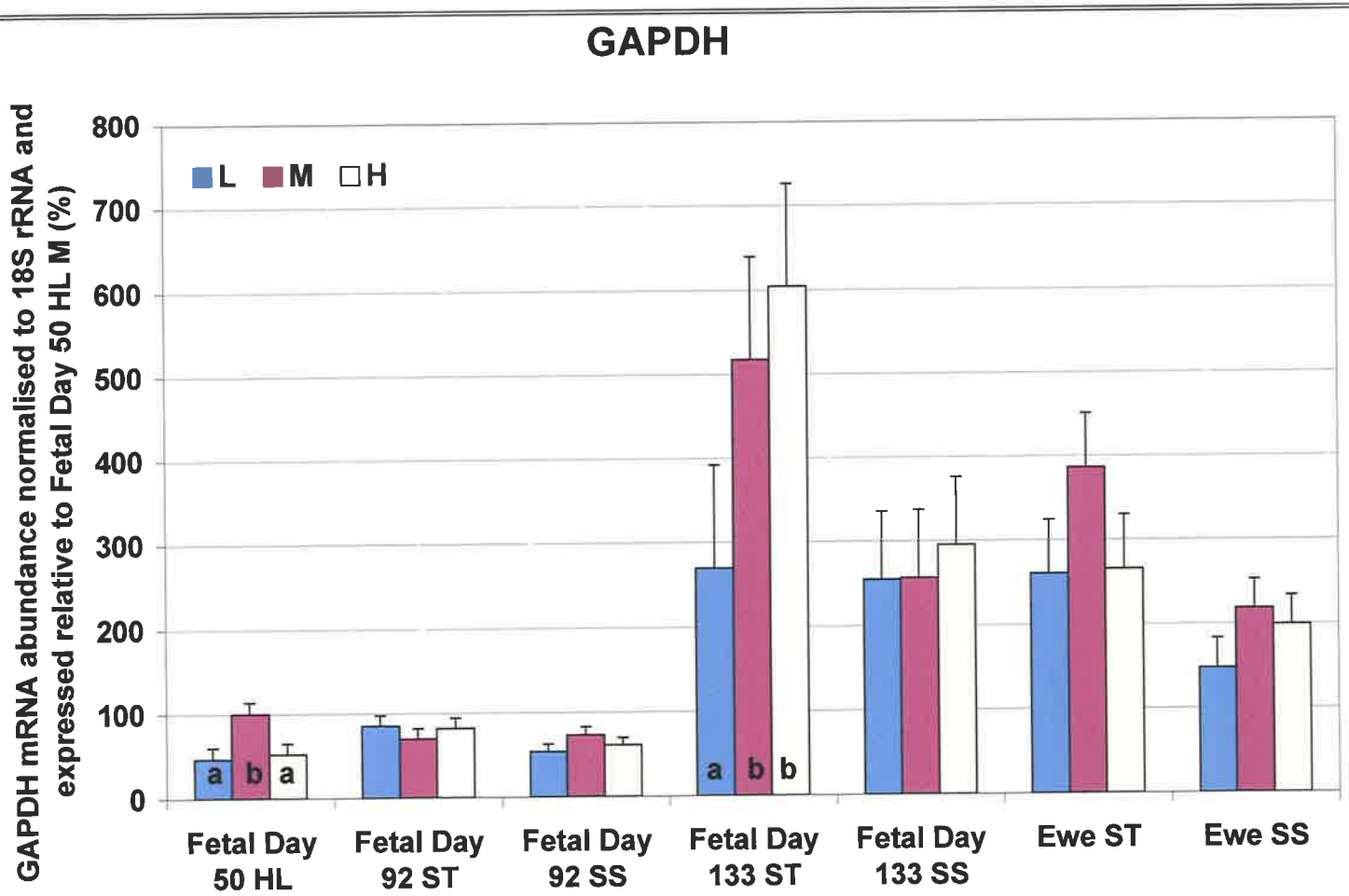


Figure 5.7: The effect of feed intake (L, M, H) on the abundance of GAPDH mRNA in the semitendinosus (ST) and supraspinatus (SS) muscles of fetuses aged 50, 92 and 133 days and mature ewes slaughtered on day 133 of gestation. All values are expressed relative to mRNA abundance in the hind-limb (HL) of maintenance fetal sheep at day 50 (100%). Different letters within each muscle at each stage of development indicate a significant difference ($p<0.05$).

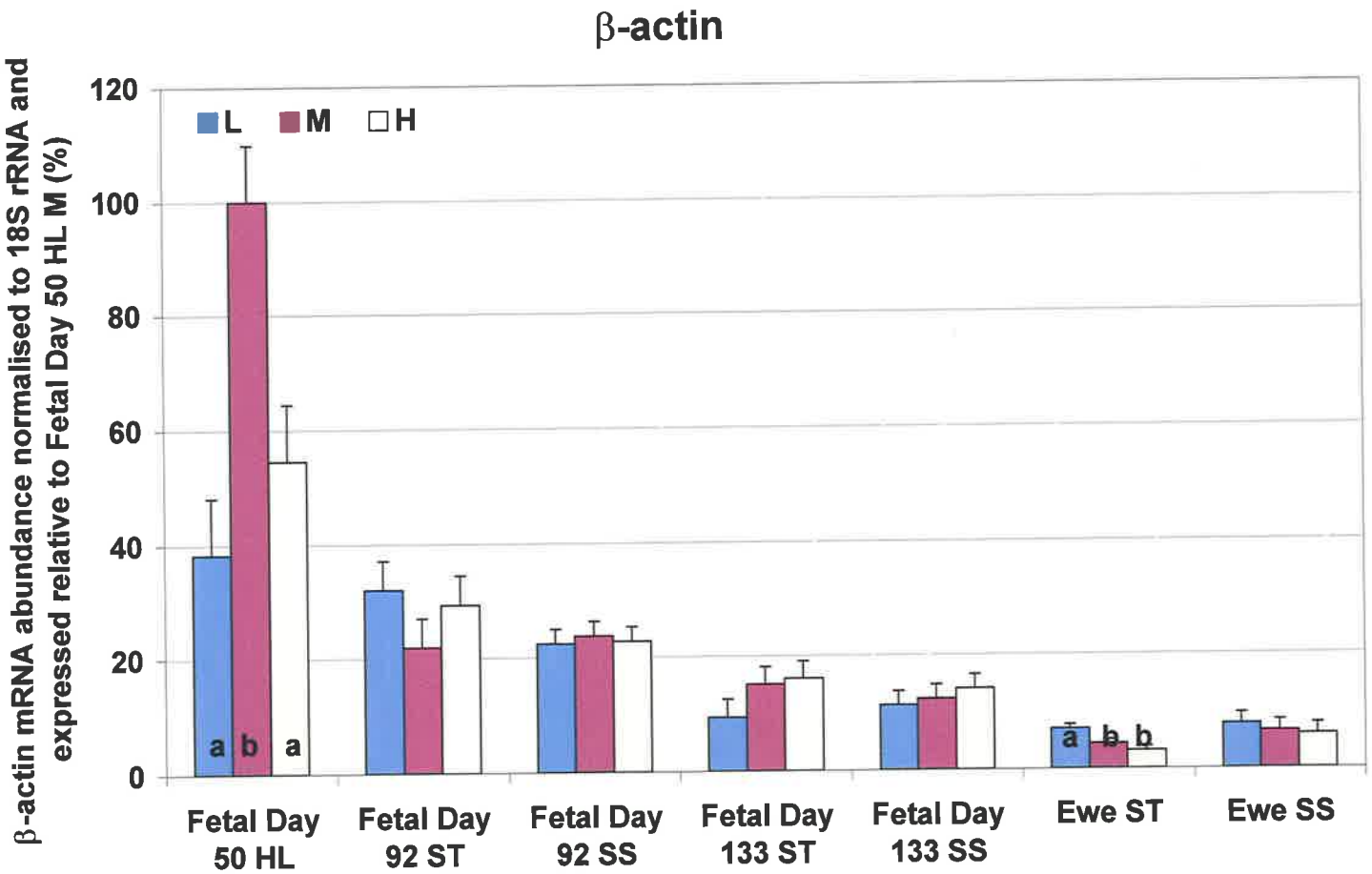


Figure 5.8: The effect of feed intake (L, M, H) on the abundance of β-actin mRNA in the semitendinosus (ST) and supraspinatus (SS) muscles of fetuses aged 50, 92 and 133 days and mature ewes slaughtered at day 133 of gestation. All values are expressed relative to mRNA abundance in the hind-limb (HL) of maintenance fetal sheep at day 50 (100%). Different letters within each muscle at each stage of development indicate a significant difference ($p<0.05$).

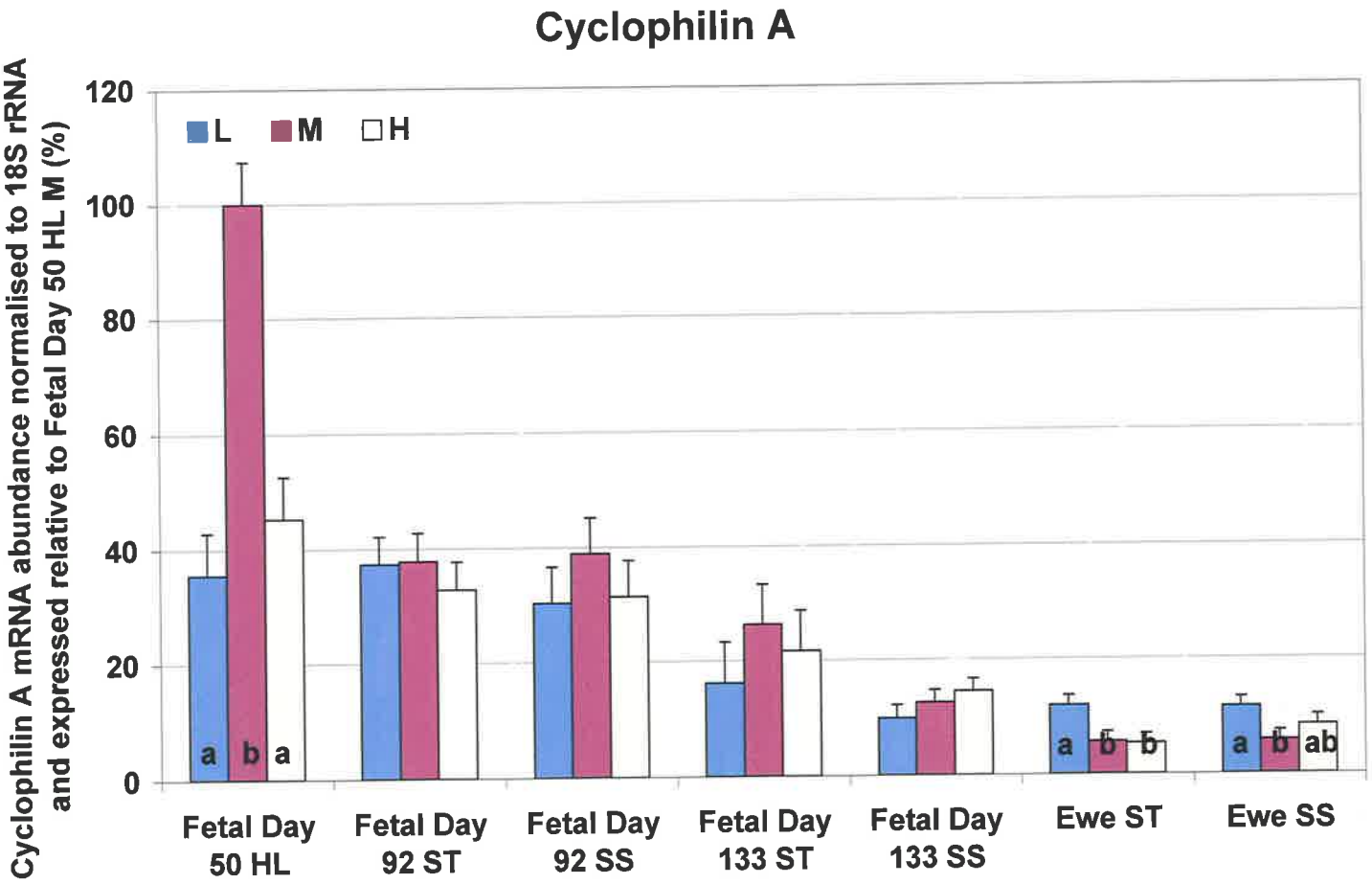


Figure 5.9: **The effect of feed intake (L, M, H) on the abundance of Cyclophilin A mRNA in the semitendinosus (ST) and supraspinatus (SS) muscles of fetuses aged 50, 92 and 133 days and mature ewes slaughtered on day 133 of gestation.** All values are expressed relative to mRNA abundance in the hind-limb (HL) of maintenance fetal sheep at day 50 (100%). Different letters within each muscle at each stage of development indicate a significant difference ($p<0.05$).

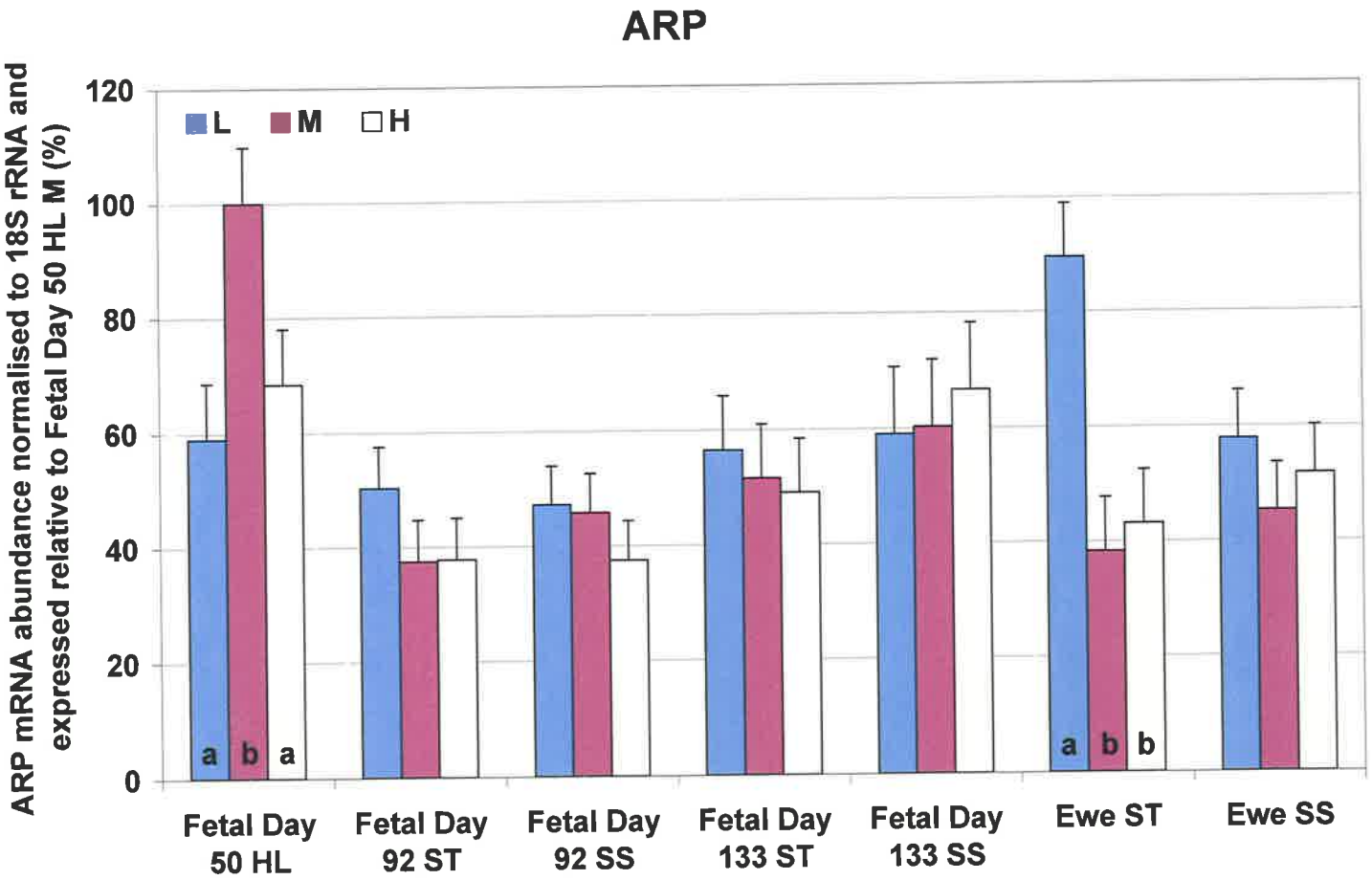


Figure 5.10: The effect of feed intake (L, M, H) on the abundance of Acidic Ribosomal Phosphoprotein mRNA in the semitendinosus (ST) and supraspinatus (SS) muscles of fetuses aged 50, 92 and 133 days and mature ewes slaughtered at day 133 of gestation. All values are expressed relative to mRNA abundance in the hind-limb (HL) of maintenance fetal sheep at day 50 (100%). Different letters within each muscle at each stage of development indicate a significant difference ($p<0.05$).

5.3.1.2.3 Abundance of myf-5, myoD, myogenin, myf-6 and myostatin mRNA in fetal and ewe skeletal muscle.

The overall analysis of variance models are presented in Appendix 1.

5.3.1.2.3.1 Stage of development

The level of myf-5, myoD and myogenin mRNA decreased with stage of development (Table 5.7). In contrast, myf-6 mRNA abundance increased with stage of development (Table 5.7). Myostatin mRNA level peaked in the skeletal muscle of day 92 fetuses and declined thereafter (Table 5.7).

5.3.1.2.3.2 Muscle type

The abundance of the MRFs, with the exception of myogenin, and myostatin mRNA was greater in the ST muscle of day 92 fetuses (Table 5.8). Myf-6 and myostatin mRNA levels tended to be greater in the ST muscle of day 133 fetuses ($p<0.1$; Table 5.8). The ST muscle of mature aged ewes had greater myoD and myostatin mRNA levels, although not significant when normalised to 18S rRNA (Table 5.8).

5.3.1.2.3.3 Feed intake

The mRNA levels of MRFs and myostatin were elevated in the muscle of maintenance fetuses at day 50 of gestation ($p<0.05$; Appendix 2). However, this observation was deemed to be an anomaly with no biological basis since it was present in all the genes examined in this study. Rather, it is probably due to a technical problem that arose during the RNA clean-up procedure (see discussion for more details). Myf-5 mRNA level was elevated in the muscles of Low fetuses at day 133 of gestation ($p<0.05$; Appendix 2). MyoD mRNA level was reduced in the muscles of High feed intake ewes ($p<0.05$; Appendix 2), in both muscles (Figure 5.12). The abundance of myf-6 mRNA was reduced in the muscles of ewes offered maintenance requirements; there was no difference myf-6 abundance in the muscles of High and Low feed intake ewes ($p<0.05$; Appendix 2). Myostatin mRNA was at lower levels in High fetuses at day 92 of gestation ($p<0.05$; Appendix 2). The results of the analysis of the effect of feed intake on the mRNA abundance of the MRFs and myostatin in each muscle type at each stage of development is presented in Figures 5.11-5.15.

Table 5.7: The abundance of myf-5, myoD, myogenin, myf-6 and myostatin mRNA in skeletal muscles (pooled averages at each stage) of fetuses aged 50, 92 and 133 days and mature ewes slaughtered on day 133 of gestation, expressed relative to abundance at day 50 (100%). *values are means ± SEM converted to percentages; data were log transformed. Different superscripts within rows indicate significantly different relative mRNA levels between stages ($p<0.05$).

	Stage of Development			
	Fetal day 50	**Fetal day 92**	**Fetal day 133**	**Mature ewe**
Myf-5	$100.0 \pm 5.6^{*a}$	51.2 ± 3.7^{b}	8.2 ± 3.7^{c}	3.2 ± 3.7^{d}
MyoD	100.0 ± 13.7^{ab}	103.0 ± 9.0^{a}	69.8 ± 9.0^{bc}	54.2 ± 9.0^{c}
Myogenin	100.0 ± 6.5^{a}	85.7 ± 4.3^{a}	34.4 ± 4.3^{b}	16.1 ± 4.3^{c}
Myf-6	100.0 ± 97.2^{a}	195.7 ± 63.6^{b}	191.5 ± 63.6^{b}	1244.1 ± 63.6^{c}
Myostatin	100.0 ± 14.5^{a}	169.5 ± 9.5^{b}	53.2 ± 9.5^{c}	33.4 ± 9.5^{d}

Table 5.8: The abundance of myf-5, myoD, myogenin, myf-6 and myostatin mRNA in the semitendinosus (ST) muscle expressed as a percentage of mRNA in the supraspinatus (SS) muscle of mid- and late gestation fetuses and mature ewes slaughtered on day 133 of gestation. *values are least-squares means ± SEM converted to percentages. Different superscripts within rows indicate significantly different mRNA levels between muscles at each stage (lowercase, $p<0.05$; uppercase, $p<0.1$).

	Fetal day 92		Fetal day 133		Mature ewe	
	SS	**ST**	**SS**	**ST**	**SS**	**ST**
Myf-5	$100.0 \pm 9.6^{*a}$	143.2 ± 9.6^{b}	100.0 ± 10.6	104.4 ± 10.6	100.0 ± 9.3^{a}	48.8 ± 9.3^{b}
MyoD	100.0 ± 14.5^{a}	150.2 ± 14.5^{b}	100.0 ± 12.9	68.7 ± 12.9	100.0 ± 20.9	118.9 ± 20.9
Myogenin	100.0 ± 11.2	121.2 ± 11.2	100.0 ± 12.9	112.9 ± 12.9	100.0 ± 15.6	77.1 ± 15.6
Myf-6	100.0 ± 11.7^{A}	133.2 ± 11.7^{B}	100.0 ± 17.8^{A}	147.3 ± 17.8^{B}	100.0 ± 13.4	100.7 ± 13.4
Myostatin	100.0 ± 14.0^{a}	168.0 ± 14.0^{b}	100.0 ± 25.8^{A}	172.5 ± 25.8^{B}	100.0 ± 34.5	124.6 ± 34.5

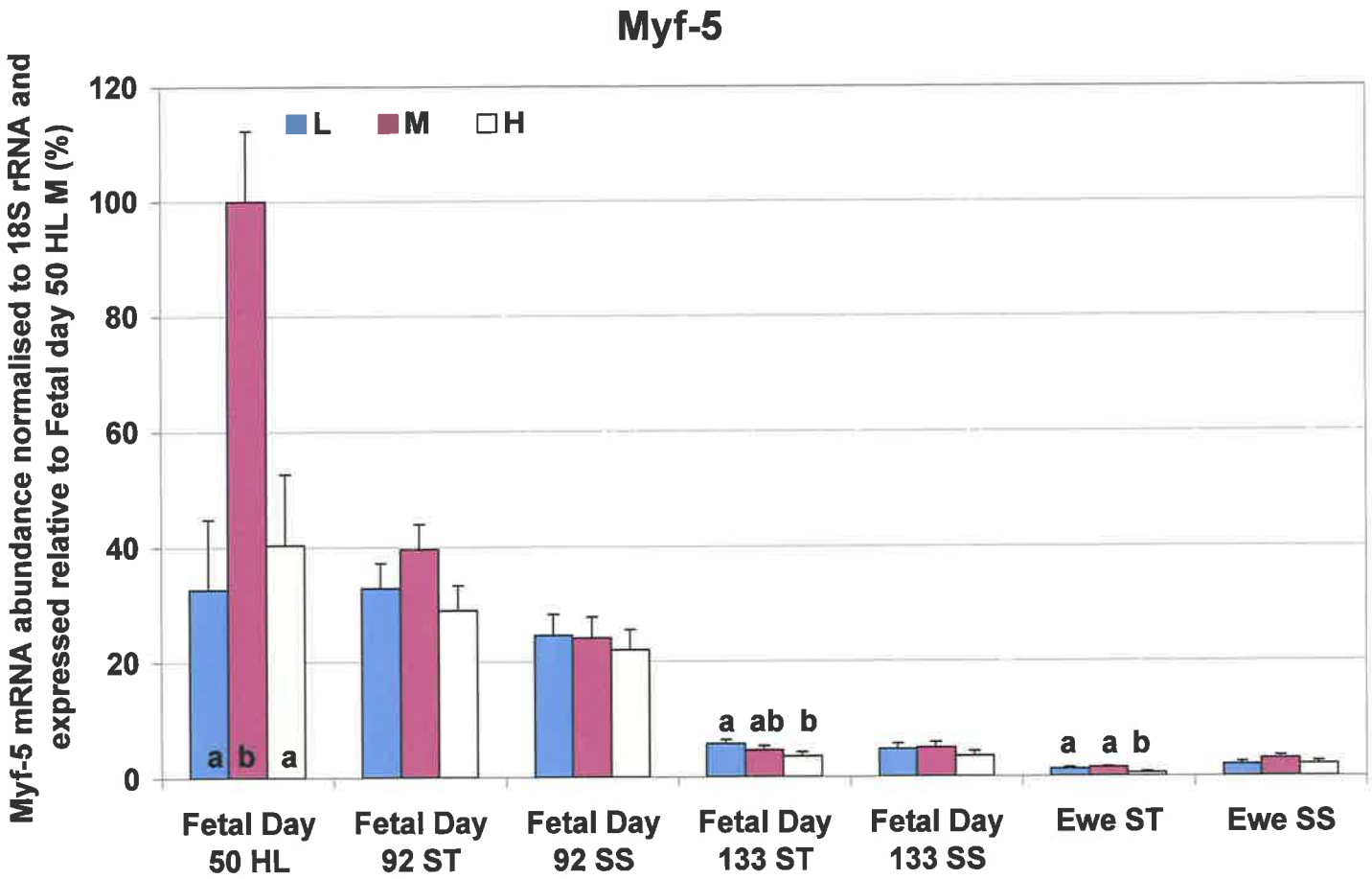


Figure 5.11: The effect of feed intake (L, M, H) on the abundance of myf-5 mRNA in the semitendinosus (ST) and supraspinatus (SS) muscles of fetuses aged 50, 92 and 133 days and mature ewes slaughtered on day 133 of gestation. All values are expressed relative to mRNA abundance in the hind-limb (HL) of maintenance fetal sheep at day 50 (100%). Different letters within each muscle at each stage of development indicate a significant difference ($p<0.05$).

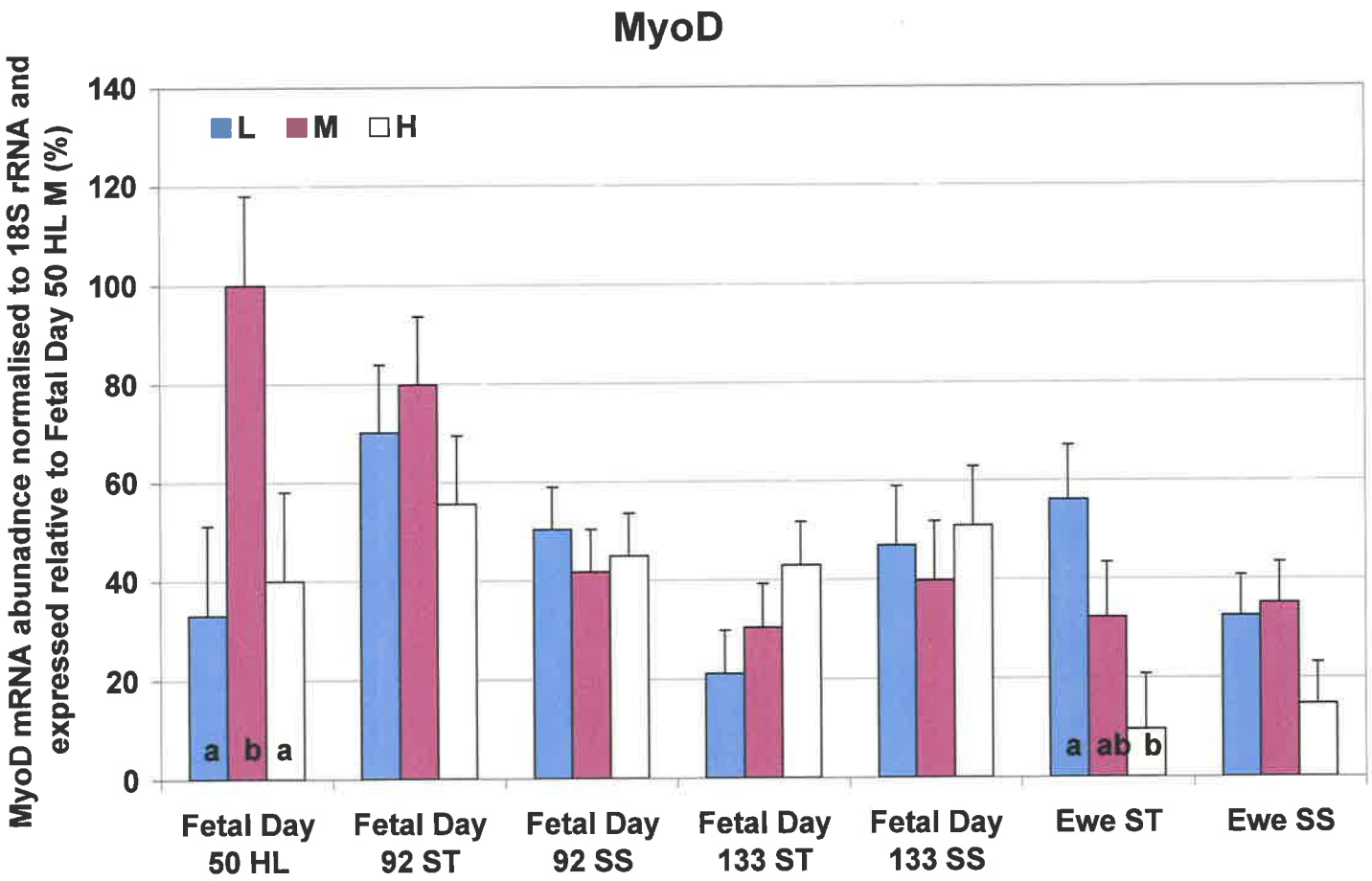


Figure 5.12: The effect of feed intake (L, M, H) on the abundance of myoD mRNA in the semitendinosus (ST) and supraspinatus (SS) muscles of fetuses aged 50, 92 and 133 days and mature ewes slaughtered on day 133 of gestation. All values are expressed relative to mRNA abundance in the hind-limb (HL) of maintenance fetal sheep at day 50 (100%). Different letters within each muscle at each stage of development indicate a significant difference ($p<0.05$).

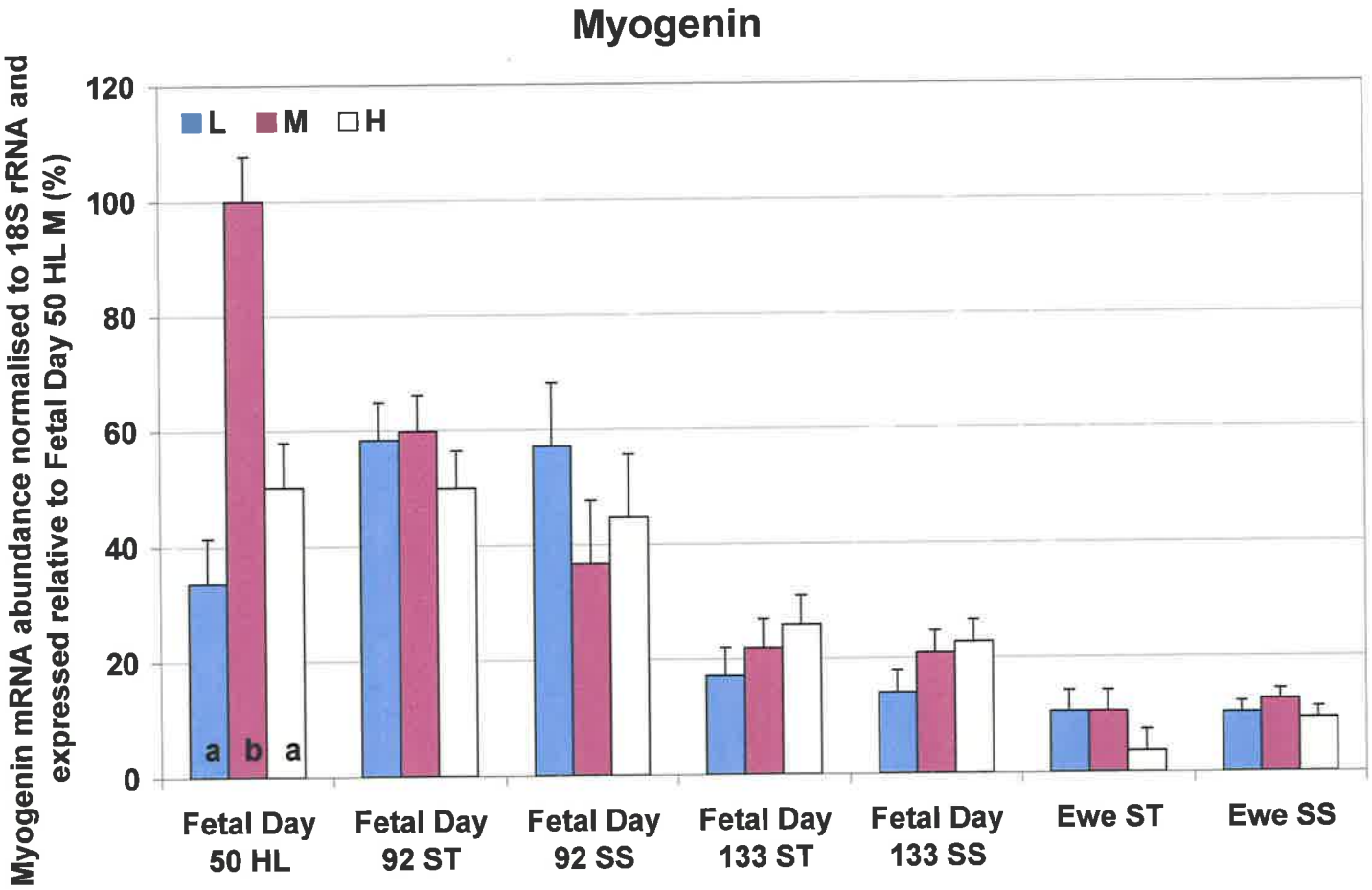


Figure 5.13: The effect of feed intake (L, M, H) on the abundance of myogenin mRNA in the semitendinosus (ST) and supraspinatus (SS) muscles of fetuses aged 50, 92 and 133 days and mature ewes slaughtered at day 133 of gestation. All values are expressed relative to mRNA abundance in the hind-limb (HL) of maintenance fetal sheep at day 50 (100%). Different letters within each muscle at each stage of development indicate a significant difference ($p<0.05$).

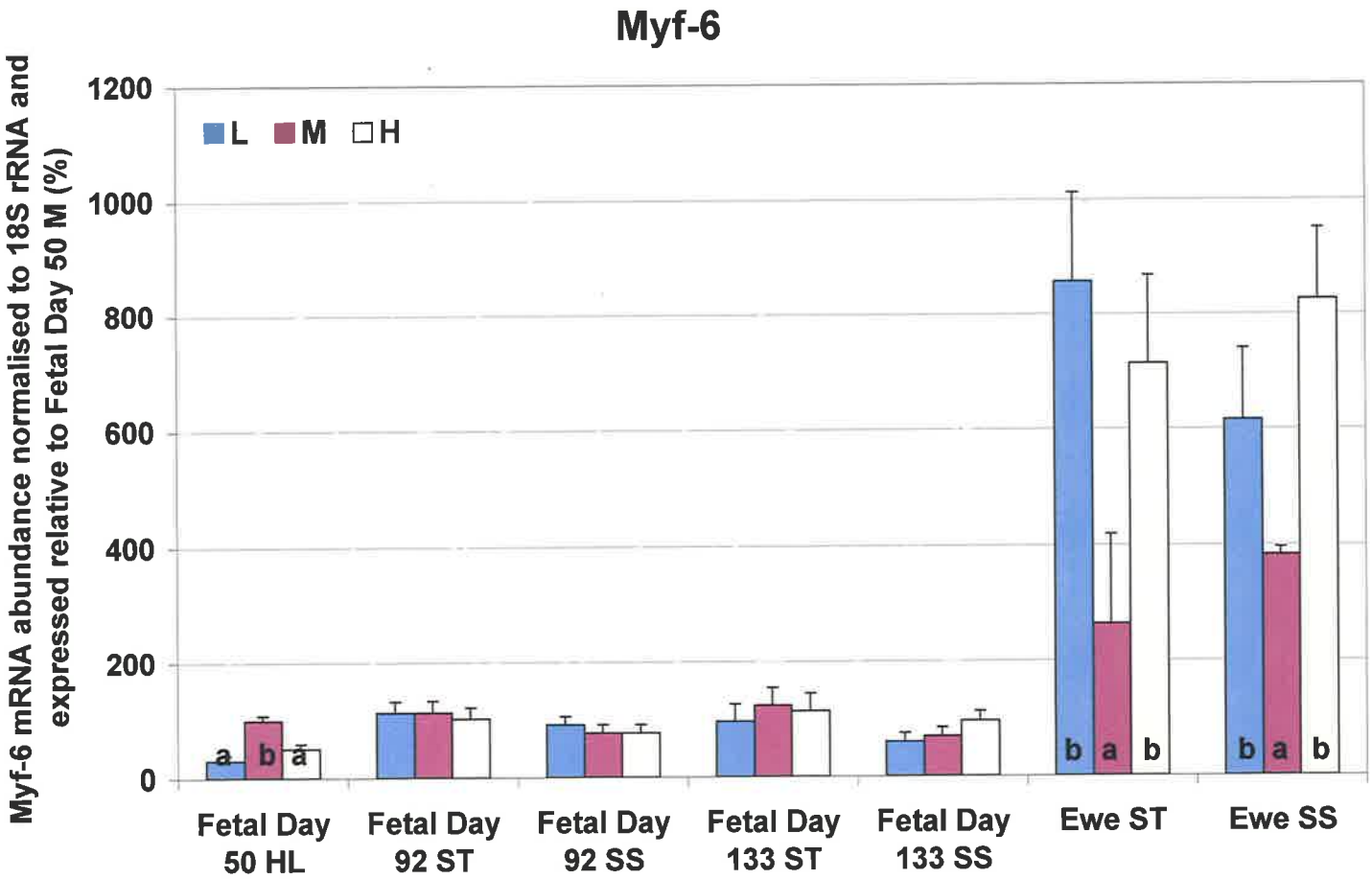


Figure 5.14: The effect of feed intake (L, M, H) on the abundance of myf-6 mRNA in the semitendinosus (ST) and supraspinatus (SS) muscles of fetuses aged 50, 92 and 133 days and mature ewes slaughtered on day 133 of gestation. All values are expressed relative to mRNA abundance in the hind-limb (HL) of maintenance fetal sheep at day 50 (100%). Different letters within each muscle at each stage of development indicate a significant difference ($p<0.05$).

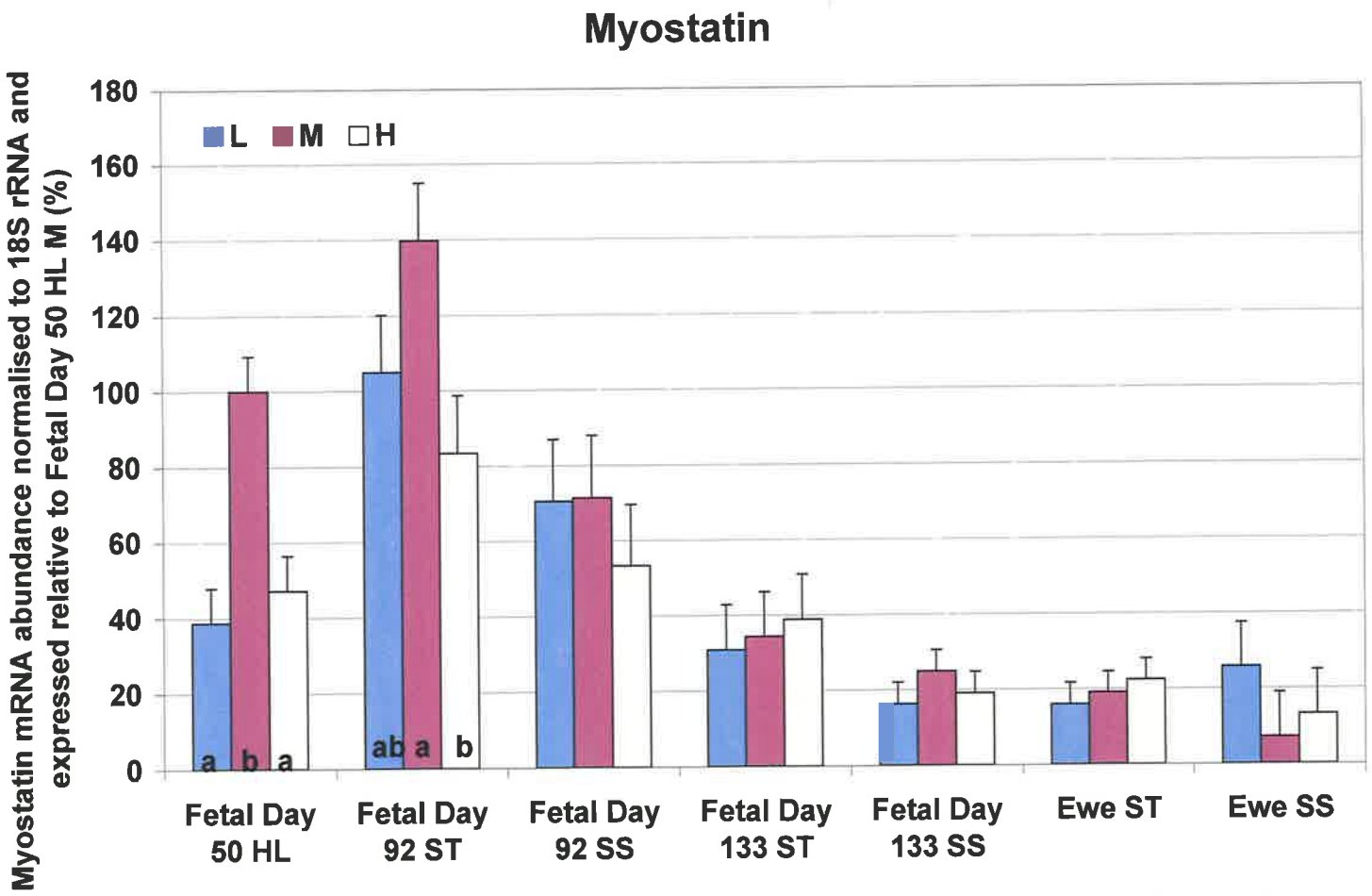


Figure 5.15: The effect of feed intake (L, M, H) on the abundance of myostatin mRNA in the semitendinosus (ST) and supraspinatus (SS) muscles of fetuses aged 50, 92 and 133 days and mature ewes slaughtered on day 133 of gestation. All values are expressed relative to mRNA abundance in the hind-limb (HL) of maintenance fetal sheep at day 50 (100%). Different letters within each muscle at each stage of development indicate a significant difference ($p<0.05$).

5.3.1.2.4 Abundance of IGF-I, IGF-II and IGF-I receptor mRNA in fetal and ewe skeletal muscle.

The overall analysis of variance models are presented in Appendix 1.

5.3.1.2.4.1 Stage of development

Skeletal muscle IGF-I and IGF-II mRNA levels peaked at day 92 of fetal development and declined thereafter (Table 5.9). The abundance of IGF-I receptor mRNA in skeletal muscle was greatest at day 50 of fetal development and least at day 133 of fetal development (Table 5.9).

5.3.1.2.4.2 Muscle type

The abundance of IGF-I mRNA did not differ between the semitendinosus and supraspinatus muscles at any stage of development (Table 5.10). There was significantly greater IGF-II mRNA in the semitendinosus muscle of day 92 fetuses, but significantly less at day 133 (Table 5.10). The semitendinosus muscle of day 92 fetuses tended to have higher IGF-I receptor mRNA than the supraspinatus muscle ($p<0.1$; Table 5.10).

5.3.1.2.4.3 Feed intake

Ewes offered a restricted diet (L) had significantly higher skeletal muscle IGF-I, IGF-II and IGF-I receptor mRNA than those allowed *ad libitum* feed intake ($p<0.05$; Appendix 2). Further, IGF-I receptor mRNA was elevated in the skeletal muscle of day 92 and 133 fetuses whose mothers had feed intake restricted ($p<0.05$; Appendix 2). The effect of feed intake on the mRNA abundance of IGF-I, IGF-II and IGF-I receptor in each muscle type at each stage of development is presented in Figures 5.16-5.18.

Table 5.9: Abundance of IGF-I, IGF-II and IGF-I receptor mRNA in skeletal muscles (pooled average at each stage) of fetuses aged 50, 92 and 133 days and mature ewes slaughtered on day 133 of gestation, expressed relative to abundance at day 50 (100%). *values are means ± SEM converted to percentages; data were log transformed. Different superscripts within rows indicate significantly different relative mRNA levels between stages ($p<0.05$).

	Stage of Development			
	Fetal day 50	**Fetal day 92**	**Fetal day 133**	**Mature ewe**
IGF-I	$100.0 \pm 9.9^{*a}$	134.8 ± 6.5^{b}	80.6 ± 6.5^{a}	12.7 ± 6.5^{c}
IGF-II	100.0 ± 21.1^{a}	235.8 ± 13.8^{b}	43.8 ± 13.8^{c}	2.3 ± 13.8^{d}
IGF-I Receptor	100.0 ± 8.0^{a}	55.3 ± 5.2^{b}	33.6 ± 5.2^{c}	83.4 ± 5.2^{d}

Table 5.10: The abundance of IGF-I, IGF-II and IGF-I receptor mRNA in the semitendinosus (ST) muscle expressed as a percentage of mRNA in the supraspinatus (SS) muscle of mid- and late gestation fetuses and mature ewes slaughtered on day 133 of gestation *values are least-squares means ± SEM converted to percentages. Different superscripts within rows indicate significantly different mRNA levels between muscles at each stage (lowercase, $p<0.05$; uppercase $p<0.1$).

	Fetal day 92		Fetal day 133		Mature ewe	
	SS	**ST**	**SS**	**ST**	**SS**	**ST**
IGF-I	$100.0 \pm 9.0^{*}$	119.8 ± 9.0	100.0 ± 14.0	111.4 ± 14.0	100.0 ± 18.8	83.8 ± 18.8
IGF-II	100.0 ± 16.6^{a}	174.6 ± 16.6^{b}	100.0 ± 9.5^{a}	52.3 ± 9.5^{b}	100.0 ± 12.9^{A}	66.9 ± 12.9^{B}
IGF-I Receptor	100.0 ± 9.5^{A}	124.7 ± 9.5^{B}	100.0 ± 10.7	83.8 ± 10.7	100.0 ± 12.1	96.7 ± 12.1

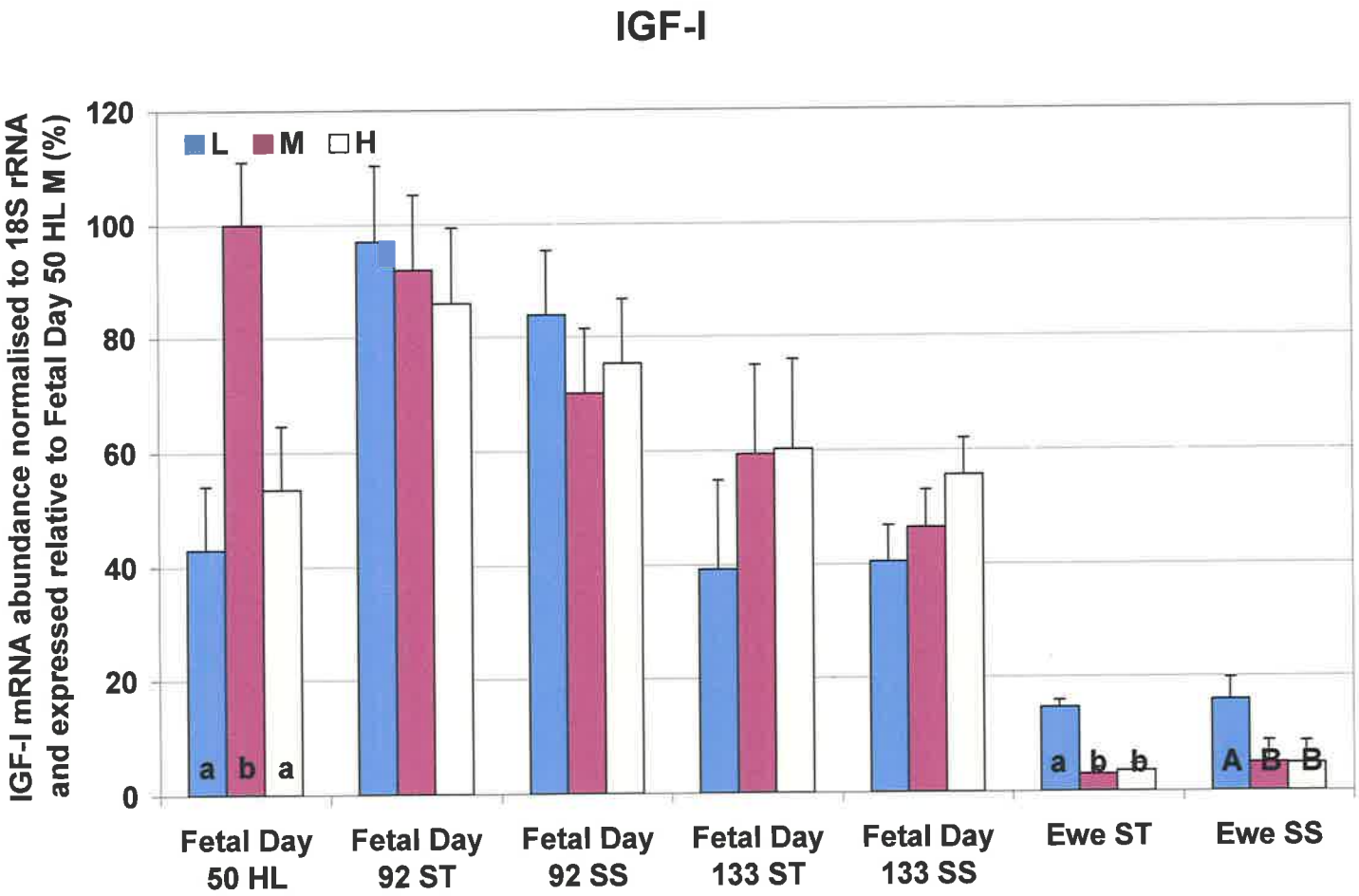


Figure 5.16: The effect of feed intake (L, M, H) on the abundance of IGF-I mRNA in the semitendinosus (ST) and supraspinatus (SS) muscles of fetuses aged 50, 92 and 133 days and mature ewes slaughtered on day 133 of gestation. All values are expressed relative to mRNA abundance in the hind-limb (HL) of maintenance fetal sheep at day 50 (100%). Different letters within each muscle at each stage of development indicate a significant difference (lowercase, $p<0.05$; uppercase, $p<0.1$).

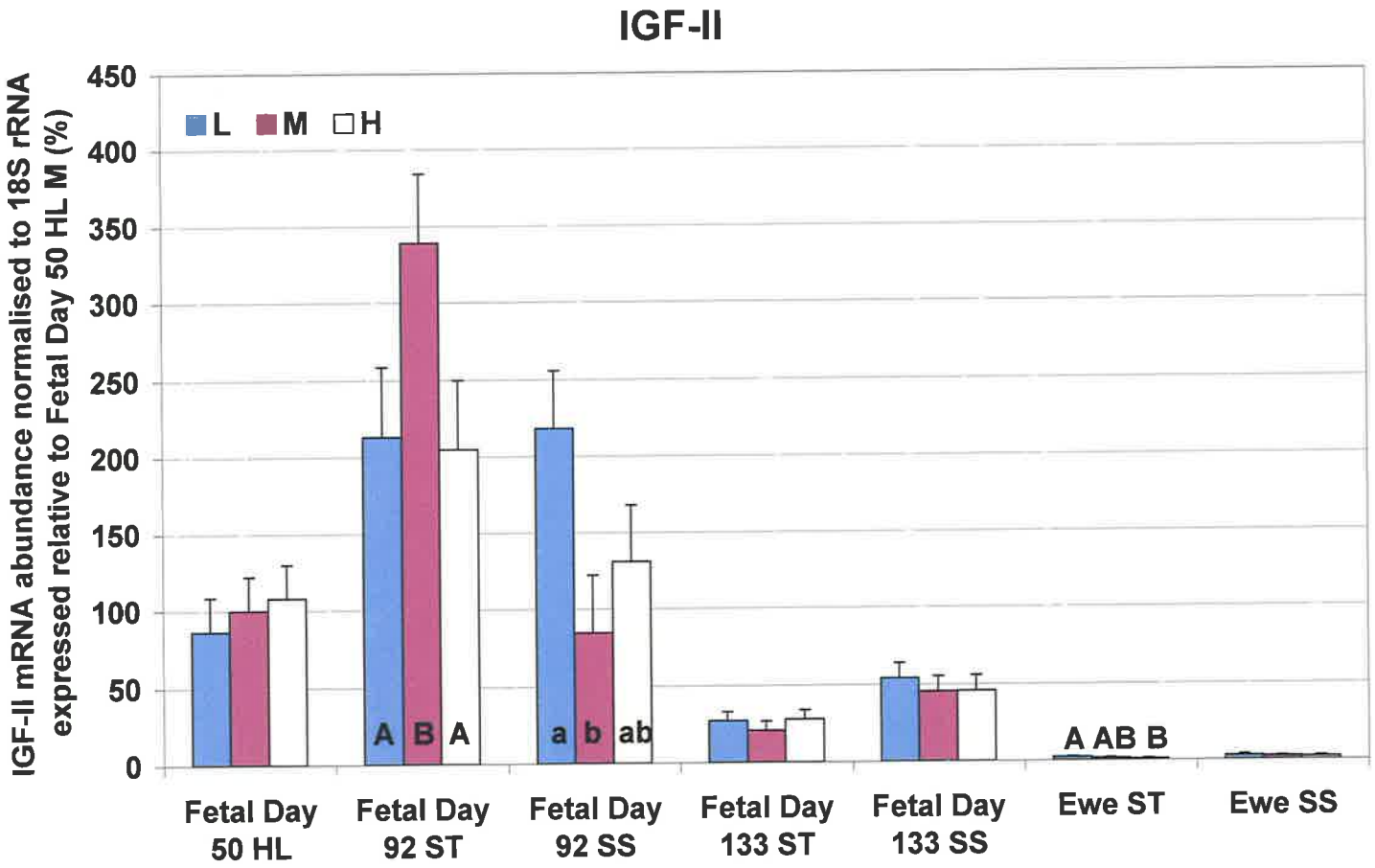


Figure 5.17: The effect of feed intake (L, M, H) on the abundance of IGF-II mRNA in the semitendinosus (ST) and supraspinatus (SS) muscles of fetuses aged 50, 92 and 133 days and mature ewes slaughtered on day 133 of gestation. All values are expressed relative to mRNA abundance in the hind-limb (HL) of maintenance fetal sheep at day 50 (100%). Different letters within each muscle at each stage of development indicate a significant difference (lowercase, $p<0.05$; uppercase, $p<0.1$).

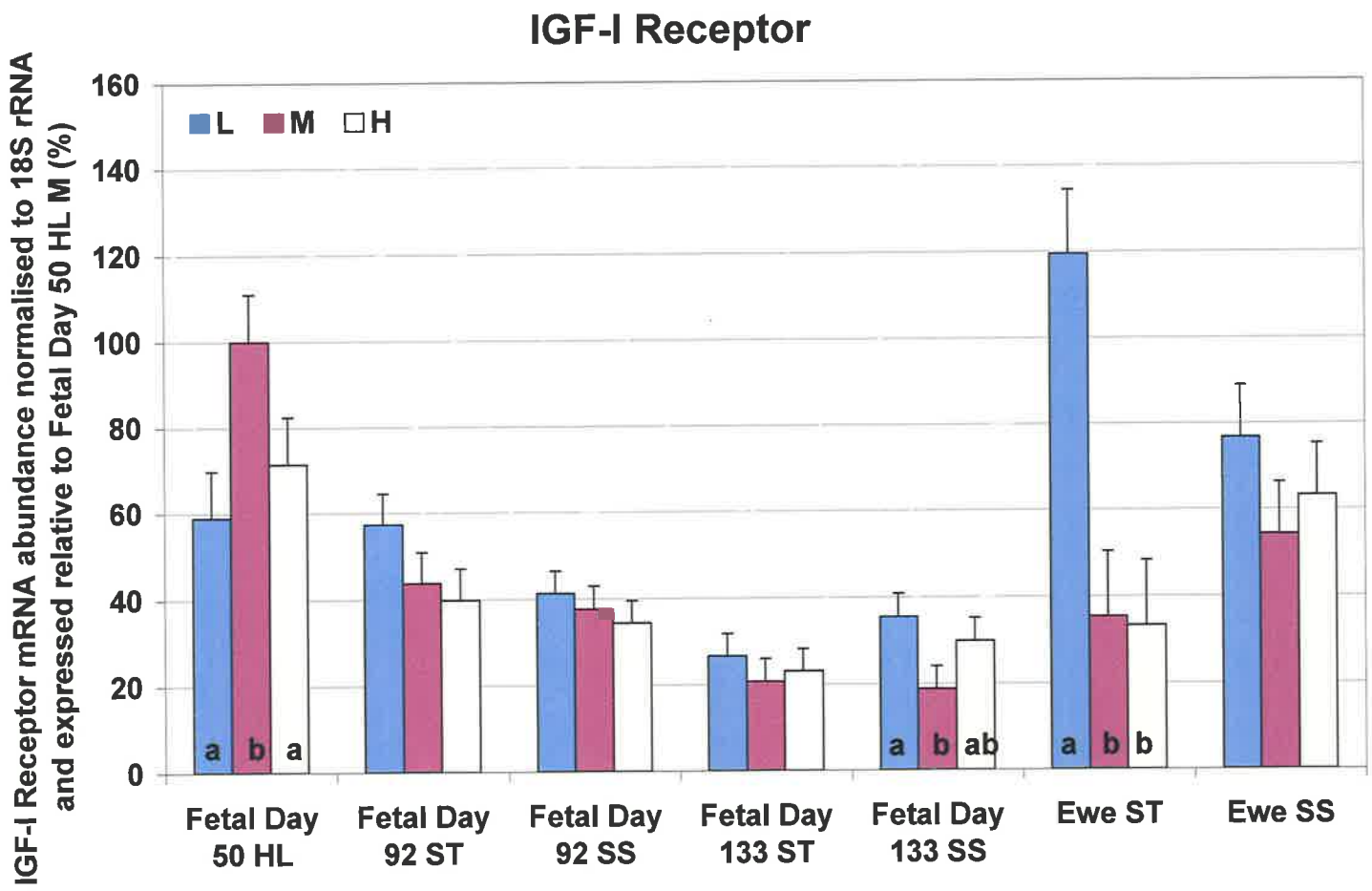


Figure 5.18 : The effect of feed intake (L, M, H) on the abundance of IGF-I receptor mRNA in the semitendinosus (ST) and supraspinatis (SS) muscles of fetuses aged 50, 92 and 133 days and mature ewes slaughtered on day 133 of gestation. All values are expressed relative to mRNA abundance in the hind-limb (HL) of maintenance fetal sheep at day 50 (100%). Different letters within each muscle at each stage of development indicate a significant difference ($p<0.05$).

5.3.1.2.5 Correlations

Abundance of candidate or housekeeping gene mRNA was not significantly correlated with fetal weight or fetal liver weight at day 50 and 92 of fetal development.

GAPDH mRNA abundance in the ST muscle of day 133 fetuses was positively correlated with fetal weight ($r=0.66$, $p<0.01$), fetal liver weight ($r=0.57$, $p<0.05$) and fetal ST weight ($r=0.61$, $p<0.05$). Similarly, ST β-actin mRNA level was positively correlated with ST weight ($r=0.70$, $p<0.01$), while ST myogenin mRNA level tended to be correlated with ST weight ($r=0.47$, $p<0.1$). IGF-I receptor mRNA level in the SS muscle of day 133 fetuses was negatively correlated with fetal weight ($r=-0.60$, $p<0.05$), fetal liver weight ($r=-0.69$, $p<0.01$) and fetal SS weight ($r=-0.55$, $p<0.05$). In contrast, SS muscle IGF-I mRNA level tended to correlate positively with fetal weight ($r=0.50$, $p=0.06$) and fetal liver weight ($r=0.46$, $p=0.09$) in day 133 fetuses.

In the mature ewe, GAPDH mRNA abundance in the ST muscle was positively correlated with ewe live weight ($r=0.51$, $p=0.052$) and condition score ($r=0.52$, $p<0.05$). In contrast, myoD mRNA abundance in the ST muscle was negatively correlated with ewe live weight ($r=-0.64$, $p<0.05$) and condition score ($r=-0.60$, $p<0.05$). Ewe live weight and condition score were negatively correlated with ST muscle IGF-I mRNA ($r=-0.70$ and $r=-0.69$, respectively, $p<0.01$), IGF-II mRNA ($r=-0.56$ and $r=-0.55$, respectively, $p<0.05$) and IGF-I receptor mRNA levels ($r=-0.73$ and $r=-0.74$, respectively, $p<0.01$). In the SS muscle similar relationships between the IGFs mRNA levels and ewe live weight and condition score existed. Further, ARP and Cyclophilin A mRNA levels in the SS muscle of ewes tended to be negatively correlated with ewe live weight and condition score ($p<0.1$).

5.3.2 Muscle protein analysis

5.3.2.1 Separation of muscle proteins

Total protein was extracted from 100mg of the semitendinosus muscle of sheep at various stages of development and separated through a 1-D SDS polyacrylamide gel with a 4-12% gradient (Section 2.4.6.1). Total protein concentration (μg /mg of tissue) increased with increasing age, as depicted in Figure 5.19. The up- and down-regulation of individual skeletal muscle-derived proteins with stage of development can be seen in Figures 5.19 and 5.20.

5.3.2.2 Myostatin westerns

Despite numerous attempts with different primary and secondary antibody concentrations, blocking procedures and a variety of different anti-myostatin primary and HRP-conjugated secondary antibodies, no conclusive evidence was ever obtained for the expression levels or size of the different myostatin protein forms in fetal sheep muscle samples. The ability of an in-house Protein A purified antibody (described in section 2.4.6.2.1) to detect myostatin was validated by detection of the myostatin C-terminus and N-terminus positive control peptides (described in section 2.4.6.2.3) expressed in *E.coli* and comparison reactivity of the with the commercially available myostatin antibodies (Bethyl MyoB and Santa Cruz, C terminus; section 2.4.6.2.1). The in-house Protein A purified antibody detected both the recombinant myostatin peptides (Figure 5.21a); this antibody was designed spanning the cleavage site, where the precursor dissociates into the latency associated peptide (LAP) and processed myostatin. The Bethyl (Figure 5.21b) and Santa Cruz (Figure 5.21c) antibodies detected the C-terminus peptide only, consistent with being designed solely to the C terminus; the Santa Cruz antibody demonstrated less reactivity with the expressed peptide when used at the same dilution as the Bethyl MyoB antibody, however, the Santa Cruz antibody was supplied at 200μg /ml while the Bethyl antibody was supplied at 1mg /ml. Further, the Bethyl antibody is designed to a human peptide with 100% conservation to the sheep protein sequence in that region while the Santa Cruz antibody was raised to an undisclosed peptide of mouse origin which may not be perfectly matched to the sheep myostatin sequence. Recombinant C-terminus peptide was detected at approximately 24kDa by all three primary antibodies; the N-terminus peptide was detected at approximately 36kDa by the in-house antibody. Therefore, the in-house antibody showed good reactivity with recombinant myostatin peptides designed in two regions of the myostatin gene sequence; the C-terminus peptide was detectable down to a 1:1000 dilution (approximately 1-5ng) after 1min exposure (Figure

5.22). Technical information obtained from the manufacturer of the ECL kit, used in these studies, suggests that the detection of the myostatin positive control at 1-5ng is at the bottom end of the kit's sensitivity range. Therefore, it was expected that the in-house myostatin antibody would detect the precursor, LAP and processed myostatin in the skeletal muscle of fetal and adult sheep.

Initial probing of muscle extracts revealed high backgrounds and cross-reactivity, which were predominantly due to secondary antibody interactions with muscle extracts. The use of a monoclonal secondary antibody decreased some non-specific binding but it had far less reactivity than the polyclonal goat anti-rabbit secondary antibody. Optimisation of blocking reagents and conditions markedly reduced the background and non-specific binding, evident by no reactivity when blots were incubated in the presence of the polyclonal goat anti-rabbit secondary antibody alone (i.e. no primary antibody; Figure 5.23a). The Santa Cruz primary was raised in goat and therefore required an anti-goat secondary, however, the same blocking conditions used with the other secondary antibodies were unable to inhibit the reactivity of the rabbit anti-goat secondary with proteins in sheep muscle and plasma extracts (Figure 5.23c). Two proteins displayed strong reactivity either in the presence or absence of the Santa Cruz primary (Figure 5.23c and d) and were common in both muscle and plasma samples. The presence of the primary antibody enhanced the reactivity and the C-terminus expressed peptide was faintly detected. Considering the relatively weak binding of Santa Cruz to the C-terminus positive control expressed peptide and the strong reactivity to the two common proteins in sheep muscle and plasma samples, the use of this antibody was stopped at this point. It was thought that these bands were due to the goat IgG secondary antibody reacting strongly with the same IgG proteins of sheep origin which would be expected to be present in muscle and plasma at the different levels which they were detected at in this study. It is of interest to note that these two bands corresponded approximately to the expected sizes of processed myostatin and the precursor, therefore it is possible that the strong reactivity of these non-specific bands was in fact masking a weaker signal generated from the desired myostatin targets. However, possible myostatin reactive proteins were detected in the skeletal muscle extracts of 5month old lambs (Figure 5.23b), which may correspond to the processed form of myostatin, the LAP and the precursor. Despite these reactive proteins being of the predicted sizes (see Appendix 4; McMahon *et al.*, 2003a), they could not be positively identified as processed myostatin, LAP and precursor as the same proteins were detected in the pre-immune serum (Figure 5.24).

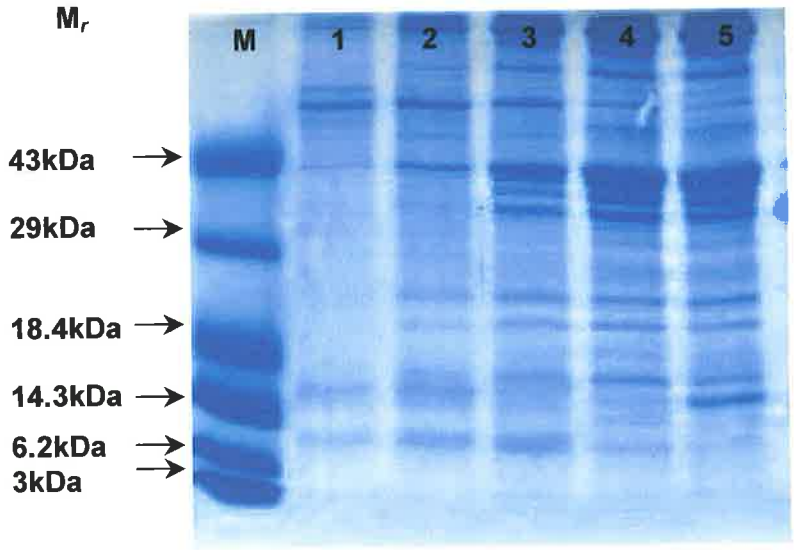


Figure 5.19: Total protein content of the semitendinosus muscle of sheep at various stages of development. Proteins were extracted in an equal w /v ratio (100mg tissue /1ml), separated through a 4-12% gel and stained with Coomassie blue. Lane M is molecular weight markers, lanes 1, 2 and 3 are muscles from 50, 92 and 133 days old fetuses, respectively; lane 4 is 5 month lamb muscle and lane 5 is adult ewe muscle.

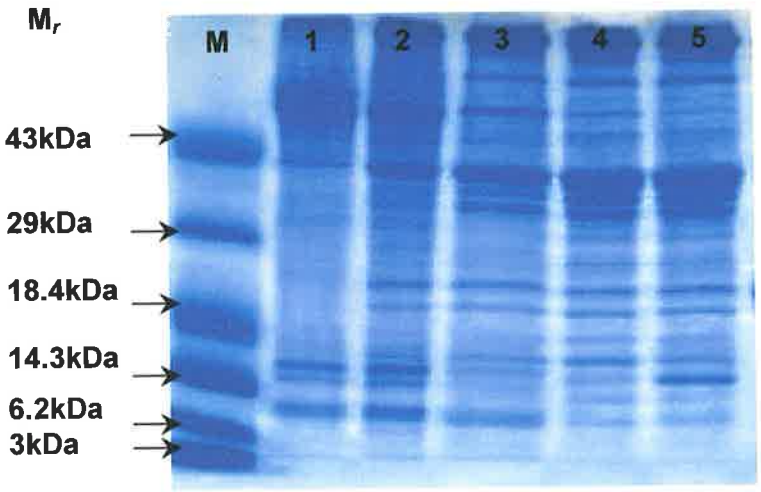


Figure 5.20: Adjusted total protein content of the semitendinosus muscle of sheep at various stages of development. An equal amount (10μg) of protein was separated through a 4-12% gel and stained with Coomassie blue. Lane M is molecular weight markers, lanes 1, 2 and 3 are muscles from 50, 92 and 133 days old fetuses, respectively; lane 4 is 5 month lamb muscle and lane 5 is adult ewe muscle.

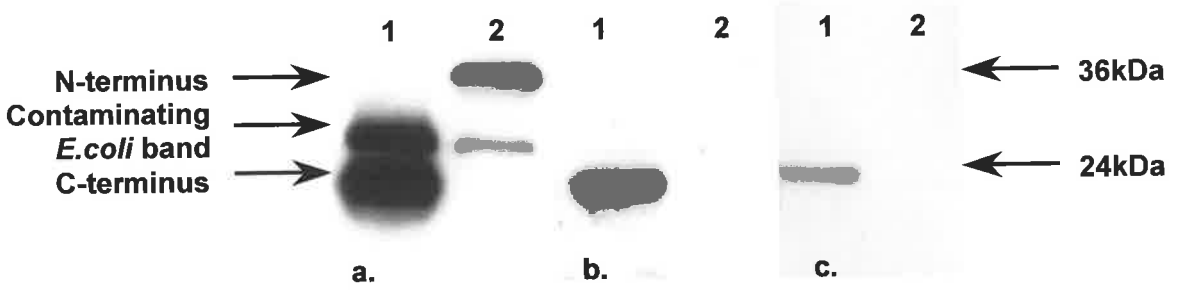


Figure 5.21: Western transfer analysis of myostatin peptides. Lane 1 = C-terminus and lane 2 = N-terminus, expressed in *E.coli*, by in-house (a.), Bethyl myoB (b.) and Santa Cruz (c.) primary myostatin antibodies. Primary antibodies were used at a dilution of 1:1000 and ECL blots were exposed to autoradiographic film for 1min (a. and b.) or 5min (c.).

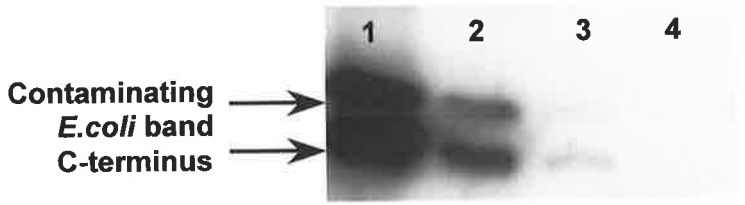


Figure 5.22: Western transfer analysis of the myostatin C-terminus positive control peptide at a range of serial dilutions. Lanes 1, 2, 3 and 4 are 1:10 (~100ng), 1:100 (~10ng), 1:1000 (~1ng) and 1:10 000 (~0.1ng) dilutions of the C-terminus peptide detected by Protein A purified myostatin antibody. Primary antibody was used at 1:1000 and ECL blots were exposed to autoradiographic film for 1min.

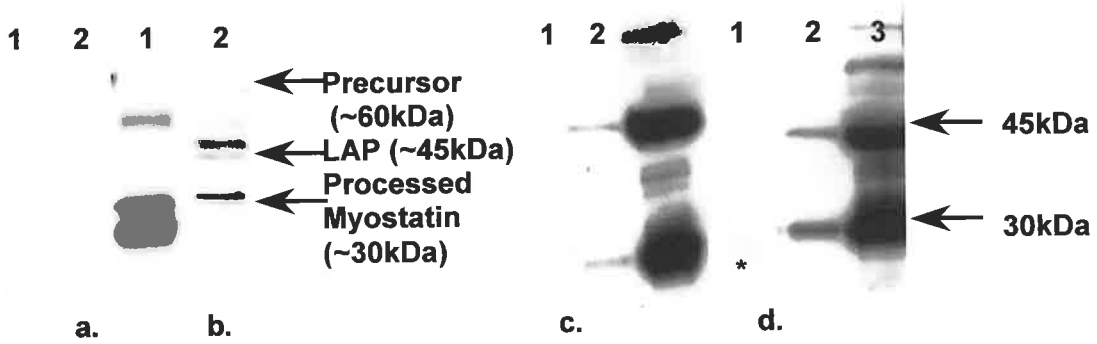


Figure 5.23: Western transfer analysis of myostatin C-terminus positive control (lane 1), muscle protein from a five month old lamb (lane 2) and sheep plasma (lane 3, blots c and d only). No primary antibody was added to some blots (a. and c.) to confirm specificity of the primary antibody. The in-house and Santa Cruz primary antibodies were added to blots b. and d., respectively. The immunoreactive proteins detected by the anti-serum are designated by arrows and may correspond to processed myostatin, the LAP and the precursor. The asterix indicates faint detection of the 1:10 dilution of the positive control. Primary antibody was used at 1:1000 and ECL blots were exposed to autoradiographic film for 1min.

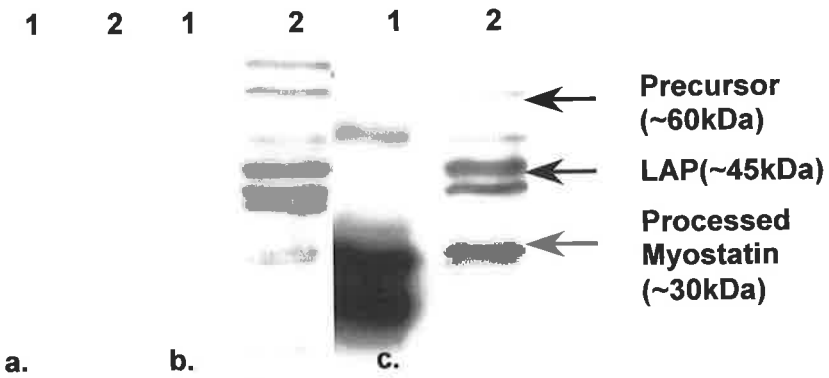


Figure 5.24: Western transfer analysis of the myostatin C-terminus positive control (lane 1) and muscle protein from a five month old lamb (lane 2). Blots were incubated with either no primary antibody (a.), pre-immune rabbit sera (b.) or Protein A purified anti-serum (c.) and exposed to autoradiographic film for 1 minute. Primary antibodies used in (b.) and (c.) were used at 1:1000.

5.4 Discussion

This study reports, for the first time, the ontogeny and the spatial and nutritional regulation of the abundance of mRNA of the MRFs, myostatin, IGFs and several housekeeping genes in the skeletal muscles of ovine fetuses, divergent in growth, and in the skeletal muscles of their mothers at late gestation. These results and their consequences are discussed in detail, as is the development of real-time quantitative PCR assays to measure mRNA expression.

In the present study, two anomalies in the results occurred which are worth discussing at the outset. The first anomaly was the consistently higher levels of mRNA detected in the muscle of day 50 maintenance fetuses compared to other fetuses at that stage of development. While this could be a real biological result, it is more likely due to a problem that occurred during RNA purification of these samples. By chance all M day 50 RNA samples were purified as one batch of RNA, the initial purification of these cDNAs failed; while cDNA synthesis was successfully repeated, differences in PCR amplification of all mRNAs were noted. Interestingly, mRNAs of most genes analysed were more abundant in maintenance samples, however, 18S rRNA abundance was significantly lower; this suggests that in some situations rRNA may not accurately reflect mRNA and therefore may not be appropriate as an internal control. While this result was probably erroneous due to a technical problem, the data has been included in the analysis presented in this thesis; it is likely that these data points will be omitted from any publications that arise from this work.

The second irregularity was the decline in 18S rRNA measurements that occurred in the SS muscle of mature ewes. Similar to the abnormal maintenance day 50 results, this decline may be a real biological result. However, it should be noted that all ewe SS measurements were done in the final batch of qPCR for each assay; there is a chance that the diluted reference samples may have lost their stability by this stage giving an inaccurate measure of mRNA abundance. When an erroneous 18S rRNA is used for normalisation it may give misleading results, so for this reason the results with no normalisation are presented in Appendix 2. The major discrepancies between the 18S rRNA and no normalisation results lie in the ewe samples. Due to the time constraints, the 18S rRNA measurements were unable to be repeated prior to submission, however, they will be revisited before publication of any of the present work.

5.4.1 Housekeeping genes

The use of an internal control gene to normalise values in mRNA quantitation experiments, has long been convention to verify equal RNA loading and reverse transcription efficiency, when PCR is used. For a gene to be considered a suitable internal control, its expression level must remain constant in response to the experimental treatments imposed. In the course of this study, the usefulness of several commonly reported "housekeeping" genes were assessed for their use as normalisers in experiments where muscle maturation and activity may be affected.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a key enzyme involved in the production of energy in the glycolytic pathway. While GAPDH is commonly used as a housekeeping gene, a large body of evidence suggests it is not suitable for normalisation in many circumstances (Suzuki *et al.*, 2000). In the present study, GAPDH mRNA levels were increased with stage of development and, hence, myogenesis, and then declined in the mature ewe. The large increase in GAPDH mRNA levels witnessed between day 92 and 133 of fetal development may indicate a transition in fibre metabolism as the muscle develops and an increase in the use of glycolytic enzymes occurs. The presumptive changes in GAPDH expression, in response to stage and nutrition, were more evident in the ST muscle compared with the SS muscle. GAPDH mRNA abundance was generally higher in the faster type II ST muscle compared with the slower type I SS muscle; this appears reasonable given the role GAPDH plays in glycolysis and the glycolytic nature of the ST muscle compared with the SS muscle. Further, feed intake status only affected GAPDH expression in the ST muscle, suggesting increasing nutrition alone was not enough to induce changes in the metabolic properties of muscle. However, in glycolytic type muscles nutrition appears to regulate the amount of GAPDH produced, which may infer that the rate of muscle glycolysis is also regulated by nutrition. For example, in the present study restriction of maternal feed intake reduced GAPDH levels in the ST muscle of day 133 fetuses, suggesting there was less nutriment and energy available to these muscles, glycolysis was reduced and, therefore, less GAPDH was required to convert glucose into energy. Whether the oxidative metabolic properties of the SS muscle are as sensitive to changes in feed intake remains to be determined, but may explain the belief that slow type muscles are less sensitive to changes in feed intake (Howells *et al.*, 1978; Dwyer and Stickland, 1992). A reduction in energy supply to the muscle may explain the delayed hypertrophic development of growth-restricted fetal muscles described in Chapter 4, as the synthesis of proteins requires a readily available source of ATP. Alternatively, lower GAPDH in ST muscles of feed restricted animals may also

be evidence of delayed muscle maturation, i.e. there was a delay in transition from oxidative to glycolytic metabolic properties. Therefore, it appears that GAPDH is an inappropriate endogenous control for normalisation in experiments that examine the abundance of mRNA in skeletal muscle as skeletal muscle GAPDH mRNA levels are variable when experimentation involves altering myofibre maturation, nutrition or muscle fibre.

β-actin, Cyclophilin A and ARP have all been used in experiments where normalisation of gene expression is required. β-actin, Cyclophilin A and ARP mRNA levels were all reduced, to some extent, as myogenesis proceeded. The down-regulation of β-actin, a cytoplasmic actin, during myoblast differentiation has been reported from studies *in vitro* (Schwartz and Rothblum, 1981) and *in vivo* with chickens (Hayward and Schwartz, 1986), in contrast to α-skeletal actin whose expression increases as differentiation proceeds. Cyclophilin A is a ubiquitous secreted protein known for its ability to bind cyclosporin A, and therefore has been implicated in mediating the negative affects of cyclosporin A on muscle differentiation in situations where oxidative stress occur (Hong *et al.*, 2002). Currently no reports of its ontogeny during normal myogenesis *in vivo* exist.

Like GAPDH, the use of β-actin, Cyclophilin A and, to a lesser extent, ARP as endogenous controls for normalisation of levels of mRNA of other genes in fetal muscle is inappropriate as their levels may vary depending on muscle maturity and experimental treatments. For example, normalisation to β-actin in the present experiment may have shown expression of the candidate genes to increase with stage of development, however the increase would actually be due to decreased β-actin expression. In addition, we found that primers for all four housekeeping genes amplified a specific product in gDNA preparations, despite being designed across introns, suggesting amplification of pseudogenes had occurred. Pseudogenes are considered to be non-functional copies of genes found in genomic DNA and the presence of pseudogenes for the housekeeping genes investigated in the present study have been reported previously (Zhang *et al.*, 2003). Due to the relatively high abundance of GAPDH, β-actin, Cyclophilin A and ARP mRNA in fetal and adult ovine muscle the amplification of possible pseudogenes approximately 10 cycles later (2^{10}=1024 fold less copies) was unlikely to interfere with our analysis. However, if these genes were used as housekeeping genes in tissues where their abundance was low or

gDNA contamination was high then the presence of pseudogenes may have given a misleading result.

Another commonly reported housekeeping gene used for normalisation is ribosomal RNA (18S and 28S rRNA). Ribosomal RNA accounts for the majority of total RNA and its use as an endogenous control for normalisation has been validated by some authors (Schmittgen and Zakrajsek, 2000; Tsuji *et al.*, 2002) and rejected by others (Tricarico *et al.*, 2002). The imbalance between mRNA and rRNA content reported by Solanas *et al.* (2001) indicates that rRNA may not necessarily reflect mRNA. Of specific interest to the present study is the finding of Habets *et al.* (1999) who reported higher 28S rRNA in type I fibres compared with type IIb fibres, suggesting ribosomal RNA, and hence total RNA, content may vary between muscles with different fibre type distributions.

In this study, the use of the 18S rRNA primer to normalise data produced qPCR results that were very similar to the results obtained from non-normalised data. The primary reason for this observation is that the amount of input RNA in each cDNA reaction is determined by spectrophotometric readings which in essence are really a measure of the ribosomal RNA content of total RNA preparations. Therefore, if the reverse transcriptase efficiencies are constant between cDNA samples and the amount of total RNA used for cDNA synthesis is the same then there is no need to measure the 18S levels by qPCR because they should not vary much at all. Moreover, measuring 18S rRNA or housekeeping genes by qPCR undoubtedly introduces another level of qPCR variation which further reduces the accuracy of target gene measurements as the same data were used to normalise all target genes. Additional problems arise when 18S rRNA is used for normalisation due to the abundance of this sequence in gDNA, which meant that additional dilution steps were required for each cDNA sample. This increases the chance of introducing more errors, and reduces the stability of the 18S rRNA due to the higher dilution level.

While any endogenous gene that remains constant under experimental treatments would make a suitable candidate for normalisation, it could take considerable screening to validate the reliability of a gene. It is unlikely that any one "housekeeping gene" will be suitable across all experimental situations, therefore, use of any internal housekeeping gene should be validated for each experimental situation.

Spiking an identical amount of an exogenous control RNA into experimental RNA samples was used to assess the issue of differing reverse transcription efficiencies between samples. Therefore, discrepancies between cDNA samples in exogenous control levels would indicate differences in reverse transcription efficiencies. Control mRNA levels were not significantly different ($p>0.05$) within cDNA preparation batches, indicating that the efficiency of reverse transcription reactions within a batch was consistent. However, levels of exogenous control differed between batches of cDNA, indicating that the reverse transcriptase efficiency varied between the three batches of cDNA. However, these results do not appear to have been caused by reduced reverse transcriptase efficiency as the 18S rRNA levels were relatively similar across the three batches of cDNA. Since equal amounts of total RNA were included in the cDNA reactions, a more likely explanation for variable control levels between the batches is probably due to the stability of the 5pg stock solution of the control RNA (i.e. traces of nucleases or adsorption of the RNA to the polypropoylene tube) which was freeze-thawed for the synthesis of each batch of cDNA.

It is concluded that, in experiments that examine developmental, spatial and nutritional aspects of muscle maturation and activity, GAPDH, β-actin, Cyclophilin A and, to a lesser extent, ARP are inappropriate candidates for normalisation. 18S rRNA was used as an endogenous gene for normalisation in this study due to accepted convention and because the intended approach with the control RNA exhibited batch variation. A major critisism for measuring housekeeping genes as the sole approach to data normalisation is their obvious variation in expression level. This approach does not take into account the number of different cells that are present in muscle samples exhibiting tissue heterogeneity, caused by different stages of gestation, muscle type or nutritional treatments or the transcriptional/translational capacity of the samples.

In our hands, the most effective method of normalisation, after confirmation of similar reverse transcription efficiencies is no normalisation, or what we refer to as input RNA normalisation. The use of accurate input RNA levels, which really are measures of the ribosomal RNA present in a total RNA sample, in combination with the use of aliquots of control RNA to confirm similar reverse transcriptase efficiencies between samples and cDNA batches will in future be our preferred method for normalisation of qPCR data. While this approach has been advocated in a recent review by Bustin (2002) it is unlikely that this view is widely accepted and it is for this reason alone that data presented here has been normalised.

5.4.2 Myogenic Regulatory Factors

The expression of the individual MRFs responded differently to stage of development, muscle type and feed intake. The ontogeny of MRF mRNA abundance in skeletal muscle of fetuses and ewes was as expected, based on previous studies *in vitro* and with other species, and demonstrated that the co-ordinated sequential activity of the MRFs is evident in sheep. Briefly, the decline in myf-5 (~92%), myoD (~30%) and myogenin (~65%) mRNA in fetal skeletal muscle as myogenesis progresses was in agreement with their reported roles in commitment, proliferation and initiation of differentiation of myoblasts, respectively. The exception was the peak in myogenin expression at day 50 compared with the defined peak at day 85 reported by Fahey *et al.* (2003). Fahey *et al.* (2003) reported relatively constant myogenin expression in the muscle of day 40, 55 and 70 sheep fetuses, increasing 3-fold at day 85 and gradually declining thereafter. It is possible that myogenin did peak at day 85 in the present study, however this stage of development was not examined. The increase in myf-6 expression between early and late gestation is in concurrence with its proposed role in maintaining terminal differentiation. Myf-5, myoD and myogenin are all reduced in the skeletal muscle from the fetal to the adult stage, indicating cell cycle activity is further reduced in older animals. The significant increase in myf-6 mRNA in adult muscle may be related to the increased size and protein content of the muscle fibres and a greater requirement to maintain steady state muscle integrity and function.

The present study was the first to explore the expression profile of the MRFs in skeletal muscles of different metabolic and contractile properties during fetal development and in adult sheep. Expression profiles of the MRF mRNA in different muscles during fetal development may provide evidence of altered muscle maturation. The ST muscle of day 92 fetuses had higher expression levels of myf-5, myoD, and myf-6, which may suggest relatively more myoblasts were committed and a greater rate of myogenesis was occurring, with possibly more or larger muscle fibres formed compared to the SS muscle, at that time. The higher myf-6 expression in the ST muscle of day 133 fetuses may infer there was a greater number or larger muscle fibres present than in the SS muscle. Therefore, there may have been a greater requirement for myf-6 to maintain the terminally differentiated state. The differences between muscle types witnessed at day 92 are more likely to be due to fibre maturation rather than contractile or metabolic properties. Javen *et al.* (1996) demonstrated that differentiation of muscle fibre type contractile properties commences after day 90 of gestation (despite differential reactions to myosin ATPase evident before this time), while Ashmore *et al.* (1972) reported no difference in succinic dehydrogenase or phophorylase activity until after

day 100 of gestation. In support of this, the ST muscle is believed to mature earlier than the SS muscle in cattle (Vestergaard *et al.*, 2000).

Higher expression of myoD mRNA in fast twitch glycolytic muscles has been reported in adult rats (Hughes *et al.*, 1993; Voytik *et al.*, 1993) and adult cattle (Muroya *et al.*, 2002) but not in post-natal pigs (Christensen *et al.*, 2000). While myogenin has previously been shown to be associated with slow twitch fibre content of muscles in rats (Hughes *et al.*, 1993; Voytik *et al.*, 1993; Hughes *et al.*, 1999), but not in adult cattle (Muroya *et al.*, 2002) this is in contrast to the present study which found no difference in myogenin mRNA expression between the ST and SS muscles at any stage of development. Voytik *et al.* (1993) reported that myf-5 and myf-6 were not differentially affected by muscle fibre contractile properties in adult rats. In the present study, myf-5 was the only MRF to be expressed differentially between muscles of adult sheep. This further supports the notion that differences witnessed between muscles in 92-day-old fetuses were due to muscle fibre maturation rather than fibre type *per se*. It would have been expected that the metabolic and contractile properties of the ST and SS muscle would have become more divergent in the adult ewe. Hence, fibre type related differences in gene expression should have been apparent at this stage of life. Therefore, associations between MRF expression and muscle fibre properties in sheep are likely to differ depending on the stage of development at which they are examined. Further, it appears that MRF expression responded to feed intake in a muscle type-dependent manner, since feed intake generally induced a change in MRF expression in the ST muscle, suggesting that the MRFs in slow twitch muscles may be less sensitive to changes in nutrition.

It was hypothesised that myf-5 mRNA would be lower in growth-retarded fetuses during early myogenesis, but be higher at a later stage of myogenesis, indicative of a slower cell cycle activity and a prolonged proliferative phase. The present study reflected this hypothesis to some extent, with elevated myf-5 expression evident in Low fetuses during late myogenesis (day 133). Fetal growth-retardation and maternal nutrient restriction results in slower cell cycle activity in the muscle of fetal sheep (Greenwood *et al.*, 1999). Given that muscle cell number was similar between nutrition treatments in the present study it is possible that myogenesis continued at a slower rate for a longer period in fetuses whose mothers received restricted feed intake. Similarly, elevated myf-5 expression was associated with increased muscle fibre number (Maxfield *et al.*, 1998a), after embryonic manipulations, suggesting enhanced cell cycle activity. The elevated myf-5 expression in growth-restricted fetuses at day 133 may have reflected

prolonged myogenesis. Alternatively, elevated myf-5 expression in growth-retarded fetuses may indicate an increased proportion of quiescent satellite cells (Beauchamp *et al.*, 2000). The higher abundance of myf-5 mRNA in underfed post-natal sheep has been implicated with reduced satellite cell activity (Jeanplong *et al.*, 2003). The effect of maternal feed intake on myf-5 mRNA abundance in late gestation fetuses was only evident in the ST muscle, further suggesting that myf-5 expression in the SS muscle is resistant to changes in nutrient supply.

Lower levels of myoD mRNA at all stages of fetal development could be anticipated in growth-retarded fetuses, indicative of slower myogenesis and reduced satellite cell activation at late gestation. However, there was no difference in myoD expression profiles between High and Low fetuses during early myogenesis. McCoard *et al.* (2001) demonstrated that the number of actively proliferating satellite cells and the total nuclei number were important determinants of muscle growth during late gestation and early post-natal life and that myoD was a suitable marker for satellite cell activity in sheep. Further, in situations where muscle fibre number is increased (i.e. double-muscled cattle) myoD expression is up-regulated, possibly contributing to an elevated cell cycle activity resulting in more myoblasts or satellite cells available for fusion or a greater requirement of myoD to initiate differentiation (Oldham *et al.*, 2001).

It was hypothesised that peak myogenin mRNA would be delayed in growth-restricted fetuses, indicative of delayed myoblast fusion and fibre differentiation. However, the present study indicated maternal feed intake had little affect on myogenin mRNA abundance during myogenesis. While myogenin has been associated with carcass characteristics in pigs (te Pas *et al.*, 1999a) it was not differentially expressed in normal and double-muscled fetal calves at various stages of myogenesis (Oldham *et al.*, 2001), suggesting that even in extreme alterations of myogenesis *in vivo* (i.e. double-muscled cattle) myogenin mRNA levels remain relatively constant.

In the mature adult ewe, it was thought that little difference in MRF expression would be evident, given that myogenesis is completed and most satellite cells are quiescent at this age. However, myoD expression was significantly elevated in feed restricted ewes compared to *ad libitum* ewes and myf-6 expression was up-regulated in both restricted and *ad libitum* ewes, compared with maintenance ewes. The elevated myoD expression in Low feed intake ewes, whose weight and muscle mass had declined, may have been suggestive of increased satellite cell activity (McCoard *et al.*, 2001). This appears unlikely unless some degree of muscle repair or regeneration was

occurring in response to severe muscle wasting due to under-nutrition, possibly to maintain muscle integrity. Interestingly, Jeanplong *et al.* (2003) reported elevated myoD in the muscle of growing lambs who were underfed for a shorter time (4 to 21 weeks) compared with the current study. Two possible reasons for the up-regulation of myoD in the muscles of ewes offered a restricted diet for over 7 months may be put forward. Firstly, severe muscle wasting for such a prolonged period may require activation of satellite cells for myofibre regeneration to maintain the muscles' most basic functions of support and mobility, even if muscle mass was being lost. Secondly, prolonged muscle wasting, due to feed restriction, may have induced a change in fibre type proportions, similar to that witnessed with hind limb suspensions (Stevens *et al.*, 2000) and weightlessness (Haddad *et al.*, 1993; Caiozzo *et al.*, 1996). Elevated myoD may have been associated with an increased proportion of type IIb fibres in response to long-term feed restriction, although the effects of nutrition on fibre type proportions in the literature are inconsistent. Somewhat in contrast, myf-5 mRNA levels were also elevated in ewes offered a restricted diet, possibly indicating greater satellite cell quiescence or an unidentified function of myf-5 in mature muscle cells. The elevated abundance of myf-6 mRNA may have been elevated in feed restricted ewes, compared to maintenance ewes, as a compensatory mechanism to maintain the structure of muscle.

The expression of the MRFs is differentially affected by stage of development, muscle type and nutrient supply. It appears that nutrient supply to the fetus may impact on myogenesis by regulating the expression of myf-5 and myoD mRNA, both of which are likely to impact on the proliferative phase of myogenesis and on satellite cell activity, respectively. Myf-5 in particular may be a good indicator of stage of myogenesis and hence muscle maturity.

5.4.3 Myostatin

Myostatin mRNA abundance peaked in day 92 fetal muscle and declined in late gestation, which is similar to that reported for cattle (Oldham *et al.*, 2001), pigs (Ji *et al.*, 1998) and chickens (Kocamis *et al.*, 1999). The peak at this time in sheep probably coincides with a time when myostatin may be both inhibiting proliferation of myoblasts (Thomas *et al.*, 2000a, b) and inhibiting the differentiation of myoblasts in forming secondary myofibres (Ríos *et al.*, 2002), thereby regulating both the number of myoblasts available for differentiation and the number of myofibres formed. The down-regulation of myostatin during late gestation allows the proliferation of satellite cells (McCroskery *et al.*, 2003) and the accretion of proteins (Taylor *et al.*, 2001) within

myofibres. The results of the present study indicate that myostatin mRNA is further down-regulated in mature aged ewes. This is in contrast with Ji *et al.* (1998), Mallidis *et al.* (1999) and Jeanplong *et al.* (2003) who found that myostatin levels increased as animals (rats, pigs and sheep, respectively) matured. In contrast to all of these findings, Welle *et al.* (2002) reported no difference in myostatin mRNA in muscles of young mature men (21-31 years of age) and old men (62-77 years of age).

The expression of elevated myostatin mRNA in fast twitch muscles was confirmed in this study, supporting the work of Carlson *et al.* (1999), Ji *et al.* (1999) and Artaza *et al.* (2002). Myostatin mRNA was relatively higher in the ST muscle of day 92 and 133 fetuses and in mature age ewes, compared with the SS muscle. The reason for the apparent accumulation of myostatin in fast twitch type muscles remains unknown. One hypothesis might be that the greater size and faster growth of fast twitch fibres (Kiessling, 1979) require more myostatin to inhibit satellite cell proliferation (McCroskery *et al.*, 2003) and muscle fibre growth. This is in line with the belief that myostatin may act as a 'chalone' (Lee and McPherron, 1999), a factor that inhibits cell activity to maintain normal proportional development of organs (Bullough, 1962). As fast type muscles appear to be more sensitive to changes in their environment, they may be likely to change mass more readily, so there is a greater requirement for myostatin to regulate the normal development of fast muscles compared with slow muscles.

The hypothesis that higher myostatin expression is evident in the fast twitch ST muscle, due to a greater requirement to regulate satellite cell activity in order to maintain normal muscle development, appears to be in contrast with the work of Beermann *et al.* (1983) and Gibson and Schultz (1983). These authors reported increased satellite cell number and proportions in the total myofibre nuclei population of slow oxidative muscles (soleus) compared with fast glycolytic muscles (extensor digitorium longus). Further, McFarland *et al.* (1997) demonstrated that greater protein synthesis and degradation and greater glucose uptake occurred in satellite cell cultures derived from predominately slow twitch, red muscles of turkeys. However, Lagord *et al.* (1998) reported that satellite cells from the soleus muscle proliferated more actively but fused into myotubes less efficiently than those from the extensor digitorium longus. It is possible then, that elevated myostatin in the ST muscle is related to maintaining normal muscle development by regulating the efficiency of satellite cell fusion (differentiation) into myofibres that are fast twitch in nature, rather than regulating proliferation.

Little difference in myostatin expression in fetal muscle was witnessed in response to maternal feed intake, although High fetuses did have lower myostatin expression at day 92 in the ST muscle only. Similarly, Carlson *et al.* (1999) and Ji *et al.* (1998) found myostatin expression to be insensitive to feed intake restrictions in pigs and mice, respectively. This may suggest that the ST muscle of H fetuses was at an advanced stage of maturity at day 92 compared with L fetuses or that myoblast and satellite cell proliferation and differentiation were inhibited by elevated myostatin in growth-restricted fetuses; either would ultimately result in greater DNA content of the semitendinosus muscle of H fetuses.

Given that myostatin is associated with another protein, the LAP, mRNA levels may not necessarily reflect its bioactivity. For example, a 40-60% reduction in processed myostatin protein expression was evident in muscles of male mice compared with females, despite no difference in myostatin mRNA expression (McMahon *et al.*, 2003b). Futher, Jeanplong *et al.* (2003) reported that processed myostatin in the ST muscle of sheep responds to feed intake differently to the LAP and precursor. Hence, as it appears that myostatin activity may be regulated at both the transcriptional and translational levels measurement of the abundance of the processed myostatin, LAP and precursor proteins was attempted. However, there is conjecture in the literature regarding the exact size of myostatin related proteins. The predicted size of processed myostatin, based on its amino acid sequence is 13kDa (McPherron *et al.*, 1997). While this has been confirmed *in vitro* (McPherron *et al.*, 1997; Lee and McPherron, 2001), processed myostatin is detected at 26 to 35kDa in most *in vivo* studies (Gonzalez-Cadavid *et al.*, 1998; Jeanplong *et al.*, 2003; McMahon *et al.*, 2003a, b). The greater size of processed myostatin *in vivo* is thought to be due to post-translational modifications, which make the protein difficult to fractionate even under reducing conditions (McMahon, Personal Communication).

In the present study, the processed form of myostatin, the LAP or the precursor could not be detected with consistency or confidence in ovine muscle or plasma with a range of primary antibodies. While proteins of expected size were detected in muscle samples with the in-house Protein A purified anti-serum, their identity could not be definitively confirmed as similar bands were detected with the pre-immune serum. Notably, both the "no primary" and "no secondary" antibody controls failed to detect any immunoreactive proteins under the optimised blocking conditions, confirming that the proteins of expected size for myostatin in the terminal and pre-immune bleeds were definitely detected by non-specific antibodies present in primary antibody preparation.

Affinity purification of the in-house antibody and the use of more sensitive detection kits may assist in confirming the identity of myostatin reactive proteins in these samples by removing some of the issues concerning non-specific binding and the possibility that myostatin is present at a concentration that is undetectable using the current methodology.

5.4.4 Insulin-like Growth Factors

IGF-I and -II are known to promote myogenic proliferation and differentiation and the accretion of proteins in skeletal muscle. IGF-I and -II mRNA levels peaked at day 92 in the skeletal muscle of fetal sheep, supporting previous work (Dickson *et al.*, 1991; O,Mahony *et al.*, 1991; Brameld *et al.*, 2000; Fahey *et al.*, 2003), suggesting that this is a critical time when myogenic activity is at its greatest. In contrast, expression of the IGF-I receptor mRNA in skeletal muscle tissue of fetal sheep peaked at day 50 and declined thereafter. To my knowledge the developmental expression profile of IGF-I receptor mRNA has not previously been reported for fetal sheep. In the ST muscle of bovine fetuses, Listrat *et al.* (1999) reported maximal IGF-I receptor expression at 110-170 days of gestation (term=280 days), declining thereafter. Elevated IGF-I and -II mRNA levels at day 92 in the present study might suggest this was the greatest period of differentiation. Evidence to support this assertion comes from *in vitro* studies. Firstly, increasing IGF-I to muscle cells in culture enhances differentiation, mediated by elevated myogenin mRNA expression (Florini *et al.*, 1991b). Secondly, suppression of IGF-II expression delays differentiation of myoblasts (Florini *et al.*, 1991c). The greater potency of IGF-I over IGF-II in stimulating myogenic differentiation (Florini *et al.*, 1986), may explain the greater up regulation of IGF-II mRNA at this time. The early peak expression of the IGF-I receptor in ovine fetal skeletal muscle appears to be in conflict with the thought that enhanced differentiation by both IGF-I and -II is mediated through the IGF-I receptor (reviewed by Florini *et al.*, 1987) and that functional inactivation of the IGF-I receptor delays differentiation (Cheng *et al.*, 2000). While IGF-II receptor abundance was not measured in the present study, it is unlikely that IGF-I and -II act through the type 2 receptor, as all mitogenic effects of the IGFs on muscle cells are targeted through the type 1 receptor *in vitro*. However, a role for skeletal muscle IGF-II receptor *in vivo* cannot be ruled out, and as such may contribute to mediating the mitogenic actions of IGF-I and IGF-II. Alternatively, other pathways may exist where the IGFs stimulate differentiation by indirectly interacting with myogenin, or perhaps the IGFs bind with greater affinity at this stage of development or are cleared from the circulation more rapidly. The large decline in skeletal muscle IGF-I and -II mRNA

expression, with relatively minor changes in IGF-I receptor suggests that IGFs may act in an endocrine rather than an autocrine or paracrine manner as muscle matures.

Expression of IGF-I and -II mRNA in different skeletal muscles of fetal sheep has not been previously reported. In the present study, differential expression of IGF-I mRNA was not witnessed between the ST and SS muscles of day 92 and 133 fetuses and mature ewes. This is in support of Welle *et al.* (2002), who found no associations between IGF-I mRNA and muscle fibre type in young and old men. In contrast, IGF-II mRNA was elevated in the ST muscle of day 92 fetuses and repressed in the ST muscles of day 133 fetuses, compared with SS levels. This may indicate the earlier maturation of the glycolytic ST muscle, as the decline in IGF-II mRNA expression in this muscle may reflect the end of myogenic differentiation. Similar to muscle type influences on the expression of the MRFs, this muscle effect on IGF-II expression is likely to be related to muscle maturity rather than fibre type. There was little difference in IGF-I receptor mRNA expression suggesting IGF-I receptor did not preferentially accumulate in the presence of glycolytic or oxidative muscle cells. This supports the work of Alexandrides *et al.* (1989) who found little difference in IGF-I receptor expression between different rat muscles, at the same stage of development.

Maternal feed intake had little affect on the expression of IGF-I and IGF-II mRNA in the skeletal muscle of fetuses at days 92 and 133 of gestation, supporting the work of Brameld *et al.* (2000). In contrast, Kind *et al.* (1995) reported decreased IGF-I mRNA in the skeletal muscle of day 121 growth-restricted fetal sheep, due to carunclectomy; placental restriction had no affect on skeletal muscle IGF-II mRNA. However, the effect of carunclectomy on fetal growth is more severe than the effect of maternal feed restriction, reported in the present study. While there is little doubt that IGF-I and -II are implicated in the regulation of myogenesis, it appears that fetal skeletal muscle IGFs are relatively insensitive to long-term nutrient restriction of the ewe with only the most severe aberrations of the fetal environment altering their expression in skeletal muscle.

In the mature age, feed intake-restricted ewe, IGF-I, IGF-II and the type 1 receptor mRNA were all up-regulated in skeletal muscle compared with *ad libitum* intake ewes. Most studies report decreased (Jeanplong *et al.*, 2003) or unchanged (Loughna *et al.*, 1992) IGF-I mRNA expression in skeletal muscle in response to nutrient restriction. However, few studies examine expression profiles in the mature nutrient restricted animal. It has been previously hypothesised that once the growth phase of development is completed the role of IGFs may shift to one of maintaining muscle

function, by inhibiting protein degradation, when humans are subjected to nutrient restriction (Clemmons *et al.*, 1992). Hence, increased local expression of IGF-I and -II mRNAs may be a compensatory adaptation to maintain muscle mass, by inhibiting protein degradation (Oddy and Owens, 1996), in the presence of reduced circulating IGFs. Alternatively, IGF-I and -II may be elevated to inhibit apoptosis of myonuclei (Napier *et al.*, 1999) to maintain muscle function. The elevated IGF-I receptor expression in undernourished ewes may be part of this adaptation to maintain muscle function. Alternatively, elevated IGF-I receptor mRNA expression may be related to increased connective tissue in muscles of feed restricted ewes. Long-term feed restriction, such as that employed in the present study, may have resulted in muscle atrophy, with myofibrillar proteins being broken-down more rapidly than connective tissue. RNA was extracted from an identical mass of muscle from all animals. If there was muscle atrophy occurring this portion of muscle may have actually contained relatively more connective tissue and therefore less myofibrillar tissue and, hence, RNA than in muscles that weren't atrophying. Oldham *et al.* (1996) reported greater expression of IGF-I receptor in the connective tissue than in the muscle bundles of the biceps femoris and gastrocnemius muscles of ewes. Even if there was the same amount of connective tissue in the muscle samples of Low and High intake ewes, Oldham *et al.* (1996) demonstrated that the expression of IGF-I receptor was higher in the connective tissues of fasted compared with fed ewes. This may also account for the elevated IGF-I receptor expression witnessed in the ST muscle of ewes. The fast twitch ST muscle would be more susceptible to changes in nutrient supply than the slow twitch SS muscle. Therefore, if feed intake did alter muscle connective tissue content, it may have been more evident in the ST muscle.

5.4.5 Gene interactions

The regulation of myogenesis involves the co-ordinated expression of the MRFs, myostatin and the IGFs (Chapter 1). Interactions between these genes have been reported elsewhere, mostly derived from *in vitro* studies that, while important, do not necessarily reflect the situation *in vivo*. The expression profiles of the candidate genes investigated in the muscles of fetal and mature sheep in the present study are summarised in Table 5.11.

Table 5.11: Trends of mRNA expression of genes in the muscles of fetal and mature sheep. (D=day of gestation. ↑ expression increases. ↓ expression decreases. – expression constant).

	mRNA expression		
	D50-92	D92-133	D133-Ewe
GAPDH	↑	↑	↑
β-actin	↓	↓	↓
Cyclophilin	↓	↓	↓
Acidic riboprotein	↓	↑	↓
Myf-5	↓	↓	↓
MyoD	–	↓	↓
Myogenin	↓	↓	↓
Myf-6	↑	↑	↑
Myostatin	↑	↓	↓
IGF-I	↑	↓	↓
IGF-II	↑	↓	↓
IGF-I receptor	↓	↓	↑

In the present study, myf-5, myoD, myogenin and IGF-I receptor all peak during early myogenesis, while myostatin, IGF-I and IGF-II peak during mid- to late myogenesis, and myf-6 peaks post-natally. These trends are somewhat in contrast to the published observations of the interactions between these genes (see Figure 1.5). It is believed that myoD is a prerequisite for myogenin expression and may also act upstream of myostatin (Spiller *et al.*, 2002). Myogenin itself is thought to be a target for myostatin (Joulia *et al.*, 2003) and IGF-I and -II mediated via the IGF-I receptor (Florini *et al.*, 1991b). Myostatin has also been shown to down-regulate myoD (Langley *et al.*, 2002). While myf-5 progressively declines in response to IGF mediated activation of myogenin (Mangiacapra *et al.*, 1992).

In the present study, it appears that myoD and myogenin are expressed independent of myostatin and the IGFs. The peak expression of myostatin may be responsible for the subsequent decline in myoD. Myf-5 expression progressively declines as the myoblast population becomes increasingly differentiated, which is negatively associated with an increase in myf-6. The major discrepancy between the present data and the published

data is the peak in IGF-I receptor expression earlier than peak IGF-I, -II and myogenin expression. The earlier peak in IGF-I receptor expression could be related to events occurring *in vivo* that are not replicated *in vitro*, or may be related to other sources of circulating IGF acting on muscle in an endocrine manner or altered bioactivity or clearance of IGFs from the circulation.

5.4.6 Real-time PCR

A major component of this work was the development of a qPCR approach to quantitate mRNA expression in the skeletal muscle of sheep. This methodology was found to be more sensitive and required less time and starting material than other methods of quantitation. Northern blots require large amounts of starting material, meaning the running of replicates of the same sample is unlikely. Further, the sensitivity of Northern blots is significantly less than that of qPCR, for example Ma *et al.* (2001) did not detect myostatin mRNA in cell culture when treated with dexamethasone, but did detect a 2 fold difference in expression by RT-PCR; Wang *et al.*, (1989) reported that RT-PCR was 1000 fold more sensitive than RNA dot blots. In the present study, 6 C_t measurements, effectively individual measurements of relative expression, were conducted on 14 genes all from a total of 3μg of total RNA, with some cDNA left over at the completion of the present qPCR experiments. The intra- and inter-assay coefficient of variation for most genes was approximately 15%, which may be considered a bit high; however the exponential nature of amplification in PCR and the number of variables within both the prepared samples and the reagents mean that only a slight delay in C_t can give a large variation among triplicate measurements for an identical sample. The main issue related to consistent measurements amongst triplicates appears to be cDNA stability. As cDNA loses its stability more variation in amplification amongst triplicates occurs, even when the PCRs are prepared with the same reagents, using the robotic pippetting system at the same time. Storing cDNA in single use aliquots would overcome this problem but is logistically difficult and requires many consumables when a large number of samples and genes are being examined. Further, the use of qPCR was relatively inexpensive, particularly when in-house reagents were used and labour costs were reduced due to the speed of experiments. In future, experience learnt from these studies will be used to refine our qPCR methodology for measuring gene expression in muscle and other tissues, reducing the variation.

The results of the qPCR assays in the present experiment are consistent with previously reported developmental and spatial expression of the genes investigated.

Further, the in-house results reflected the trends measured using the commercially available master mix. The consistency between the *in vivo* results reported in this chapter, the preliminary data obtained using the Applied Biosystems machine, consumables and reagents and the results of studies *in vitro* and with other species studies, provides confidence that the assays developed are useful for quantitating mRNA abundance in ovine muscle. However, it should be noted that the development of qPCR assays for all gene transcripts was a continual learning experience with many subtle changes being employed to improve the efficiency and repeatability of the system. Improvements in factors such as those affecting degradation of fluorescent dyes and stability of cDNA and standards will continue to increase the accuracy of the system.

One major problem still to be overcome in *in vivo* experiments is the heterogeneity of the sample from which RNA is obtained. For example, in the current experiment skeletal muscle tissue contained a mixed population of primary /secondary and fast /slow fibre types that often existed in close proximity to each other. These fibres, with different metabolic and contractile properties, may react differently depending on the stage of development or the treatments imposed. Therefore, the results presented here describe gene expression at the whole muscle level rather than gene expression of distinct cell populations, which would collectively contribute to total muscle development in a co-ordinated manner. In the current experiment, we were unable to distinguish the expression of different genes within distinct myofibre populations and wether they respond differentially with stage of development or to environmental cues. One solution to this problem would be to sample homogeneous muscles, however, these muscles are often of little significance to animal production. The use of in situ hybridisation techniques would assist in localising the abundance of gene transcripts in distinct myofibre populations. However, it was not employed in the present study primarily due to time and resource constraints. An alternative option that may soon become readily available is the use of Laser Capture Microdissection (LCM). LCM allows the isolation of intact cells from tissue samples; similar cells (e.g. all type IIb fibres) could be pooled and RNA extracted from each cell type within a heterogeneous tissue. Given that only a small amount of RNA will be obtained from single cells, the use of qPCR is a much more suitable method for transcript profiling than other techniques, such as Northern transfers. However, issues associated with sample collection, preparation and storage for LCM will need to be addressed.

5.5 Summary

The present study describes the mRNA abundance of the MRFs, myostatin, the IGFs and several housekeeping genes in different skeletal muscles of sheep at different stages of fetal development and in mature ewes, as determined by quantitative real-time PCR. Further, the effect of maternal feed intake on expression levels was also examined and discussed in conjunction with the altered myogenesis evident in growth-retarded fetuses. GAPDH, β-actin, Cyclophilin A and ARP mRNA levels were altered by stage of development, muscle type or nutrient supply and as such were unsuitable as housekeeping genes for normalization in the current experiment. The expression of candidate genes in sheep muscle occurred in a coordinated manner reflecting their reported roles from *in vitro* studies and other species. Differential expression of genes occurred between different muscles, which may be related to fibre type content or rate of muscle maturation. Key times during muscle development were identified when gene expression may be altered directly by nutrition, independently altering myogenesis. In summary, it appears that delayed myogenesis in growth-retarded fetuses may be related to energy supply, as indicated by repressed GAPDH mRNA, or delayed differentiation as indicated by elevated myf-5 and myostatin mRNA in restricted fetuses at day 92 and 133, respectively. Whether changes in myf-5 and myostatin mRNA expression are causative or a result of altered myogenesis remain to be determined. Whether the differences in mRNA transcript levels measured between different stages of development, muscles and feed intakes are reflected at the protein level requires further investigation. It would also be of interest to determine the roles of different splice variants of IGF-I. While real-time PCR provided a rapid quantitative measure of expression of our candidate genes, "the usual suspects" in sheep muscle, it would be best used in conjunction with other approaches that identify novel genes as candidates. However, the establishment of the generic technique described in this thesis is readily amenable to most experimental situations and gene expression to be quantitated.

CHAPTER 6.

6 THE EFFECTS OF SHORT-TERM MATERNAL FEED INTAKE DURING THE PERI-CONCEPTION PERIOD ON MUSCLE DEVELOPMENT AT MID-GESTATION.

6.1 Introduction

In Chapters 3 and 4, it was demonstrated that long-term regulation of maternal feed intake, prior to and throughout gestation, resulted in altered fetal development and altered muscle cell hypertrophy, with no change in muscle fibre number. In contrast, Everitt (1965) reported reduced muscle fibre number in fetuses whose mothers had feed intake restricted after conception and throughout gestation. Therefore, it was thought that the period prior to and immediately after mating might influence subsequent muscle development.

There is an increasing awareness that the pre-implantation period of embryo development, particularly the time of activation of the embryonic genome, can influence phenotypic outcome. This knowledge has resulted primarily from studies, in both the sheep and cow, involving manipulation of the embryo or its environment. Exogenous progesterone administration (Kleemann *et al.*, 1994, 2001), asynchronous embryo transfer (Wilmut and Sales, 1981; Maxfield *et al.*, 1998a) or *in vitro* culture of embryos (Walker *et al.*, 1992; Farin and Farin, 1995; Maxfield *et al.*, 1998b; Crosier *et al.*, 2002), have resulted in increased fetal growth (Walker *et al.*, 1992; Kleemann *et al.*, 1994; Crosier *et al.*, 2002), increased and disproportionate muscle growth (Kleemann *et al.*, 2001), increased muscle fibre density and size (Maxfield *et al.*, 1998a) and increased secondary to primary fibre ratio (S:P) (Maxfield *et al.*, 1998a; Crosier *et al.*, 2002).

These perturbations in myogenesis could result from a progesterone-mediated alteration of placental function (Hartwich *et al.*, 1995; Kleemann *et al.*, 2001) and hence fetal development or possibly from epigenetic modifications hence programming of the genetic determinants that regulate embryonic and myogenic development (Crosier *et al.*, 2002). Altered myf-5 protein (Maxfield *et al.*, 1998a) and myostatin mRNA (Crosier *et al.*, 2002) abundance have been associated with altered muscle development after manipulation of the embryo or its environment.

Some consequences of the manipulations mentioned above may be influenced by nutrition, possibly through circulating progesterone concentration, which can vary with feed intake (Parr *et al.*, 1982; Williams and Cumming, 1982). Kakar *et al.* (In Press) demonstrated that differential maternal feed intake for as little as six days after ovulation influences cell allocation within the blastocyst, leading to speculation that alterations in cell lineage differentiation might be one means whereby embryonic or environmental manipulation is able to influence subsequent development. The objective of this experiment was to determine if a nutritionally mediated alteration of the peri-conception environment is able to influence subsequent muscle development of the fetal sheep.

Therefore, the hypothesis tested were that

- Maternal nutrition during the peri-conception period may alter myogenic programming and may account for some discrepancies reported in regard to experiments examining maternal nutrient supply and fetal muscle development.
- Restricting maternal feed intake during the peri-conception period may enhance fetal and muscle development, mediated by enhanced placental development at mid-gestation.

6.2 Experimental

All procedures were conducted according to the guidelines of the Australian Code of Practice for the Use of Animals for Scientific Purposes and were approved by the PIRSA Animal Ethics Committee.

6.2.1 Location

The experiment was conducted at Turretfield Research Centre and the University of Adelaide, Roseworthy Campus, South Australia. Donor ewes were individually penned indoors at Turretfield, while recipient ewes were managed as one group in a paddock at Roseworthy.

6.2.2 Animals, feeding and experimental design

6.2.2.1 Donor ewes

Mature age South Australian Merino ewes (n=24) were used as embryo donors. Ewes were stratified on live weight and randomly allocated to two blocks (n=12 /block). Ewes were further allocated to two nutritional treatments within blocks, and offered either high (H=1.5 x maintenance, n=6) or restricted (L=0.5 x maintenance, n=6) amounts of feed. Protocols and measurements between blocks commenced one week apart. Donor ewes were individually penned and fed roughage /grain pellets (90.1% dry matter; 10.1MJ ME /kg DM; 19.1% crude protein; 23.9% acid detergent fibre) (Johnsons Stockfeed, Kapunda, South Australia) at 9:00h each day with *ad libitum* access to water. After a two-week acclimatisation period, treatment diets were offered for eighteen days prior to mating (day -18 to 0) and for six days after ovulation (day 0 to 6). This 24-day period of treatment is subsequently referred to as the peri-conception period.

6.2.2.2 Recipient ewes

Mature aged Merino ewes (n=60) were stratified on live weight and randomly assigned as recipient ewes to treatments within blocks. Recipients were managed as one group to maintain body weight at pasture with *ad libitum* access to water.

6.2.3 Mating and embryo transfer

Synchrony of oestrus and ovulation in donor ewes was achieved using a progestagen pessary (45mg flugestone acetate; Intervet, Paris, France) for 12 days followed by treatment with follicle stimulating hormone (FSH; Ovagen, ICP, Auckland, New Zealand). Ewes were injected (i.m) twice daily with FSH on three consecutive days, commencing 48h prior to pessary removal (total dose of 10ml /ewe). Pregnant mare

serum gonadotrophin (Pregnecol; 500 IU, Horizon, New South Wales, Australia) was given (i.m.) at the first FSH treatment. Ewes were administered synthetic gonadotrophin releasing hormone 27h after pessary removal (Fertagyl; 50μg /ewe, Intervet).

Laparoscopic insemination of donor ewes occurred approximately 40h after pessary removal using fresh semen collected from a single proven ram. On day 6, embryos were collected from donor ewes under general anaesthesia. Either one or two embryos were transferred to each recipient with the aid of a laparoscope.

6.2.4 Pregnancy diagnosis

Pregnancy and litter size were determined by ultrasonography (Echo Camera SSD-500, 3.5MHz probe; Aloka Co Ltd, Tokyo, Japan) on day 45 of gestation. Non-pregnant ewes were subsequently removed from the experiment.

6.2.5 Sampling schedule

High and Low feed intake pregnant ewes, within each block, were randomly assigned to consecutive days during gestation for fetal collection (day 75 or 76). However, due to a poor pregnancy rate in block two, only one day was required for fetal sampling (day 75). Therefore, the final experimental sampling was considered as three replicates (Table 6.1).

Table 6.1: Description of final experimental design with nutritional treatment (Low, High) represented within each of these replicates.

Replicate	Block	Gestational age (days)
1	1	75
2	1	76
3	2	75

6.2.6 Fetal recovery

The average age of fetuses, at collection, was 75 days; sex, litter size and dimensions were recorded and the major organs weighed. Individual placentomes were separated from the membranes and weighed. The semitendinosus (ST), longissimus dorsi (LD) and supraspinatus (SS) muscles were dissected from the left and right side of each fetus. Samples from the left side were weighed and then mounted on cork blocks with 5% (w /v) tragacanth gum (Sigma; Castle Hill, New South Wales) and frozen by immersion in isopentane (Sigma) cooled over liquid nitrogen. The ST muscle from the right side was wrapped in foil and frozen in liquid nitrogen.

6.2.7 Muscle fibre analysis

Serial sections (10μM) were cut across the mid-belly of the ST muscle using a cryostat (2800 Frigocut E, Reichert-Jung); box -25°C and object -18°C. Sections were reacted for ATPase activity (section 2.2.2.2) after pre-incubation at pH 4.5 and 10.3 (Figure 6.1) or stained with hematoxylin and eosin (section 2.2.2.1) (Figure 6.2). The ST muscle cross-section area (MCSA) was determined as described earlier (section 2.2.3.1). Apparent number of muscle fibres and ratio of secondary to primary fibres were determined by counting fibres in 40.6 ± 10.1 (mean ± standard deviation) fields (x400) across the total MCSA using a random field generator approach, as described earlier (section 2.2.3.2). Fibres that displayed centrally located vacuoles or reacted less intensely to ATPase at pH 10.3 were identified as primary fibres; smaller, more intensely stained fibres without vacuoles were classified as secondary fibres (Figure 6.1). The mean fibre cross-sectional areas (FCSA) of primary and secondary fibres were determined in at least five fields that represented the mean muscle fibre number and ratio of secondary to primary fibre types for that muscle. For each muscle, FCSA was measured for greater than 50 primary and 450 secondary muscle fibres by tracing the perimeter of individual fibres using a mouse and computer monitor attached to a microscope, as described in section 2.2.3.4.

6.2.8 Muscle dry matter, nucleic acid and protein content

Muscle dry matter content was determined as described in section 2.3.8. RNA, DNA and protein concentration were determined as described earlier (section 2.3.1-2.3.7). The intra- and inter-assay coefficients of variation are given in Table 6.2.

Table 6.2: Coefficient of variation (CV%) of assays measuring the dry matter, DNA, RNA and protein content of the semitendinosus muscle of mid-gestation fetal sheep.

	CV%	
	Intra-assay	Inter-assay
Dry Matter %	2.87	*
DNA	7.98	*
RNA	5.18	*
Protein	3.47	5.13

* one assay only

6.2.9 Statistical analysis

The final experimental population and the population of singletons available for analysis are presented in Tables 6.3 and 6.4, respectively.

Initially, the main effects of maternal peri-conception feed intake, litter size, fetal sex and day of sampling and first-order interactions on fetal body dimensions and organ and muscle weights were analysed in the overall model (GLM procedure, SAS). Due to low numbers and uneven distribution of twin fetuses between treatment groups and sample day replicates (Table 6.3) it was decided to omit twins from the data set and analyse singleton fetuses only. The effect of maternal feed intake was always retained in the final model, while non-significant ($p>0.1$) main effects and interactions were sequentially removed in order of least significance. Day of sampling (i.e. rep) had a significant effect on most fetal parameters and was always retained in the final model, when significant.

Data are presented as least-squares means ± standard error of the mean (SEM). Different alphabetical superscripts indicate a significant effect (lower case, $p<0.05$), or tendency to significance (upper case, $p<0.1$) of peri-conception maternal feed intake on placental and fetal parameters and indices of muscle development of singleton fetuses at mid-gestation.

Table 6.3: Fetal population sampled at mid-gestation (day 75, 76) after maternal nutritional treatments during the peri-conception period.

		Maternal Feed Intake				
		Low		High		Total
Sample day rep	Fetal age (d)	1[a]	2[b]	1	2	
1	75	3	0	3	4	10
2	76	4	0	3	2	9
3	75	7	2	1	0	10
	Total	14	2	7	6	**29**
		M[c]	**F[d]**	**M**	**F**	**Total**
1	75	0	3	3	4	10
2	76	4	0	3	2	9
3	75	4	5	0	1	10
	Total	8	8	6	7	**29**

[a] singleton fetus [b] twin fetus [c] male fetus [d] female fetus

Table 6.4: Population of singleton fetuses sampled at mid-gestation (day 75, 76) after maternal nutritional treatments during the peri-conception period.

		Maternal Feed Intake				
Sample day rep	Fetal age (d)	Low		High		Total
1	75	3		3		6
2	76	4		3		7
3	75	7		1		8
	Total	14		7		**21**
		M[a]	**F[b]**	**M**	**F**	**Total**
1	75	0	3	2	1	6
2	76	4	0	1	2	7
3	75	4	3	0	1	8
	Total	8	6	3	4	**21**

[a] male fetus [b] female fetus

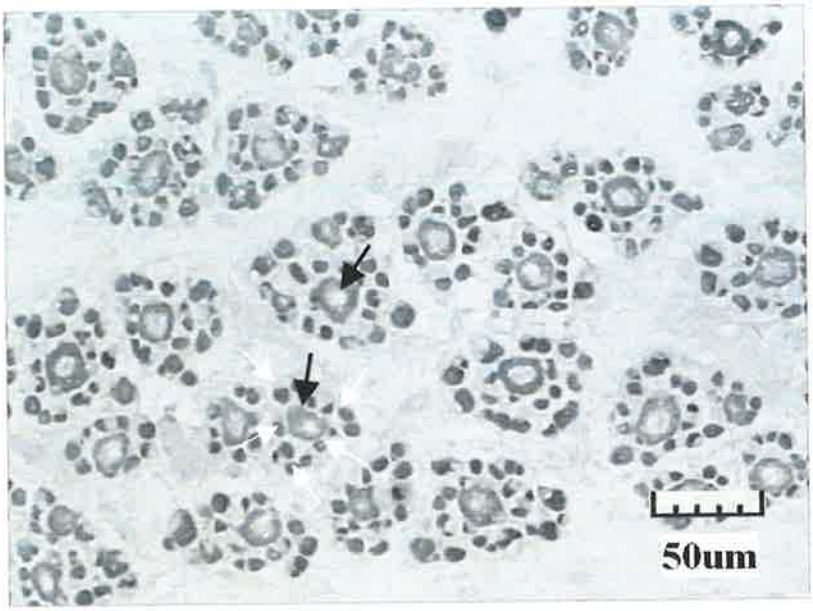


Figure 6.1: Typical muscle fibre population in the semitendinosus muscle of mid-gestation fetuses. Stained for ATPase activity after basic pre-incubation. Dark arrows indicate primary fibres; White arrows indicate secondary fibres (x200).

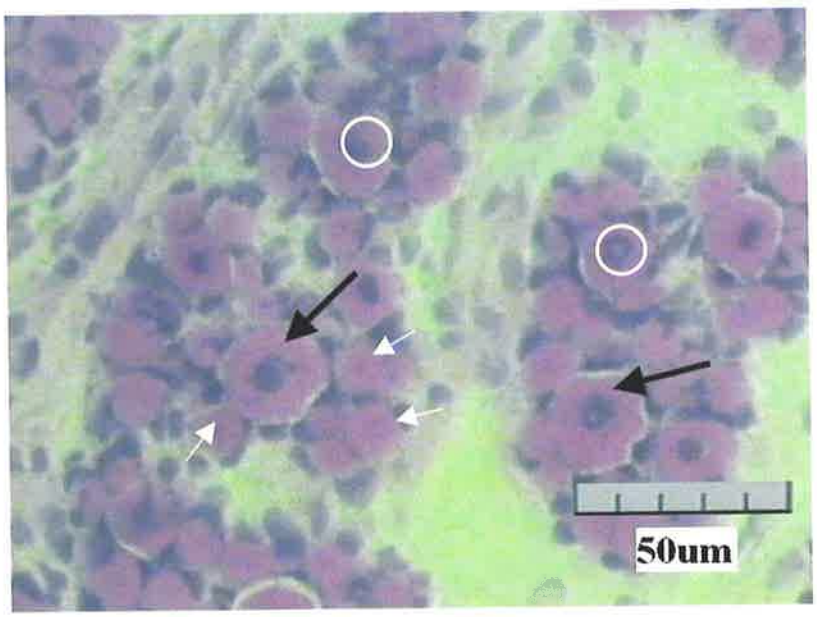


Figure 6.2: Typical muscle fibre population in the semitendinosus muscle of mid-gestation fetuses. Stained with H and E. Dark arrows indicate primary fibres; White arrows indicate secondary fibres (x400). Nuclei stain dark blue (e.g. circled). Muscle fibres are pink.

6.3 Results

6.3.1 Placental characteristics

Maternal feed intake during the peri-conception period had no affect on the number of placentomes (p=0.28), total placentome weight (p=0.70), mean placentome weight (p=0.40) or the ratio of fetal to placental weight (p=0.39) at mid-gestation (day 75) in singleton bearing ewes. Placental characteristics were unaffected by replicate.

6.3.2 Fetal body dimensions and organ weights of singleton fetuses

Maternal feed intake during the peri-conception period did not influence fetal weight (222.2 ± 7.2 and 216.5 ± 5.2g, H and L respectively, p=0.54), CRL (215.2 ± 3.5 and 217.1 ± 2.5mm, H and L respectively, p=0.68) and other body dimensions (Appendix 1). Fetal organ weights developed in proportion to fetal body dimensions and were unaffected by maternal peri-conception feed intake (Appendix 1). Similarly fetal body dimensions and organ development were unaffected by fetal sex (Appendix 1). However, day of sampling had a significant affect on fetal body dimensions and organ weights (Appendix 1). Generally, fetuses sampled on day 76 were 17-25% larger, with greater organ weights than fetuses sampled on day 75.

6.3.3 Muscle weights of singleton fetuses

Mid-gestation fetal muscle weights developed in proportion to fetal weight and were unaffected by maternal feed intake during the peri-conception period (Table 6.5). Mid-gestation fetal muscle weights were unaffected by fetal sex but tended to be greatest on day 76 of sampling (Appendix 1).

6.3.4 Muscle fibre size, number and arrangement

Cross-sectional area of primary (176.1 ± 14.0 vs. 163.4 ± 8.4mm^2, H and L, respectively) and secondary (35.3 ± 2.8 vs. 37.1 ± 1.7mm^2, H and L respectively) fibres were unaffected by maternal feed intake during the peri-conception period

The number of primary fibres was similar between L and H fetuses (p=0.75), while there were significantly more secondary fibres in H fetuses (p<0.05) resulting in a greater total muscle fibre number (p=0.06) (Figure 6.3). Hence, the number of secondary fibres per primary fibre (S:P) was also greater in the ST muscle of H (11.0 ± 0.8) compared to L fetuses (8.7 ± 0.5, p<0.05).

Generally, fibres were arranged in small bundles of 10-20 cells. Bundles typically consisted of a large primary fibre, identified by a large vacuole /centrally located nuclei (Ashmore *et al.*, 1972), surrounded by a larger population of smaller secondary cells, with peripheral nuclei. There was some evidence for differential staining in response to ATPase activity, where primary fibres stained less intensely than secondary fibres after pre-incubation at basic pH.

6.3.5 Muscle DNA, RNA and protein content

The effect of maternal peri-conception feed intake on the total amount, or concentration, of DNA, RNA and protein in the ST muscle of singleton fetuses at mid-gestation was not significant. Similarly, RNA:DNA and protein:RNA were unaffected by maternal nutrition. However, there was approximately 28% more protein per unit of DNA (protein:DNA) in the ST muscle of H fetuses (39.0 ± 3.9) compared with L fetuses (30.5 ± 2.4, $p<0.1$).

Table 6.5: Muscle weights of mid-gestation (day 75, 76) singleton fetuses obtained from ewes offered different levels of peri-conception feed intake.

	Maternal feed intake			
	Low	High	Low	High
	Absolute muscle weight (g)		Relative muscle weight (g /kg fetal weight)	
Longissimus dorsi	2.54 ± 0.10	2.53 ± 0.14	10.96 ± 0.44	10.86 ± 0.61
Supraspinatus	0.57 ± 0.02	0.59 ± 0.03	2.45 ± 0.11	2.53 ± 0.15
Semitendinosus	0.39 ± 0.02	0.38 ± 0.02	1.68 ± 0.07	1.65 ± 0.11
Semitendinosus CSA (mm^2)	46.81 ± 2.53	55.35 ± 4.21		

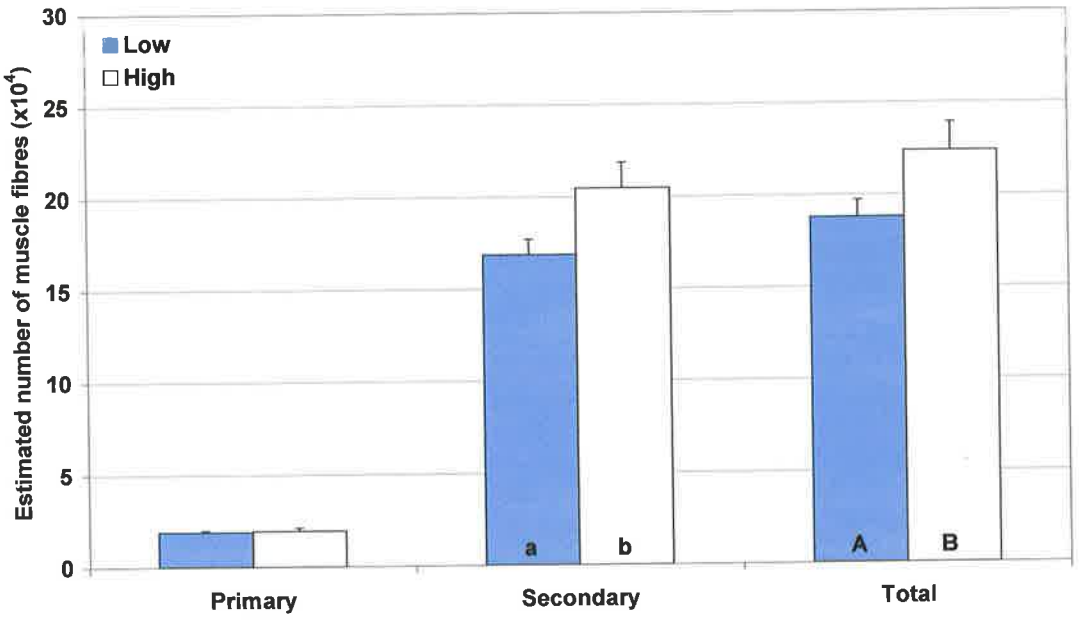


Figure 6.3: Estimated number of primary, secondary and total fibres at the mid-belly of the semitendinosus muscle of mid-gestation fetuses from ewes offered High or Low feed intake during the peri-conception period. Different letters within each fibre type indicate significant effect of maternal feed intake (lowercase, $p<0.05$; uppercase $p<0.1$).

6.4 Discussion

Evidence from this study, together with the findings of Maxfield *et al.* (1998a) and Crosier *et al.* (2002), indicate that the early embryonic environment may program events that determine subsequent myogenic activity. In the present study, total muscle fibre number and protein:DNA ratio tended to be greater in fetuses whose mothers had a high feed intake over the peri-conception period compared to those who had a low intake, although no change in muscle weight was observed. The tendancy to increased muscle fibre number in fetuses of well-fed mothers was due to an increased number of secondary fibres, resulting in a greater secondary to primary fibre ratio. This is in concurrence with the belief that changes in muscle fibre number are due to changes in the secondary fibre population with primary fibre number being resistant to treatments (Wigmore and Stickland, 1983). The increased S:P ratio evident here, as the result of an early embryonic nutritional intervention, is consistent with, but not as extreme as changes reported in response to artificial manipulations (Maxfield *et al.*, 1998a; Crosier *et al.*, 2002). Further, the lower protein:DNA ratio in L fetuses may be indicative of delayed myogenic development. Therefore, it is possible that increased maternal feed intake during the peri-conception period increased the rate of secondary fibre hyperplasia in the ST muscle of singleton fetuses at mid-gestation or conversely, that reduced maternal intake over the peri-conception period delayed myogenesis.

The results reported here support my first hypothesis; maternal feed intake during the peri-conception period did alter subsequent muscle development, therefore the nutrient status of the mother at this time may account for discrepancies between results of different experiments. However, the result did not support my second hypothesis; maternal feed restriction over the peri-conception period did not enhance fetal muscle development at mid-gestation.

Whether the altered fetal muscle development observed in this study, is permanent or transient remains to be elucidated. Examination of time points later in gestation, when myogenesis is completed, may reveal whether myogenesis continues longer in L fetuses, allowing them to attain normal potential for muscle growth. Although, evidence indicates that even after myogenesis is completed, there are still marked differences in muscle fibre characteristics between different artificial treatments in both sheep (Maxfield *et al.*, 1998a) and cattle (Crosier *et al.*, 2002) suggesting that the early embryonic period may irreversibly program myogenic potential. It should be noted that the fetuses generated in the present study were derived from artificial insemination and embryo transfer protocols. Whether the responses of the embryo to nutritional

perturbations presented in the current experiment are reflected in a natural mating system remain to be determined.

The underlying mechanisms responsible for altered myogenesis described here and elsewhere remain unclear. It is known that myf-5 and myostatin abundance are involved in regulating muscle cell commitment, proliferation and differentiation (Ott *et al.*, 1991; Thomas *et al.*, 2000a, b; Langley *et al.*, 2002), and their expression levels can be altered by earlier manipulation of the embryo or its environment (Maxfield *et al.*, 1998a; Crosier *et al.*, 2002). However, it remains to be determined what events occur during the early pre-implantation period that are able to influence subsequent gene expression patterns. This study did not distinguish between pre- and post-ovulation events, although Kakar *et al.* (In Press) found that maternal nutrition for six days after ovulation had a similar affect on blastocyst development as the combined pre- and post-ovulation nutrition treatments used in the present study. The observation of Kakar *et al.* (In Press) indicates that the period between fertilisation and blastocyst development is particularly sensitive to changes in nutrition and it is possible that perturbations in myogenesis observed in this study resulted from differing feed intake at this time.

Altered myogenesis, resulting from a nutritional perturbation of the embryonic environment, may be a consequence of changes in factors or influences external to the developing embryo, which alter its immediate environment and development, via either non-genetic mechanisms (e.g. oviductal environment, progesterone concentrations) or through epigenetic modifications of the embryo itself, both with persistent consequences.

Several of these potential pathways could involve the effects of altered progesterone production and clearance and abundance as these vary with intake. Elevated progesterone concentration over the first three days of pregnancy has been implicated with altered placental morphology and enhanced fetal growth at mid-gestation in sheep (Kleemann *et al.*, 2001). In the present study, neither progesterone concentrations nor functional capacity of the placenta were determined. Although, in a similar experiment utilising identical nutritional treatments, Kakar *et al.* (In Press) reported that oviductal progesterone concentrations were significantly elevated in ewes offered a restricted feed intake. Endogenous circulating progesterone concentrations are also inversely responsive to nutrition, during the pre- and post-implantation periods in the ewe (Parr *et al.*, 1982; Williams and Cumming, 1982). In the present study, maternal feed intake

during the peri-conception period did not affect the development of the placenta to mid-gestation in gross terms, even though Kakar *et al.* (In Press) reported that similar nutritional treatments significantly increased the relative number of trophectoderm cells. It is also possible that nutritionally altered circulating and tissue progesterone concentrations may alter myogenesis by inducing conditions that enhance embryonic development, through interactions with other growth factors, or by regulating embryo migration from the oviduct to the uterus.

The insulin-like growth factors are important modulators of embryonic development and are regulated by nutrition. Altered myogenesis at mid-gestation following altered peri-conception nutrition could therefore be related to the interaction between oviductal /uterine and embryonic IGFs. Kakar (personal communication) determined that oviductal IGF-I concentrations were lower in sheep when feed intake was restricted over the peri-conception period. Expression of IGF-I, -II and type-I receptor mRNA have been detected in the oviduct (Stevenson and Wathes, 1996) and from the one-cell stage of the developing embryo (Watson *et al.*, 1994). *In vitro* studies have demonstrated that the addition of IGF-I and -II to culture media can enhance the development of embryos (Harvey and Kaye, 1992a, b), while knockout experiments in mice clearly demonstrate the importance of all members of the IGF family to subsequent fetal development (reviewed by Watson *et al.*, 1999). Further, IGF-II and IGF-II receptor levels have been associated with overgrowth of *in vitro* derived bovine (Blondin *et al.*, 2000) and ovine (Young *et al.*, 2001) fetuses, respectively. Restricting feed intake over the peri-conception period may have reduced oviductal IGF-I levels resulting in delayed embryonic and consequently fetal development, including myogenesis in the latter. Conversely, if embryonic IGFs were elevated with high maternal feed intake and if elevated levels were maintained for a prolonged period, myogenesis could be directly affected. IGF-II and IGF-II receptor have been shown to be elevated at later stages of gestation in bovine and ovine fetuses after *in vitro* culture of embryos (Blondin *et al.*, 2000; Young *et al.*, 2001). IGF-II and IGF-I receptor mRNA have been detected in the neural tube, while IGF-I is systemic in the developing embryo (Pirskanen *et al.*, 2000); all have been implicated in initiating and promoting myogenesis within the somite (Pirskanen *et al.*, 2000). In mice, IGF-II has been shown to promote mesoderm formation, possibly through the IGF-I receptor (Morali *et al.*, 2000), and myogenic differentiation (Prelle *et al.*, 2000). Investigation of IGF expression profiles in ovine embryos exposed to different peri-conception maternal nutritional treatments is required. Further, it would be of interest to determine if altered

IGF expression does occur, and whether changes are permanent or transient in response to nutrition.

Epigenetic mechanisms could be one pathway whereby permanent changes in gene expression could be induced in the embryo by altered peri-conception nutrition, and are known to affect IGF bioavailability with major consequences for fetal growth and development, including that of muscle. Embryonic manipulations have resulted in epigenetic changes in the expression of specific imprinted alleles involved in regulating fetal development (e.g. IGF-II and IGF-II receptor). Epigenetic changes have been implicated in the abnormal development of offspring (e.g. the large offspring syndrome) and are thought to arise from a lack of methylation of DNA sites, resulting in reduced IGF-II receptor expression (Young *et al.*, 2001), possibly affecting the bioavailability and action of IGF-II. The possible role of nutrient supply on epigenetic modifications that influence subsequent development has received scant attention and requires further investigation.

In the present study, muscle weights, fetal body dimensions and organ development were generally unaffected by maternal feed intake. This is in contrast to the differences in fetal development that occur in response to artificial manipulations of the early embryonic environment (Farin and Farin 1995; Kleemann *et al.*, 2001; Crosier *et al.*, 2002). However, the unresponsiveness of gross fetal development to peri-conception nutrition in the current study is similar to that reported by others (Oliver *et al.*, 2001; Edwards and McMillan, 2002). However, it is possible that programming of specific events that may influence subsequent development and health did occur but were not evident from gross measurements conducted at mid-gestation. For example, Gallaher *et al.* (1998) and Edwards and McMillan (2002) and demonstrated that peri-conception nutrition irreversibly affected the development of the hypothalmic-pituitary adrenal and IGF axis, respectively, which were not influenced by subsequent nutrition during late gestation.

There are several limitations to this experiment, meaning the data should be viewed with caution. Firstly, only a small number of fetuses were generated. Secondly, it would have been of interest to confirm the effect of feed intake treatments on maternal metabolic status and progesterone concentrations. Finally, examination of fetal muscle development at day 75 does not indicate whether peri-conception feed intake affects the long-term muscle growth potential. Examination of muscle development throughout and after myogenesis is required to determine if the results witnessed here are transient or permanent. It would have been of interest to examine the gene expression

profiles of the candidate genes investigated earlier in this thesis, however there was insufficient muscle tissue and financial resources available.

6.5 Summary

The effect of short-term maternal feed intake during the peri-conception period on the development of fetal muscle at mid-gestation was investigated. Maternal intake did not affect fetal body or organ development. Fetal muscle weights were also unaffected by maternal feed intake. However, estimated total muscle fibre number tended to be greater in fetuses whose mothers had higher intakes, which resulted from an increased S:P ratio. This indicates secondary fibre formation was occurring at a greater rate in these fetuses. Therefore, it appears that restricting maternal feed intake over the peri-conception period may delay myogenesis in fetal sheep. Further investigation of the underlying mechanisms involved in early embryonic development and programming of muscle cell lineage and the post-natal implications of maternal peri-conception intake on the growth and health of individuals is required.

CHAPTER 7.

7 GENERAL DISCUSSION

7.1 Introduction

In precocial species, such as sheep, development is well advanced prior to birth; the influence of the pre-natal environment on post-natal survival, health and productivity has been well documented. Perturbations of the *in utero* environment (such as manipulating maternal feed intake) may alter the development of the organs and tissues of the fetal sheep. Greenwood *et al.* (1999, 2000a), Everitt (1965) and Maxfield *et al.* (1998a) reported altered myogenesis in fetal sheep because of placental insufficiency, maternal feed restriction and manipulations of the early embryonic environment, respectively. However, these studies demonstrated that the timing and type of treatment imposed may determine the means and extent by which myogenesis is altered. The underlying mechanisms responsible for altered muscle development witnessed in fetal sheep have received less attention. The studies reported in the present thesis investigated changes in the cellularity and gene expression of muscles of fetuses obtained from ewes exposed to varying levels of nutrition. Two models were developed. Firstly, ewes were offered variable feed intake for 3 months prior to mating and throughout gestation. In the second model, ewes were offered variable feed intake for 18 days prior to mating and 6 days after (peri-conception period).

7.2 Model One: Variable maternal feed intake prior to and throughout gestation

The long-term restriction of maternal nutrient supply prior to and throughout gestation failed to alter fetal development during early and mid-gestation, demonstrating the significance of the maternal, placental and fetal adaptations to maintain relatively normal fetal growth. During late gestation growth-retardation was evident in fetuses whose mothers had their intakes restricted. Growth-retarded fetuses were lighter and thinner but of a similar length to those counterparts whose mothers were offered sufficient feed to maintain or increase body condition. Fetal growth-restriction in the present study was thought to result from a glucose mediated effect on circulating IGF-I levels; it is possible that reduced substrate supply to growth-restricted fetuses inhibited maturation of the IGF axis during late gestation which could have consequences for subsequent post-natal growth (Greenwood *et al.*, 2002).

The hyperplastic phase of muscle development, which probably determines the phenotypic potential of subsequent muscle development, appears to be relatively unaffected by maternal feed intake in this model, as indicated by similar myofibre numbers and types between treatment groups. In contrast, hypertrophic muscle fibre growth appears to be restricted in growth-retarded fetuses, evident by smaller muscle fibres, lower protein concentration and lower muscle mass. This is probably due to reduced DNA content within the muscle, the result of slower cell cycle activity and a reduction in the activation and replication of satellite cells and their incorporation into the myofibre, which is required for hypertrophic muscle development. It suggests that myogenesis has evolved to occur during the early stages of gestation when fetal development is uncompromised by external influences allowing the main structural components of the muscle to develop, such that by term muscles have matured enough to provide support and mobility, and allow the neonate to follow the mother soon after birth to avoid predation. Whether the reduction in satellite cell activity and reduced hypertrophic muscle growth witnessed in the present study is permanent or is simply a delay that can be overcome if substrate supply is realimentated remains to be determined. Nevertheless, it is likely that growth-restricted lambs may take longer or require more inputs to attain their potential rates of muscle growth, therefore making them less efficient in a meat production system.

The expression of the MRFs and myostatin mRNA occurred in a differential and coordinated manner as myogenesis proceeded, in accordance with their roles in regulating myogenesis, proposed from *in vitro* and gene knockout studies. Myf-5, myoD and myogenin mRNA expression all declined with increased age; myostatin mRNA expression peaked in the muscle of day 92 fetuses and declined thereafter; myf-6 expression increased as age increased. Some evidence of nutritional regulation of expression existed, namely elevated expression of myostatin and myf-5 in the ST muscle of mid- and late gestation growth-restricted fetuses, respectively. This may suggest that myostatin and myf-5 mRNA expression is implicated in the inhibition of satellite cell proliferation and muscle protein accretion at mid-gestation and a delayed myoblast proliferative phase continuing into late gestation in growth-restricted fetuses. Myf-5 in particular may be a good candidate gene for measurement in any treatments that affect myogenesis, as its expression may be directly related to stage of muscle development. The implications of altered myostatin and myf-5 expression in response to nutrient supply may be significant. Myostatin is a negative regulator of muscle development, and perturbation of its function results in dramatic changes in muscle

mass (Kambadur *et al.*, 1997; McPherron *et al.*, 1997) and myf-5 expression has been associated with growth rates in cattle (Li *et al.*, 2004) and pigs (te Pas *et al.*, 2000).

Skeletal muscle IGF-I and -II mRNA expression peaked at mid-gestation in agreement with other studies and their proposed roles in stimulating myoblast proliferation and differentiation in a co-ordinated manner. The expression of IGF-I and IGF-I receptor was elevated in adult ewes fed a restricted diet. IGF-I receptor was also elevated in the skeletal muscle of growth-restricted fetuses at mid- and late gestation. Elevation of IGF-I receptor in the skeletal muscle of growth-restricted fetuses is in contrast with its proposed role as a mediator of the stimulatory effects of IGF-I and IGF-II on myoblast proliferation and differentiation in knockout mice, and further demonstrates the complexities of *in vivo* systems compared with *in vitro* systems or direct gene modification studies.

In the present study, the use of some commonly reported "housekeeping" genes (GAPDH, β-actin, Cyclophilin A and acidic ribosomal protein) for normalisation was considered inappropriate. The mRNA expression of all these genes varied with stage of development in muscle, while GAPDH also varied with feed intake treatment and muscle type; in fact the measurement of these genes may be useful as indicators of some aspects of muscle development, structure and metabolism.

From an applied point of view under-feeding ewes is of detriment to fetal development, and probably neonatal survival and post-natal productivity. In addition, there appears little value in overfeeding ewes throughout pregnancy for enhancing fetal development. Fetal development and fetal muscle weights were similar between High and Maintenance fetuses, suggesting excess supply of nutrients was partitioned toward maternal energy reserves. Therefore, it may appear that overfeeding pregnant ewes throughout pregnancy may be inefficient, however, the implications of feeding to increase condition over pregnancy on subsequent lactational stresses may be significant.

7.3 Model Two: Variable maternal feed intake during the peri-conception period.

Variable short-term feed intake during the peri-conception period did not influence placental characteristics of ewes or fetal development at mid-gestation. However, muscle fibre number was reduced, due to a decreased S:P ratio in fetuses from donor

ewes whose feed intake was restricted during the peri-conception period. This suggests that hyperplasia may have been modified due to altered substrate supply to the embryo and its environment. It has previously been proposed that advanced fetal development could be due to elevated progesterone concentration (Kleemann *et al.*, 2001). Progesterone concentration was not measured in the present study, however others (Parr *et al.*, 1982; Williams and Cumming, 1982) demonstrated that progesterone concentration was elevated in response to under-nutrition. It therefore seems unlikely that progesterone was a contributing factor in inhibiting myogenesis in the present study, although it cannot be ruled out that peri-conception feed intake altered the bioactivity or clearance of progesterone. If the alteration in myogenesis witnessed is permanent, it is possible that myogenic programming occurred independent of progesterone concentration, via epigenetic modifications of the embryo. Epigenetic modifications of *in vitro* cultured sheep embryos have been associated with large for age fetuses (Young *et al.*, 2001). To my knowledge, no studies have investigated the role nutrient supply may play in programming subsequent developmental events.

Caution must be used in making a recommendation to increase feed intake over the peri-conception period to enhance fetal muscle development. Over-nutrition during early gestation has resulted in poor embryo survival, associated with reduced plasma progesterone concentration (Parr *et al.*, 1987) and may have a negative affect on embryo quality (Yaakub *et al.*, 1999).

7.4 Future work

More work is required to develop a complete understanding of the regulation of myogenesis *in vivo*. Six main extensions of the work reported here are warranted for consideration in the future.

Firstly, further studies aimed at developing a better understanding of peri-conception influences on subsequent development are necessary. The current work reported in this thesis relating to the peri-conception period requires a further replicate to confirm the results of this study, which were based on a small number of animals. The experiment also needs to be repeated in a natural mating system, to determine if the altered myogenesis witnessed in this study occurs independent of the superovulation and embryo transfer techniques employed in the present experiment. If these two studies confirmed altered myogenesis in response to maternal feed intake during the peri-conception period, then investigation into the underlying mechanisms responsible

for such modifications would be required. Experiments could examine the molecular and cellular responses of the embryo and factors within the uterine environment, to maternal feed intake over the peri-conception period.

Secondly, it would be of interest to determine whether critical times during myogenesis in sheep exist, when muscle development might be altered. I believe that there is a dearth of species-specific information regarding the development of muscle in large animals, particularly in the periods prior to and during early myogenesis (e.g. days 15 to 50 in fetal sheep), which may be very significant as much of the commitment of cells to myogenic lineage probably occurs during this period. A complete analysis of the morphological, metabolic and contractile properties of developing myofibres is required as is a need for basic information on the timing and genetic regulation of events, "a myogenic map". This may involve more frequent sample collection (e.g. every two-five days) throughout gestation, commencing at an early stage of development (e.g. day 20 of gestation). For example, studies in the pig and guinea pig have identified critical times when myogenesis appears to be sensitive to external interventions such as maternal feed intake, dietary composition and growth hormone administration during the early stages of myogenesis. The identification of such critical times during sheep myogenesis may be significant. I believe that if a critical stage of myogenesis, which is sensitive to nutrition, is identified, muscle development probably will not be enhanced in fetuses whose growth is normal by increasing nutrition to ewes that are in good condition. Rather, nutrient supplementation could be used during the critical stage to maintain normal muscle development in fetuses that may be candidates for IUGR when ewes are in poor condition.

Thirdly, extension of the understanding of the genetic regulation of biological processes may involve a broader genome-wide examination of mRNA and protein expression levels, rather than focus on the "usual suspects". The complex nature of biological systems are rarely under the control of one gene, more likely they are regulated by the coordinated interactions of many different genes at many different levels. This regulation may involve gene-gene interactions, post-translational modifications or epigenetic factors, which should all be examined in developing a more detailed understanding of myogenesis *in utero*.

Fourthly, investigation of the post-natal outcomes of both the models presented in this thesis may determine whether the effects reported are permanent or transient and whether muscle growth rates, muscle yield and meat quality are altered. From an

animal production perspective the influence of both models on post-natal growth rate, feed efficiency and meat quality are of particular interest and are worthy of further study. Whether long-term severe feed restriction throughout pregnancy permanently affects post-natal production, aside from meat production, also warrants further investigation.

Both models would also be of considerable interest from a human health perspective. The effect of IUGR on health later in life has generated considerable interest recently. The incidence of obesity, cardiovascular disease, non-insulin dependent diabetes and high blood pressure are increased later in life in individuals who undergo some form of pre-natal growth aberration. In contrast, relatively little attention has been focused on peri-conception events and their influence on later health.

Fifthly, the development of standardised approaches for muscle fibre type classification and enumeration would allow for greater consistency in interpreting results across experiments. Standardisation of techniques to classify and enumerate muscle fibre types would greatly assist workers in this field. At present, many inconsistencies exist in methodologies used and the way data are reported, making it difficult to compare and contrast results between different studies. Automation of the techniques used in cell sorting, classification and enumeration will assist in standardising methods across laboratories. Once more automated methodologies are developed they will be an attractive option to many workers. Firstly, they will remove the laborious nature of current manual histology techniques employed. Secondly, they will remove the effect of operator inconsistencies and subjectiveness that may vary between laboratories.

Finally, further time spent optimising conditions for enzymatic and immuno categorisation of myofibres during the development of fetal sheep would enhance our understanding of the transitions that occur in myofibre contractile, metabolic and structural properties and how these properties may influence muscle development. This may enhance our knowledge of how different fibre type populations (primary and secondary; fast and slow twitch) combine to regulate muscle development in a co-ordinated manner. In addition, development of convenient methods to identify satellite cells would enhance our understanding of their role in muscle growth. Much work is required to comprehend the process of satellite cell formation and fate, and factors that regulate the proliferation and differentiation of the satellite cell population.

7.5 Overall conclusions

Differential alterations in myogenesis occur in response to variable maternal feed intake for either a long-term prior to and throughout gestation or a short-term during the peri-conception period. Long-term maternal feed intake appears to affect cell cycle activity during late myogenesis, where restricted feed intake probably results in slower cell cycle activity and delayed activation of satellite cells. This is probably related to substrate supply, which may alter the expression of specific genes involved in regulating myogenesis at critical stages of development (i.e. myf-5, myostatin and the type I IGF receptor). Short-term maternal feed intake during the peri-conception period may program subsequent myogenic events, as muscle fibre number was reduced in fetuses whose mothers were offered restricted feed intake. The two results are somewhat conflicting as the treatments imposed in the long-term model encompassed the timing of the treatments imposed in the short-term model, but did not elicit a similar response in fetal muscles. This conflict may be attributed to the use of superovulation and embryo transfer in the short-term model, possibly making the embryo more sensitive to nutrition, or that the effects due to peri-conception feed intake were transient and not evident after mid-gestation. Alternatively, the time when maternal feed intake was altered may have been more important than the length and severity of the treatment imposed.

APPENDIX 1.

OVERALL STATISTICAL MODELS

Table A1.1: Least squares analysis of variance, with degrees of freedom (df) and probability (p) values, of live weight and condition score of ewes offered variable feed intake prior to and throughout pregnancy.

	Ewe live weight		Ewe condition score	
	df	p	df	p
Feed Intake (FI)	2	<0.0001	2	<0.0001
Stage	4	<0.0001	4	0.0031
FI*Stage	8	<0.0001	8	<0.0001
Error	344		342	

Table A1.2: Least squares analysis of variance, with degrees of freedom (df) and probability (p) values, of plasma metabolite concentrations of ewes offered variable feed intake prior to and throughout pregnancy. a. Glucose, albumin and Urea. b. NEFA, cholesterol and triglycerides (ns is not significant).

a.

	Glucose		Albumin		Urea	
	df	p	df	p	df	p
Feed Intake (FI)	2	0.0034	2	<0.0001	2	0.8407
Stage	2	<0.0001	2	0.8358	2	<0.0001
Litter Size (LS)	1	0.7486	1	0.7879	1	0.2448
FI*Stage	.	ns	4	0.0938	.	ns
FI*LS	.	ns	.	ns	.	ns
Stage*LS	.	ns	.	ns	.	ns
Error	74		70		74	

b.

	NEFA		Cholesterol		Triglycerides	
	df	p	df	p	df	p
Feed Intake (FI)	2	0.0415	2	0.0147	2	0.8312
Stage	2	0.0003	2	0.0011	2	0.4518
Litter Size (LS)	1	0.0075	1	0.8246	1	0.4200
FI*Stage	.	ns	.	ns	4	0.0030
FI*LS	2	0.0434	.	ns	.	ns
Stage*LS	2	0.0012	.	ns	.	ns
Error	70		74		70	

Table A.1.3: Least squares analysis of variance, with degrees of freedom (df) and probability (p) values, of placental characteristics from ewes offered variable feed intake prior to and throughout pregnancy (data was log transformed; ns not significant).

	Placentome Number		Total Placentome Weight		Mean Placentome Weight	
	df	p	df	p	df	p
Feed intake (FI)	2	0.1552	2	0.1658	2	0.5622
Stage	2	0.4238	2	<0.0001	2	<0.0001
Litter Size (LS)	1	<0.0001	1	<0.0001	1	<0.0001
FI*Stage	.	ns	.	ns	.	ns
FI*LS	.	ns	.	ns	.	ns
Stage*LS	.	ns	2	0.0967	2	0.0087
Error	75		73		73	

Table A.1.4: Least squares analysis of variance, with degrees of freedom (df) and probability (p) values, of fetal characteristics from ewes offered variable feed intake prior to and throughout pregnancy (data was log transformed; ns not significant).

	Total Fetal Weight		Fetal: Placentome Weight	
	df	p	df	p
Feed intake (FI)	2	0.0106	2	0.0727
Stage	2	<0.0001	2	<0.0001
Litter Size (LS)	1	<0.0001	1	0.0016
FI*Stage	.	ns	.	ns
FI*LS	.	ns	.	ns
Stage*LS	2	0.0044	.	ns
Error	73		73	

Table A1.5: Least squares analysis of variance, with degrees of freedom (df) and probability (p) values, of proportion of placentomes of each morphology from ewes offered variable feed intake prior to and throughout pregnancy (data was arcsin transformed; ns is not significant).

	Proportion type A		Proportion type B		Proportion type C		Proportion type D	
	df	p	df	p	df	p	df	p
Feed Intake (FI)	2	0.0434	2	0.3487	2	0.0015	2	0.0861
Stage	1	0.2117	1	0.0130	1	0.0598	1	0.4044
Litter Size (LS)	1	0.2149	1	0.1692	1	0.0156	1	0.8349
FI*Stage	.	ns	.	ns	.	ns	.	ns
FI*LS	.	ns	.	ns	2	0.0011	.	ns
Stage*LS	.	ns	.	ns	.	ns	.	ns
Error	48		48		46		48	

Table A1.6: Least squares analysis of variance, with degrees of freedom (df) and probability (p) values, of weight of placentomes of each morphology from ewes offered variable feed intake prior to and throughout pregnancy (data was arcsin transformed; ns is not significant).

	Weight Type A		Weight Type B		Weight Type C		Weight Type D	
	df	p	df	p	df	p	df	p
Feed Intake (FI)	2	0.7043	2	0.5044	2	0.5746	2	0.5696
Stage	1	0.0135	1	0.0053	1	0.1676	1	0.5149
Litter Size (LS)	1	0.3237	1	0.732	1	0.4301	1	0.3828
FI*Stage	.	ns	.	ns	.	ns	.	ns
FI*LS	.	ns	.	ns	.	ns	.	ns
Stage*LS	.	ns	.	ns	.	ns	.	ns
Error	47		31		27		22	

Table A1.7: Least squares analysis of variance, with degrees of freedom (df) and probability (p) values, of body dimensions of fetuses obtained from ewes offered variable feed intake prior to and throughout pregnancy. a. Fetal body weight and crown-rump length. b. Abdominal girth, thoracic girth, skull width and skull length (data was log transformed; ns is not significant).

a.

	Fetal weight		CRL	
	df	p	df	p
Feed Intake (FI)	2	0.0034	2	0.4849
Stage	2	0.0001	2	0.0001
Litter Size (LS)	1	0.0009	1	0.3957
Sex	1	0.1129	1	0.6349
FI*Stage	4	0.0053	4	0.0242
FI*LS	.	ns	.	ns
FI*Sex	.	ns	2	0.0663
Stage*LS	2	0.0001	.	ns
Stage*Sex	.	ns	.	ns
LS*Sex	.	ns	.	ns
Error	97		99	

b.

	Abdominal girth		Thoracic girth		Skull width		Skull length	
	df	p	df	p	df	p	df	p
Feed Intake (FI)	2	0.0532	2	0.0001	2	0.0143	2	0.0045
Stage	2	0.0001	2	0.0001	2	0.0001	2	0.0001
Litter Size (LS)	1	0.0005	1	0.0178	1	0.0007	1	0.0683
Sex	1	0.3437	1	0.0092	1	0.7336	1	0.5137
FI*Stage	.	ns	.	ns	.	ns	.	ns
FI*LS	.	ns	2	0.0476	.	ns	.	ns
FI*Sex	.	ns	.	ns	.	ns	.	ns
Stage*LS	2	0.0001	2	0.0009	2	0.0003	2	0.0158
Stage*Sex	.	ns	.	ns	.	ns	.	ns
LS*Sex	.	ns	.	ns	.	ns	.	ns
Error	102		101		102		102	

Table A1.8: Least squares analysis of variance, with degrees of freedom (df) and probability (p) values, of absolute organ weights of fetuses obtained from ewes offered variable feed intake prior to and throughout pregnancy. a. Liver and brain weight and Brain: Liver ratio. b. Heart, lung and kidney weight (ns is not significant).

a.

	Liver weight		Brain weight		Brain: Liver	
	df	p	df	p	df	p
Feed Intake (FI)	2	0.0914	2	0.1595	2	0.5952
Stage	2	0.0001	1	0.0001	1	0.0001
Litter Size (LS)	1	0.0075	1	0.0851	1	0.0105
Sex	1	0.025	1	0.1809	1	0.0876
FI*Stage	4	0.0116	.	ns	.	ns
FI*LS	2	0.034	.	ns	2	0.0211
FI*Sex	.	ns	.	ns	.	ns
Stage*LS	2	0.0001	1	0.0993	1	0.0018
Stage*Sex	.	ns	.	ns	.	ns
LS*Sex	.	ns	.	ns	.	ns
Error	97		66			

b.

	Heart weight		Lung weight		Kidney weight	
	df	p	df	p	df	p
Feed Intake (FI)	2	0.4914	2	0.0953	2	0.3854
Stage	2	0.0001	2	0.0001	2	0.0001
Litter Size (LS)	1	0.0218	1	0.0359	1	0.0064
Sex	1	0.0431	1	0.2508	1	0.0057
FI*Stage	.	ns	.	ns	.	ns
FI*LS	.	ns	2	0.0861	.	ns
FI*Sex	.	ns	.	ns	.	ns
Stage*LS	2	0.0154	2	0.0015	2	0.0001
Stage*Sex	.	ns	.	ns	2	0.0057
LS*Sex	.	ns	.	ns	.	ns
Error	103		98		101	

Table A1.9: Least squares analysis of variance, with degrees of freedom (df) and probability (p) values, of organ weights relative to fetal weight of fetuses obtained from ewes offered variable feed intake prior to and throughout pregnancy. a. Liver and brain weight. b. Heart, lung and kidney weight (ns is not significant).

a.

	Liver (g/ kg)		Brain (g/ kg)	
	df	p	df	p
Feed Intake (FI)	2	0.0002	2	0.6026
Stage	2	0.0001	1	0.0001
Litter Size (LS)	1	0.0867	1	0.2216
Sex	1	0.7862	1	0.1978
FI*Stage	.	ns	.	ns
FI*LS	.	ns	.	ns
FI*Sex	.	ns	.	ns
Stage*LS	.	ns	.	ns
Stage*Sex	2	0.0219	.	ns
LS*Sex	.	ns	.	ns
Error	101		67	

b.

	Heart (g/ kg)		Lung (g/ kg)		Kidney (g/ kg)	
	df	p	df	p	df	p
Feed Intake (FI)	2	0.0533	2	0.6918	2	0.797
Stage	2	0.0001	2	0.0001	2	0.0001
Litter Size (LS)	1	0.5748	1	0.726	1	0.9806
Sex	1	0.0962	1	0.084	1	0.8578
FI*Stage	.	ns	.	ns	.	ns
FI*LS	.	ns	.	ns	.	ns
FI*Sex	.	ns	.	ns	.	ns
Stage*LS	.	ns	.	ns	.	ns
Stage*Sex	.	ns	.	ns	.	ns
LS*Sex	.	ns	.	ns	.	ns
Error	103		100		103	

Table A1.10: Least squares analysis of variance, with degrees of freedom (df) and probability (p) values, of plasma metabolite concentration of fetuses obtained from ewes offered variable feed intake prior to and throughout pregnancy (ns is not significant).

	Glucose		Albumin		Urea	
	df	p	df	p	df	p
Feed Intake (FI)	2	0.0440	2	0.2334	2	0.1362
Stage	1	<0.0001	1	0.0001	1	0.1237
Litter Size (LS)	1	0.0242	1	0.6466	1	0.5570
Sex	1	0.2272	1	0.0588	1	0.9791
FIxStage	.	ns	.	ns	2	0.0051
FIxLS	2	0.0584	.	ns	.	ns
FIxSex	.	ns	.	ns	.	ns
StagexLS	.	ns	.	ns	.	ns
StagexSex	.	ns	.	ns	.	ns
LSxSex	.	ns	.	ns	.	ns
Error	64		66		64	

Table A1.11: Least squares analysis of variance, with degrees of freedom (df) and probability (p) values, of plasma IGF-I and IGF-II concentration of fetuses obtained from ewes offered variable feed intake prior to and throughout pregnancy (ns is not significant).

	IGF-I		IGF-II	
	df	p	df	p
Feed Intake (FI)	1	0.0008	1	0.7257
Stage	1	0.0028	1	0.0724
Litter Size (LS)	1	0.0319	1	0.4366
Sex	1	0.6869	1	0.892
FIxStage	1	0.0437	.	ns
FIxLS	.	ns	.	ns
FIxSex	.	ns	.	ns
StagexLS	.	ns	.	ns
StagexSex	.	ns	.	ns
LSxSex	.	ns	.	ns
Error	32		33	

Table A.1.12: Least squares analysis of variance, with degrees of freedom (df) and probability (p) values, of absolute weight of the semitendinosus, longissimus dorsi and supraspinatus muscles of late-gestation fetuses obtained from ewes offered variable feed intake prior to and throughout pregnancy (ns is not significant).

	Absolute ST weight		Absolute LD weight		Absolute SS weight	
	df	p	df	p	df	p
Feed Intake (FI)	2	0.06	2	<0.01	2	0.07
Litter Size (LS)	1	0.25	1	<0.01	1	<0.01
Sex	1	0.33	1	0.43	1	0.12
FI*LS	.	ns	.	ns	.	ns
FI*Sex	.	ns	.	ns	.	ns
LS*Sex	.	ns	1	0.01	1	<0.01
Error	30		27		29	

Table A.1.13: Least squares analysis of variance, with degrees of freedom (df) and probability (p) values, of the semitendinosus, longissimus dorsi and supraspinatus muscle weights relative to fetal weight of late-gestation fetuses obtained from ewes offered variable feed intake prior to and throughout pregnancy (ns is not significant).

	Relative ST weight		Relative LD weight		Relative SS weight	
	df	p	df	p	df	p
Feed Intake (FI)	2	0.26	2	0.24	2	0.49
Litter Size (LS)	1	0.61	1	0.42	1	0.57
Sex	1	0.59	1	0.74	1	0.53
FI*LS	.	ns	.	ns	.	ns
FI*Sex	.	ns	.	ns	.	ns
LS*Sex	.	ns	.	ns	1	0.08
Error	30		28		29	

Table A.1.14: Least squares analysis of variance, with degrees of freedom (df) and probability (p) values, of the number and type of muscle fibres in the semitendinosus muscle of small and large fetuses obtained from ewes offered variable feed intake prior to and throughout pregnancy (ns is not significant).

	Total Fibre Number		Slow Fibre Number		Fast Fibre Number		Fast to Slow Ratio	
	df	p	df	p	df	p	df	p
Fetal Size (FS)	1	0.1	1	0.79	1	0.17	1	0.62
Stage	1	<0.01	1	<0.05	1	<0.01	1	0.93
Sex	.	ns	.	ns	.	ns	.	ns
FSxStage	.	ns	.	ns	.	ns	.	ns
FSxSex	.	ns	.	ns	.	ns	.	ns
Stage*Sex	.	ns	.	ns	.	ns	.	ns
Error	34		24		24		24	

Table A.1.15: Least squares analysis of variance, with degrees of freedom (df) and probability (p) values, of the cross-sectional area (CSA) of muscle fibres in the semitendinosus muscle of small and large fetuses obtained from ewes offered variable feed intake prior to and throughout pregnancy (ns is not significant).

	Total Fibre CSA		Primary Fibre CSA		Secondary Fibre CSA	
	df	p	df	p	df	p
Fetal Size (FS)	1	<0.01	1	0.054	1	<0.01
Stage	1	<0.01	1	<0.01	1	<0.01
Sex	.	ns	.	ns	.	.
FSxStage	1	<0.01	.	ns	1	0.17
FSxSex	.	ns	.	ns	.	ns
Stage*Sex	.	ns	.	ns	.	ns
Error	33		27		26	

Table A.1.16: Least squares analysis of variance, with degrees of freedom (df) and probability (p) values, of the dry matter percentage and the concentration of DNA, RNA and Protein in the semitendinosus and supraspinatus muscles of small and large fetuses obtained from ewes offered variable feed intake prior to and throughout pregnancy (ns is not significant).

	Dry Matter Percentage		DNA Concentration		RNA Concentration		Protein Concentration	
	df	p	df	p	df	p	df	p
Fetal Size (FS)	1	0.28	1	0.94	1	0.05	1	0.03
Stage	1	<0.01	1	<0.01	1	0.04	1	<0.01
Muscle	1	0.02	1	0.03	1	0.23	1	<0.01
FSxStage	.	ns	1	0.02	.	ns	1	0.09
FSxMuscle	1	0.09	.	ns	.	ns	1	0.08
Stage*Muscle	.	ns	1	0.06	1	<0.01	.	ns
Error	34		34		34		34	

Table A.2.17: Least squares analysis of variance, with degrees of freedom (df) and probability (p) values, of the ratio of nucleic acids and protein in the semitendinosus and supraspinatus muscles of small and large fetuses obtained from ewes offered variable feed intake prior to and throughout pregnancy (ns is not significant).

	RNA: DNA		Protein: DNA		Protein: RNA	
	df	p	df	p	df	p
Fetal Size (FS)	1	0.81	1	0.048	1	0.002
Stage	1	0.001	1	<0.0001	1	<0.0001
Muscle	1	0.013	1	0.4	1	0.002
FSxStage	1	0.024	1	0.003	.	ns
FSxMuscle	.	ns	.	ns	.	ns
Stage*Muscle	.	ns	1	0.013	1	0.001
Error	34		34		34	

Table A1.18: Least squares analysis of variance, with degrees of freedom (df) and probability (p) values, of the total DNA, RNA and protein content in the semitendinosus and supraspinatus muscles of small and large fetuses at late-gestation (ns is not significant).

	Total DNA content		Total RNA content		Total Protein content	
	df	p	df	p	df	p
Fetal Size (FS)	1	0.008	1	<0.0001	1	<0.0001
Muscle	1	0.59	1	0.31	1	0.35
FSxMuscle	.	ns	.	ns	.	ns
Error	17		16		17	

Table A1.19: Least squares analysis of variance, with degrees of freedom (df) and probability (p) values, of exogenous control mRNA and 18S rRNA abundance in the semitendinosus and supraspinatus muscles of fetal and adult sheep offered different feed intakes (data was log transformed; ns is not significant).

	Control		18S rRNA	
	df	p	df	p
Feed Intake (FI)	2	0.36	2	<0.1
Stage	3	<0.05	3	<0.01
Muscle	1	0.60	1	0.12
Batch	2	<0.01	2	<0.01
FI*Stage	.	ns	6	<0.05
FI*Muscle	.	ns	.	ns
Stage*Muscle	.	ns	.	ns
FI*Batch	.	ns	.	ns
Stage*Batch	.	ns	.	ns
Muscle*Batch	.	ns	.	ns
Error	96		90	

Table A1.20: Least squares analysis of variance, with degrees of freedom (df) and probability (p) values, of GAPDH, β-actin, Cyclophilin A and ARP mRNA expression, normalised to 18S rRNA, in the semitendinosus and supraspinatus muscles of fetal and adult sheep offered different feed intakes (data was log transformed; ns is not significant).

	GAPDH		β-actin		Cyclophilin A		ARP	
	df	p	df	p	df	p	df	p
Feed Intake (FI)	2	<0.01	2	0.11	2	0.11	2	0.42
Stage	3	<0.01	3	<0.01	3	<0.01	3	<0.01
Muscle	1	<0.01	1	0.68	1	0.13	1	0.51
FI*Stage	.	ns	6	<0.01	6	<0.01	6	<0.05
FI*Muscle	.	ns	.	ns	.	ns	.	ns
Stage*Muscle	.	ns	.	ns	.	ns	.	ns
Error	98		92		92		92	

Table A1.21: Least squares analysis of variance, with degrees of freedom (df) and probability (p) values, of myf-5, myoD, myogenin, myf-6 and myostatin mRNA expression, normalised to 18S rRNA, in the semitendinosus and supraspinatus muscles of fetal and adult sheep offered different feed intakes (data was log transformed; ns is not significant).

	Myf-5		MyoD		Myogenin		Myf-6		Myostatin	
	df	p	df	p	df	p	df	p	df	p
Feed Intake (FI)	2	<0.01	2	<0.05	2	<0.05	2	0.41	2	0.55
Stage	3	<0.01	3	<0.01	3	<0.01	3	<0.01	3	<0.01
Muscle	1	0.25	1	0.92	1	0.68	1	0.055	1	<0.01
FI*Stage	6	<0.01	6	<0.01	6	<0.01	6	<0.01	.	ns
FI*Muscle	.	ns	.	ns	.	ns	.	ns	.	ns
Stage*Muscle	2	<0.01	.	ns	2	<0.05	2	ns	.	ns
Error	90		92		90		92		98	

Table A1.22: Least squares analysis of variance, with degrees of freedom (df) and probability (p) values, of IGF-I, IGF-II and IGF-I receptor mRNA expression, normalised to 18S rRNA, in the semitendinosus and supraspinatus muscles of fetal and adult sheep offered different feed intakes (data was log transformed; ns is not significant).

	IGF-I		IGF-II		IGF-I receptor	
	df	p	df	p	df	p
Feed Intake (FI)	2	0.25	2	0.19	2	0.054
Stage	3	<0.01	3	<0.01	3	<0.01
Muscle	1	0.87	1	0.24	1	0.54
FI*Stage	6	<0.01	.	ns	6	<0.01
FI*Muscle	.	ns	.	ns	.	ns
Stage*Muscle	.	ns	2	<0.01	.	ns
Error	92		96		92	

Table A1.23: Least squares analysis of variance, with degrees of freedom (df) and probability (p) values, of placental characteristics of ewes offered variable feed intake during the peri-conception period (ns is not significant).

	Placentome Number		Total Placentome Weight		Average Placentome Weight		Fetal: Placentom Weight	
	df	p	df	p	df	p	df	p
Feed Intake (FI)	1	0.2836	1	0.7041	1	0.4015	1	0.385
Rep	.	ns	.	ns	.	ns	.	ns
FI*Rep	.	ns	.	ns	.	ns	.	ns
Error	19		19		19		19	

Table A1.24: Least squares analysis of variance, with degrees of freedom (df) and probability (p) values, of body dimensions of fetuses obtained from ewes offered variable feed intake during the peri-conception period (ns is not significant). a. Fetal body weight and crown-rump length. b. Abdominal girth, thoracic girth, skull width and skull length.

a.

	Fetal weight		CRL	
	df	p	df	p
Feed Intake (FI)	1	0.54	1	0.68
Rep	2	<0.01	2	<0.01
Sex	.	ns	.	ns
FI*Rep	.	ns	.	ns
FI*Sex	.	ns	.	ns
Error	17		17	

b.

	Abdominal girth		Thoracic girth		Skull width		Skull length	
	df	p	df	p	df	p	df	p
Feed Intake (FI)	1	0.19	1	0.26	1	0.68	1	0.36
Rep	2	<0.01	2	<0.01	2	<0.01	2	<0.01
Sex	.	ns	.	ns	.	ns	.	ns
FI*Rep	.	ns	.	ns	.	ns	.	ns
FI*Sex	.	ns	.	ns	.	ns	.	ns
Error	17		17		17		17	

Table A1.25: Least squares analysis of variance, with degrees of freedom (df) and probability (p) values, of absolute organ weights of fetuses obtained from ewes offered variable feed intake during the peri-conception period. a. Liver and brain weight. b. Heart, lung and kidney weight (ns is not significant).

a.

	Liver weight		Brain weight	
	df	p	df	p
Feed Intake (FI)	1	0.86	1	0.33
Rep	2	<0.01	2	<0.05
Sex	.	ns	.	ns
FI*Rep	.	ns	.	ns
FI*Sex	.	ns	.	ns
Error	17		17	

b.

	Heart weight		Lung weight		Kidney weight	
	df	p	df	p	df	p
Feed Intake (FI)	1	0.6500	1	0.72	1	0.58
Rep	2	<0.01	2	<0.01	2	<0.01
Sex	.	ns	.	ns	.	ns
FI*Rep	.	ns	.	ns	.	ns
FI*Sex	.	ns	.	ns	.	ns
Error	17		17		17	

Table A1.26: Least squares analysis of variance, with degrees of freedom (df) and probability (p) values, of organ weights relative to fetal weight of fetuses obtained from ewes offered variable feed intake during the peri-conception period. a. Liver and brain weight. b. Heart, lung and kidney weight (ns is not significant).

a.

	Liver (g /kg)		Brain (g /kg)	
	df	p	df	p
Feed Intake (FI)	1	0.85	1	0.33
Rep	2	<0.01	2	<0.05
Sex	.	ns	.	ns
FI*Rep	.	ns	.	ns
FI*Sex	.	ns	.	ns
Error	17		17	

b.

	Heart (g /kg)		Lung (g /kg)		Kidney (g /kg)	
	df	p	df	p	df	p
Feed Intake (FI)	1	0.66	1	0.72	1	0.59
Rep	2	<0.01	2	<0.01	2	<0.01
Sex	.	ns	.	ns	.	ns
FI*Rep	.	ns	.	ns	.	ns
FI*Sex	.	ns	.	ns	.	ns
Error	17		17		17	

Table A1.27: Least squares analysis of variance, with degrees of freedom (df) and probability (p) values, of absolute weights of the semitendinosus, longissimus dorsi and supraspinatus muscles of fetuses obtained from ewes offered variable feed intake during the peri-conception period (ns is not significant).

	ST weight (g)		LD weight (g)		SS weight (g)	
	df	p	df	p	df	p
Feed Intake (FI)	1	0.63	1	0.93	1	0.63
Rep	2	<0.01	2	<0.01	2	<0.01
Sex	.	ns	.	ns	.	ns
FI*Rep	.	ns	.	ns	.	ns
FI*Sex	.	ns	.	ns	.	ns
Error	17		17		17	

Table A2.28: Least squares analysis of variance, with degrees of freedom (df) and probability (p) values, of weights of the semitendinosus, longissimus dorsi and supraspinatus muscles relative to fetal weight of fetuses obtained from ewes offered variable feed intake during the peri-conception period (ns is not significant).

	ST weight (g /kg)		LD weight (g /kg)		SS weight (g /kg)	
	df	p	df	p	df	p
Feed Intake (FI)	1	0.7900	1	0.9	1	0.67
Rep	2	<0.01	2	<0.01	2	<0.01
Sex	.	ns	.	ns	.	ns
FI*Rep	.	ns	.	ns	.	ns
FI*Sex	.	ns	.	ns	.	ns
Error	17		17		17	

Table A1.29: Least squares analysis of variance, with degrees of freedom (df) and probability (p) values, of the number and type of muscle fibres in the semitendinosus muscle fetuses obtained from ewes offered variable feed intake during the peri-conception period (ns is not significant).

	Total Fibre Number		Primary Fibre Number		Secondary Fibre Number		Secondary: Primary Ratio	
	df	p	df	p	df	p	df	p
Feed Intake (FI)	1	<0.10	1	0.75	1	<0.05	1	<0.05
Rep	2	<0.05	.	ns	2	<0.05	.	ns
Sex	.	ns	.	ns	.	ns	.	ns
FI*Rep	.	ns	.	ns	.	ns	.	ns
FI*Sex	.	ns	.	ns	.	ns	.	ns
Error	15		17		15		17	

Table A1.30: Least squares analysis of variance, with degrees of freedom (df) and probability (p) values, of the cross-sectional area (CSA) of muscle fibres in the semitendinosus muscle fetuses obtained from ewes offered variable feed intake during the peri-conception period (ns is not significant).

	Primary Fibre CSA		Secondary Fibre CSA	
	df	p	df	p
Feed Intake (FI)	1	0.45	1	0.58
Rep	.	ns	.	ns
Sex	.	ns	.	ns
FI*Rep	.	ns	.	ns
FI*Sex	.	ns	.	ns
Error	17		17	

Table A1.31: Least squares analysis of variance, with degrees of freedom (df) and probability (p) values, of the concentration of DNA, RNA and Protein in the semitendinosus muscle of fetuses obtained from ewes offered variable feed intake during the peri-conception period (ns is not significant).

	DNA concentration		RNA concentration		Protein concentration	
	df	p	df	p	df	p
Feed Intake (FI)	1	0.87	1	0.84	1	0.3
Rep	2	<0.01	2	<0.01	.	ns
Sex	.	ns	.	ns	.	ns
FI*Rep	.	ns	.	ns	.	ns
FI*Sex	.	ns	.	ns	.	ns
Error	17		17		18	

Table A1.32: Least squares analysis of variance, with degrees of freedom (df) and probability (p) values, of the ratios of nucleic acids and protein in the semitendinosus muscle of fetuses obtained from ewes offered variable feed intake during the peri-conception period (ns is not significant).

	RNA: DNA		Protein: DNA		Protein: RNA	
	df	p	df	p	df	p
Feed Intake (FI)	1	0.89	1	<0.10	1	0.14
Rep	2	<0.01	2	<0.01	2	<0.10
Sex	.	ns	.	ns	.	ns
FI*Rep	.	ns	.	ns	.	ns
FI*Sex	.	ns	.	ns	.	ns
Error	17		17		17	

Table A1.33: Least squares analysis of variance, with degrees of freedom (df) and probability (p) values, of the total DNA, RNA and protein content in the semitendinosus muscle of fetuses obtained from ewes offered variable feed intake during the peri-conception period (ns is not significant).

	Total DNA		Total RNA		Total Protein	
	df	p	df	p	df	p
Feed Intake (FI)	1	0.84	1	0.95	1	0.36
Rep	2	<0.01	2	<0.01	2	<0.10
Sex	.	ns	.	ns	.	ns
FI*Rep	.	ns	.	ns	.	ns
FI*Sex	.	ns	.	ns	.	ns
Error	17		17		17	

APPENDIX 2.

ADDITIONAL GENE EXPRESSION DATA

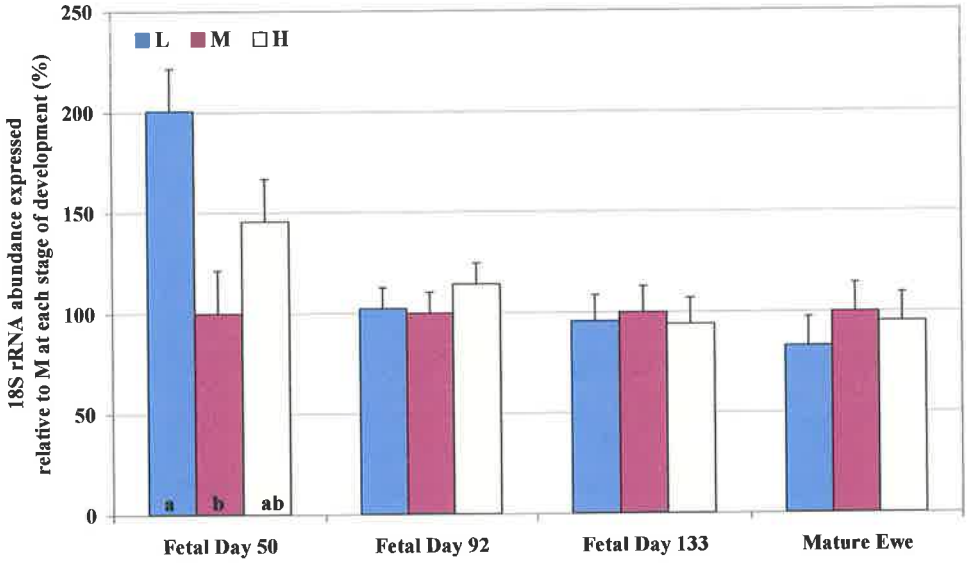


Figure A2.1: Effect of maternal feed intake on 18S rRNA expression at each stage of development. Different superscripts within a stage indicate significant differences ($p<0.05$).

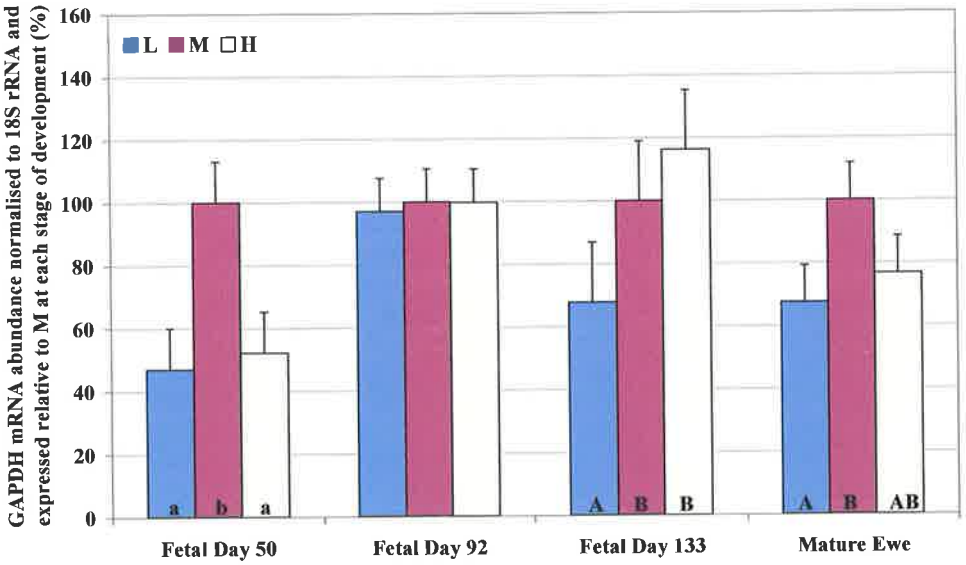


Figure A2.2: Effect of maternal feed intake on GAPDH mRNA expression normalised to 18S rRNA at each stage of development. Different superscripts within a stage indicate significant differences (lowercase, $p<0.05$; uppercase, $p<0.1$).

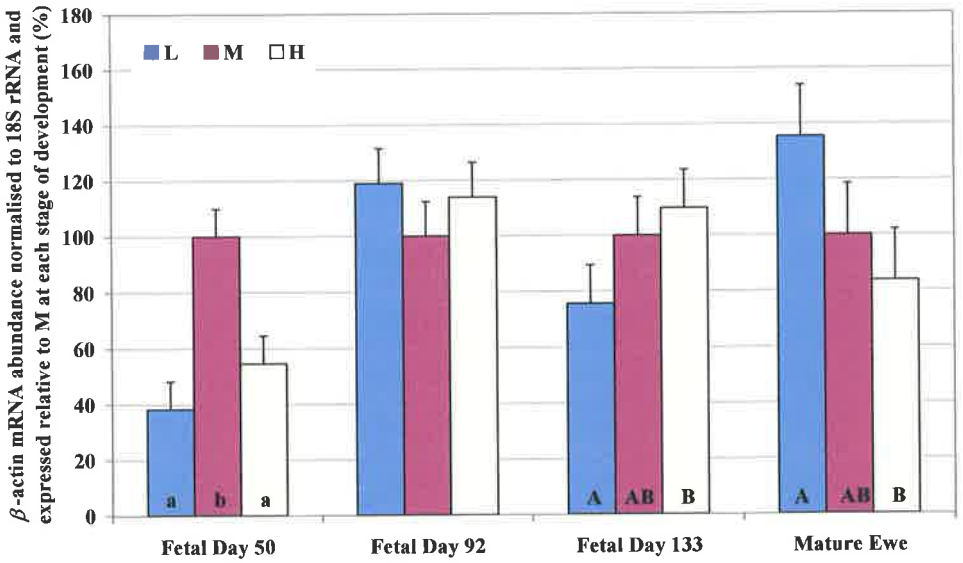


Figure A2.3: Effect of maternal feed intake on β-actin mRNA expression normalised to 18S rRNA at each stage of development. Different superscripts within a stage indicate significant differences (lowercase, $p<0.05$; uppercase, $p<0.1$).

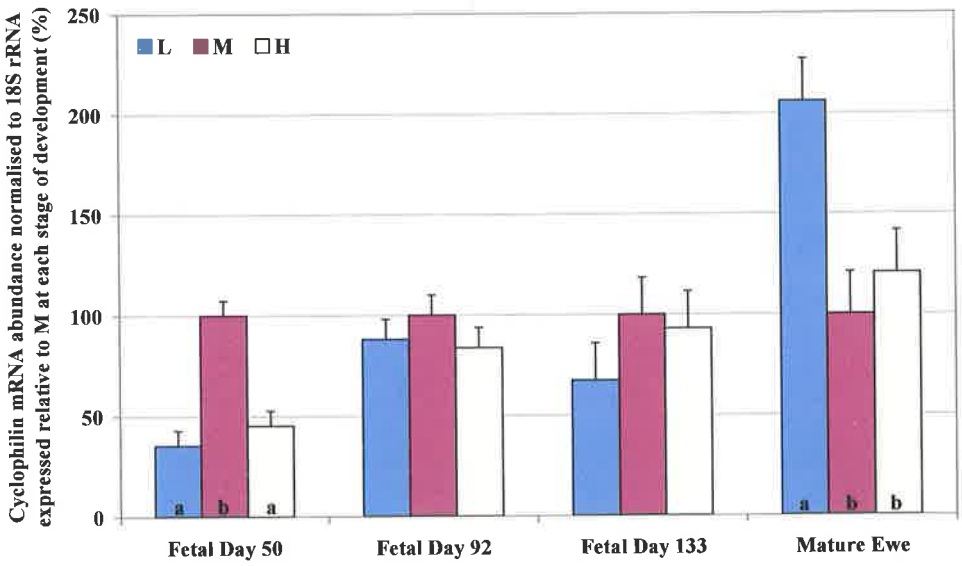


Figure A2.4: Effect of maternal feed intake on Cyclophilin A mRNA expression normalised to 18S rRNA at each stage of development. Different superscripts within a stage indicate significant differences ($p<0.05$).

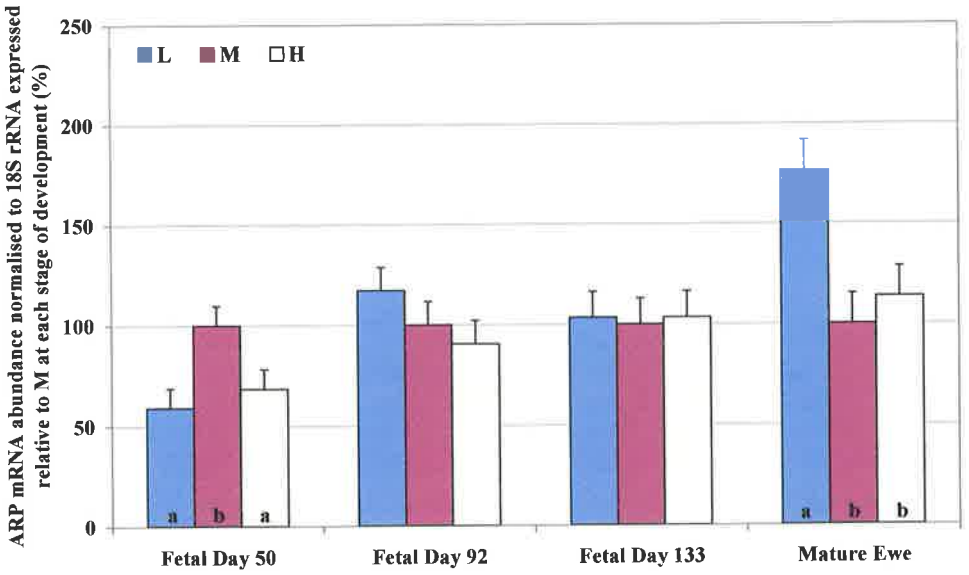


Figure A2.5: Effect of maternal feed intake on Acidic Ribosomal Phosphoprotein (ARP) mRNA expression normalised to 18S rRNA at each stage of development. Different superscripts within a stage indicate significant differences ($p<0.05$).

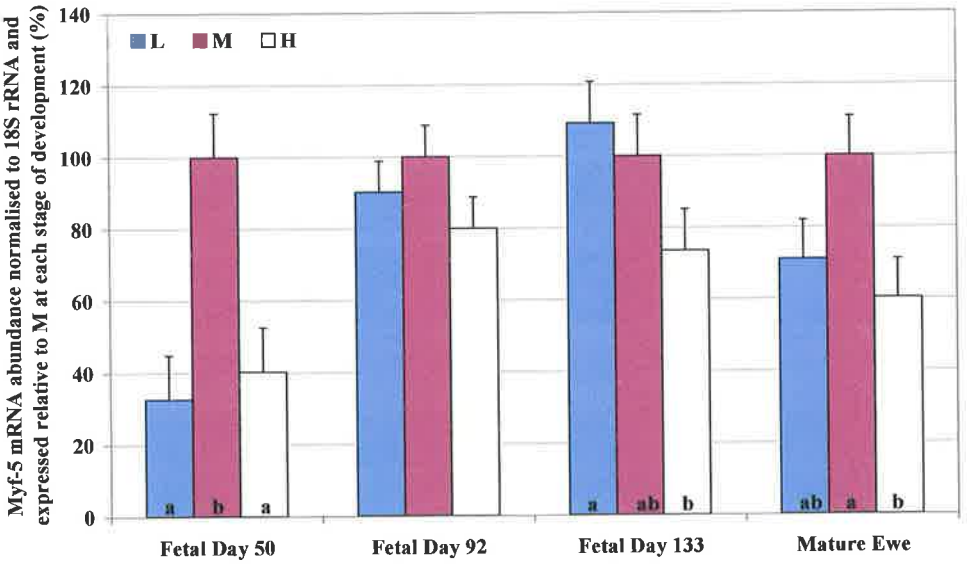


Figure A2.6: Effect of maternal feed intake on Myf-5 mRNA expression normalised to 18S rRNA at each stage of development. Different superscripts within a stage indicate significant differences ($p<0.05$).

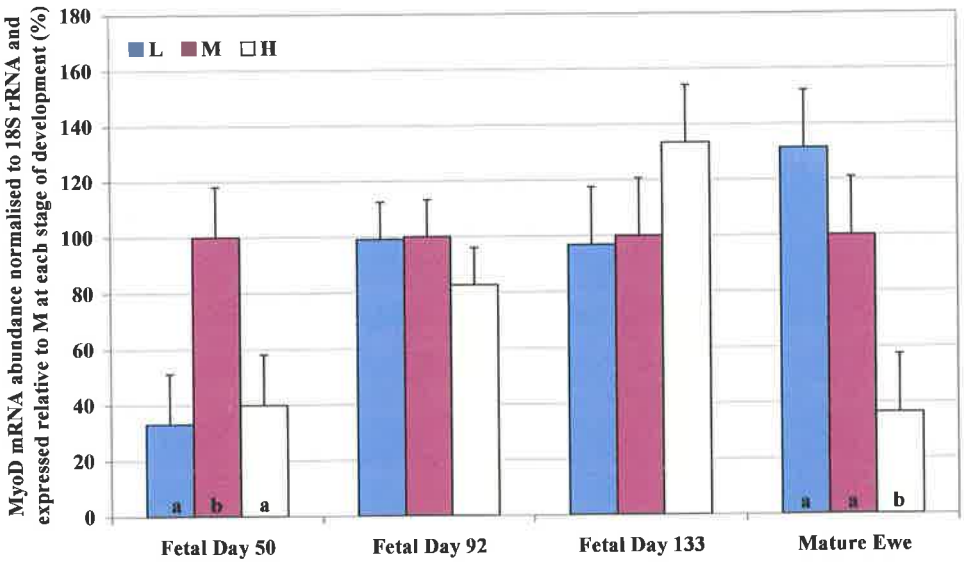


Figure A2.7: Effect of maternal feed intake on MyoD mRNA expression normalised to 18S rRNA at each stage of development. Different superscripts within a stage indicate significant differences ($p<0.05$).

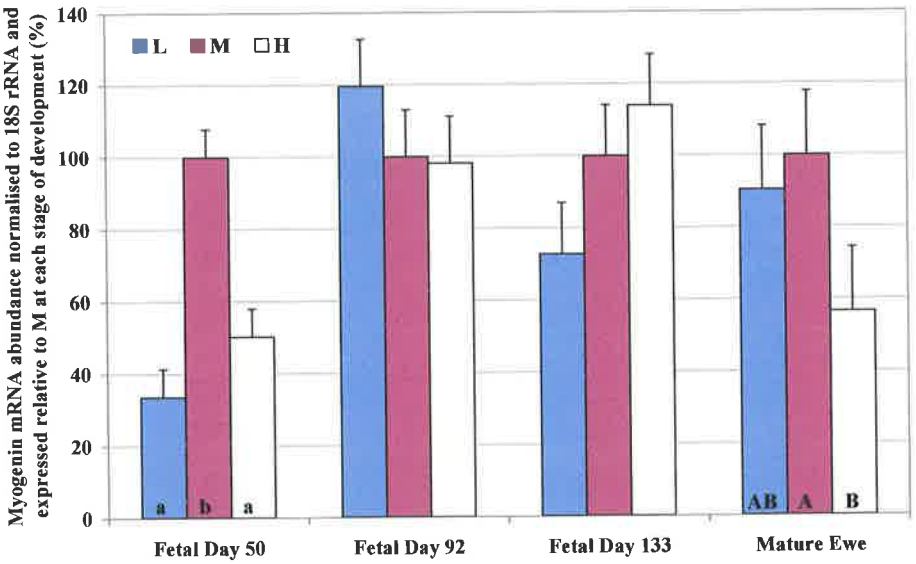


Figure A2.8: Effect of maternal feed intake on myogenin mRNA expression normalised to 18S rRNA at each stage of development. Different superscripts within a stage indicate significant differences (lowercase, $p<0.05$; uppercase, $p<0.1$).

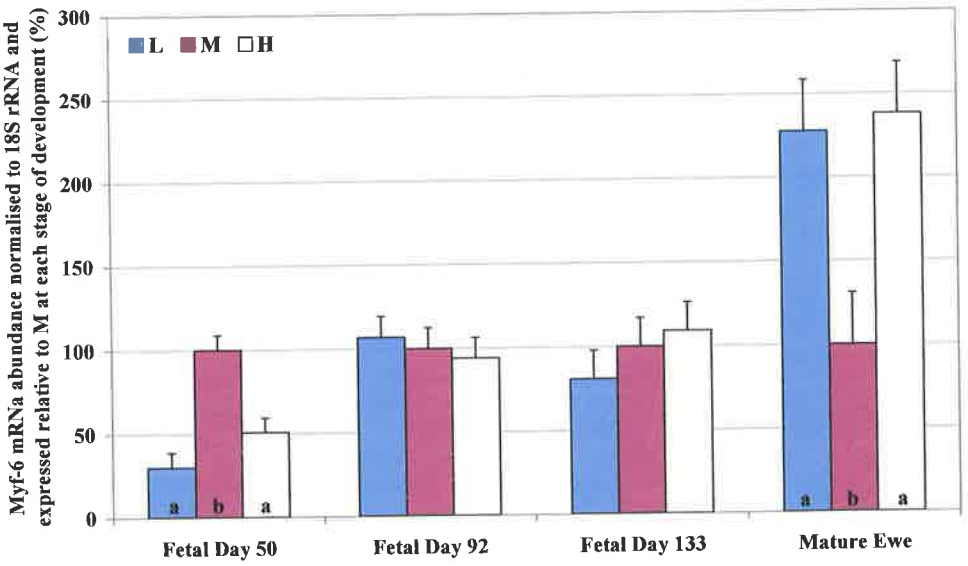


Figure A2.9: Effect of maternal feed intake on Myf-6 mRNA expression normalised to 18S rRNA at each stage of development. Different superscripts within a stage indicate significant differences ($p<0.05$).

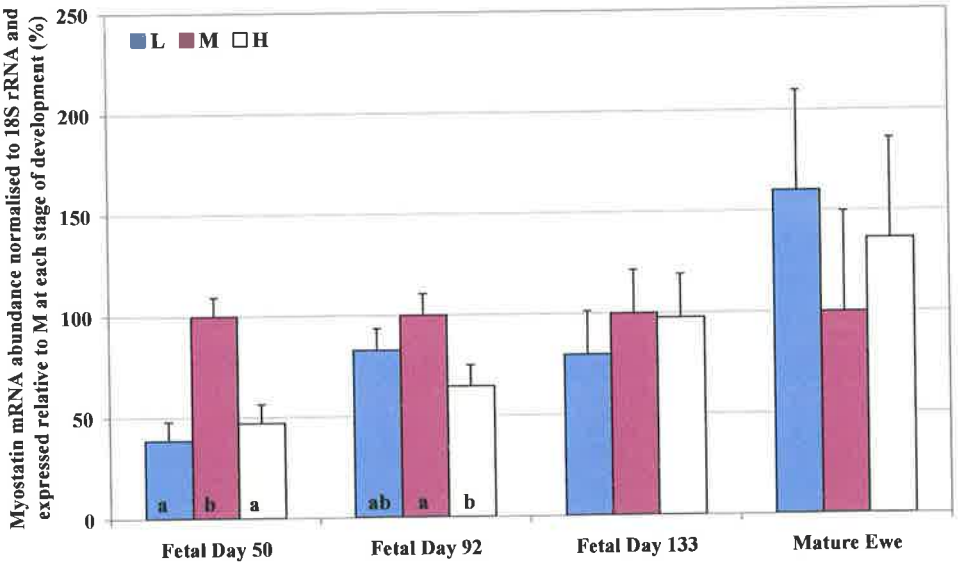


Figure A2.10: Effect of maternal feed intake on Myostatin mRNA expression normalised to 18S rRNA at each stage of development. Different superscripts within a stage indicate significant differences ($p<0.05$).

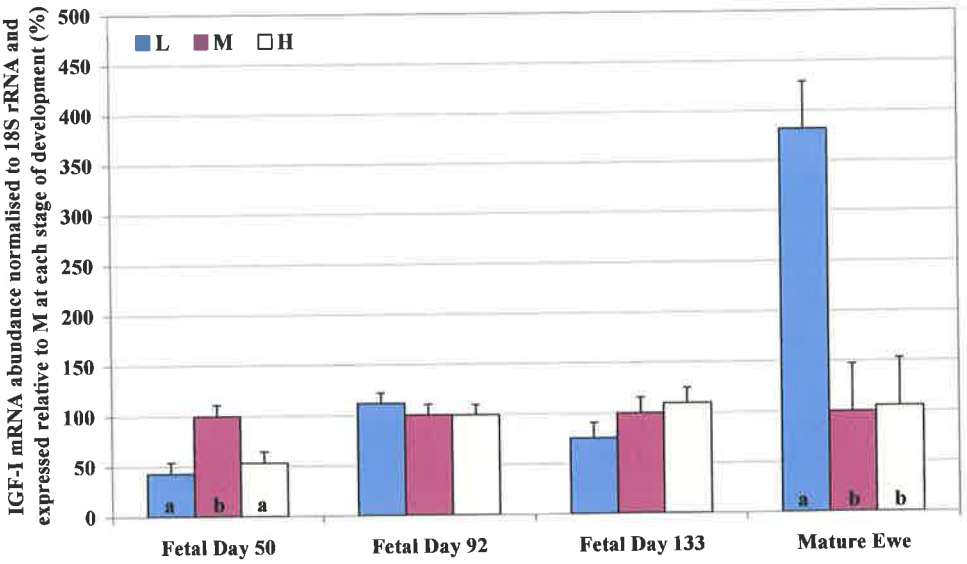


Figure A2.11: Effect of maternal feed intake on IGF-I mRNA expression normalised to 18S rRNA at each stage of development. Different superscripts within a stage indicate significant differences ($p<0.05$).

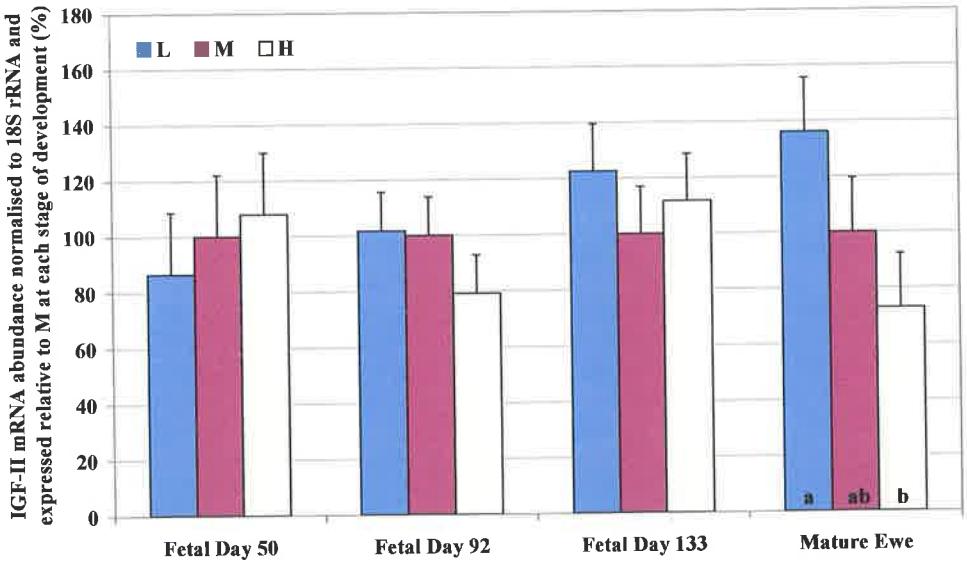


Figure A2.12: Effect of maternal feed intake on IGF-II mRNA expression normalised to 18S rRNA at each stage of development. Different superscripts within a stage indicate significant differences ($p<0.05$).

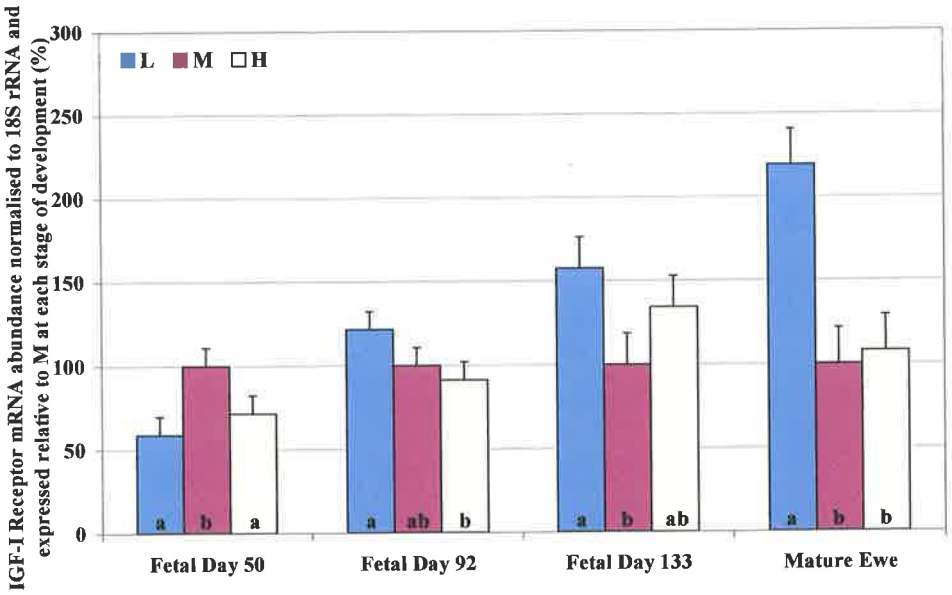


Figure A2.13: Effect of maternal feed intake on IGF-I receptor mRNA expression normalised to 18S rRNA at each stage of development. Different superscripts within a stage indicate significant differences ($p<0.05$).

Table A2.1: The effect of stage of development on the expression of housekeeping genes without normalisation. Different superscripts within rows indicate significant difference between stages of development ($p<0.05$). *values are LSmeans ± standard error of the mean.

	Stage of Development			
	Fetal day 50	Fetal day 92	Fetal day 133	Mature ewe
18S rRNA	$100.0 \pm 8.6^{*a}$	102.1 ± 8.3^{a}	55.9 ± 7.9^{b}	40.8 ± 7.1^{b}
GAPDH	100.0 ± 273.3^{a}	749.9 ± 178.9^{b}	3355 ± 178.9^{c}	1811.6 ± 178.9^{d}
β-actin	100.0 ± 4.7^{a}	39.6 ± 3.1^{b}	17.5 ± 3.1^{c}	5.5 ± 3.1^{d}
Cyclophilin A	100.0 ± 3.9^{a}	58.8 ± 2.6^{b}	24.2 ± 2.6^{c}	9.2 ± 2.6^{d}
Acidic Riboprotein	100.0 ± 5.0^{a}	53.8 ± 3.3^{b}	64.3 ± 3.3^{b}	45.6 ± 3.3^{c}

Table A2.2: The effect of stage of development on the expression of the MRFs and myostatin without normalisation. Different superscripts within rows indicate significant difference between stages of development ($p<0.05$). *values are LSmeans ± standard error of the mean.

	Stage of Development			
	Fetal Day 50	Fetal Day 92	Fetal Day 133	Ewe
Myf-5	$100.0 \pm 4.5^{a*}$	52.1 ± 2.9^{b}	7.5 ± 2.9^{c}	2.3 ± 2.9^{c}
MyoD	100.0 ± 11.8^{a}	110.3 ± 7.7^{a}	62.6 ± 7.7^{b}	35.6 ± 7.7^{c}
Myogenin	100.0 ± 5.2^{a}	84.6 ± 3.4^{b}	30.7 ± 3.4^{c}	10.6 ± 3.4^{d}
Myf-6	100.0 ± 254.5^{a}	553.4 ± 166.6^{a}	484.6 ± 166.6^{a}	2473.0 ± 166.6^{b}
Myostatin	100.0 ± 10.7^{a}	164.3 ± 7.0^{b}	47.3 ± 7.0^{c}	21.7 ± 7.0^{d}

Table A2.3: The effect of stage of development on the expression of the IGFs without normalisation. Different superscripts within rows indicate significant difference between stages of development ($p<0.05$). *values are LSmeans ± standard error of the mean.

	Stage of Development			
	Fetal day 50	**Fetal day 92**	**Fetal day 133**	**Mature ewe**
IGF-I	$100.0 \pm 8.4^{a*}$	133.6 ± 5.5^{b}	67.6 ± 5.5^{c}	7.5 ± 5.5^{d}
IGF-II	100.0 ± 16.4^{a}	213.7 ± 10.7^{b}	35.5 ± 10.7^{c}	1.3 ± 10.7^{d}
IGF-I receptor	100.0 ± 4.5^{a}	54.0 ± 2.9^{b}	28.5 ± 2.9^{c}	50.1 ± 2.9^{d}

Table A2.4: The effect of muscle on the expression of housekeeping genes without normalisation. Different superscripts within rows indicate significant difference between stages of development ($p<0.05$). *values are LSmeans ± standard error of the mean.

	Fetal day 92		Fetal day 133		Mature ewe	
	SS	**ST**	**SS**	**ST**	**SS**	**ST**
18S rRNA	$100.0 \pm 7.8^{*}$	98.5 ± 7.8	100.0 ± 11.7	116.7 ± 11.7	100.0 ± 15.7^{a}	149.0 ± 15.7^{b}
GAPDH	100.0 ± 7.4^{a}	124.8 ± 7.4^{b}	100.0 ± 16.0^{a}	181.4 ± 16.0^{b}	100.0 ± 19.3^{a}	261.2 ± 19.3^{b}
β-actin	100.0 ± 9.2	115.1 ± 9.2	100.0 ± 8.0	111.2 ± 8.0	100.0 ± 11.1	110.7 ± 11.1
Cyclophilin A	100.0 ± 7.2	107.6 ± 7.2	100.0 ± 10.3^{a}	167.3 ± 10.3^{b}	100.0 ± 16.5	133.3 ± 16.5
Acidic Riboprotein	100.0 ± 6.5	97.2 ± 6.5	100.0 ± 7.6	92.7 ± 7.6	100.0 ± 10.9^{a}	163.74 ± 10.9^{b}

Table A2.5: The effect of muscle on the expression of the MRFs and myostatin without normalisation. Different superscripts within rows indicate significant difference between stages of development (lowercase, $p<0.05$; uppercase, $p<0.1$). *values are LSmeans ± standard error of the mean.

	Fetal day 92		Fetal day 133		Mature ewe	
	SS	ST	SS	ST	SS	ST
Myf-5	$100.0 \pm 7.5^{*a}$	142.1 ± 7.5^{b}	100.0 ± 10.3	118.0 ± 10.3	100.0 ± 24.1	59.7 ± 24.1
MyoD	100.0 ± 14.4^{a}	143.8 ± 14.4^{b}	100.0 ± 10.5	74.2 ± 10.5	100.0 ± 19.8^{a}	161.1 ± 19.8^{b}
Myogenin	100.0 ± 7.4^{a}	126.3 ± 7.4^{b}	100.0 ± 11.2	120.7 ± 11.2	100.0 ± 18.9	108.5 ± 18.9
Myf-6	100.0 ± 5.3^{a}	131.3 ± 5.3^{b}	100.0 ± 13.3^{a}	145.1 ± 13.3^{b}	100.0 ± 22.6^{A}	164.5 ± 22.6^{B}
Myostatin	100.0 ± 9.7^{a}	175.0 ± 9.7^{b}	100.0 ± 22.9^{a}	187.6 ± 22.9^{b}	100.0 ± 40.0^{a}	278.1 ± 40.0^{b}

Table A2.6: The effect of muscle on the expression of the IGFs without normalisation. Different superscripts within rows indicate significant difference between stages of development ($p<0.05$). *values are LSmeans ± standard error of the mean.

	Fetal day 92		Fetal day 133		Mature ewe	
	SS	ST	SS	ST	SS	ST
IGF-I	$100.0 \pm 8.3^{*}$	121.5 ± 8.3	100.0 ± 8.3	106.7 ± 8.3	100.0 ± 15.8	124.2 ± 15.8
IGF-II	100.0 ± 8.3^{a}	180.5 ± 8.3^{b}	100.0 ± 7.7^{a}	56.9 ± 7.7^{b}	100.0 ± 13.1	96.9 ± 13.1
IGF-I receptor	100.0 ± 5.8^{a}	124.14 ± 5.8^{b}	100.0 ± 6.6	91.7 ± 6.6	100.0 ± 10.8^{a}	141.7 ± 10.8^{b}

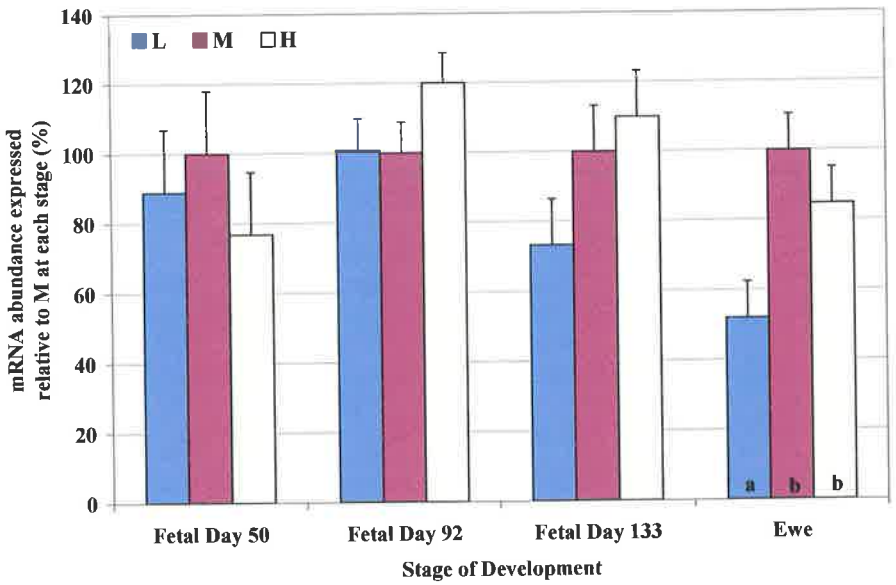


Figure A2.14: Effect of maternal feed intake on GAPDH mRNA expression without normalisation at each stage of development. Different superscripts within a stage indicate significant differences ($p<0.05$).

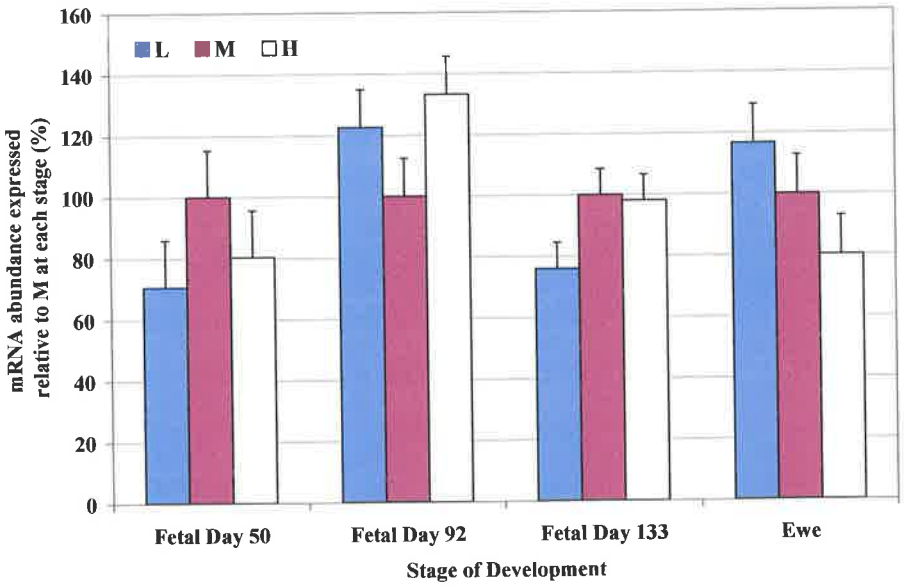


Figure A2.15: Effect of maternal feed intake on β-actin mRNA expression without normalisation at each stage of development.

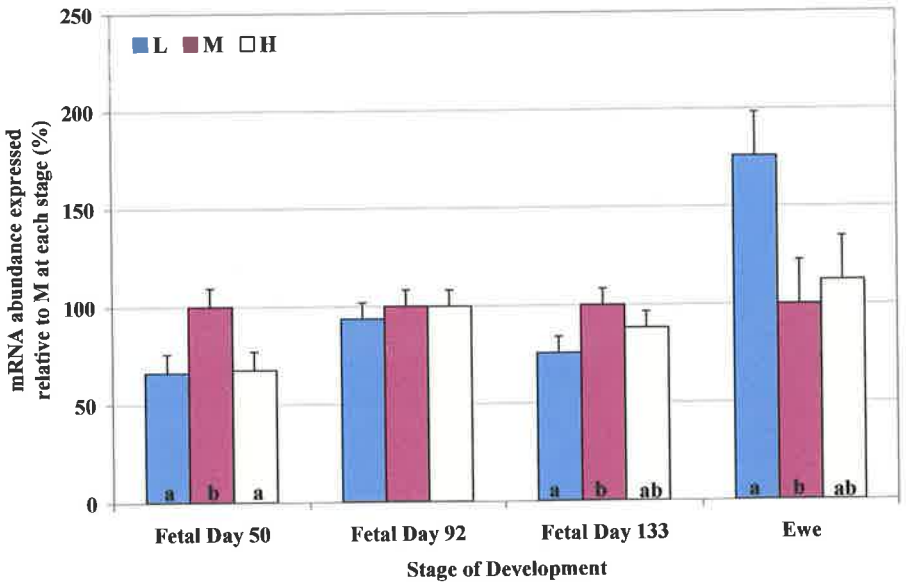


Figure A2.16: Effect of maternal feed intake on Cyclophilin A mRNA expression without normalisation at each stage of development. Different superscripts within a stage indicate significant differences ($p<0.05$).

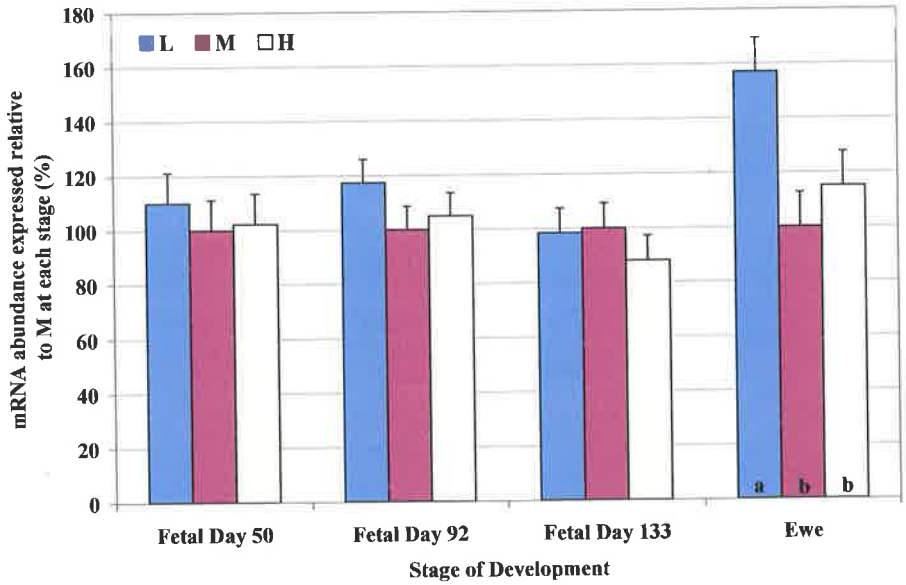


Figure A2.17: Effect of maternal feed intake on Acidic Ribosomal Phosphoprotein (ARP) mRNA expression without normalisation at each stage of development. Different superscripts within a stage indicate significant differences ($p<0.05$).

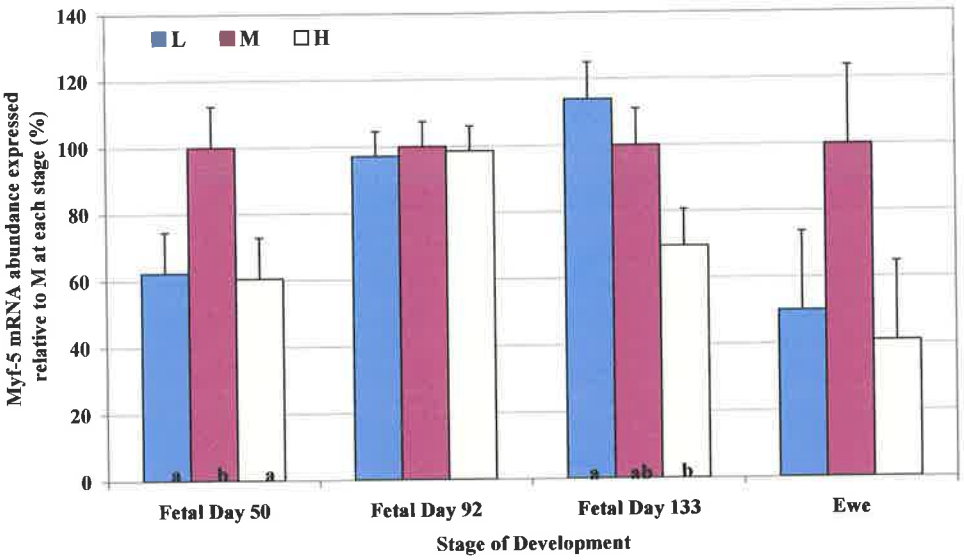


Figure A2.18: Effect of maternal feed intake on Myf-5 mRNA expression without normalisation at each stage of development. Different superscripts within a stage indicate significant differences ($p<0.05$).

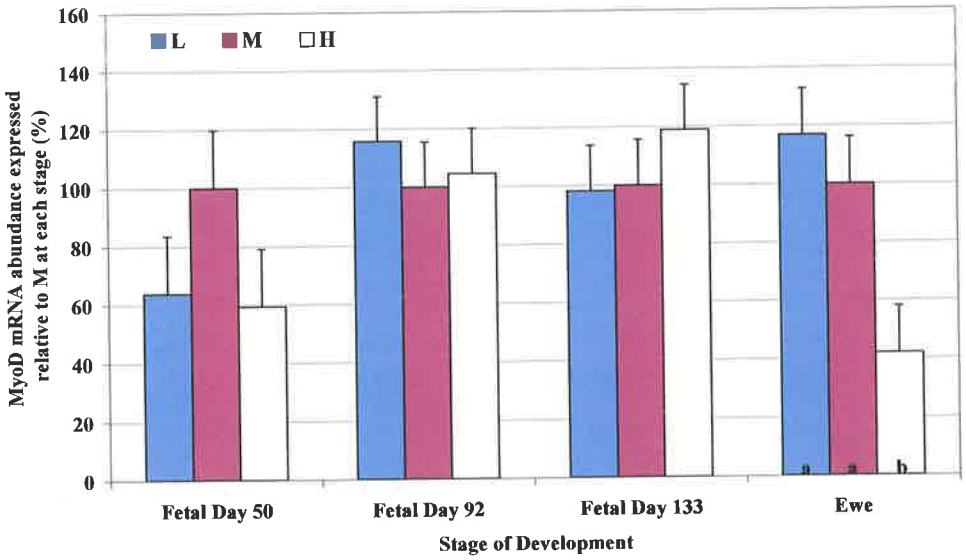


Figure A2.19: Effect of maternal feed intake on MyoD mRNA expression without normalisation at each stage of development. Different superscripts within a stage indicate significant differences ($p<0.05$).

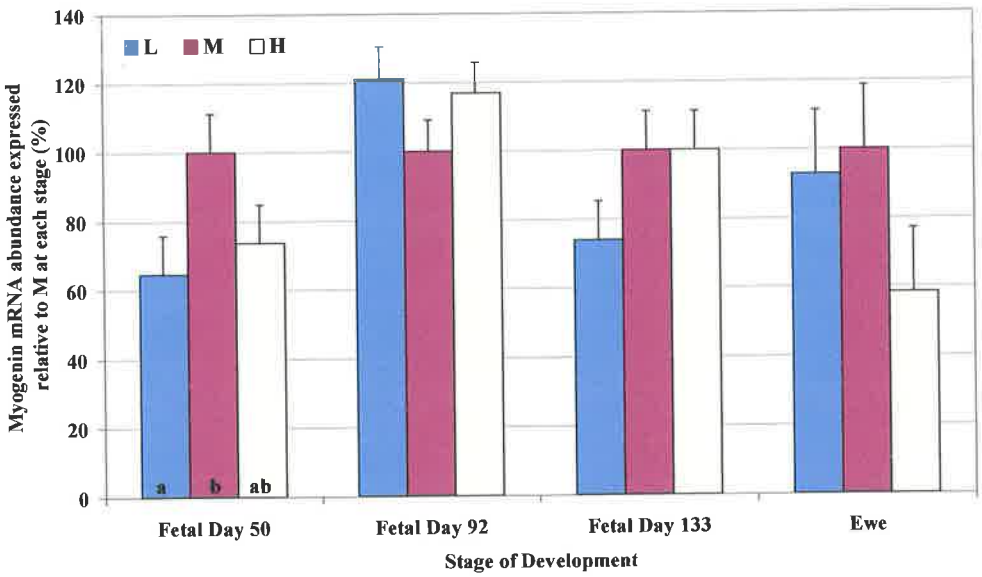


Figure A2.20: Effect of maternal feed intake on Myogenin mRNA expression without normalisation at each stage of development. Different superscripts within a stage indicate significant differences ($p<0.05$).

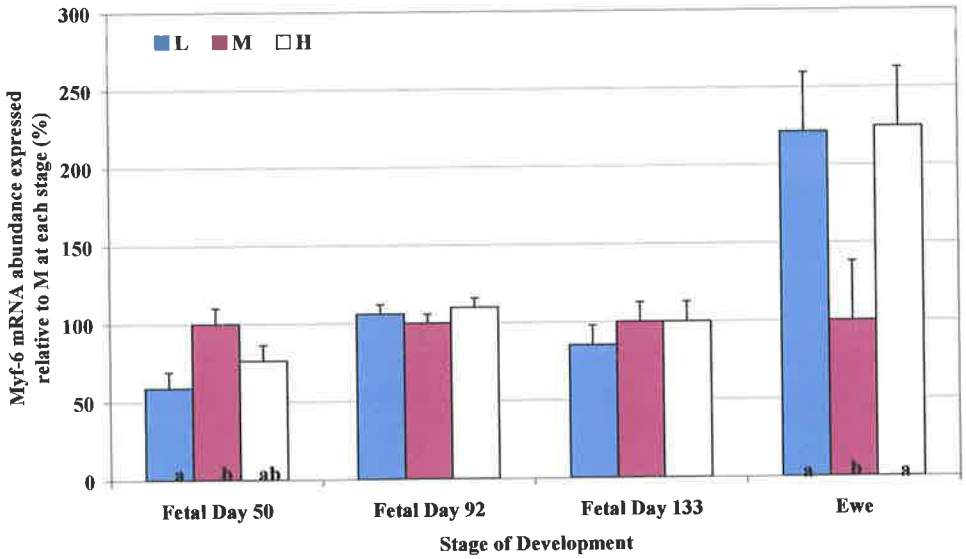


Figure A2.21: Effect of maternal feed intake on Myf-6 mRNA expression without normalisation at each stage of development. Different superscripts within a stage indicate significant differences ($p<0.05$).

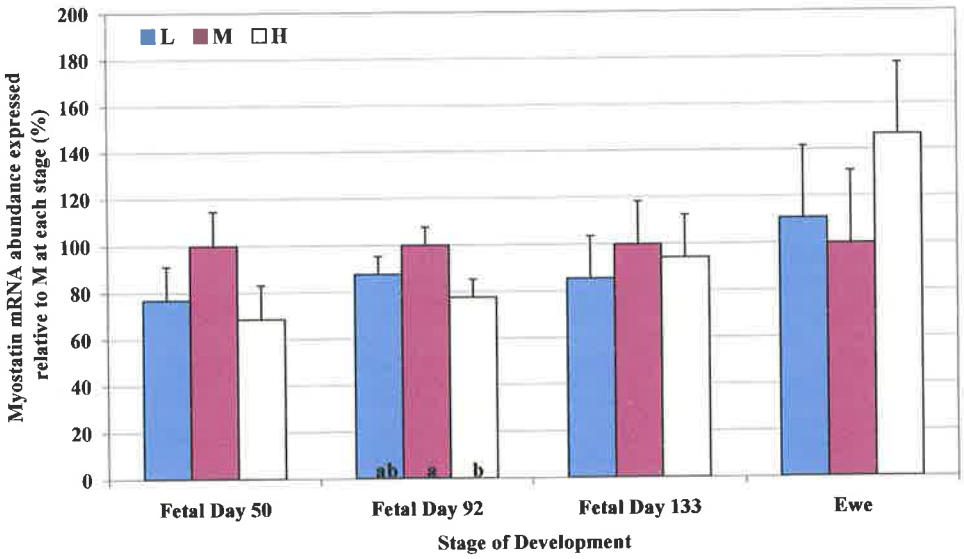


Figure A2.22: Effect of maternal feed intake on Myostatin mRNA expression without normalisation at each stage of development. Different superscripts within a stage indicate significant differences ($p<0.05$).

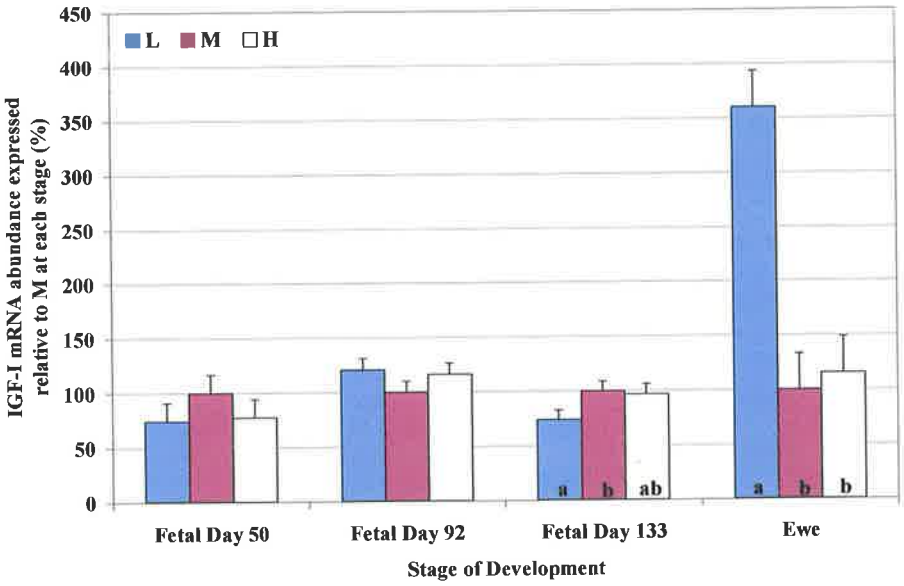


Figure A2.23: Effect of maternal feed intake on IGF-I mRNA expression without normalisation at each stage of development. Different superscripts within a stage indicate significant differences ($p<0.05$).

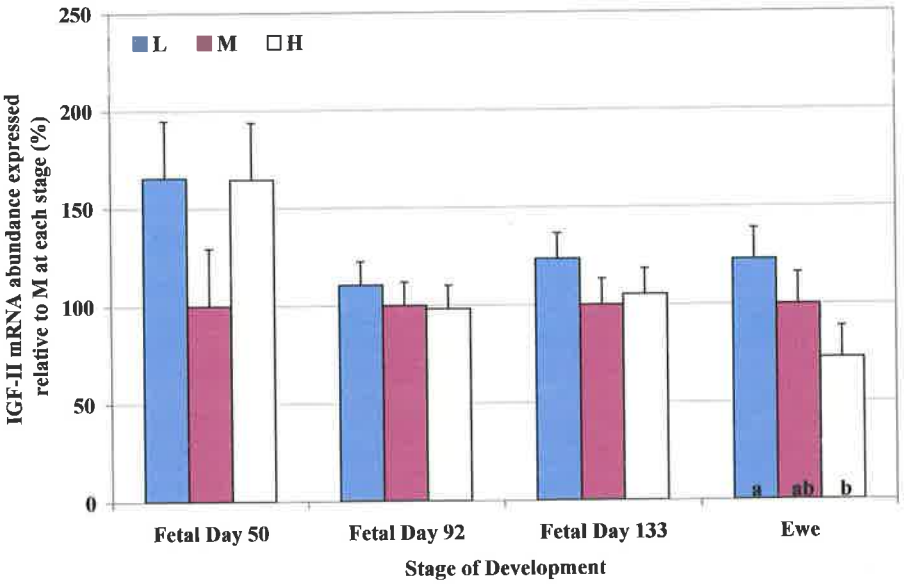


Figure A2.24: Effect of maternal feed intake on IGF-II mRNA expression without normalisation at each stage of development. Different superscripts within a stage indicate significant differences ($p<0.05$).

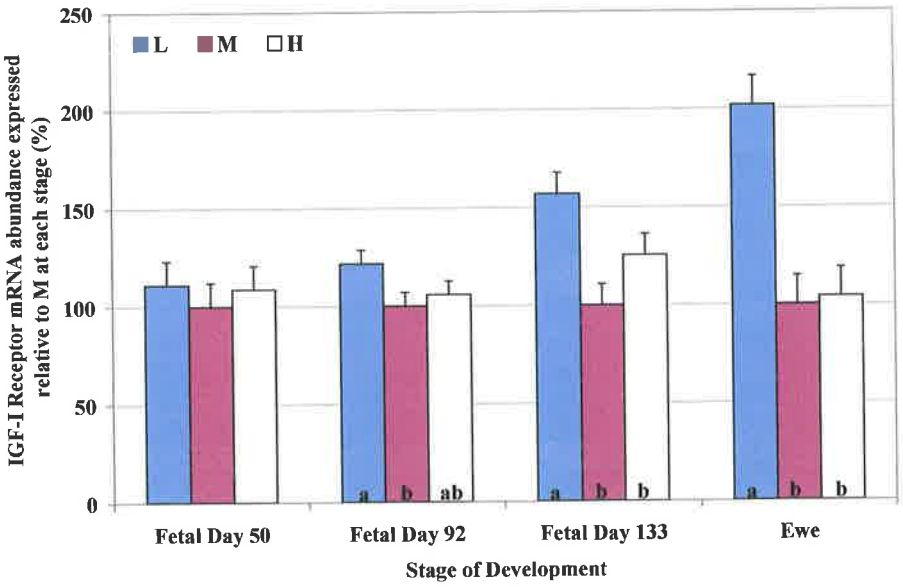


Figure A2.25: Effect of maternal feed intake on IGF-I Receptor mRNA expression without normalisation at each stage of development. Different superscripts within a stage indicate significant differences ($p<0.05$).

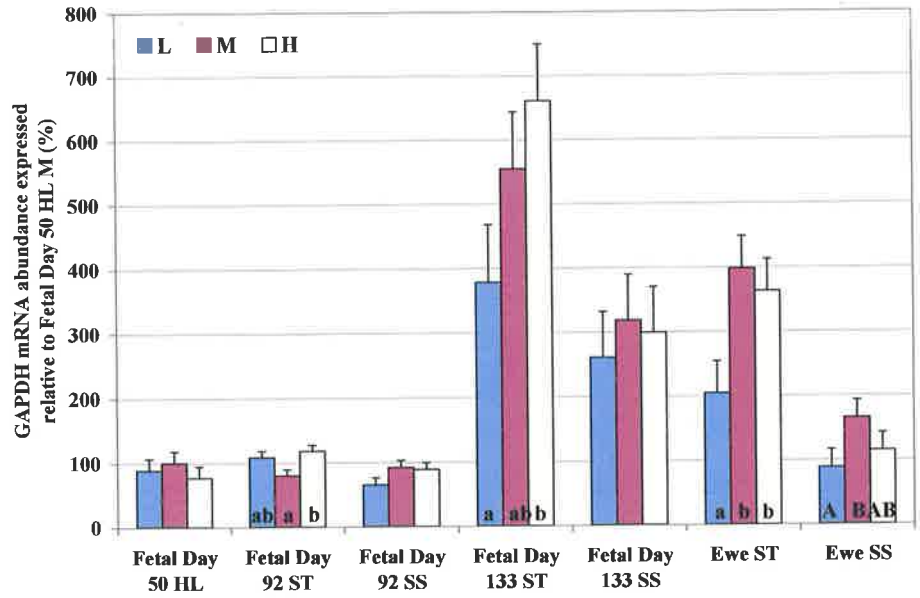


Figure A2.26: The overall trend of GAPDH mRNA expression without normalisation. Different letters within a muscle type at each stage of development indicate significant difference (lowercase, $p<0.05$; uppercase, $p<0.1$).

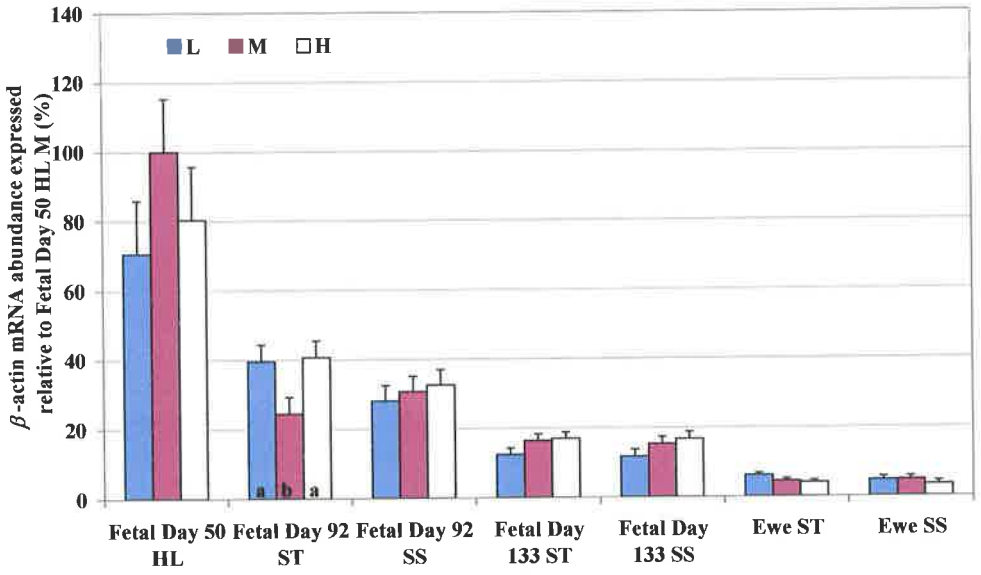


Figure A2.27: The overall trend of β-actin mRNA expression without normalisation. Different letters within a muscle type at each stage of development indicate significant difference ($p<0.05$).

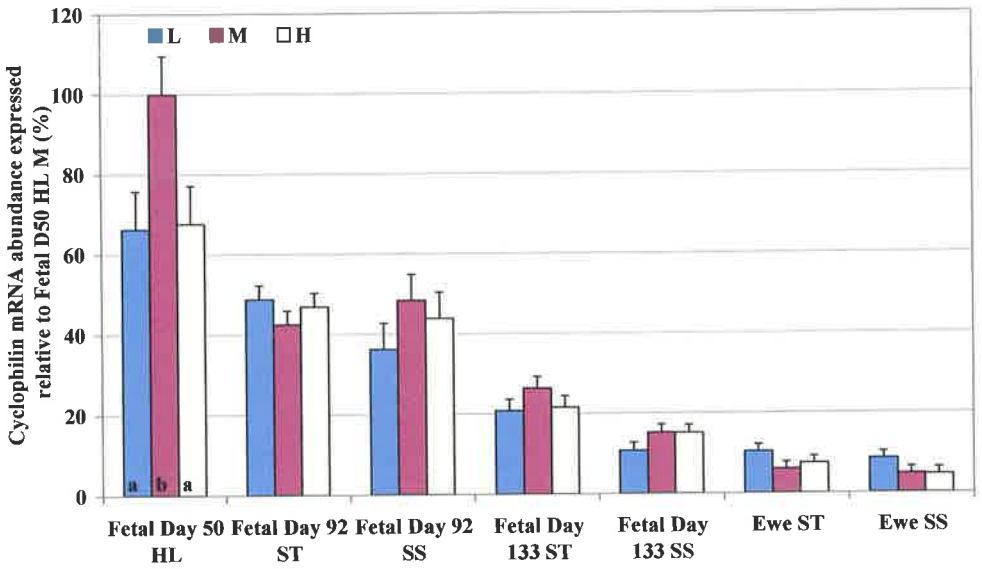


Figure A2.28: The overall trend of Cyclophilin A mRNA expression without normalisation. Different letters within a muscle type at each stage of development indicate significant difference ($p<0.05$).

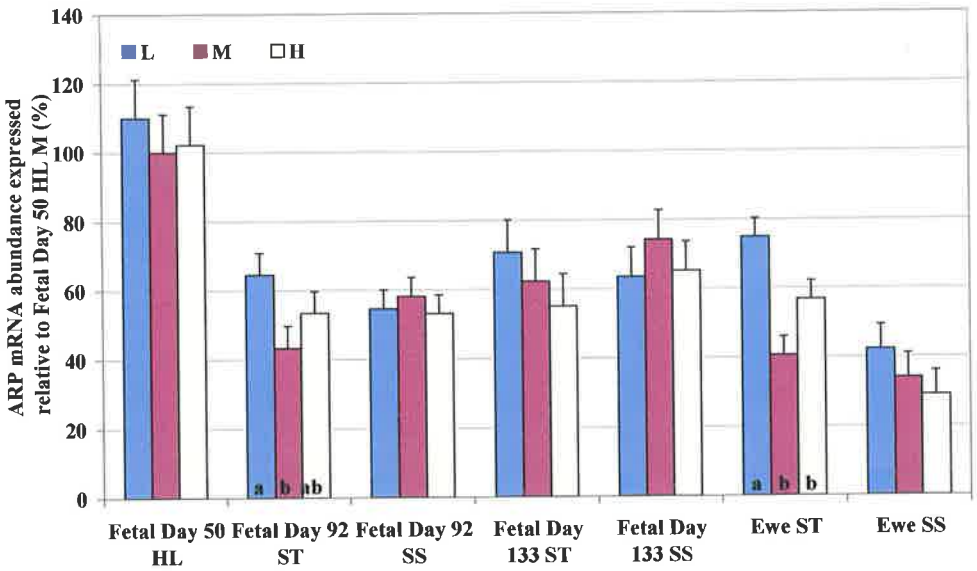


Figure A2.29: The overall trend of Acidic Ribosomal Phosphoprotein (ARP) mRNA expression without normalisation. Different letters within a muscle type at each stage of development indicate significant difference ($p<0.05$).

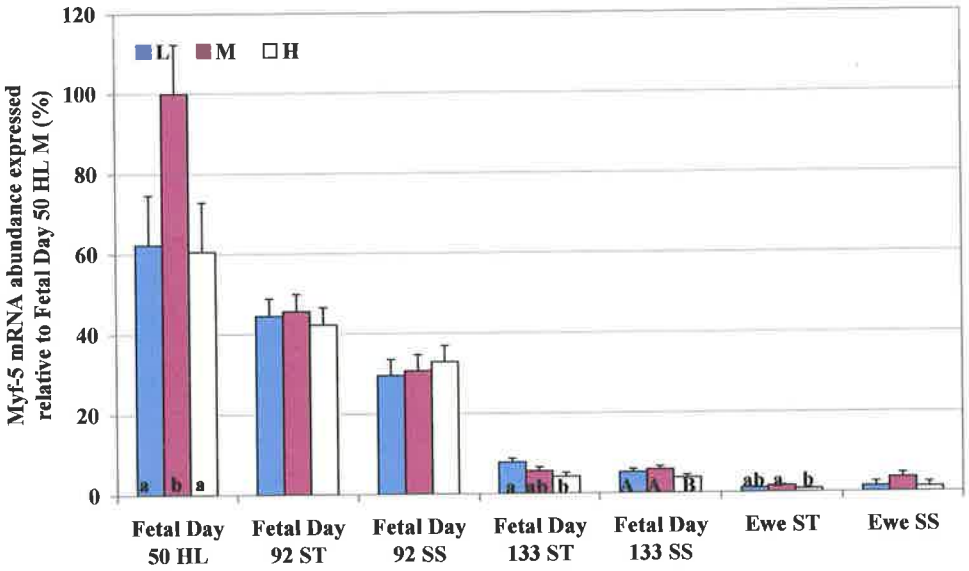


Figure A2.30: The overall trend of Myf-5 mRNA expression without normalisation. Different letters within a muscle type at each stage of development indicate significant difference (lowercase, $p<0.05$; uppercase, $p<0.1$).

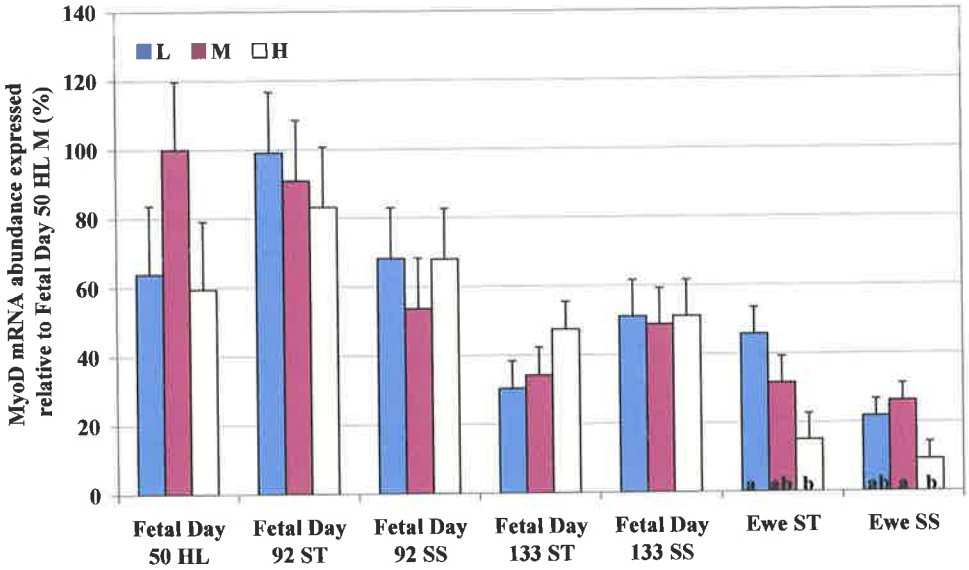


Figure A2.31: The overall trend of MyoD mRNA expression without normalisation. Different letters within a muscle type at each stage of development indicate significant difference ($p<0.05$).

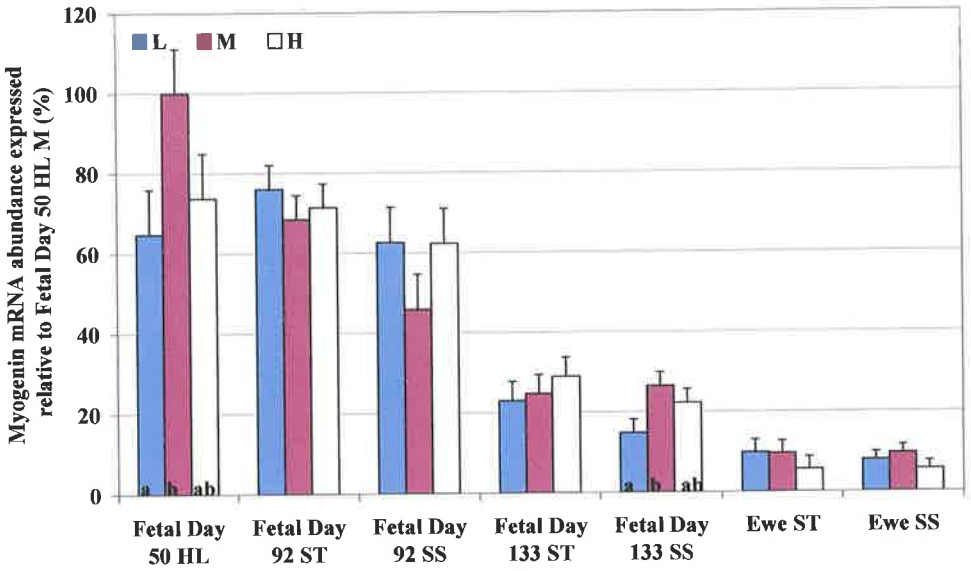


Figure A2.32: The overall trend of Myogenin mRNA expression without normalisation. Different letters within a muscle type at each stage of development indicate significant difference ($p<0.05$).

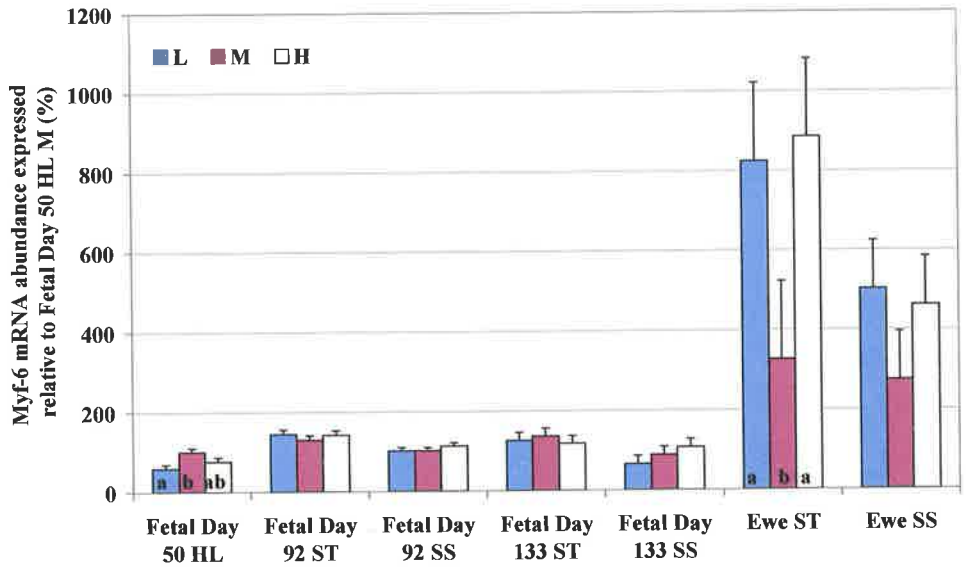


Figure A2.33: The overall trend of Myf-6 mRNA expression without normalisation. Different letters within a muscle type at each stage of development indicate significant difference ($p<0.05$).

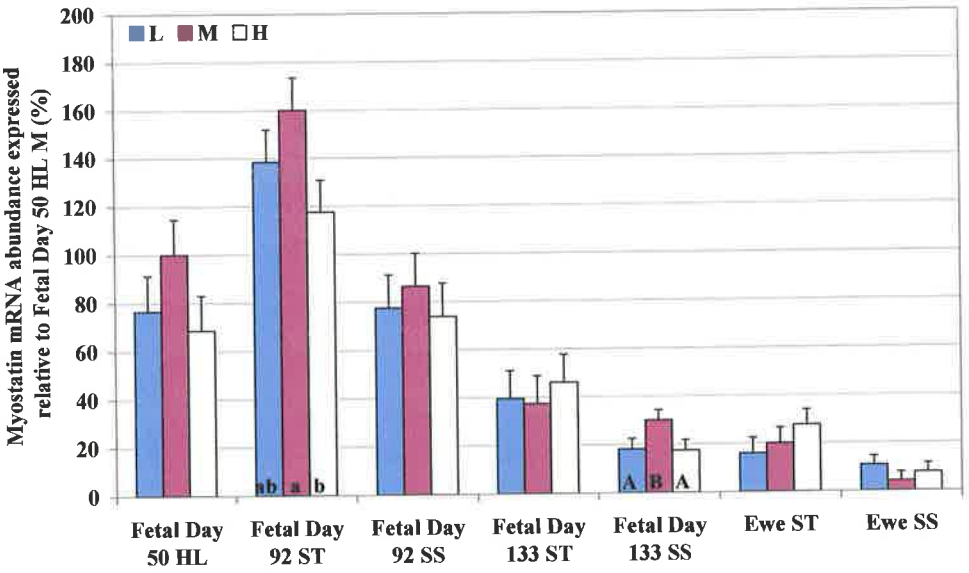


Figure A2.34: The overall trend of Myostatin mRNA expression without normalisation. Different letters within a muscle type at each stage of development indicate significant difference (lowercase, $p<0.05$; uppercase, $p<0.1$).

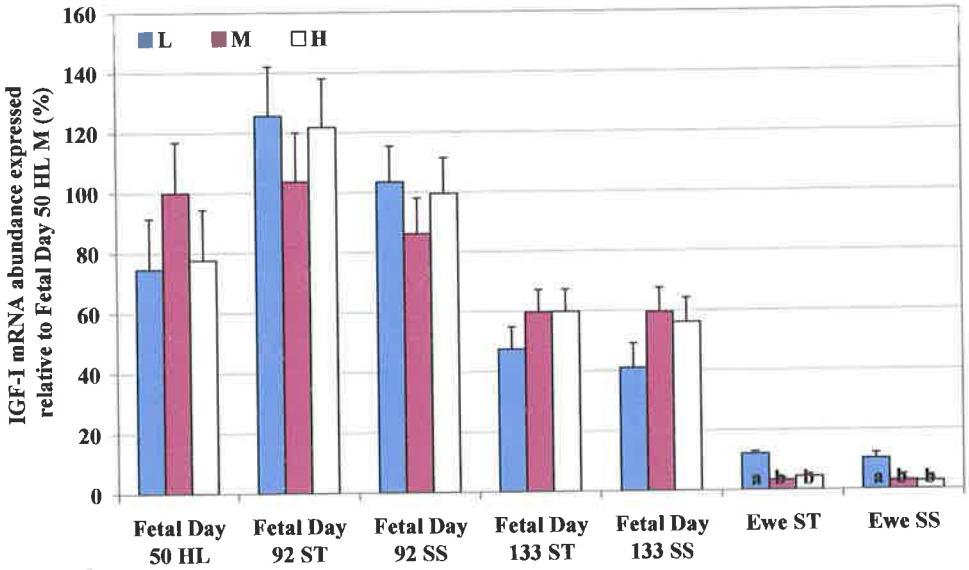


Figure A2.35: The overall trend of IGF-I mRNA expression without normalisation. Different letters within a muscle type at each stage of development indicate significant difference ($p<0.05$).

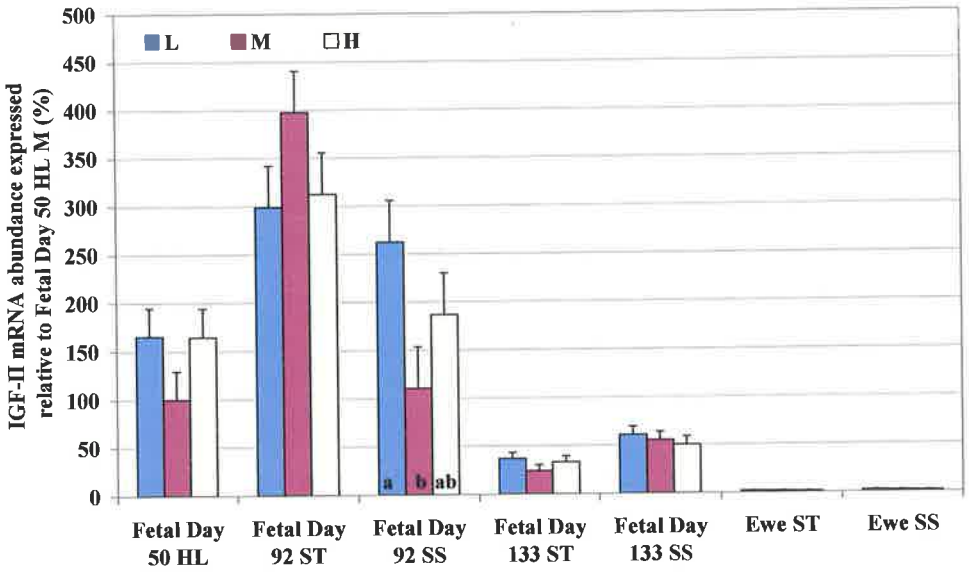


Figure A2.36: The overall trend of IGF-II mRNA expression without normalisation. Different letters within a muscle type at each stage of development indicate significant difference ($p<0.05$).

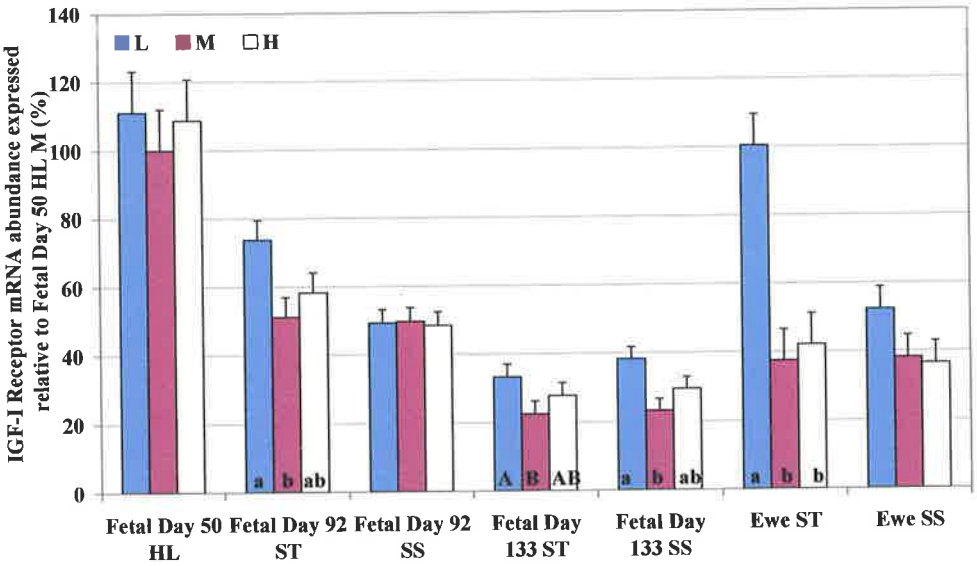


Figure A2.37: The overall trend of IGF-I mRNA Receptor expression without normalisation. Different letters within a muscle type at each stage of development indicate significant difference ($p<0.05$).

APPENDIX 3.

BUFFERS AND REAGENTS

A3.1. HISTOLOGICAL REAGENTS AND BUFFERS

0.5% Lillee Mayers' hematoxylin (1L)

5g haemotoxylin
50g aluminium ammonium sulphate
300ml glycerol
700ml water
1g sodium iodate
20ml glacial acetic acid

1% acid-ethanol (1L)

10ml HCl
990ml 70% ethanol

0.1% Eosin (500ml)

50ml 1% aqueous eosin yellowish
390ml 95% ethanol
5ml 1% aqueous phloxine
2ml glacial acetic acid

Glycine buffer (200ml)

1.13g glycine
0.87g NaCl
200ml MQ water

Basic pre-incubation solution (pH 10.3)*

0.088g $CaCl_2$
17.5ml glycine buffer
7.5ml 0.15M NaOH

0.2M Barbital acetate buffer (500ml)

9.7g sodium acetate

14.7g sodium barbital

500ml MQ water

Acidic pre-incubation solution*

20ml 0.2M Barbital acetate buffer

40ml 0.1M HCl

32ml MQ water

divided into three equal volumes with pH adjusted to 4.3, 4.4 and 4.5 respectively

Sodium barbital buffer (500ml)

1.0g $CaCl_2$

2.06g sodium barbital

500ml MQ water

ATP incubation solution (pH 9.4) (100ml)

150mg Adenosine 5`-triphosphate (ATP; Sigma)

100ml sodium barbital buffer

0.2M Tris buffer (pH 7.4) (200ml)

4.84g Tris base (Tris [hydroxymethyl] amino-methane; Sigma)

200ml MQ water

NADH incubation medium*

8mg β-nicotinamide adenine dinucleotide (Sigma)

16mg nitro blue tetrazolium (NBT, 2,2`-Di-p-nitrophenyl-5,5`-diphenyl-3,3`-[3,3`-dimethoxy-4,4`-diphenylene] ditetrazolium chloride; Sigma)

20ml of Tris pH 7.4

GlyceroGel

Glycerol gelatin (59% w/v glycerol, 0.9% w/v gelatin, 1% w/v phenol); Sigma).

0.2M phosphate buffer (pH 7.4)

1.088g KH_2PO_4

7.76g K_2HPO_4

210ml MQ water

GPD incubation medium*

160mg α-glycerophosphate (Sigma)

20mg NBT

8mg Menadione (Vitamin K_3; 2-methyl-1, 4-naphthoquinone; Sigma)

20ml 0.2M phosphate buffer

*volume for a coplin jar containing 10 slides

A3.2. BUFFERS AND REAGENTS USED FOR NUCLEIC ACID AND PROTEIN CONTENT DETERMINANTIONS

PBS-EDTA buffer, pH 7.2 (1L)

1.16g Na_2HPO_4
0.22g NaH_2PO_4
0.37g EDTA di sodium salt
0.20g NaN_3
8.10g NaCl
make to 1L with MQ water

0.3M NaOH (1L)

12g NaOH
make to 1L with MQ water

2.3M perchloric acid (PCA) (1L)

200ml PCA (70% w/w)
800ml MQ water

0.4M PCA (1L)

34.5ml PCA (70% w/w)
965.5ml MQ water

0.22M PCA (1L)

19ml PCA (70% w/w)
981ml MQ water

Orinol Reagent (100ml)

100ml HCl (12M)
100mg $FeCl_3$
100mg Orcinol

Diphenylamine Reagent (100ml)

1500mg Diphenylamine
100ml Glacial Acetic Acid (17.4M)
1.4ml Sulphuric acid (93% w/w)
10.25μl Acetaldehyde in 0.5ml MQ water

Bradford Reagent (500ml)

50mg Commassie Blue (G250 Sigma)

25ml Ethanol

50ml 85% (w/v) Orthophosphoric acid

Made up in MQ water and filtered (Whatman No. 1).

A3.3. BUFFERS, REAGENTS, MEDIA AND ENZYMES USED IN MOLECULAR ANALYSIS

Solution D

4M guanidinium thiocyanate

25mM sodium citrate, pH 7

0.5% sarcosyl

0.1M β-mercaptoethanol

Luria Broth (LB) Media (1L)

10g NaCl

10g Bacto-tryptone

5g Bacto-yeast extract

For plates add 15g Agar to the LB before the solution is autoclaved.

Ampicillin

Stock solution (100mg /ml) made with MQ water.

LB media and plates contained a final ampicillin concentration of 100μg /ml.

Transformation Buffer

10mM PIPES

55mM $MnCl_2$

15mM $CaCl_2$

250mM KCl

pH 6.7

TE buffer

10mM Tris-HCl pH 8

0.1M EDTA Buffer

Formamide Load buffer (2xFLB)

95% Formamide

20mM EDTA

0.05% Bromophenol blue

0.05% Xylene cyanol

Agarose Load buffer (1xALB)

5% (v/v) Glycerol
10mM EDTA
0.01% SDS
0.01% Bromophenol blue
0.01% xylene cyanol

Super Dooper Buffer (10xSDB)

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

Klenow DNA polymerase buffer (10x)

500mM Tris-HCl (pH 7.2)
100mM $MgSO_4$
1mM DTT

AmpliTag DNA polymerase buffer (5x)

500mM KCl
100mM Tris-HCl (pH 8.3)

Tris Acetate EDTA (20xTAE)

800mM Tris-acetate
20mM EDTA

Protein Lysis buffer

390mM NaCl
15mM Na_2HPO_4
15mM $NaH_2PO_4.H_2O$
0.05% Nonidet P-40

3xPBS

Combine 50ml - 390mM NaCl /30mM Na_2HPO_4
- 390nM NaCl / 30nM $NaH_2PO_4.H_2O$

Adjust pH to 7.2 and autoclave

1xPBS + Nonidet

20ml 3xPBS

40ml MQ water

30μl Nonidet P-40

Working aliquot

10ml 1xPBS + Nonidet

1 Complete Protease Inhibitor tablet (Roche)

Protein Gel buffer (1.5M Tris, 0.4% SDS, pH 8.8)

18.2g Tris

4.0 ml 10% SDS

MQ water to 100ml

Protein Load buffer (x2) (100μl) (8% glycerol, 3% SDS, 187.5mM Tris-HCl 8.8, 0.05% bromophenol blue, 10% β-mercaptoethanol)

10μl 80% Glycerol

30μl 10% SDS

12.5μl Gel buffer (x4)

5μl 1% Bromophenol blue

10μl β-mercaptoethanol

Acrylamide stock 30% (38:1)

29.231g Acrylamide

0.769g bis-acrylamide

MQ water to 100ml

Towbin buffer (1x) (25mM Tris, 192mM Glycine, 20% Methanol, 0.1% SDS)

3.03g Tris

14.4g Glycine

10ml 10%SDS

200ml Methanol

MQ water to 1L

TBS with tween (TBS-T), pH 7.6 (136mM NaCl, 20mM Tris, 0.1% Tween-20)

8g NaCl

10ml 10% Tween-20

20ml 1M Tris-HCl, 7.6

MQ water to 1L, pH to 7.6

TGS running buffer (1xLaemlli Buffer), pH 8.3 (Biorad)

25mM Tris

192mM glycine

0.1% SDS

Commassie Blue stain (500ml)

0.5g Coomassie Brilliant Blue (R-250) (Sigma)

250ml Methanol

50ml Glacial acetic acid

200ml MQ water

Ponceau S red (200ml) (0.1% Ponceau S, 5% Acetic acid)

200mg Ponceau S

10ml Acetic acid

MQ water to 200ml

APPENDIX 4.

MYOSTATIN WESTERNS

Bovine	MQKLQISVYI YLFMLIVAGP VD*LNENSEQK ENVEKEGLCN*	40
Ovine	MQKLQIFVYI YLFMLLVAGP VDLNENSEQK ENVEKKGLCN	40
Human	MQKLQLSVYI YLFMLIVAGP VDLNENSEQK ENVEKEGLCN	40
Bovine	*ACLWRENTTS SRLEAIKIQI LSKLRLETAP NISKDAIRQL*	80
Ovine	ACLWRQNNKS SRLEAIKIQI LSKLRLETAP NISKDAIRQL	80
Human	ACTWRQNTKS SRIEAIKIQI LSKLRLETAP NISKDVIRQL	80
Bovine	*LPKAPPLLEL IDQFDVQRDA SSDGSLEDDD YHARTETVIT*	120
Ovine	LPKAPPLREL IDQYDVQRDD SSDGSLEDDD YHVTTETVIT	120
Human	LPKAPPLREL IDQYDVQRDD SSDGSLEDDD YHATTETIIT	120
Bovine	*MPTESDLLTQ VEGKPKCCFF KFSSKIQYNK LVKAQLWIYL*	160
Ovine	MPTESDLLAE VQEKPKCCFF KFSSKIQHNK VVKAQLWIYL	160
Human	MPTESDFLMQ VDGKPKCCFF KFSSKIQYNK VVKAQLWIYL	160
Bovine	*RPVKTPATVF VQILRLIKPM KDGTRYTGIR SLKLDMNPGT*	200
Ovine	RPVKTPTTVF VQILRLIKPM KDGTRYTGIR SLKLDMNPGT	200
Human	RPVETPTTVF VQILRLIKPM KDGTRYTGIR SLKLDMNPGT	200
Bovine	*GIWQSIDVKT VLQNWLKQPE SNLGIEIKAL DENGHDLVAT*	240
Ovine	GIWQSIDVKT VLQNWLKQPE SNLGIEIKAL DENGHDLVAT	240
Human	GIWQSIDVKT VLQNWLKQPE SNLGIEIKAL DENGHDLVAT	240
Bovine	*FPEPGEDGLT PFLEVKVTDT PK***RSRR***DFGL DCDEHSTESR*	280
Ovine	FPEPGEEGLN PFLEVKVTDT PK**RSRR**DFGL DCDEHSTESR	280
Human	FPGPGEDGLN PFLEVKVTDT PK**RSRR**DFGL DCDEHSTESR	280

Bovine	CCRYPLTVDF EAFGWDWIIA PKRYKANYCS GECEFVFLQK	320
Ovine	CCRYPLTVDF EAFGWDWIIA PKRYKANYCS GECEFLFLQK	320
Human	CCRYPLTVDF EAFGWDWIIA PKRYKANYCS GECEFVFLQK	320
Bovine	YPHTHLVHQA NPRGSAGPCC TPTKMSPINM LYFNGEGQII	360
Ovine	YPHTHLVHQA NPKGSAGPCC TPTKMSPINM LYFNGKEQII	360
Human	YPHTHLVHQA NPRGSAGPCC TPTKMSPI***NM LYFNGKEQII***	360
Bovine	YGKIPAMVVD RCGCS.	376
Ovine	YGKIPGMVVD RCGCS.	376
Human	***YGK***IPAMVVD RCGCS.	376

Figure A.4.1: The human, bovine and ovine myostatin amino acid sequences. Boxed residues in the bovine and ovine sequences differ from human. The RSRR splice-cite is in bold. The peptide used to raise the Bethyl human myoB antibody is in bold italics. The bovine myostatin peptide used for immunization of rabbits and subsequent antibody production and the C-terminus positive control peptide is underlined. The N-terminus positive control peptide is in italics.

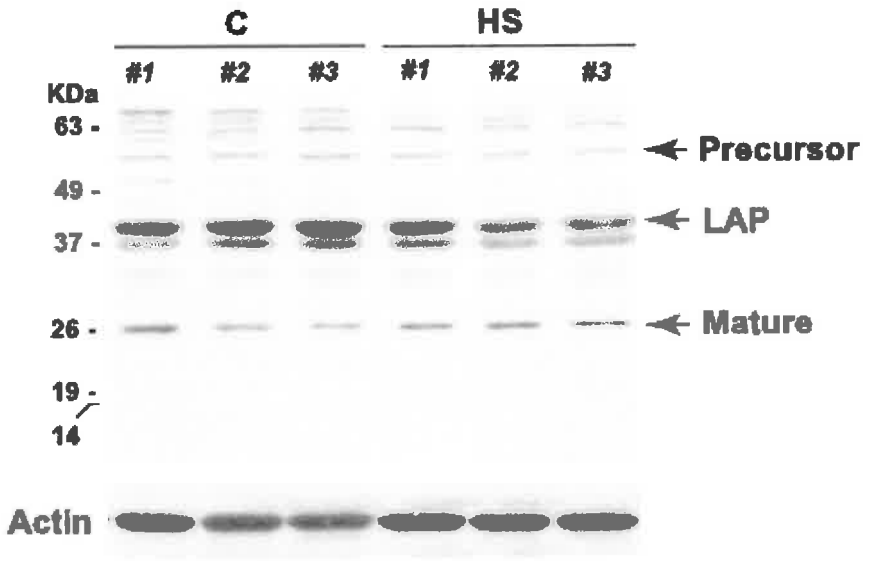


Figure A.4.2: Example of a myostatin western blot, showing the processed myostatin, latency associate peptide and precursor proteins in the quadriceps femoris muscle of control (C) and hind-limb suspended (HS) mice (from McMahon *et al.*, 2003a).

Table A4.1: Summary of published myostatin western studies.

Author	Year	Species	Treatment	Tissue	Detected Size (kDa)		
					Processed	LAP*	Precursor
Berry *et al*.	2002	Cattle	Double muscled phenotype (Piedmontese)	Muscle; Plasma	26	40	52
Gonzalez-Cadavid *et al*.	1998	Human	HIV associated muscle atrophy	Muscle; Sera	30	-	-
Jeanplong *et al*.	2003	Sheep	Undernutrition	Muscle	30	45	63
Lalani *et al*.	2000	Rats	Microgravity environment	Muscle	28	-	49
Ma *et al*.	2003	Rats	Glucorticoid treatment	Muscle	30	-	-
Ma *et al*.	2001	Human	Dexamethasone	Muscle	30	-	-
McMahon *et al*.	2003a	Mice	Myostatin-deficient; Hindlimb suspension	Muscle	26	40	52
McMahon *et al*.	2003b	Mice	Sexual dimorphism	Muscle; Plasma	26	40	52
Mott and Ivarie	2002	Poultry	Growth selected lines	Muscle	14	-	-
Roberts and Goetz	2003	Trout	Developmental stages	Muscle	14	-	42
Sharma *et al*.	1999	Cattle	Double muscled phenotype (Belgian Blue)	Muscle	26/ ND	-	52 /ND
Sharma *et al*.	1999	Sheep	Myocardial infarction	Heart	-	-	52
Vianello *et al*.	2003	Zebrafish	Developmental stages	Muscle	15	-	40
Wehling *et al*.	2000	Rats	Hindlimb suspension	Muscle	-	37	-
Zimmers *et al*.	2002	Mice	Myostatin knockout	Sera	12.5	-	-

* Latency Associated Peptide

ND - not detected in Belgian Blue

- Either not measured or not reported

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