Amino acids and skeletal muscle growth in lambs

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ABSTRACT

The objectives of this thesis were three-fold. Firstly, to identify whether reduced muscle growth in twin compared to singleton lambs during late pregnancy and early post-natal life was associated with changes in the concentration of intracellular free amino acids (FAA) that may play a role in the regulation of pathways involved in muscle growth. Secondly, to evaluate if supplementation with a specific amino acid improved muscle growth in twin fetuses/lambs, and thirdly to examine the role of mTOR signalling. The first objective was investigated by examining the differences in muscle FAA concentrations between singleton and twin fetuses in late pregnancy from either heavy or light ewes, under a maintenance or *ad libitum* feeding regimen. Twin fetuses had lower intracellular FAA concentrations of arginine (Arg), leucine, valine, glutamine, while muscle mass was positively associated only with Arg concentration. A further study characterised the FAA concentrations of singleton and twin well-fed lambs at 140 days pregnancy and at weaning. High levels of Arg and glutamine were associated with muscle growth during pregnancy; however several FAA appeared to be associated with muscle growth to weaning. Objective 2 was tested by examining the effects of maternal Arg administration on fetal muscle growth and mTOR signalling. Well-fed twin-bearing ewes, received either an intravenous bolus of Arg or saline solution 3 times daily from 100 days of pregnancy to parturition. Female lambs from supplemented ewes had increased birth weight and muscle mass at market weight, associated with increased ribosome number and mTOR abundance at P140 and increased ribosome number at weaning, compared to control females. An additional experiment supplemented twin-born lambs with Arg via fortification of colostrum and milk replacer from birth to 28 days or from birth to 70 days of life. Supplementation increased body growth between 7 and 21 days of life. Only supplemented females expressed higher muscle weight at 70 days, compared with control females. Collectively, these results indicate that singleton and twin muscle differs in Arg concentration, and the use of Arg during pregnancy and early neonatal life improves muscle growth in females. This action potentially occurs through mTOR signalling, and ameliorates reduced females weight in at birth and growth from birth to weaning.

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ABBREVIATIONS

4E-BP1 eukaryotic initiation factor 4E binding protein

AA Amino acid

AMP-activated protein kinase AMPK

Arg Arginine

BCAA Branched-chain amino acid

EAA Essential amino acid

FAA Free amino acid

i.v. intravenous

IGF-I/PI(3)K insulin growth factor-1/ Phosphatidylinositide 3-kinases

mTOR mechanistic target of rapamycin

NEAA Non-essential amino acid

NO nitric oxide

P70S6K1 ribosomal protein S6 kinase S6Ks

PI(3)K-Akt phophatidylinositol-3 kinase/Akt kinase

ST Semitendinosus

GENERAL INTRODUCTION

The improvement of lambing percentage has been one of the key factors leading to increased productivity and profitability for New Zealand sheep farmers. The increase of the national lambing percentage of over 26% in the last 40 years, reaching an average of 124% (Anon, 2013a) and the increase of market weight of lambs have both compensated for the 32% decrease in the national sheep stock number during the last 15 years, and maintained the productivity as measured by kg of meat produced (Anon, 2013b). However, while the increase in litter size and thus the number of multiple-born lambs can impact positively on farm productivity, there are important constraints compared to singletons. Reduced fetal growth, higher mortality rates in the first 3 weeks of life (Scales et al., 1986; Greenwood et al., 2000b; Morris and Kenyon, 2004; Everett-Hincks et al., 2005; Gootwine, 2005; Gootwine et al., 2007), reduced neonatal growth rate and lower muscle mass (Bennett et al., 1991; McCoard et al., 1997; McCoard et al., 2010), are key factors in multiple-born lambs for which farmers demand feasible solutions. When considering that fetal skeletal muscle accounts for 25–30% of body mass at term of pregnancy (Forhead et al., 2002), and up to 45% of the animal body weight post-birth (Teleni, 1993), reduced muscle growth in twins compared to singletons becomes a key component behind the lower productivity in twins. However, the mechanisms resulting in differences between singles and twins are not clear.

Nutrition during pregnancy and post-natally plays a crucial role in skeletal muscle growth. The relevance of nutrition has been enhanced by the discovery that amino acids (AA) have roles other than as building blocks of proteins and other nitrogenous substances, glucose and fatty acids (Galli, 2007; Grillo and Colombatto, 2007; Li et al., 2007; Sugita et al., 2007). Changes in intracellular concentration of specific AA can also regulate cellular signalling pathways, such as the mechanistic target of rapamycin (mTOR), which controls protein accretion and therefore, muscle growth (Jobgen et al., 2006; Kim et al., 2007). Whether reduced muscle growth in twin lambs during pregnancy and post-natally is associated with lower intracellular AA concentrations in muscle and down regulation of mTOR signalling is not known. The objectives of this thesis were therefore to, firstly, identify possible intracellular AA which could act as limiting factors for muscle growth during late pregnancy and post-natally in twin sheep, secondly, to evaluate the *in vivo* the effect of supplementing a potentially regulatory AA on muscle

growth, and thirdly to examine the role of mTOR signalling in mediating any observed effects on muscle growth. The objectives of each chapter in this thesis were:

Chapter 1: Review the existing literature on the role of AA in the regulation of the development and growth of skeletal muscle.

Chapter 2: Identify intracellular FAA in muscle of singleton and twin fetuses at term, in order to determine which FAA could be acting as limiting signalling AA for skeletal muscle growth in twin fetuses.

Chapter 3: Identify intracellular FAA in muscle of singleton and twin fetuses at term and at weaning, in order to determine which ones could be acting as limiting signalling AA for skeletal muscle growth in twin lambs.

Chapter 4: Arginine (Arg) was shown in Chapter 2 and Chapter 3 to be associated with muscle mass during pregnancy. This chapter evaluated the effect of maternal Arg supplementation during mid-late pregnancy on fetal muscle growth and the effect on mTOR signalling. Further, this chapter also evaluated post-natal skeletal muscle growth to determine possible carry-over effects of maternal Arg supplementation on post-natal muscle growth stage in the offspring.

Chapter 5: To determine if oral Arg supplementation of twin-born lambs, had an effect on skeletal muscle growth while the lamb is a monogastric (from birth until 28 days of life) and from birth until 70 days of life, and determine if mTOR plays a role on muscle growth.

Chapter 6: A summary of the main findings, potential limitations and future work which could complement the results of the research presented in this thesis.

Chapter 1: Literature review

1.1 INTRODUCTION

The development of technologies which increase the number of lambs weaned per ewe and the growth and skeletal muscle content in the carcass of the offspring would have major impacts in the sheep meat industry. However, increasing litter size results in higher lamb mortality and reduced growth rates compared to singletons (Morris and Kenyon, 2004; Everett-Hincks et al., 2005; Gootwine et al., 2007). Although some advances have been made to solve these problems, such as pre-lambing shearing (Morris, 1999) or pregnancy scanning (Fogarty, 1992) producers still demand new knowledge/technologies to further mitigate these effects.

It is well established that twin lambs have lower skeletal muscle mass during pregnancy and after birth, compared to singletons (Bennett et al., 1991; McCoard et al., 1997; McCoard et al., 2010). Thus, increasing muscle mass in twins could be a means to improve their performance, as muscle mass constitutes over 40% of body mass (Teleni, 1993). It is generally accepted that plane of maternal nutrition during pregnancy and nutrition post-natally affects skeletal muscle development and growth (Greenwood et al., 2000a; Zhu et al., 2004). However, even under a well-fed maternal nutrient regimen during pregnancy (Freetly and Leymaster, 2004), and a high level of post-natal nutrition (Hohenboken et al., 1976), differences in muscle growth between singletons and twins are still evident. Research into the role of nutrition and mechanisms regulating muscle growth during pregnancy and after birth in monogastrics has advanced in the last few years (Davis and Fiorotto, 2009; Wu et al., 2010a). The effect of pre- and post-natal nutrition, the use of high- and low-energy and protein feeding and the use of specific nutrients has been the focus of a large amount of research (Rehfeldt et al., 2010). However, during the last decade there has been an increased recognition of the role of specific amino acids (AA) in muscle growth. That AA act not only as building blocks for protein formation, but also as signalling molecules controlling protein synthesis (Wu, 2009), has increased the understanding of the mechanisms controlling protein accretion and has opened a new area of specific intervention research. In contrast, the effect of AA in muscle growth in ruminants, is not clear.

The discovery of the mechanistic target of rapamycin (mTOR), a protein complex which acts as a key integrator of environmental signals regulating protein synthesis, and therefore tissue growth, has opened a new area of research (Laplante and Sabatini, 2012).

Factors, such as hormones, AA, energy and stress can modulate the activation of mTOR (Dibble and Manning, 2013). However, AA have been proposed as the most essential stimulus leading to mTOR activation (Jewell and Guan, 2013). The regulatory effect of specific AA on mTOR signalling and therefore, muscle growth has been clearly demonstrated in monogastrics during pregnancy and post-natally (Suryawan et al., 2008; Tan et al., 2009; Murgas Torrazza et al., 2010). Therefore, it is plausible to hypothesise that the use of AA in strategic nutritional intervention protocols in sheep could result in increased protein synthesis and muscle growth.

The goal of this review is to examine the role of AA in the regulation of the development and growth of skeletal muscle. It is hoped that a better understanding of the role of AA in muscle growth, will lead to improved fetal and neonatal muscle growth, resulting in higher birth weight and therefore increased survival and also enhanced lean tissue growth from birth to weaning in multiple-born lambs.

1.2 MULTIPLE BIRTHS: PRODUCTION AND ECONOMIC CONSIDERATIONS FOR FARMERS

1.2.1 Economic significance of the sheep industry in New Zealand

The sheep meat industry in New Zealand accounts for approximately 50% of the value of all exported meat, and 13% of the total income from New Zealand's pastoral sector (Anon, 2012). In a global context, New Zealand accounts for 33% of the world trade in sheep meat (Anon, 2013c). With a total population of 31 million sheep in New Zealand (Anon, 2013d), the sheep industry, together with beef cattle farming continues to be the dominant agricultural area of land use in New Zealand (MPI, 2013). Together, these statistics highlight the economic relevance of the sector to New Zealand.

1.2.2 Production impact of multiple births

Lamb meat production efficiency can be defined as the number and weight of lambs weaned per ewe exposed for breeding (Schoenian and Burfening, 1990). Implementation of nutritional, genetic, management and health strategies by New

Zealand sheep farmers, has resulted in improved number of lambs born per ewe mated. Over the last 30 years, lambing percentage has reached an average of 124%, up from a 100% in 1990 (Morel et al., 2008), with a range of 92% on high country to 130% on intensive finishing country (Morris and Kenyon, 2014), with the potential of 200%, under more intense management New Zealand (Demmers et al., 2011; Shorten et al., 2013). Higher lambing percentages are associated with an increase in the proportion of twin- and triple-born lambs (Schoenian and Burfening, 1990; Amer et al., 1999). The total litter weight of twins is greater than singletons (Freetly and Leymaster, 2004), which can increase farm profit (Gootwine et al., 2007), however, reduced lamb survival and growth from birth to weaning are two of the major constraints with multiple born lambs, which represent lost opportunities for sheep meat production.

Lamb survival plays a major role in farm profit (Amer et al., 1999). In general, twins have reduced birth weight, and it is well known that lower birth weight in twins leads to higher mortality rates at lambing compared to singletons (Scales et al., 1986; Greenwood et al., 2000b; Morris and Kenyon, 2004; Everett-Hincks et al., 2005; Gootwine, 2005; Gootwine et al., 2007). Impaired fetal growth (Mellor and Murray, 1982), and lower muscle mass (McCoard et al., 1997), both contributes to the lower birth weight of twins compared to singletons, where decreased muscle mass is a major factor in low birth weight (Hediger et al., 1998). Mortality values of up to 20% in twins at birth have been reported under New Zealand conditions (Kenyon et al., 2006; Morel et al., 2008), while values up to 44% for mortality from mid pregnancy to tail docking are described for Australian sheep flocks (Kleemann and Walker, 2005). Therefore, any attempt to increase birth weights of twin lambs should increase survival rates.

Multiple-born lambs require a longer time to reach market weight (Nordby et al., 1987; Dimsoski et al., 1999). Delays of up to four weeks compared to singles have been reported (Nordby et al., 1987). This negatively impacts the overall farm system, as twin lambs use pasture which could be used to grow other stock or improve maternal weight gain after weaning, which in turn improves body condition and increases future fertility/fecundity (Kenyon et al., 2004; Kenyon et al., 2010).

From an economic point of view, muscle is the most important tissue in the carcass (Du et al., 2011). However, twins have a lighter and leaner carcass compared to singletons (Afolayan et al., 2007; McCoard et al., 2010). Lambs reared as twins also have

correspondingly lower carcass measurements compared with lambs reared as singletons, with significantly lower leg lengths and eye muscle area (Bennett et al., 1991; McCoard et al., 2010). The effects of twinning on fetal growth, carcass traits and muscle growth reinforce the need for a better understanding of mechanisms influencing the differences in muscle growth, both *in utero* and from birth to weaning, between singletons and twins, which could lead to the generation of new strategies to positively impact twin productivity.

1.3 SKELETAL MUSCLE DEVELOPMENT AND GROWTH

In sheep, fetal skeletal muscle accounts for 25–30% of body mass at term (Forhead et al., 2002); up to 45% of the animal body weight post-natally (Teleni, 1993) and up of 70% carcass weight, considering only the saleable meat (Hopkins and Fogarty, 1998). Muscle fibres are the main components of muscle, with the number and size of these fibres being the main determinant of muscle mass. The number of fibres in precocial species like sheep is established during prenatal myogenesis (Ashmore et al., 1972; Picard et al., 2002; Brameld et al., 2003) and this process influences the growth pattern post-natally. The focus of this literature review will be the last third of pregnancy and the post-natal period. Myogenesis will only be briefly covered to understand the processes occurring during muscle hypertrophy.

1.3.1 Myogenesis

Post-natal skeletal muscle growth, and thus total muscle mass in the mature animal, depends on fibre number (Bass et al., 2000). Myogenesis has been previously described in vertebrates (Buckingham, 2001; Picard et al., 2002; Buckingham et al., 2003), and more specifically in sheep (Ashmore et al., 1972; Wilson et al., 1992). The basic events of myogenesis are stem cell commitment, proliferation and apoptosis of myoblasts, differentiation and fusion of myoblasts, and finally, maturation of muscle fibres (Rehfeldt and Kuhn, 2006).

Briefly, before skeletal muscle formation, myoblasts undergo an extensive proliferation increasing the number of cells (Buckingham et al., 2003). Following myogenin expression, fibre formation is initiated (Andrés and Walsh, 1996), which

occurs in two main migration waves. Embryonic myoblasts fuse to form primary fibres (Buckingham et al., 2003), which are observed at 32 to 38 days of fetal life in sheep (Wilson et al., 1992). Fetal myoblasts use primary fibres as a structural framework to form secondary fibres around the primary myotube innervations sites (Duxson and Sheard, 1995). Fusion of mononucleated myoblasts form the secondary myotubes (Duxson et al., 1989), which are observed from day 38 to 62 days in the sheep fetus (Wilson et al., 1992), and account for the majority of skeletal muscle fibres (Beermann et al., 1978). The ratio of primary to secondary fibres differs between species, and is about 1:70 in the sheep (Wilson et al., 1992). In fetal sheep, the majority of muscle differentiation takes place around day 85 (Fahey et al., 2005b). Tertiary muscle formation occurs from day 62 of fetal life (Wilson et al., 1992). Tertiary fibres are associated with secondary fibres and appear to be derived from different populations of satellite cells (Kalhovde et al., 2005) and contribute to increased total number of fibres (Maltin et al., 2001). For some muscles, such as the *M. peroneus longus*, myogenesis is completed by about 100 to 115 days of pregnancy (Greenwood et al., 1999). However, other muscles continue to increase in myofibre number after this age (McCoard et al., 2000a). After myogenesis is completed, muscle growth will result from fibre hypertrophy, and is dependent on the total number of muscle fibres within a muscle (Rehfeldt et al., 2000).

1.3.2 Hypertrophy

During late pregnancy (Du et al., 2011) and post-natally in sheep (Brameld et al., 1998; Rehfeldt et al., 2000), muscle fibre growth occurs through an increase in the size of the fibres. Muscle hypertrophy is associated with two events. Firstly, an increase in DNA content due to satellite-cell fusion with existing muscle (Brameld et al., 1998) and secondly, an increase in protein deposition (Davis et al., 1989).

There are a number of studies demonstrating the role of nuclei addition to myofibres in muscle hypertrophy (reviewed by Adams, 2006). Studies comparing singleton and twin lambs before and after lambing, showed that twins have lighter muscle weight, with a lower content of total nuclei, possibly related to a decreased hypertrophy capacity (McCoard et al., 2001). Similarly, Greenwood et al. (2000a) described a reduced concentration of DNA in muscle of low birth weight lambs, which persisted during

neonatal growth and was regulated by nutrient supply, reinforcing the role of myonuclei accumulation in sheep muscle growth.

Protein accretion results when the balance between protein synthesis and degradation favours net synthesis (Oksbjerg et al., 2004; Vary and Lynch, 2007). The rate of muscle growth during the neo-natal period is higher than at any other stage after birth (Davis and Fiorotto, 2009) and is associated with a high rate of protein synthesis (Davis et al., 1989). The rate of protein synthesis is determined by the abundance of RNA and efficiency for protein production (Lobley, 1998). Considering that >80% of the RNA in muscle is rRNA (Iadevaia et al., 2012a), it is acceptable to propose that protein synthesis will depend on ribosome content and their translation efficiency. Consistent with this notion, studies in rats have shown skeletal muscle growth rapidly decreases with age during the early post-natal period, in association with a reduced ribosome number (Davis et al., 1989; Davis et al., 1996; Fiorotto et al., 2000). Similarly, in growing animals, the fractional protein synthesis rate decreases from birth to weaning, however, the reduction in translation efficiency is more marked (reviewed by Lobley, 1993). In lambs, fractional rates of protein synthesis decrease from one to five weeks of age and is associated with decreased capacity for protein synthesis (Attaix et al., 1988).

1.3.3 Regulatory factors involved in skeletal muscle development and growth

Genetic factors (Freking et al., 1998; Rehfeldt et al., 2000; Brameld et al., 2003), hormones, such as thyroid hormone (Finkelstein et al., 1991; Forhead et al., 2002), , growth hormone (Breier et al., 2000) and leptin (Yuen et al., 1999), and growth factors such as insulin like growth factor (IGF-I and IGF-II, (Glass, 2003; Miyazaki, 2013), all play a role in the regulation of muscle development and growth. However, nutrition during pregnancy (Barker and Clark, 1997) and post-natally (Joubert, 1956) plays the most critical role in influencing muscle growth.

During fetal development, skeletal muscle has lower priority, in terms of nutrient partitioning, compared to other tissues such as brain, heart and liver, resulting in muscle being more vulnerable to nutrient deficiency (Zhu et al., 2006). The effect of nutrition on fetal and muscle growth depends not only on the level, but also on the timing of the

restriction (Fahey et al., 2005a). Severe maternal restriction during early pregnancy (<80 days) may be associated with a decrease in the number of secondary fibres, affecting fetal muscle growth (Fahey et al., 2005a; Zhu et al., 2006; Costello et al., 2008), with carryover effects post-natally (Zhu et al., 2006). Restricted fetal nutrition in late pregnancy (>100 days) resulting from placental insufficiency, such as occurs with twins, can reduce muscle weight (McCoard et al., 1997; McCoard et al., 2000b; Fahey et al., 2005a), and is associated with reduced DNA accretion, restricted muscle protein synthesis (Greenwood et al., 2000b; McCoard et al., 2001) and fibre density (Costello et al., 2008). Reduced muscle development in twins can be associated with a lower birth weight and reduced post-natal growth, altered body composition and reduced lifetime production performance (Barker, 1998; Greenwood et al., 1998).

The effect of nutrition on the rate of protein turnover in muscle post-natally, has been extensively described (Harris et al., 1992; Lobley et al., 1992; Lobley, 1998). Previous studies have shown that while body protein mass remains stable in energy-restricted lambs, under a protein-restricted regimen, there is a reduction in body protein (Drouillard et al., 1991), highlighting the importance of protein for lamb growth. Reduced protein content in the diet also affects muscle growth in lambs (Johns and Bergen, 1976). Further, energy and protein restriction results in lower muscle weight coupled with changes in DNA, RNA and protein content (Greenwood et al., 2000a). There are a limited number of studies evaluating the mechanisms explaining the differences in muscle hypertrophy between singletons and twins. Of all nutrients, AAs have received special attention during the last few years, as they are not only the building blocks for protein synthesis, but also are key regulators of metabolic pathways which control development and growth (Wu, 2009). Amino acids have long been known to participate in the regulation of skeletal muscle mass by stimulating protein synthesis and reducing protein degradation (Louard et al., 1995; Kimball, 2007). Therefore, AAs might play an important role in the differences in muscle growth between singletons and twins during pregnancy and from birth to weaning.

1.4 ROLE OF AMINO ACIDS IN MUSCLE DEVELOPMENT AND GROWTH

1.4.1 Pregnancy

Amino acid transfer and metabolism: from the mother to the fetal muscle

Fetal growth relies on a maternal supply of nutrients to the fetus via the placenta. During pregnancy, 35-40% of the fetal energy is taken up as glucose, however, 55% is taken up as free AA, highlighting the role of AA in fetal growth (see Bell et al., 2005). Most AAs are delivered to the fetus in greater amounts than required for net rate of accretion (Lemons et al., 1976; Marconi et al., 1989), in an energy-dependant process (Smith et al., 1992; Regnault et al., 2005), resulting in fetal AA concentrations being higher than maternal AA concentrations (Ashworth et al., 2011). The relationship between maternal and fetal flux of AA has previously been reviewed (Battaglia, 2002). Briefly, fetal AA uptake depends on the maternal AA concentration, and is mediated by AA transporters (Battaglia and Regnault, 2001). In addition, the flux is particular to each AA (Paolini et al., 2001). There is no fetal uptake of maternal glutamic acid, aspartic acid and serine and these AA are produced by fetal tissue (Lemons et al., 1976; Holzman et al., 1979; Cetin et al., 1991; Battaglia, 1992; Chung et al., 1998). The fetal liver produces glutamic acid, which is taken up by the placenta, to produce glutamine and then returns to the fetus (Battaglia, 2000). Maternal serine is transformed in the placenta into fetal glycine, some of which is delivered into the fetal circulation (Moores et al., 1993; Chung et al., 1998), resulting in no transport of serine from the mother to the fetus. Instead, serine is produced mainly by the fetal liver (Cetin et al., 1991). In addition, nutrient and AA flow from the placenta can be enhanced by specific AA such as arginine. Arginine is a precursor for compounds such as nitric oxide, which is a potent vasodilator and stimulates angiogenesis, increasing blood flow across the placenta and, in turn, increasing the transfer of nutrients from the mother to the fetus (Oksbjerg et al., 2013).

Developmental changes occur in maternal and fetal plasma AA concentrations during pregnancy in sheep. For example, between P40 and P140, changes in the concentrations of all AA, except for proline and tyrosine are observed in maternal plasma, while changes in the concentrations of AA, except for alanine, aspartate, isoleucine,

leucine, phenylalanine, tryptophan, and valine, are observed in fetal plasma (Kwon et al., 2003). Factors other than stage of pregnancy may influence maternal and fetal AA profiles. For example, maternal nutrient status can influence both maternal and fetal plasma AA concentrations (Kwon et al., 2004; Jobgen et al., 2008; Satterfield et al., 2010). In the pregnant ewe, prolonged maternal infusion of a mix of essential and non-essential AA has been shown to only increase branched-chain AA (BCAA) and phenylalanine concentration in the fetus (Józwik et al., 1999). In contrast, reduced maternal plasma AA, produced by maternal hypoaminoacidemia decreases fetal plasma concentrations of primarily essential AA (EAA) (Thureen et al., 2000). Further, pregnancy rank results in differences between singleton and twin fetuses, where twin fetuses at 140 days of pregnancy have lower concentrations of histidine and glutamine, and tend to have lower arginine and leucine concentrations in plasma compared to singletons (van der Linden et al., 2012). Interestingly, intrauterine growth restriction (IUGR) resulting from increased litter-size in sheep (Gootwine et al., 2007), is associated with changes in the transport of specific AA, such as leucine, across the placenta (Regnault et al., 2005). In addition to maternal nutrition, fetal and maternal plasma AA concentration can also be influenced by maternal breed (Ashworth et al., 2011).

Skeletal muscle plays an active role in the flux of AA in the fetus, and this process is influenced by maternal nutrition. In a maternal *ad libitum* fed state, the fetal hindlimb takes up most of the AA (Wilkening et al., 1994). In contrast, during maternal fasting, several AA, including glutamine and alanine are released by the muscle (Liechty and Lemons, 1984), in association with increased uptake of BCAA by the fetal hindlimb (Liechty et al., 1987a; Liechty et al., 1987b). Released glutamine (Cetin, 2001) and alanine (Prior and Christenson, 1977) from muscle can potentially be used to generate glucose in the fetal liver. The flux of AA from fetal muscle to fetal plasma, in response to maternal nutrition, reinforces the active role muscle plays during fetal development. However, it is unclear if changes in the concentration of specific AA occurring in the muscle of twin fetuses could potentially explain the differences in muscle growth when compared to singletons.

Amino acids in fetal muscle growth

Amino acids act as precursors of nitrogenous substances, such as polyamines and nitric oxide (NO) which likely mediate growth and development of muscle fibres (Wu et

al., 2010a). In addition, AA exert a signalling effect on the regulating factors controlling myogenesis (Yoon and Chen, 2013), affecting the primary phase of myofibre formation and modifying the number of myofibres (Bérard and Bee, 2010). In later stages of pregnancy, skeletal muscle growth increases rapidly (Lewis et al., 1984), and the fetus responds to infusion of specific (e.g. arginine) or a mixture of AAs by increasing protein synthesis (Liechty et al., 1999; De Boo et al., 2005). This response during fetal life appears to be associated with the plasma level of insulin in the fetus, as shown in pigs (Brown et al., 2009) and sheep (Brown and Hay, 2006). However, in IUGR sheep models, when AA are infused directly into the fetus, net fetal protein accretion increases independently of insulin changes (Brown et al., 2012).

1.4.2 Post-natal

Amino acid transfer and metabolism in muscle

The arterio-venous difference technique has shown that sheep muscle releases alanine, glutamine, and tyrosine and uptakes serine, glutamic acid, and possibly lysine, while in starved animals, there is a net output of most AA from muscle (Ballard et al., 1976). The flux of AA through skeletal muscle is influenced by the level of nutrition of growing lambs. The use of 0.5 to 2.5 times maintenance levels of nutrition to quantify AA kinetics in the growing lamb, showed a linear relationship in most of the AA evaluated. However, several AA (e.g. phenylalanine, lysine, leucine, isoleucine and tyrosine) had a curvilinear responses (Savary-Auzeloux et al., 2003). The effect of feed intake on AA flux in the ovine hindlimb has been shown to elicit a similar linear response (Hoskin et al., 2001; Hoskin et al., 2003), highlighting the relationship between level of nutrition and muscle AA flux.

Amino acids in neonatal muscle growth

Studies in rats have demonstrated the role of AA in protein synthesis. For example, a short-term fasting depresses protein accretion (Emery et al., 1983). This result is reversed when rats are fed a diet containing AA, while no response is elicited feeding an AA-free diet (Yoshizawa et al., 1998). In skeletal muscle, protein synthesis is stimulated by AA infusion independently of insulin availability (Watt et al., 1992; O'Connor et al., 2003a). Contrary to what occurs with insulin (McNulty et al., 1993; Wray-Cahen et al., 1998), protein synthesis stimulation by AA in skeletal muscle is age independent (Volpi et al., 1998).

Increasing the concentration of a mix of AA (Davis et al., 2002) or BCAA (O'Connor et al., 2003b) in the diet of neonate pig results in increased protein synthesis in skeletal muscle. In contrast, reduced protein content in the diet in growing pigs, decreases muscle weight, diminishes protein content in the muscle, and reduces plasma and intracellular free AA (FAA) concentrations. Intracellular alanine and glutamine concentrations increase as a result of muscle tissue protein breakdown and decreased utilisation of indispensable FAA by the muscle (Guay and Trottier, 2006). Similar effects have been reported for calves, in which the increased protein content in the diet increased intracellular concentration of leucine, methionine and valine, while reducing lysine, alanine and aspartate levels in muscle, although no effect on muscle weight was reported (Rius et al., 2012). Variations in the concentration of FAA in muscle of singleton and twin lambs during the neonatal period has not been addressed, but could explain some of the differences in muscle growth. The effect of specific AA supplementation, such as leucine (Anthony et al., 2000; Escobar et al., 2006; Suryawan et al., 2008; Escobar et al., 2010; Torrazza et al., 2010; Wilson et al., 2010; Yin et al., 2010; Suryawan et al., 2011; Wilson et al., 2011; Suryawan et al., 2012; Boutry et al., 2013) and arginine (Kim et al., 2004; Yao et al., 2008; Tan et al., 2009) on muscle growth has been described in pigs and rats, and their effect is associated with the activation of mTOR signalling.

1.5 THE MTOR PATHWAY: A NUTRIENT SENSING PATHWAY SIGNALLED BY AMINO ACIDS

Three principal intracellular metabolic pathways are associated with protein formation. These include insulin growth factor-1/ Phosphatidylinositide 3-kinases (IGF-I/PI(3)K), AMP-activated protein kinase (AMPK) and mTOR (Sandri, 2008; Polak and Hall, 2009) pathways. For the purposes of the research outlined in this thesis, the focus will be only on mTOR for two reasons. Firstly, mTOR acts as a central coordinator of the signals generated by growth factors via the phophatidylinositol-3 kinase/Akt kinase (PI(3)K-Akt) signalling pathway and is negatively regulated by low cellular energy via AMPK. Secondly, mTOR is activated by AA (Polak and Hall, 2009), which is the main focus of this thesis.

1.5.1 mTOR overview

The protein kinase mTOR is critical for both sensing nutrient availability and for nutrient-stimulated muscle growth (Bodine et al., 2001; Pallafacchina et al., 2002; Sakamoto et al., 2003). Many processes are controlled by mTOR, including translation, ribosome biogenesis, protein synthesis, nutrient transport and mitochondrial metabolism (Inoki et al., 2005; Hall, 2008). mTOR also plays an essential role in skeletal myogenesis (Erbay et al., 2003; Brown et al., 2009), controlling the initiation of myoblast differentiation through the regulation of IGF-II expression (Erbay et al., 2003).

mTOR occurs in two different complexes, mTORC1 and mTORC2 (Loewith et al., 2002) each with its own functions. mTORC1, a complex sensitive to the macrolid rapamycin (Kim et al., 2002), is formed by mTOR, mammalian lethal with Sec13 protein 8 (mLST8) and raptor (Liao et al., 2008), and has been mainly associated with cell growth. The mTORC2 complex, initially described as insensitive to rapamycin (Jacinto et al., 2004), can be inhibited under an over 24 h rapamycin treatment (Sarbassov et al., 2006), and is formed by rictor, PRR5 (piroline-rich protein 5), mSIN1 (mammalian SAPK interacting protein) and mLST8. mTORC2 is associated with the mediation of spatial control of cell growth, by regulating the actin cytoskeleton (Hall, 2008).

Compared to mTORC1, understanding of the activators of mTORC2 is very limited (Sparks and Guertin, 2010). mTORC1 responds to signals, such as growth factors (insulin, IGF) (Lai et al., 2004), energy (Hardie and Sakamoto, 2006) and nutrients, in which AA play a major role (Hall, 2008), and therefore will be the main focus of this review, where mTOR will be used as an abbreviated descriptor for mTORC1 throughout the thesis.

1.5.2 AA regulation of mTOR

Amino acids activate mTOR (Hara et al., 1998), however, the mechanisms through which AA modulate the activation of mTOR are not fully understood. Recently, a model has been proposed whereby mTOR activation occurs in the lysosome, mediated by AA sufficiency (Long et al., 2005; Sancak et al., 2008) and this has been recently reviewed (Dibble and Manning, 2013; Jewell and Guan, 2013; Jewell et al., 2013). Briefly, AA stimulates the recruitment of mTOR to the lysosome (Sancak et al., 2010).

In the lysosome mTOR interacts with Rheb (Ras homologue enriched in brain) and a multimeric complex called Ragulator (Rag; Sancak et al., 2010). Through unknown mechanisms, AA activate Rag allowing mTOR to be docked to the lysosome which then interacts with Rheb-GTP, allowing activation (Sancak et al., 2008) (Figure 1.1).

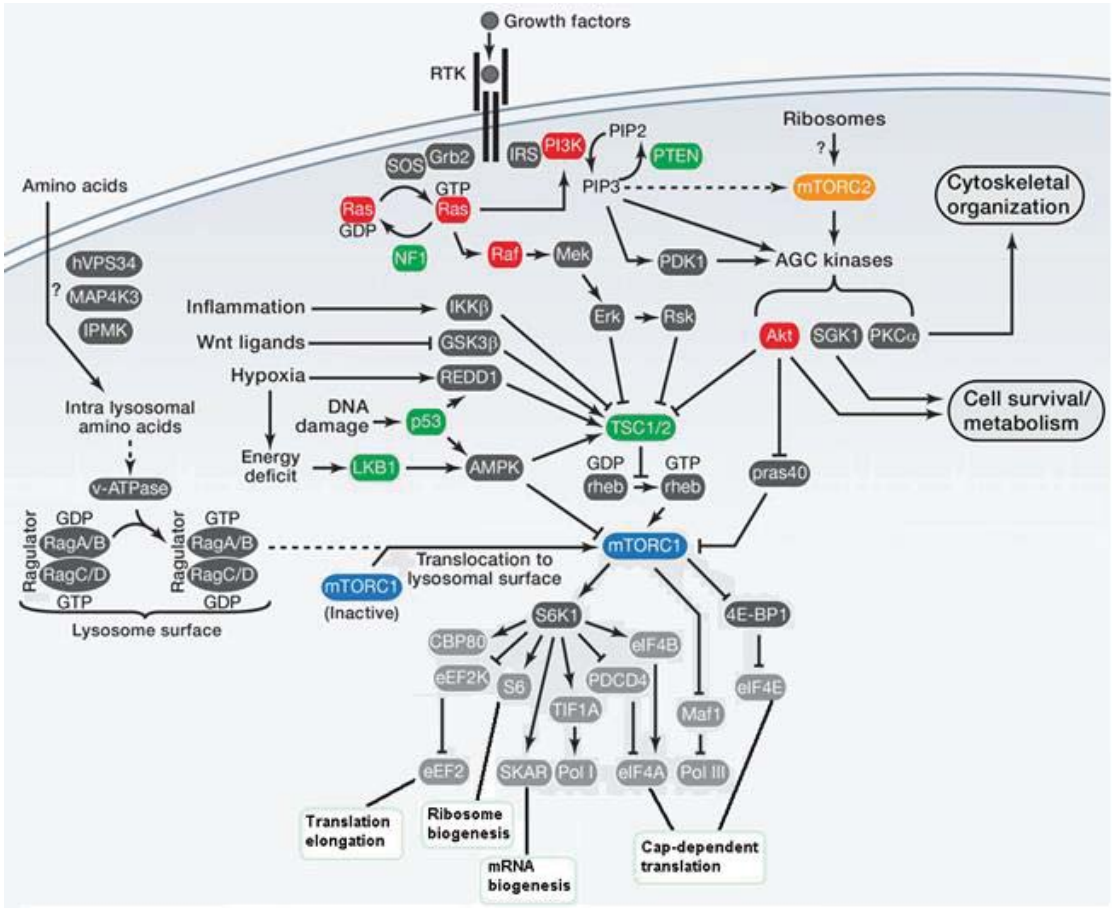

Figure 1.1. The mTOR pathway. Adapted from (Laplante and Sabatini, 2012).

1.5.3 Downstream effectors of mTOR

Protein synthesis is the best-characterised process controlled by mTOR. mTOR regulates muscle growth through the phosphorylation of proteins that regulate protein translation (Bolster et al., 2003). Once mTOR is activated, it phosphorylates two specific downstream targets involved in translation initiation, the eukaryotic initiation factor 4E binding protein (4E-BP1) and ribosomal protein S6 kinase S6Ks ($P70^{S6K1}$) (reviewed by Ma and Blenis, 2009). The phosphorylation of 4E-BP1, prevents its binding to the protein

eIF4E, enabling it to form a complex with eIF4F and initiate translation (Hay and Sonenberg, 2004). Phosphorylation of S6Ks results in the phosphorylation of several targets, linked to protein synthesis. One target is ribosomal protein S6 (Wang et al., 2001), which is directly associated with cell size (reviewed in Ruvinsky and Meyuhas 2006). eIF4B is also activated, which promotes the activity of eIF4A (Shahbazian et al., 2006). In addition, mTOR regulates ribosome biogenesis (Jastrzebski et al., 2007), impacting on the rate of protein synthesis (Figure 1.1).

1.5.4 mTOR and muscle development

The association of mTOR with fetal muscle development has been demonstrated in fetal sheep following a 50% restriction of nutritional requirements from day 28 to 78 of pregnancy. Maternal nutrient restriction does not decrease the total concentration of mTOR, but rather the phosphorylated form of mTOR and ribosomal protein S6 phosphorylation. Reduced activation of mTOR signalling reduces the ratio of secondary to primary muscle fibres, as lower activation of mTOR in nutrient-restricted fetuses may reduce the proliferation of myoblasts. This reduces the formation of secondary myofibres, affecting muscle development (Zhu et al., 2004). In contrast, maternal over-nutrition (1.5 times requirements), negatively affects the activation of fetal mTOR in skeletal muscle and reduces the density of muscle cells (Zhu et al., 2008). These results highlight the relevance of mTOR as a nutrient sensor and the association between nutritional status and pathway regulation.

Many studies have demonstrated that increasing the overall availability of AA during the neonatal phase in pigs can increase protein synthesis in skeletal muscle by enhancing the activities of positive regulators of translation initiation factors including mTOR (Davis et al., 2000; O'Connor et al., 2003a; Wang et al., 2007). Further, specific AA such as leucine (O'Connor et al., 2003a) and Arg (Yao et al., 2008) can stimulate the mTOR pathway and thus muscle protein synthesis. It is not clear whether mTOR senses individual AA or the total intracellular pool of AA (Dibble and Manning, 2013). Intracellular concentrations of AA can regulate mTOR signalling (Christie et al., 2002; Beugnet et al., 2003; Proud, 2004b) (Figure 1.2). Therefore, it can be hypothesised that changes in the concentration of specific AA may result in altered muscle growth in singletons and twins. To date this has not been explored.

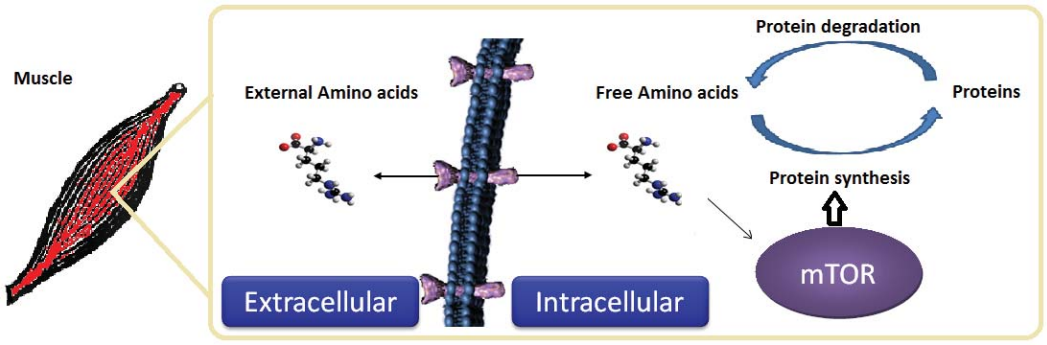

Figure 1.2. Graphical representation of the relationship between AA concentration in muscle and mTOR signalling.

1.6 EVIDENCE FOR AA ACTIVATION OF MTOR AND SKELETAL MUSCLE GROWTH IN LIVESTOCK

1.6.1 Monogastrics

Dietary Arg supplementation to gestating sows has been shown to increase fetal growth and survival of fetal pigs (Mateo et al., 2008). Post-natal Arg supplementation to piglets between 7 and 14 days of age, has been shown to influence the mTOR pathway in pigs, resulting in increased rates of fractional protein synthesis, average daily gain and overall body weight (Yao et al., 2008). Furthermore, supplementing the diet of growing-finishing pigs with Arg, decreases body white-fat and increases muscle protein synthesis, resulting in an overall live weight gain in a tissue-specific manner; an effect mediated via the mTOR pathway (Tan et al., 2009). Arginine supplementation has also been associated with an increase in the internal deposition of white fat and an increase in muscle pH post mortem, both of which can influence meat quality and meat processing (Tan et al., 2009).

Leucine can activate mTOR and its downstream factors in skeletal muscle, leading to mRNA translation (Escobar et al., 2005, 2006; Suryawan et al., 2008). However, this response depends on animal age, as increased protein synthesis and the phosphorylation

of mTOR, RPS6K-1, and 4E-BP1 are higher in 6-day-old compared to 26-day-old pigs (Suryawan et al., 2007; Suryawan et al., 2008). Translation decreases rapidly due to the reduction in the concentration of other AA in circulation (Wilson et al., 2010). When the total AA concentration is restored, protein synthesis in muscle is maintained, resulting in a longer effect of leucine supplementation (Wilson et al., 2010).

1.6.2 Ruminants

There is limited literature examining the association between AA supplementation and mTOR activation in ruminants. In cattle, restricted maternal nutrition to 70% of metabolisable net energy and 86% of metabolisable protein requirements, from 30 days after conception to 125 days of pregnancy reduced the phosphorylation of mTOR and RPS6, inhibiting protein synthesis in fetal muscle (Du et al., 2005).

One of the few studies in sheep that established an effect of AA on the mTOR pathway through the activation of the downstream target S6K, was a study performed by Brown *et al.* (2009). A direct parenteral supplementation of a mix of AA to the fetus, resulted in the activation of S6K, but this effect was dependent on insulin concentration as AA infusion upregulates S6K only when an AA-stimulated increase in insulin occurs. Recently, a study compared muscle from twin and singleton fetuses in late pregnancy. Ewes were fed on a maintenance pasture-only nutritional regimen from day 21 to 140 of pregnancy, and grazed such that the total increase in maternal body weight during pregnancy approximately equalled the expected conceptus mass at term. The study showed a down-regulation of mTOR signalling, leading to reduced ribosome number and abundance of the translational machinery (Sciascia et al., 2010). This was proposed as a possible mechanism for the retardation of myofibre hypertrophy and reduced muscle mass in twins.

Other studies in sheep showed that ewe lambs (5 to 8 months old) subjected to a 10-day infusion directly into the blood of a mixture of 6 AA (arginine, lysine, histidine, threonine, methionine and cysteine in the ratio found in bovine milk) supplying an extra 343 mg of $N \cdot kg^{-0.75} \cdot d^{-1}$, or oral supplementation of fishmeal which supplied an extra 719 mg of $N \cdot kg^{-0.75} \cdot d^{-1}$ compared to controls, resulted in improved rate of initiation of mRNA translation. In this study, the activation of mTOR or an increase in IGF-I were proposed

as the possible triggers (Connors et al., 2009). In nutritionally restricted singleton-bearing ewes (fed of 50% NRC recommendation), parenteral administration of Arg in doses of 155μmol/kg body weight^{-1}, three times daily from day 60 of pregnancy to parturition, ameliorated fetal growth restriction and increased birth weight (Lassala et al., 2010). Similar increases in birth weight were observed in ewes carrying quadruplets and supplemented with 345 mmol Arg-HCl/kg body weight^{-1} 3 times daily, from day 100 to 121 of pregnancy (Lassala et al., 2011). However, no effect was found on twin- or triple-bearing ewes and the mechanisms explaining the effect on quadruplets were not studied.

Collectively, these observations highlight the potential for the involvement of the mTOR pathway in the regulation of fetal and post-natal muscle growth. However, the role AA and mTOR have on muscle development in sheep is not clear.

1.7 RATIONALE FOR THESE STUDIES

This PhD program aims to investigate the role of AA in the developmental programming of skeletal muscle growth in twin-born lambs and potential mediation via the mTOR pathway. The central hypothesis is that restricted skeletal muscle hypertrophy in twin-born lambs is mediated, at least in part, by changes in intracellular concentration of specific free AA, affecting the abundance or activation of mTOR signalling, and that these effects can be ameliorated through specific AA supplementation.

The research questions are:

- Is muscle hypertrophy, and thus muscle mass, in late-pregnancy fetal and post-natal lambs associated with differential intracellular free AA profiles and mTOR signalling?
- What are the critical time windows for AA regulation of future capacity for muscle hypertrophy?
- Does supplementation with specific AA stimulate muscle hypertrophy in fetal/neonatal twin lambs, and are these effects mediated by mTOR signalling?

The target outcome of this research program is to contribute to a better understanding of the mechanisms associated with muscle growth in sheep and the potential role of AA. This study will try to establish a baseline for future work, leading to potential solutions that could directly increase skeletal muscle growth in twin lambs, thereby providing a cost-effective technology for extensive production systems enhancing lamb survival and meat productivity.

Chapter 2: Muscle free amino acid profiles are related to differences in skeletal muscle growth between single and twin ovine fetuses near term

General overview of the chapter:

Amino acids play a major role as building blocks and signalling molecules for protein accretion. Competition between fetuses in twin pregnancy may lead to AA deficiencies, which could be associated with restricted muscle growth. This chapter intends to determine which AA could be acting as limiting signalling AA for skeletal muscle growth in twin fetuses in late pregnancy. The information generated will contribute to the design of an *in vivo* trial, to demonstrate the effect of supplementation of such AA on fetal muscle growth in twins in late pregnancy.

Chapter 2 has been published: Sales F, Pacheco D, Blair H, Kenyon P, McCoard S (2013) Muscle free amino acid profiles are related to differences in skeletal muscle growth between single and twin ovine fetuses near term. SpringerPlus 2 (1):1-9

The format has been adjusted to the general format of the thesis. Table and figure numbers were kept as in publication.

2.1 ABSTRACT

Twin sheep fetuses have reduced skeletal muscle weight near birth relative to singles as a result of restricted muscle hypertrophy. In monogastrics intracellular free amino acids (FAA) are reported to regulate metabolic pathways which control muscle protein accretion, whereby reduced intracellular content of specific FAA may reduce their activation and therefore, muscle hypertrophy. The aim of this study was to determine whether differences in muscle weight between singleton and twin fetuses, under different maternal conditions is associated with reduced concentration of specific FAA. The FAA content in the *M. semitendinosus* (ST) in singleton and twin fetuses (rank) at 140 days of pregnancy from heavy (H) or light (L) ewes fed *ad libitum* (A) or maintenance (M) level of nutrition was measured. Muscle weight was reduced in twin fetuses compared to singletons in all groups. Reduced concentrations of leucine, threonine and valine, but higher concentrations of methionine, ornithine, lysine and serine were found in twin fetuses compared to singletons. Maternal size and nutrition interaction with rank resulted in reduced glutamine in twins from HM-ewes (H-ewes under M nutrition) compared to their singleton counterparts. Maternal weight interaction with pregnancy rank was associated with reduced concentration of arginine in muscles of twins, with a larger effect in foetuses from H-ewes compared with L-ewes. Maternal size interaction with pregnancy rank was observed in twins from M-ewes, having lower alanine, while twins from A-ewes had lower aspartic acid concentration compared to singletons. The ST muscle weight was positively correlated only with arginine concentration after taking into account rank, size and nutrition. The present results indicate that reduced concentrations of specific intracellular FAA, such as arginine, leucine, valine, glutamine, which are known to play a role in muscle growth, could be acting as limiting factors for muscle hypertrophy in twin fetuses during late pregnancy. Ewe size and nutrition can influence the concentration of specific FAA in muscle and should be considered in any intervention plan to improve twin fetal muscle growth.

2.2 INTRODUCTION

Increasing prolificacy is an effective way to improve profitability in sheep production systems (Gootwine et al., 2001). However, birth weight, post-natal survival, growth, body composition and lifetime production performance may be reduced as litter size increases (Barker, 1998; Greenwood et al., 1998; Morel et al., 2009). Reduced fetal weight near term in twins compared to singletons is associated with decreased skeletal muscle hypertrophy, leading to reduced muscle mass (McCoard et al., 2001). Although maternal undernutrition has a direct effect on fetal and skeletal muscle growth during pregnancy (Fahey et al., 2005a), reduced fetal weight and muscle weight in twins compared to singles is observed even in well-nourished ewes (Freetly and Leymaster, 2004). This suggests maternal nutrition is not the only factor to impact fetal and muscle growth as litter size increases.

It is well established that fetal growth is influenced by fetal amino acid (AA) availability (Liechty et al., 1999; Kwon et al., 2004; De Boo et al., 2005). Studies in sheep indicate that the rate of protein accretion in the fetus can be stimulated through the fetal infusion of a mix of AA (Liechty et al., 1999; De Boo et al., 2005). Importantly, AA also have the capacity to signal to metabolic pathways which regulate muscle growth (Hara et al., 1998; Brown et al., 2009) via changes in the intracellular concentration of specific AA (Christie et al., 2002; Beugnet et al., 2003; Sancak et al., 2008). For example, AA signalling plays an important role in the regulation of skeletal muscle hypertrophy in monogastrics, through the activation of specific cell signalling pathways (e.g. mechanistic target of rapamycin, mTOR), which controls protein synthesis (Yao et al., 2008; Tan et al., 2009). However, the potential for specific AA to act as signalling molecules to regulate skeletal muscle hypertrophy during pregnancy in ruminants is not well understood.

In sheep, we have preliminary evidence (Pacheco et al., 2010) that concentrations of specific intracellular AA in skeletal muscle (e.g. arginine and glutamine), differ between single and twin fetuses in late pregnancy in nutritionally-restricted ewes. The purpose of this study was to further explore the potential relationship between skeletal muscle mass and intracellular free AA concentration in twins compared to singletons, by testing two hypotheses. Our first hypothesis is that reduced skeletal muscle weight in twin, compared to single fetuses in late pregnancy, is associated with reduced

concentration of specific free AA in muscle, such as glutamine and arginine. Our second hypothesis is, that dam nutrition and body size influence the relationship between skeletal muscle AA and fetal muscle weight, between pregnancy ranks (single *vs.* twin). To test these hypotheses, the concentration of free AA from the *M. semitendinosus* (ST) muscle collected from twin and singleton fetuses at 140 days of pregnancy from heavy and light ewes fed two differing planes of nutrition were compared.

2.3 MATERIALS AND METHODS

2.3.1 Animals

All procedures described in the present study were approved by the Animal Ethics Committee of Massey University, Palmerston North, New Zealand.

This study was part of a larger study where animal selection protocols, feeding regimens (Kenyon et al., 2009; Blair et al., 2011; Kenyon et al., 2011) and euthanasia procedures (Blair et al., 2011) were previously described. Briefly, the study design utilised two different maternal sizes, according to their weight and corresponding to the heaviest (H; 60.8 ± 0.18 kg, condition score 3.02 ± 0.03 (1-5 scale; Jefferies, 1961) and lightest (L; 42.5 ± 0.17 kg, condition score 1.97 ± 0.03) Romney ewes, selected from a commercial flock. The ewes were randomly allocated to either a maintenance (M) or *ad libitum* (A) nutritional plane on pasture from day 21 after insemination until 140 days of pregnancy. Maintenance nutrition was designed to ensure that total ewe live weight increased in pregnancy at a level similar to that of the expected conceptus mass (Rattray et al., 1974; Rattray, 1986). The maternal weight change in the maintenance group was coincident with the conceptus mass, which suggest ewes were close to maintenance (Blair et al., 2011; Kenyon et al., 2011). The *ad libitum* plane was designed to provide unrestricted herbage intake under grazing conditions. To achieve these nutritional regimens, ewes were grazed using a rotational system, as described by Kenyon et al. (2009). Ewes were pregnancy scanned via ultrasound and their pregnancy rank determined (single and twin).

At 140 days of pregnancy, animals were euthanised and the ST muscle was excised from each fetus, weighed, and snap frozen in liquid nitrogen and stored at -80ºC.

The numbers of fetuses in each group, according to maternal size, maternal nutrition and pregnancy rank were: HM/single: 10; HM/twin: 10; HA/single: 9; HA/twin: 10; LM/single: 10; LM/twin: 10; LA/single: 10 and LA/twin: 10. The effect of ewe size, plane of nutrition and pregnancy rank on fetal weights have previously been reported by Firth et al. (2008), Kenyon et al. (2011) and Blair et al. (2011) as part of the same research program. To date, no comparison between single and twin muscle weight and AA concentration in muscle has been previously described for these animals.

2.3.2 Intracellular free AA (FAA) profiles in fetal ST tissue

Amino acids were determined by ion-exchange chromatography using post-column derivatisation with ninhydrin. Approximately 150 mg of ST tissue from each animal was homogenised in 1.75 mL of Seraprep (Pickering Laboratories, Alphatech Systems Ltd, Auckland, New Zealand) containing 20 µL of L-2-Amino-3-guanidinopropionic acid hydrochloride (25 µM/mL) as an internal standard (Calbiochem-Behring Corp., La Jolla, CA, USA). Samples were left in ice for 20 min, and then 40 µL 5.88 M lithium hydroxide buffer (BDH Chemical, Poole, England) added, followed by centrifugation at 8,000 g for 10 min. After centrifugation, samples were filtered using a 0.45 µm cellulose acetate filter membrane (Advantec, Toyo Roshi, Tokyo, Japan). Supernatant was analysed for FAA using a Shimadzu LC10Ai high-performance liquid chromatography (HPLC) (Shimadzu Oceania Ltd., Auckland, New Zealand), fitted with a high-efficiency lithium-ion exchange column (3 mm ID x 150 mm; Pickering Laboratories, Shimadzu Oceania Ltd., Auckland, New Zealand) and a Pickering PCX 3100 post-column reaction module (Pickering Laboratories, Shimadzu Oceania Ltd, Auckland, New Zealand). Injected volumes were 10 µL, at a flow rate of 0.3 mL/min and a run time of 162 min between injections, using Li buffers as eluants and ninhydrin post-column derivatisation (Csapó et al., 2008). Detection was performed at 570 nm for all FAA, except proline which was read at 440 nm. Amino acids in samples were quantified on the basis of known amounts of standards (Shimadzu Oceania, Pickering Laboratories, USA) and their retention times, using LC Solution ver. 1.22 SP1 software (Shimadzu, Kyoto, Japan).

2.4 STATISTICAL ANALYSIS

Fetal ST muscle weight and FAA concentration were analysed by ANOVA, using the MIXED procedure (SAS, 2006) with a linear model, which included the fixed effects of pregnancy rank (single *vs.* twin), ewe size (H *vs.* L) and ewe nutrition (A *vs.* M) and their two- and three-way interactions. Individual ewe tag was used as a random effect to adjust for twinning. Differences among least squares means were analysed using the PDIFF option of the MIXED procedure. To examine whether fetal ST weight was proportional to fetal weight, fetal weight was used as covariate in a separate analyses. Both adjusted and unadjusted values are presented for comparison. Sex of fetus had no effect on any traits of interest and was removed from the ST muscle and FAA models. Means are presented as least square means with least significant differences (LSD, 5%). Isoleucine, asparagine and cystine were detected in only some animals, therefore these FAA were omitted from analysis.

To determine the correlation between ST muscle weight and intracellular FAA concentration, partial correlations using the CORR procedure (SAS, 2006) were estimated on the residual of the AA concentration and muscle mass, after accounting for the effects of pregnancy rank, maternal size and nutritional treatments and results consider all animals. For all analysis, statistical significance was set at a probability value of $P \leq 0.05$.

2.5 RESULTS

A three-way interaction between pregnancy rank, size and nutrition was observed for ST weight ($P = 0.04$, Fig. 1). Twins from HM-ewes had lower ST weights compared with twins from HA-ewes, whereas singletons from LM-ewes had lower ST weight compared with LA singletons (Fig. 1). After adjusting for fetal body weight, only a pregnancy rank effect was observed, whereby twins had 17% lower ST weight compared to singles (8.3 ± 0.3 g *vs.* 10.0 ± 0.3 g, $P = 0.002$). No maternal size, maternal nutrition or interactions with maternal size or nutrition effects were observed for ST weight (data not shown).

A three-way interaction between pregnancy rank, maternal size and plane of nutrition was observed for ST intracellular concentrations of free glutamine and tyrosine (Table 1). Twin fetuses from HM-ewes had 37% lower (P = 0.0003) glutamine concentrations compared to their singleton counterparts. Twins from LA-ewes had 32% lower (P = 0.02) tyrosine concentration compared to their singleton counterparts. No differences were observed for tyrosine in the other groups.

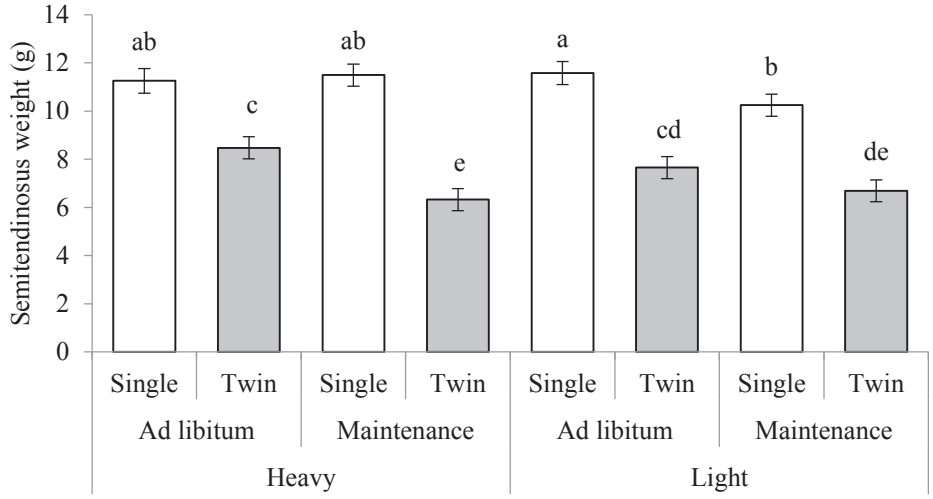

Figure 2.1. The bars graphic represent the *M. semitendinosus* weight (g) of the eight groups of fetuses (singletons and twins from Heavy and Light ewes offered an *ad libitum* or maintenance feeding regime) at 140 days pregnancy, not adjusted by fetal weight. Data are presented as least square means and standard error of the mean (SEM). Bars with different letters are significantly different at $P \leq 0.05$.

A two-way interaction between pregnancy rank and maternal size was observed for arginine, glutamic acid, glycine and proline in ST muscle (Table 2). Twins from H-ewes had 40% lower (P < 0.0001) arginine concentration than their singleton counterparts, whereas twins from L-ewes had 9% lower (P = 0.03) arginine concentration relative to singletons. Twins from L-ewes had 51% higher (P < 0.0001) concentration of glutamate compared to singletons, while no difference between pregnancy ranks was observed for fetuses from H-ewes. Twin fetuses had 47% higher (P < 0.0001) glycine concentrations compared to singletons in the H-ewes, while no differences were observed between twins and singletons from L-ewes. Twins had 25% lower (P = 0.02) proline

concentration in H-ewes while no difference between pregnancy ranks was observed for fetuses from L-ewes.

A pregnancy rank by nutrition interaction was observed for alanine and aspartic acid concentration in ST muscle (Table 2). Twin fetuses had 13% lower ($P = 0.02$) alanine concentration compared to singletons in the M-ewes, while no difference was observed between pregnancy ranks in the A-ewes. For aspartic acid, twin fetuses from A-ewes had 33% lower ($P < 0.0001$) concentration compared to singletons from A-ewes, while no difference was observed between ranks in the M-ewes group.

Compared to singletons, twins had lower ST muscle concentration of leucine (19%), threonine (23%), valine (36%) and total EAA (11%) (Table 2), but higher concentrations of methionine (67%), ornithine (19%), lysine (23%), serine (33%) and total non EAA (5%) (Table 2).

Table 2.1. Three-way interaction between maternal size, (S, Heavy *vs.* Light) plane of nutrition (N, *Ad libitum vs.* *vs.* Twin) for the concentration (nmol/g wet tissue) of free amino acids in *M. semitendinosus* of fetuses at 140 d square mean (LSM). The average of the difference of the least square means (LSD, α = 0.05) and probability (RxSxN) are presented.

	Heavy ewes				Light ewes			
	Ad libitum		Maintenance		*Ad libitum*		Maintena	
	Single	Twin	Single	Twin	Single	Twin	Single	
Essential amino acids								
Histidine	225	242	201	232	199	261	227	
Leucine	60	52	54	40	50	42	60	
Lysine	109	115	80	81	67	88	70	
Methionine	48	79	42	64	46	86	46	
Phenylalanine	41	50	47	37	44	43	48	
Threonine	1252	823	968	841	1222	839	1212	
Valine	175	98	119	69	150	121	191	
Total EAA	1690	1423	1455	1272	1763	1462	1707	
Non-essential amino acids								
Alanine	2393	2495	2239	1858	2137	2164	2400	
Arginine[1]	410	239	424	263	392	263	340	
Aspartic Acid	465	302	132	85	500	343	423	
Carnosine	1407	1379	1297	1124	1157	1432	1148	
Citrulline	75	53	59	52	87	72	92	
Cystathionine	140	97	218	157	153	169	287	
Glutamic acid	1483	1240	1191	1201	1045	1807	1365	
Glutamine	3202[a]	3276[a]	3111[a]	1973[b]	3302[a]	3539[a]	3043[a]	
Glycine	1844	2794	2010	2868	1888	2215	2134	
Ornithine	102	99	93	119	69	92	90	
Proline	352	246	370	295	319	285	322	
Serine	1353	1520	995	1474	772	1365	1239	
Taurine	5501	6046	5786	5770	6170	6728	6485	
Tyrosine	51[a]	57[a]	60[a]	52[a]	64[a]	44[b]	68[a]	
Total NEAA	18634	19640	17665	17238	17766	19831	19361	
TotalL	20359	21118	19177	18590	19567	21358	21114	

[1]Deemed as conditionally essential (Wu, 2009).

Table 2.2. Rank effect (Single (S) *vs.* Twin (T)), two-way interaction between pregnancy rank and maternal s pregnancy rank and plane of nutrition (*Ad libitum* (A) *vs.* Maintenance (M)) for the concentration (nmol/g w *semitendinosus* of fetuses at 140 days pregnancy. Values are expressed as least square mean (LSM). The averag (LSD, α = 0.05) is presented.

	Rank				Rank x Size						
	Single	**Twin**	**LSD**	***P***	**H-S**	**H-T**	**L-S**	**L-T**	**LSD**	***P***	**A**
Essential amino acids											
Histidine	213	249	38	0.07	213	237	213	260	54	0.56	2
Leucine	56	45	8	0.01	57	46	55	44	11	0.95	5
Lysine	82	101	18	0.04	95	98	69	104	26	0.09	8
Methionine	45	76	8	<0.001	45	72	46	80	12	0.39	4
Phenylalanine	45	44	7	0.85	44	44	46	45	9	0.94	4
Threonine	1163	892	143	<0.001	1110	832	1217	952	203	0.93	12
Valine	159	102	19	<0.001	147	83	171	120	27	0.49	1
Total EAA	1654	1467	93	0.05	1572	1347	1735	1588	131	0.68	17
Non-essential amino acids											
Alanine	2292	2170	184	0.19	2316	2176	2268	2164	260	0.85	22
Arginine[2]	392	274	44	<0.001	417[a]	251[b]	366[a]	298[a]	62	0.03	4
Aspartic Acid	380	269	48	<0.001	299	194	461	344	68	0.80	4
Carnosine	1252	1256	162	0.97	1352	1251	1152	1260	229	0.20	12
Citrulline	78	69	14	0.16	67	53	90	84	19	0.52	8
Cystathionine	200	158	51	0.11	179	127	220	189	72	0.69	1
Glutamic acid	1271	1521	184	0.01	1337[a]	1220[a]	1205[a]	1821[b]	261	<0.001	12
Glutamine[1]	3165	3075	305	0.56	3156	2624	3173	3527	431	<0.001	32
Glycine	1969	2482	272	<0.001	1927 [a]	2831 [b]	2011 [a]	2133 [a]	384	0.01	18
Ornithine	89	106	14	0.02	98	109	80	102	20	0.42	8
Proline	341	304	53	0.17	361[a]	270[b]	321[ab]	338[ab]	74	0.05	3
Serine	1090	1453	191	<0.001	1174	1497	1006	1408	270	0.68	10
Taurine	5985	6371	674	0.26	5644	5908	6327	6834	953	0.72	58
Tyrosine[1]	61	56	8	0.27	55	55	66	58	12	0.33	5
Total NEAA	18357	19234	448	0.05	18150	18439	18564	20029	633	0.19	18
Total	20054	20767	471	0.14	19768	19854	20341	21679	666	0.19	19

[1]Refer to Table 1 due to the existence of a three way interaction.
[2]Deemed as conditionally essential (Wu, 2009).

A positive association was observed between ST muscle weight and arginine concentration after partial correlation analysis (Fig. 2). In contrast, a negative correlation was found between ST muscle weight and intracellular concentration of taurine (Fig. 3). No correlations were found for any other FAA with ST muscle weight (data not shown).

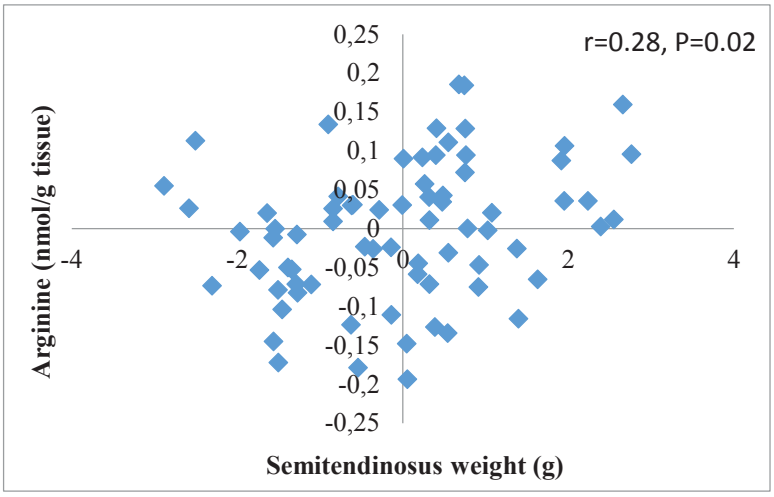

Figure 2.2. Partial correlation plot for *M. semitendinosus* (ST) weight with arginine concentration. The plot graphic shows the partial correlation analysis for ST muscle weight (g) with arginine concentration (nmol/g wet tissue). The analysis considered pooled data of all fetuses and was performed after accounting for the effects of pregnancy rank, maternal size and nutrition.

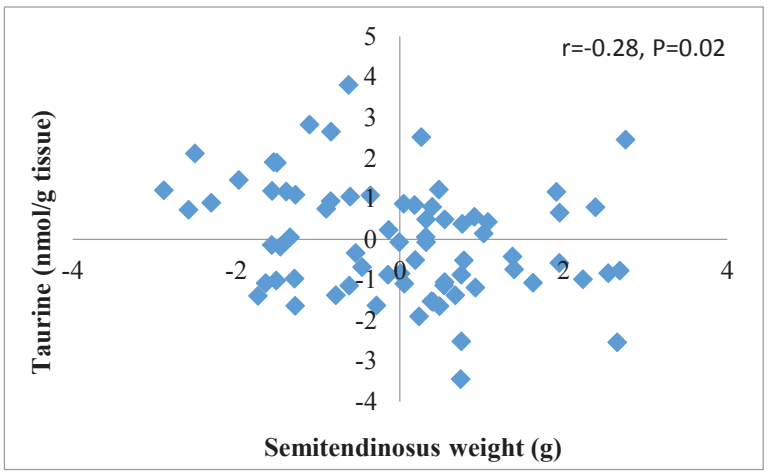

Figure 2.3. Partial correlation plot for *M. semitendinosus* (ST) weight with taurine concentration. The plot graphic shows the partial correlation analysis for ST muscle weight (g) with taurine concentration (nmol/g wet tissue). The analysis considered pooled data of all fetuses and was performed after accounting for the effects of pregnancy rank, maternal size and nutrition.

2.6 DISCUSSION

The objective of this study was to explore the potential relationship between skeletal muscle weight and intracellular FAA concentration in single compared to twin fetuses in late pregnancy sheep. An additional objective of this study was to establish the effect of maternal size and nutrition on fetal muscle FAA concentration, as an approach to understand some of the possible mechanisms explaining the lower muscle mass normally observed in twin fetuses (McCoard et al., 2001). Reduced ST mass in twins compared to singleton fetuses at 140 days pregnancy was associated with changes in the concentration of specific but not total intracellular FAA concentration. Notably, the concentrations of arginine, leucine, valine, and glutamine, known to influence pathways which regulate protein synthesis (Wu, 2009), were lower in muscle of twin compared to single fetuses. While other AA were affected by pregnancy rank, maternal size or nutrition, their role in fetal muscle growth, beyond being the building blocks for protein synthesis, is unclear. Arginine concentration, irrespective of pregnancy rank, maternal size and maternal nutrition, was the only AA positively correlated with fetal skeletal muscle mass. These results suggest that arginine may be important for skeletal muscle growth in the late-pregnancy ovine fetus.

Irrespective of maternal size or maternal nutrition, twin fetuses had lower ST muscle mass compared to singletons, in agreement with a previous study (McCoard et al., 2001). In the present study, the effect of maternal nutrition on muscle mass was influenced by maternal size. Twin fetuses from HM-ewes had disproportionately smaller ST muscle compared to their single counterparts. This indicates that heavy twin-bearing ewes fed a maintenance plane of nutrition were unable to meet the nutritional requirements to maintain not only fetal weight (Blair et al., 2011), but also muscle growth, when compared to their counterparts carrying singletons. Competition for limited nutrients between twins (McCoard et al., 2000a) or the reduced priority of nutrient partitioning to fetal skeletal muscle development compared with other organs during maternal nutrient restriction (Du et al., 2010) could explain the lower muscle mass in twins from ewes at a restricted feeding level. In contrast, the lower ST muscle weight observed in singles and twins from LM-ewes, in comparison with their single and twin counterpart from LA-ewes, could indicate that lighter ewes fed a maintenance level of nutrition are unable to provide the nutrient requirements either for a single or twin pregnancy. These results support the notion that skeletal muscle growth in twins is more

sensitive to maternal nutritional constraint than in singles, which is in agreement with a previous study (Gootwine et al., 2007). However, our results also suggest muscle growth can be compromised in singleton pregnancies under maternal nutrient restriction, as reported previously (Quigley et al., 2008).

Free AA play a major role not only as building blocks for protein synthesis, but they also regulate key metabolic pathways which are necessary for cell maintenance and growth (Wu, 2009). The function of FAA as signalling molecules is associated with changes in the intracellular concentration of specific FAA (Christie et al., 2002; Beugnet et al., 2003; Sancak et al., 2008). Intracellular pools are therefore critical to accomplish the signalling function of FAA (Nobukuni et al., 2005). The size and composition of the intracellular FAA pool depends on different processes, including availability of circulating FAA, and an increased AA influx or efflux between muscle and the plasma resulting from utilisation (e.g., by protein synthesis) or catabolism (protein turnover) (Proud, 2004b; Hundal and Taylor, 2009). In this study it is unclear what the contribution is of each of these processes to the observed differences in intracellular FAA profiles. Unfortunately, fetal plasma was not available in the present study to relate the plasma FAA profile with intracellular muscle FAA profile in single and twin fetuses. However, we have previously shown that twins from *ad libitum*-fed ewes have lower plasma concentration of glutamine, arginine and leucine compared with singletons at day 140 of pregnancy (van der Linden et al., 2012). Others have also reported a decreased concentration of arginine family members and branched-chain amino acids (BCAA) in fetal plasma when restricting maternal nutrition to 50% of their requirement (Kwon et al., 2004). It has also been proposed that reduced muscle mass in sheep fetuses exposed to maternal nutrient restriction may be associated with reduced plasma FAA, particularly serine, arginine-family AA, and BCAA (Zhu et al., 2006). Therefore, it is feasible that changes in specific FAA concentration in twins muscle were related to changes in circulating AA availability. The possible association between pregnancy rank and maternal nutrition on fetal plasma AA concentration and how this affects intracellular muscle FAA concentrations and muscle mass, is yet to be established.

Skeletal muscle growth in fetuses utilizes both EAA and non-essential AA (NEAA) (Wilkening et al., 1994). However, during fetal stress, such as maternal fasting, AA catabolism increases in muscle, resulting in release to the circulation of gluconeogenic precursors such as glutamine (Liechty and Lemons, 1984), due to an

increased metabolism of BCAA (Liechty et al., 1987b). In the present study, the reduced concentration of the BCAA leucine and valine in ST muscle of twins compared to singletons could suggest higher muscle protein breakdown in twins, resulting in a lower muscle mass compared to singles. In addition, protein breakdown may have also contributed to the observed reduction in glutamine concentration in ST muscle of twin fetuses from HM-ewes. This may have led to the higher growth limitation of muscle from fetuses in the HM group, reinforcing the idea of a greater degree of nutritional restriction in this group. Whether the difference in intracellular concentration of FAA is related to changes in FAA transport or a result of catabolism, warrants further investigation.

Arginine is considered a conditionally indispensable AA for the fetus (Wu et al., 2009) and participates in the synthesis of proteins, nitric oxide, polyamines, creatine, some AA and agmatine (Wu and Morris Jr, 1998), playing a major role in skeletal muscle growth (Wu et al., 2000). The exacerbated reduction in arginine concentration observed in twins compared to singles from H-ewes compared to their L-ewes counterparts, may be associated with a more stressful fetal environment. Reduced concentration of arginine family members as a result of maternal undernutrition, has been previously described in fetal plasma (Kwon et al., 2004) and in *gastrocnemius* muscle of sheep fetuses (Wu et al., 2006), which supports our findings. The reduced concentration of arginine, could be due to a higher catabolism of this AA, which would explain the increase in ornithine concentration observed in twins compared with singletons, according to Wu and Morris Jr. (1998). The higher concentration of arginine found in singletons compared to twins and the positive partial correlation between intracellular muscle arginine with muscle mass, suggests that arginine may act as a limiting AA for fetal muscle growth in twin fetuses.

Protein synthesis in muscle is controlled by specific signalling pathways, including phosphatidylinositol-3 kinase (Schiaffino and Mammucari, 2011), 5'-AMP-activated protein kinase (Bolster et al., 2002) and mitogen-activated protein kinase (Williamson et al., 2003). However, mTOR is accepted as the major pathway regulating muscle protein synthesis (Du et al., 2005). Intracellular FAA can activate mTOR (Beugnet et al., 2003), whereas a decrease in the intracellular AA concentration reduces the mTOR signalling (Sancak et al., 2008). Specific AA such as leucine (Escobar et al., 2006; Suryawan et al., 2008; Suryawan et al., 2011) and arginine (Yao et al., 2008) activate mTOR in muscle of monogastrics, as well as glutamine in cell culture models

(Nicklin et al., 2009; Chiu et al., 2012). Preliminary evidence indicates a reduced abundance of mTOR downstream targets in muscle of twin fetuses, compared with singletons at late pregnancy (Sciascia et al., 2010). Therefore, it is possible that reduced intracellular concentrations of glutamine, leucine and arginine in muscle of twins compared to singles in this study, could have resulted in a decreased mTOR signalling, and therefore, reduced muscle mass. This potential mechanism is part of future research.

The increase in methionine concentration in ST muscle from twins compared to singles is a novel finding. Methionine is an EAA, used to initiate protein synthesis (Kozak, 1983); it is involved in DNA methylation (Waterland, 2006), participates in oxidative processes (Hoshi and Heinemann, 2001) and has other metabolic functions (Brosnan and Brosnan, 2006). Previous studies in rats under starvation have shown the increased concentration of methionine in muscle as a result from high protein breakdown (Millward, 1970; Millward et al., 1974). However, reduced utilisation from lower protein accretion cannot be excluded.

2.7 CONCLUSIONS

Reduced concentrations of specific FAA, which are known to play a role in muscle growth, could act as limiting factors for muscle hypertrophy in twin fetuses in late pregnancy. The effect of decreased concentration of leucine, valine, glutamine, and especially arginine on fetal skeletal muscle growth in late pregnancy requires further investigation. The consequences of a maintenance maternal nutrition and maternal weight on fetal muscle mass and concentration of some FAA, reinforces their importance for fetal muscle growth. Altogether, these findings establish a baseline for new studies to further define the role of AA in fetal muscle growth in sheep, and open new possibilities for future strategic nutritional interventions to improve skeletal muscle development.

Chapter 3: Identification of amino acids associated with skeletal muscle growth in late pregnancy and at weaning in lambs of well-nourished sheep

General overview of the chapter:

Differences in the concentration of specific AA in twin fetal muscle, compared to singletons, was identified in Chapter 2. In addition, a positive relationship between arginine and muscle growth was observed at 140 days of gestation, which may potentially be important for signalling. This chapter aims to establish a potential carryover effect in terms of the relationship between intracellular FAA and muscle mass at weaning. Results from this study will be the baseline for a later *in vivo* study on the effect of supplementation of a specific AA in skeletal muscle growth of twin lambs, from birth to weaning.

Chapter 3 was submitted in part for publication to the Journal of Animal Science.

Chapter 3 was presented in part at the 2012 ADSA-ASAS Joint Annual Meeting. Phoenix, AR, USA: F. A. Sales, B. P. Treloar, D. Pacheco, H. T. Blair, P. R. Kenyon, G. Nicholas, M. Senna Salerno, and S. A. McCoard. 2012. Identification of key amino acids associated with fetal skeletal muscle growth in sheep. J. Anim. Sci. Vol. 90, Suppl. 3/J. Dairy Sci. Vol. 95, Suppl. 2

Chapter 3 was presented in part at the 2012 meeting of the New Zealand Society of Animal Production, Christchurch, New Zealand: Sales FA, Treloar BP, Pacheco D, Blair HT, Kenyon PR, Nicholas G, Senna Salerno M. 2012. Brief Communication: Relationship between profiles of free amino acids in fetal and maternal plasma with those in skeletal muscle, in twin and single fetuses from *ad libitum* fed ewes in late pregnancy Proceedings of the New Zealand Society of Animal Production, Volume 72, pp 213-215.

3.1 ABSTRACT

The objective of this study was to determine the association between intracellular free amino acid (FAA) profiles in skeletal muscle with muscle growth in twin and singleton fetuses in late-pregnancy and at weaning, under an *ad libitum* feeding regime of the dam. Plasma AA profiles from singleton- (n = 9) and twin-bearing (n = 10) ewes at d140 of pregnancy, and FAA concentration in the *M. semitendinosus* (ST) from the corresponding fetuses were studied. At weaning, intracellular ST FAA concentrations were compared between twins at the same age (Twin(age), n = 17) and at the same weight (Twin(wt), n = 17) to that of singletons (n = 20). Twin fetuses had 15% lower body weight and a 20% lighter ST ($P < 0.05$) compared to singletons. Maternal plasma FAA concentrations were similar ($P > 0.05$) between singleton- and twin-bearing ewes. Twin fetuses had higher ($P < 0.05$) plasma concentrations of glutamine, histidine and methionine and lower ($P < 0.05$) concentrations of aspartate, citrulline, glutamate and ornithine compared with singletons. In fetal ST, twins had lower ($P < 0.05$) concentration of aspartate and valine and higher ($P < 0.05$) concentration of methionine. Correlations were found between fetal ST weight and intracellular concentrations of arginine (r = 0.66, $P < 0.01$) and glutamine (r = 0.49, $P < 0.01$). Compared to singletons at weaning, Twins(age) were 16% lighter ($P < 0.05$) and the ST weight was proportionately 16% lighter ($P < 0.05$). For Twin(wt), the magnitude of the difference for ST weight was reduced to 8% lighter ($P < 0.05$). Twin(age) lambs had greater ($P < 0.05$) intracellular concentrations of glutamine, histidine, threonine, asparagine, alanine, serine and glutamate while reduced concentration of taurine compared to singletons. The differences in FAA concentrations were lower between Twin(wt) and singletons than between Twin(age) and singletons. Positive correlations were found between leucine, lysine, methionine, phenylalanine, proline, threonine and tyrosine concentration in muscle and ST weight at weaning. Males differed from females in intracellular FAA concentrations both in late pregnancy and at weaning. Twins had reduced RNA content during pregnancy and at weaning, suggesting a lower capacity for protein accretion. These data suggest that specific FAA concentrations, notably arginine and glutamine, are associated with differences in muscle growth during late pregnancy, and reduced protein synthesis capacity. However, the relevance of specific FAA varies according to stage of development and sex of the lamb.

3.2 INTRODUCTION

Reduced skeletal muscle mass in twin lambs compared to singletons normally arises during late pregnancy (McCoard et al., 2000a). Post-natally, twins continue to have lower muscle weight compared to singletons (Bennett et al., 1991; McCoard et al., 2010), even if raised as singletons (Hohenboken et al., 1976). The mechanisms associated with reduced muscle growth in twin lambs compared to singletons remains poorly understood.

Amino acids (AA) promote protein accretion by acting as protein substrates (Bell et al., 1989; Davis et al., 2002), but also by regulating protein synthesis via specific signalling pathways (Wu, 2009). Changes in the intracellular concentration of specific free AA (FAA) from the arginine family and leucine (Wu, 2009), can induce a protein synthesis response through activation of the mTOR pathway (Beugnet et al., 2003; Wullschleger et al., 2006; Sancak et al., 2008). Studies in neonatal and weaned piglets have shown arginine and leucine stimulates protein synthesis in muscle via activation of translation initiation factors, and therefore, influence muscle growth (Escobar et al., 2007; Yao et al., 2008; Tan et al., 2009). In the growing sheep fetus, the association between maternal and fetal plasma AA concentration undergoes substantial changes during fetal development (Kwon et al., 2003). Similarly after birth, plasma and muscle AA profiles change according to protein content in the diet (Bergen et al., 1973). However, it is not clear if differences in muscle mass during late gestation and at weaning in twin compared to singleton lambs are associated with differences in the intracellular concentration of specific FAA.

The objectives of this study were to (1) identify whether differences in muscle mass between singletons and twins at 140 days of pregnancy and at weaning, either at the same age or weight as singletons, were associated with differences in the intracellular concentration of FAA and (2) establish the association between maternal and fetal FAA plasma profile and FAA concentration in muscle tissue.

3.3 MATERIALS AND METHODS

All experimental procedures were approved by the AgResearch Ruakura Animal Ethics Committee, Hamilton, New Zealand, according to the Animal Welfare Act 1999 of New Zealand (Approval number 11844).

3.3.1 Animals

Mixed age ewes were randomly selected from a commercial population (Rissington Breedline Primera®). Ewes were naturally mated and pregnancy scanned at 80 days to identify singleton- and twin-bearing ewes and pregnancy age. A group of 30 singleton- and 27 twin-bearing ewes were selected into one group and fed *ad libitum* (2200-2500 kg/DM per ha pre-grazing allowance) on pasture to maximize growth, considering the intake in sheep is not limited above 1400 kg DM/ha (Morris and Kenyon, 2004) At approximately 140 days of pregnancy (P140), singleton- (n = 9) and twin-bearing (n = 10) ewes were euthanised by stunning with captive bolt and exsanguination. Fetal blood samples were collected via cardiac puncture after removal of the fetuses from the uterus and ewes were blood sampled from the jugular vein prior to euthanasia. Previous studies showed that the concentration of AA in fetal plasma is similar between fetuses of a twin pair (Kwon et al., 2003). Therefore, fetal plasma from each singleton and one member of a twin-pair were compared. Fetal weight and sex were recorded. The *M. semitendinosus* (ST) was collected from all fetuses, snap frozen in liquid nitrogen and stored at -80ºC.

The remaining ewes (n = 20 singleton; n = 17 twin-bearing) were allowed to lamb. Birth weight and sex of lambs were recorded immediately after lambing. Lambs were maintained with their dams under a pasture grazing regimen. Body weight was recorded weekly from birth until sampling at weaning and average daily gain (ADG) calculated per week. At 85 days of age (weaning), 20 singles and one randomly selected twin lamb of a twin pair (Twin(age); i.e. at same age as singles, n = 17), were euthanised to evaluate potential differences in intracellular FAA concentration between singles and twins at the same age. The remaining siblings of each twin pair (Twin(wt); i.e. euthanised at same weight as singles, n = 17) were maintained with the dam until reaching a similar weight to singletons at 85 days. Previous to euthanasia by stunning with captive bolt and exsanguination, lambs were blood sampled for metabolite profile. Lamb body weight was recorded and ST muscle collected, weighed and stored at -80ºC for later analysis.

3.3.2 Free amino acid determination

Maternal and fetal plasma FAA were analysed using high-performance liquid chromatography (HPLC), as previously described by van der Linden *et al.* (2012). Free AA in muscle from P140 fetuses and lambs at weaning (singletons, Twin(age) and Twin(wt) lambs) were determined by the fluorometric HPLC method involving pre-column derivatisation. Approximately 150 mg of ST tissue from each animal was homogenised in 1.75 mL of Seraprep (Pickering Laboratories, Alphatech Systems Ltd, Auckland, New Zealand) and 20 µL of L-2-Amino-3-guanidinopropionic acid hydrochloride (25 µM/mL) (Calbiochem-Behring Corp., La Jolla, CA) was added as an internal standard. Samples were left in ice for 20 min, and then 40 µL of 5.88 M lithium (Li) hydroxide buffer (BDH Chemical, Poole, England) was added, followed by centrifugation at 8,000 x g for 10 min. After centrifugation, samples were filtered using a 0.45 µm cellulose acetate filter membrane (Advantec, Toyo Roshi, Tokyo, Japan). The supernatant was analysed for FAA using a Shimadzu LC10Ai HPLC (Shimadzu Oceania Ltd., Auckland, New Zealand), fitted with a high-efficiency Li-ion exchange column (3 mm ID x 150 mm; Pickering Laboratories, Shimadzu Oceania Ltd., Auckland, New Zealand) and a Pickering PCX 3100 post-column reaction module (Pickering Laboratories, Shimadzu Oceania Ltd, Auckland, New Zealand). Injected volumes were 10 µL, with a reagent flow rate of 0.3 mL/min and a run time of 162 min between injections, using Li buffers as eluants and ninhydrin post-column derivatisation. Detection was performed at 570 nm for all FAA, except proline which was read at 440 nm. Free AA in samples were quantified on the basis of known amounts of standards (Shimadzu Oceania, Pickering Laboratories, CA, USA), using LC Solution ver. 1.22 SP1 software (Shimadzu, Kyoto, Japan). Within and between assays variation was lower that 4% and 2%, respectively, for plasma and muscle assays.

3.3.3 Plasma metabolites

Blood samples were centrifuged to separate plasma (1,120 × g for 10 min at 4°C) and frozen at −80°C before analysis. Plasma was analysed for non-esterified fatty acids (NEFA), β-hydroxybutyrate (BHBA), glucose, and urea using a Hitachi Modular P800 analyser (Roche, Basel, Switzerland) at 30°C by Gribbles Veterinary Pathology Ltd. (Palmerston North, New Zealand), as previously described by Higgs et al (2013). The inter- and intra-assay coefficients of variation were <2% for all evaluated metabolites.

3.3.4 Biochemical indices: DNA, RNA and protein content

Total RNA, DNA and protein were extracted from 100 mg of frozen muscle tissue from each lamb at P140 and weaning, using Trizol reagent (Invitrogen, Auckland, New Zealand) according to the manufacturer's instructions. Extraction of RNA and DNA was performed in triplicate and quantified using a NanoDrop Spectrophotometer ND-1000 (Nanodrop Technologies, Wilmington, DE, USA) at wavelengths of 230, 260, and 280 nm. Purity of RNA and DNA was verified by measurement of optical density ratio 260/280 (>1.8) and 230/260 (>1.85) (Sambrook et al., 1989). The integrity of RNA was verified by running samples on 1.0% non-denaturing agarose gel electrophoresis, stained with SYBR® Safe (Invitrogen). Protein content was determined by the Bradford method (Bradford, 1976). Total RNA, DNA and protein content were determined by multiplying the concentration by total ST weight. The ratios of RNA to DNA, an indicator of protein production capacity, protein to DNA, a rough measure of the myonuclear domain, which is directly proportional to fibre cross-sectional area (Haddad et al., 2003) and the protein to RNA ratio, an indicator of protein production efficiency (Lobley, 1993) were determined.

3.4 STATISTICAL ANALYSIS

Body weight, ST weights, FAA concentration in maternal and fetal plasma, fetal and lamb muscle and DNA, RNA and protein content in muscle were analysed by ANOVA, using the MIXED procedure of SAS (Inst. Inc., Cary, NC, 2006). A linear model considering pregnancy rank (singleton and twin), sex of fetus and their interaction were used as fixed effects for P140 fetal body weight, birth weight, FAA concentration, fetal plasma to maternal plasma and muscle to fetal plasma FAA ratios. A linear model considering group (singleton, Twin(age) and Twin(wt)), sex of lamb and their interaction as fixed effects were used for weaning weight and plasma metabolites. Results for the sex of lamb effect are presented and discussed when significant.

Weight of the fetus at P140 and lamb weight at weaning were used as covariates to define the proportionality of the ST muscle in fetal and post-natal lamb, respectively, to body weight between groups. Change of lamb body weight of singleton and twin groups during the trial was analysed using repeated measurement analysis by ANOVA,

with the MIXED procedure of SAS (2006) using a linear model that included group and sex of lamb as fixed effects and birth weight and date of birth as covariates. For ADG, a linear model considering rank (singleton and twin), sex of lamb and their interaction for three periods. The three periods were birth to 28 days of life, which accounted for the pre-ruminant stage (Wardrop and Coombe, 1961), 29 days to 80, which corresponded to the time all lambs were raised together and 81 to 98 days, corresponded to the time only Twin(wt) lambs were maintained with the ewes before sampling. The relationship between muscle weight and FAA concentration in muscle at P140 and at weaning were described using partial correlations analysis, considering all animals after accounting for the effects of rank and sex of the lamb.

Means separation was performed with the PDiff option of the LSMeans statement in SAS. The assumption of normality of the data was tested by evaluating the normality of residuals using the Shapiro-Wilk's test (Proc Univariate, SAS, 2006). Concentrations of NEFA were not normally distributed, therefore data were $\log_{10}$ transformed. Back-transformed least-square means with the corresponding confidence intervals are presented. For other metabolites, least-square means are shown. Statistical significance was set at a probability value of $P \leq 0.05$ and a trend when $P \leq 0.10$. Because of the likelihood of generating type I errors in analysing the AA data, only values with $P \leq 0.05$ are discussed.

3.5 RESULTS

3.5.1 Body and muscle weight

Twins were lighter ($P < 0.05$) and had lower ST muscle weight compared to singles at P140 (Table 3.1). No sex of fetus effect or pregnancy rank by sex of fetus interaction for either body or fetal muscle weight was observed. The effect of litter size on ST muscle weight was proportional to fetal weight (Table 3.1).

At birth, twins were lighter ($P < 0.05$) than singletons (Table 3.1). Overall, males and females had a similar birth weight (4.9 ± 0.1 *vs.* 4.7 ± 0.1 kg, respectively, $P > 0.05$). At weaning, twins had lower live weight than singletons when compared at the same age. After adjusting for birth weight, twins had a lower ($P < 0.05$) body weight compared to singletons from day 26 until day 85 at euthanasia (Figure 3.1). Twins had lower ADG

from birth until day 28, and from day 29 to day 80 compared to singletons (Figure 3.2). The Twin(wt) group had a lower ADG from day 81 to day 98 compared to singletons, but was similar to their Twin(age) counterparts (Figure 3.2). At weaning, male lambs were heavier than females (33.9 ± 0.6 *vs.* 31.6 ± 0.7 kg, respectively, $P < 0.05$), and this difference was maintained after adjusting for birth weight (33.8 ± 0.6 *vs.* 31.8 ± 0.6 kg, respectively, $P < 0.05$). Twin(age) and Twin(wt) animals had proportionally lower ($P < 0.05$) ST muscle weight compared to singles (Table 3.1). Males had proportionally heavier ST muscle compared to females (98.5 ± 2.0 *vs.* 92.5 ± 2.3, respectively, $P < 0.05$). No group by sex of lamb interaction for ST muscle weight was observed.

3.5.2 Free AA Concentration

Day 140 pregnancy. No difference was observed in total or individual FAA concentration in the maternal plasma between twin- and singleton-bearing ewes (Table 3.2). In fetal plasma, twin fetuses had higher concentrations of glutamine, histidine and methionine and lower concentrations of aspartate, citrulline, glutamate and ornithine compared with singleton fetuses. Total plasma FAA concentration did not differ between twins and singletons (Table 3.2). In ST muscle, twin fetuses had lower intracellular concentration of aspartate and valine, and higher concentration of methionine compared with singles (Table 3.2). Twins had higher fetal to maternal plasma FAA ratios for histidine, but lower glutamate ratio compared with singletons (Table 3.3). In addition, twins had higher muscle to fetal plasma FAA concentration ratio for citrulline and ornithine, but lower histidine ratio compared to singletons.

Table 3.1. Effect of birth rank of the fetus/lamb on body (kg) and *M. semitendinosus* weight (ST; g) at 140 days o twin (n = 20) fetuses and at weaning, considering singleton (n = 20) and twin lambs (Twin(age), n = 17) at same a n = 17) compared to singletons.

	P140			Birth		
	Body weight (kg)	ST weight (g)		Weight (kg)	Body weight (kg	
		Unadjusted	Adjusted[1]		Unadjusted	Adju
Rank						
Singleton	6.1 ± 0.32[a]	11.0 ± 0.82[a]	9.7 ± 0.53	5.6 ± 0.20[a]	35.1 ± 0.73[a]	33.8 ±
Twin(age)	5.2 ± 0.18[b]	8.7 ± 0.46[b]	9.2 ± 0.29	4.4 ± 0.10[b]	29.6 ± 0.83[b]	30.8 ±
Twin(wt)					33.5 ± 0.79[a]	33.9 ±

Data are given as least square means and standard error (SE). [1]Adjusted by fetal weight as covariate; [2] Adjusted by birth as covariate.

.[a,b,c] Different superscripts within columns are significantly different ($P \leq 0.05$).

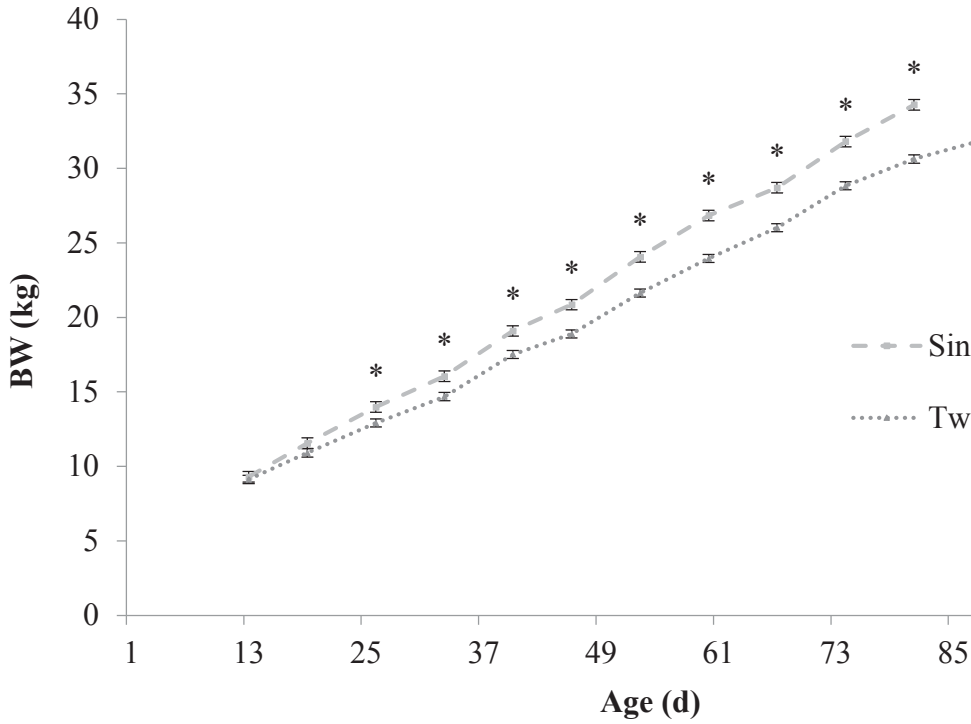

Figure 3.1. Pre-weaning live weight of singleton (n = 20) and twin lambs (n = 34) from 13 days after birth to slau
Data for twins at 81 days corresponds to all lambs, while at 98 days it represents the live weight of Twin(wt) (n = 1
means ± standard error of the mean (SEM). * $P \leq 0.05$.

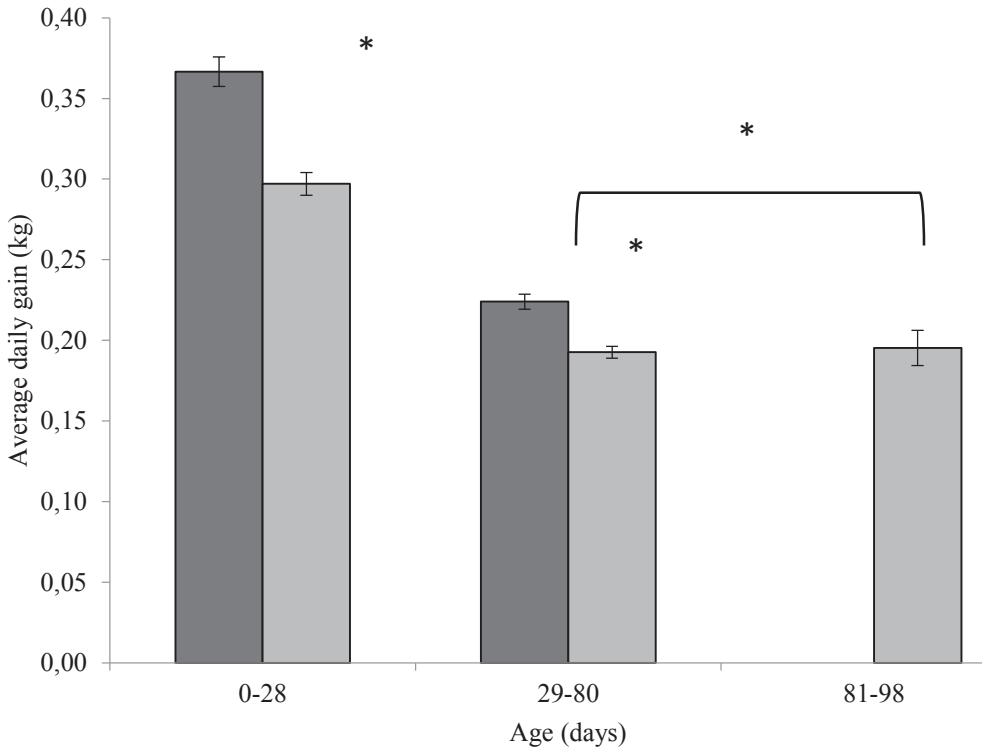

Figure 3.2. Average daily gain (kg) of singleton and twin lambs, adjusted by birth weight and age as covariates, f for the pre-ruminant stage [(1)], 29 days to 80, which corresponded to the time all lambs were raised together and Twin(wt) lambs were maintained with their dam before slaughter. Data for twins at 81 days corresponds to all lan daily gain of Twin(wt) lambs. Data are expressed as least squares means ± standard error of the mean (SEM). * A

Male fetuses had higher plasma concentration of histidine (131 ± 18 *vs.* 75 ± 7, *P* = 0.04) and proline (449 ± 38 *vs.* 277 ± 82, *P* = 0.05) and lower concentration of arginine (129 ± 11 *vs.* 190 ± 21, *P* = 0.02) and isoleucine (77 ± 5 *vs.* 100 ± 7, *P* = 0.03) compared with females. In ST muscle, males had higher concentrations of asparagine (68 ± 9 *vs.* 34 ± 5, *P* = 0.03) and citrulline (107 ± 14 *vs.* 77 ± 7, *P* = 0.05) compared with females. All other FAA concentration values were not significant between the sexes.

A positive correlation between maternal and fetal plasma FAA concentration in twins was observed for phenylalanine, valine and tyrosine, while a negative correlation was found for asparagine. A trend towards a positive correlation for lysine, threonine, cystine and ornithine and a negative trend for aspartate in twins was observed, while only a positive trend was observed in singletons for leucine and negative for methionine (Table 3.4).

A partial correlation analyses showed a positive association between muscle weight and intracellular concentration of arginine (r = 0.66, P < 0.01) and glutamine (r = 0.49, *P* < 0.01). In contrast, a negative correlation was observed with serine (r = -0.41, *P* = 0.03) concentration. No significant correlations were found for any other FAA with ST muscle weight (data not shown).

A positive correlation between fetal plasma and muscle FAA concentration was found for methionine, threonine, alanine and glycine in both singleton and twins. In singletons, citrulline was positively correlated between fetal plasma and muscle FAA concentration, while in twins, valine and serine showed a positive correlation. A trend for a positive correlation between fetal plasma and muscle FAA was found for glutamine and ornithine in singles and for arginine, histidine and glutamate in twins (Table 3.4).

Table 3.2. Free amino acid (AA) concentration (µMol/L) in maternal plasma, fetal plasma and intracellular fre *semitendinosus* from singleton and twin fetal lamb at 140 days of pregnancy. Values are expressed as mean ± sta

	Ewe plasma (µMol/L)			Fetal plasma (µMol/L)		
	Singleton	**Twin**	***P*-value**	**Singleton**	**Twin**	***P*-value**
n	**9**	**10**		**9**	**10**	
Essential AA						
Histidine	89 ± 6	86 ± 7	0.75	89 ± 9	140 ± 24	0.04
Isoleucine	114 ± 6	122 ± 11	0.56	83 ± 6	84 ± 7	0.97
Leucine	154 ± 14	157 ± 17	0.89	166 ± 16	150 ± 11	0.43
Lysine	221 ± 17	234 ± 22	0.66	164 ± 11	151 ± 9	0.34
Methionine	49 ± 2	55 ± 3	0.17	106 ± 10	153 ± 12	<0.01
Phenylalanine	80 ± 9	83 ± 10	0.85	116 ± 7	119 ± 9	0.83
Threonine	150 ± 22	167 ± 27	0.65	385 ± 29	362 ± 46	0.69
Tryptophan	50 ± 4	52 ± 9	0.85	N.D	N.D	
Valine	287 ± 32	292 ± 39	0.94	353 ± 19	316 ± 26	0.28
Non-essential AA						
Alanine	226 ± 8	227 ± 15.9	0.97	463 ± 32	457 ± 34	0.89
Arginine[1]	203 ± 16	199 ± 21	0.86	150 ± 21	140 ± 13	0.62
Asparagine	52 ± 14	56 ± 10	0.78	43 ± 4	45 ± 6	0.87
Aspartate	22 ± 3	24 ± 4	0.76	69 ± 4	59 ± 2	0.05
Carnosine	13 ± 1	11 ± 1	0.07	13 ± 2	11 ± 1	0.45
Citrulline	244 ± 24	210 ± 15	0.26	152 ± 16	112 ± 10	0.05
Cysteine	10 ± 3	6 ± 2	0.36	6 ± 1	4 ± 1	0.51
Glutamate	100 ± 16	130 ± 14	0.21	217 ± 31	127 ± 15	0.02
Glutamine	266 ± 12	291 ± 21	0.17	402 ± 26	489 ± 30	0.04
Glycine	544 ± 44	575 ± 34	0.56	715 ± 100	732 ± 60	0.88
Ornithine	116 ± 9	95 ± 10	0.17	169 ± 18	122 ± 10	0.04
Proline	330 ± 33	343 ± 23	0.70	393 ± 54	457 ± 42	0.36
Serine	114 ± 28	120 ± 15	0.83	495 ± 67	530 ± 55	0.66
Taurine	30 ± 3	25 ± 2	0.18	34 ± 4	25 ± 2	0.09
Tyrosine	101 ± 7	102 ± 9	0.94	153 ± 14	162 ± 15	0.69
Total	3514 ± 189	3608 ± 187	0.74	4907 ± 213	4902 ± 182	0.98

[1]Deemed as conditionally essential for fetal growth (Wu, 2009).
N.D.; Not detected.

Table 3.3. Ratio of fetal to maternal plasma amino acid (AA), and *M. semitendinosus* to fetal plasma AA concentration for singleton and twin fetuses at 140 days pregnancy.

	Fetal:Ewe Ratio			Muscle:Fetal Ratio		
	Singleton	**Twin**	***P*-value**	**Singleton**	**Twin**	***P*-value**
Essential						
Histidine	0.97 ± 0.22	1.44 ± 0.19	0.03	4.25 ± 0.57	2.52 ± 0.50	0.05
Isoleucine	0.77 ± 0.07	0.79 ± 0.06	0.86	3.37 ± 1.14	2.75 ± 0.78	0.53
Leucine	1.11 ± 0.14	1.06 ± 0.12	0.67	0.23 ± 0.04	0.19 ± 0.04	0.56
Lysine	0.76 ± 0.08	0.68 ± 0.07	0.26	0.65 ± 0.10	0.74 ± 0.08	0.12
Methionine	2.09 ± 0.29	2.67 ± 0.26	0.19	0.64 ± 0.06	0.75 ± 0.05	0.25
Phenylalanine	1.60 ± 0.18	1.54 ± 0.15	0.92	0.40 ± 0.06	0.37 ± 0.05	0.67
Threonine	2.89 ± 0.51	2.25 ± 0.44	0.42	2.67 ± 0.17	2.38 ± 0.16	0.24
Valine	1.44 ± 0.10	1.18 ± 0.09	0.20	0.41 ± 0.05	0.39 ± 0.05	0.49
Non-essential						
Alanine	2.03 ± 0.20	1.93 ± 1.20	0.84	4.11 ± 0.34	3.84 ± 0.29	0.67
Arginine[1]	0.89 ± 0.13	0.79 ± 0.10	0.76	2.14 ± 0.42	1.97 ± 0.36	0.39
Asparagine	1.09 ± 0.40	0.99 ± 0.40	0.92	1.20 ± 0.26	1.00 ± 0.26	0.25
Aspartate	3.58 ± 0.60	3.03 ± 0.50	0.53	6.56 ± 0.79	6.27 ± 0.68	0.17
Carnosine	1.13 ± 0.30	1.05 ± 0.20	0.75	4.91 ± 1.27	4.93 ± 0.83	0.38
Citrulline	0.62 ± 0.09	0.58 ± 0.08	0.23	0.53 ± 0.21	1.13 ± 0.18	0.01
Cystine	0.78 ± 0.16	0.50 ± 0.11	0.11	1.76 ± 1.33	3.18 ± 1.41	0.34
Glutamate	3.05 ± 0.52	1.17 ± 0.44	0.03	6.85 ± 2.36	10.04 ± 2.03	0.46
Glutamine	1.47 ± 0.18	1.60 ± 0.15	0.62	8.17 ± 0.66	7.59 ± 0.57	0.43
Glycine	1.14 ± 0.18	1.23 ± 0.16	0.74	2.54 ± 0.17	2.56 ± 0.14	0.88
Ornithine	1.43 ± 0.22	1.38 ± 0.22	0.39	0.43 ± 0.09	0.66 ± 0.07	0.01
Proline	1.10 ± 0.28	1.09 ± 0.24	0.77	0.98 ± 0.11	0.72 ± 0.12	0.31
Serine	5.74 ± 1.47	4.50 ± 1.26	0.65	2.44 ± 0.65	2.03 ± 0.59	0.53
Taurine	1.02 ± 0.23	1.12 ± 0.17	0.61	181.47 ± 25.02	185.32 ± 25.02	0.66
Tyrosine	1.64 ± 0.21	1.63 ± 0.19	0.98	0.50 ± 0.08	0.40 ± 0.07	0.31
Total	1.38 ± 0.12	1.33 ± 0.10	0.72	3.71 ± 0.30	3.56 ± 0.26	0.50

[1]Deemed as conditionally essential for fetal growth (Wu, 2009).

Table 3.4. Partial correlation between fetal plasma and *M. semitendinosus* free amino acid concentration for singleton and twin fetuses at 140 days pregnancy.

	Maternal: fetal plasma ratio				Fetal plasma:muscle ratio			
	Singleton		Twin		Singleton		Twin	
	r	*P*-value	r	*P*-value	r	*P*-value	r	*P*-value
Essential								
Histidine	0.08	0.85	0.49	0.15	-0.38	0.32	0.61	0.08
Isoleucine	0.46	0.25	0.26	0.46	0.16	0.71	0.21	0.61
Leucine	0.64	0.09	0.32	0.37	-0.07	0.86	0.40	0.38
Lysine	0.09	0.82	0.57	0.08	0.42	0.26	0.19	0.59
Methionine	-0.60	0.09	0.01	0.99	0.85	<0.01	0.75	0.01
Phenylalanine	0.02	0.97	0.82	0.01	-0.28	0.51	0.17	0.65
Threonine	-0.37	0.33	0.56	0.10	0.73	0.03	0.82	0.01
Valine	0.28	0.46	0.78	0.01	-0.05	0.91	0.91	<0.01
Non-essential								
Alanine	0.29	0.44	-0.07	0.85	0.79	0.01	0.70	0.02
Arginine[1]	-0.22	0.57	0.22	0.55	0.16	0.68	0.58	0.08
Asparagine	-0.19	0.62	-0.67	0.05	0.26	0.58	0.31	0.50
Aspartate	-0.26	0.50	-0.61	0.06	0.14	0.71	0.18	0.61
Carnosine	-0.43	0.33	-0.39	0.34	0.29	0.53	-0.24	0.54
Citrulline	-0.02	0.96	0.22	0.54	0.87	<0.01	-0.36	0.34
Cystine	-0.92	0.25	0.76	0.08	-0.71	0.29	-0.77	0.44
Glutamate	-0.21	0.60	-0.42	0.23	-0.07	0.85	0.60	0.07
Glutamine	-0.56	0.12	0.44	0.20	0.65	0.06	0.28	0.43
Glycine	0.12	0.77	0.40	0.26	0.83	0.01	0.86	<0.01
Ornithine	-0.19	0.62	0.61	0.08	0.63	0.09	0.40	0.25
Proline	-0.27	0.48	0.09	0.80	-0.34	0.37	0.53	0.18
Serine	-0.09	0.81	-0.43	0.22	0.37	0.33	0.89	<0.01
Taurine	0.19	0.76	-0.02	0.96	0.13	0.77	-0.19	0.66
Tyrosine	-0.52	0.18	0.82	0.01	0.11	0.79	0.52	0.12

[1]Deemed as conditionally essential for fetal growth (Wu, 2009).

Weaning. Twins had higher intracellular concentration of glutamine in ST muscle compared with singletons, while no difference was observed between the two twin groups (Table 3.5). Concentration of histidine in muscle was higher in Twin(age) compared with singletons and Twin(wt) animals. Threonine concentration was higher in Twin(age) animals than in singletons ($P < 0.05$), which had a higher threonine concentration than Twin(age) animals ($P < 0.05$). Twin(age) animals had higher concentration of asparagine compared with singletons and Twin(wt) animals (Table 3.5). Twins from both groups had higher concentration of alanine and reduced concentration of taurine compared to singletons (Table 3.5). Higher concentration of serine was found for the Twin(age) group compared with the other two groups. Increased concentration of glutamate was found in Twin(age) animals compared with singletons and Twin(wt) animals. Males had higher muscle concentrations of arginine, glutamine and alanine compared with females (Table 3.5). In contrast, males had reduced concentrations of isoleucine, leucine valine and taurine compared with females.

Partial correlation analysis showed a positive association between ST muscle weight and intracellular concentration of leucine (r = 0.3, $P = 0.02$), lysine (r = 0.3, $P = 0.01$), methionine (r = 0.4, $P < 0.01$), phenylalanine (r = 0.3, $P = 0.02$), proline (r = 0.3, $P = 0.05$), threonine (r = 0.4, $P < 0.01$), tyrosine (r = 0.4, $P < 0.01$) and total FAA concentration (r = 0.3, $P = 0.03$). The remaining correlations between FAA and ST muscle weight were not significant (see Appendix A: Table A.1).

Table 3.5. Intracellular free amino acid concentration (nMol/g) in *M. semitendinosus* at weaning for singlet (Twin(age)) at the same age (85 days of life) and the other twin lamb at same weight (Twin(wt), 98 days of life). V of mean (SEM). The effects of treatment and sex of lamb are shown.

	Treatment				S
	Singleton	**Twin(age)**	**Twin(wt)**	***P*-value**	**Male**
n	**20**	**17**	**17**		**30**
Essential					
Histidine	92 ± 5[a]	120 ± 8[b]	90 ± 6[a]	<0.01	107 ± 6
Isoleucine	42 ± 1[a]	43 ± 1[a,b]	45 ± 1[b]	0.07	42 ± 1
Leucine	81 ± 3	80 ± 2	79 ± 3	0.94	76 ± 2
Lysine	69 ± 3	71 ± 3	68 ± 2	0.73	71 ± 2
Methionine	22 ± 1	21 ± 1	21 ± 1	0.98	21 ± 1
Phenylalanine	38 ± 1	40 ± 1	40 ± 1	0.29	39 ± 1
Threonine	194 ± 12[a]	226 ± 11[b]	151 ± 11[c]	<0.01	194 ± 10
Valine	140 ± 7	137 ± 4	129 ± 5	0.35	129 ± 4
Non-essential					
Alanine	1654 ± 78[a]	1958 ± 91[b]	1894 ± 75[b]	0.02	1921 ± 6
Arginine[1]	105 ± 6	102 ± 6	101 ± 7	0.86	113 ± 5
Asparagine	19 ± 4[a]	34 ± 5[b]	25 ± 3[a]	0.03	27 ± 4
Aspartate	424 ± 13	398 ± 25	403 ± 20	0.59	398 ± 20
Carnosine	4118 ± 123	4423 ± 133	4174 ± 167	0.28	4303 ± 11
Glutamate	74 ± 5[a]	96 ± 5[b]	85 ± 6[a]	0.04	82 ± 5
Glutamine	1385 ± 121[a]	1886 ± 155[b]	1846 ± 192[b]	0.04	1895 ± 13
Glycine	732 ± 46[a,b]	844 ± 46[a]	697 ± 33[b]	0.06	790 ± 38
Ornithine	41 ± 2	44 ± 3	41 ± 2	0.60	43 ± 2
Proline	103 ± 9[a,b]	134 ± 19[a]	83 ± 16[b]	0.06	106 ± 14
Serine	92 ± 7[a]	122 ± 7[b]	87 ± 9[a]	0.01	103 ± 7
Taurine	7177 ± 529[a]	5705 ± 433[b]	5400 ± 464[b]	0.01	5562 ± 32
Tyrosine	50 ± 2	53 ± 2	52 ± 2	0.76	51 ± 2
Total	16411 ± 386[a]	15928 ± 534[a,b]	15082 ± 378[b]	0.09	15545 ± 3

[a,b,c] Different superscripts between columns for each factor are significantly different ($P \leq 0.05$).

[1]Deemed as conditionally essential in growing animals (Wu, 2009).

3.5.3 Biochemical indices: DNA, RNA and protein content

At P140, twins had higher DNA concentration and tended to have lower RNA concentration compared to singletons (Table 3.6). Total content of DNA in the ST muscle was similar between singleton and twin fetuses. In contrast, total RNA was lower in twin fetuses and twins tended to have lower total protein content. Twins had lower RNA:DNA ratio compared to singletons at P140.

At weaning, DNA and RNA concentration and total DNA were similar between all three groups (Table 3.6). Protein concentration was higher in the Twin(wt) group compared to the other two groups. Singleton has greater RNA content compared to Twin(age) animals with Twin(wt) intermediate. Total protein content of the ST muscle was lower in the Twin(age) group compared to singleton and Twin(wt) animals. No difference was observed either for RNA:DNA or Protein:DNA ratios between birth ranks. Twin(wt) had a greater Protein:RNA ratio compared to singleton and Twin(age), while Twin(age) had lower Protein:RNA ratio compared to singleton. Males had higher total RNA content compared to females (35.0 ± 0.7 *vs.* 32.5 ± 0.9 respectively, $P = 0.04$).

Table 3.6. Biochemical indices (DNA, RNA, protein and ratios) of *M. semitendinosus* in singleton and twin fetuses considering singleton and one of twin lamb of a twin-pair (Twin(age)) at same the age and the other twin lamb singletons. Data are presented as least square means ± standard error of the mean (SEM).

	P140				
	Singleton	**Twin**	***P*-value**	**Singleton**	**Twin**
n	**9**	**10**		**20**	1
Concentration					
DNA (mg/g ST)	1.1 ± 0.05	1.3 ± 0.06	0.04	0.2 ± 0.03	0.3 ±
RNA (mg/g ST)	1.3 ± 0.05	1.2 ± 0.03	0.08	0.3 ± 0.01	0.4 ±
Protein (mg/g ST)	41.2 ± 2.86	44.5 ± 1.60	0.61	20.3 ± 0.85[a]	18.0 ±
Total					
DNA (mg)	12.4 ± 1.07	11.2 ± 0.60	0.31	26.0 ± 3.10	20.7 ±
RNA (mg)	14.1 ± 0.99	10.3 ± 0.44	<0.01	35.3 ± 1.30[a]	30.3 ±
Protein (mg)	448.3 ± 33.72	391.9 ± 25.64	0.09	2106 ± 119[a]	1590
Ratios					
RNA:DNA	1.1 ± 0.09	0.9 ± 0.05	0.02	2.4 ± 0.40	2.1 ±
Protein:DNA	36.6 ± 3.81	35.5 ± 2.13	0.37	120.7 ± 17.20	95.8 ±
Protein:RNA	32.0 ± 2.97	38.4 ± 1.66	0.14	59.9 ± 2.51[a]	52.5 ±

[a,b,c] Different superscripts between columns for each factor are significantly different ($P \leq 0.05$).

3.5.4 Plasma metabolites

Plasma metabolites did not differ between birth ranks for BHBA, glucose or urea (Table 3.7), with the exception of a trend ($P = 0.09$) for singleton and Twin(age) animals to have higher NEFA compared with Twin(wt). Females had lower plasma concentration of BHBA, compared with males at weaning (0.21 ± 0.01 *vs.* 0.27 ± 0.01, $P < 0.01$) while no other sex effect was observed (data not shown).

Table 3.7. Blood β-hydroxybutyrate (BHBA), glucose, non-esterified fatty acids (NEFA) and urea concentration for twin lambs at the same age (Twin(age), 85 days of life) or weight (Twin(wt), 98 days of life) compared to singletons. Least square means ± standard error of mean (SEM) are presented. Back transformed values and CI (95%) are shown for NEFA.

	Singleton	Twin(age)	Twin(wt)	*P*-value
n	20	17	17	
BHBA , mmol/L	0.25 ± 0.01	0.24 ± 0.01	0.23 ± 0.01	0.79
Glucose mmol/L	5.14 ± 0.09	5.23 ± 0.09	5.04 ± 0.09	0.34
NEFA, mmol/L	0.11 (0.08-0.16)[a]	0.11 (0.08-0.16) [a]	0.04 (0.03-0.06) [b]	0.09
Urea, mmol/L	5.79 ± 0.28	5.22 ± 0.32	6.18 ± 0.31	0.13

[a,b]Different superscript $P < 0.10$.

3.6 DISCUSSION

Reduced skeletal muscle mass in twin lambs compared to singletons arises during late pregnancy (McCoard et al., 2000a) and tends to continue post-natally (Bennett et al., 1991; McCoard et al., 2010). In the current study, reduced body weight and ST muscle mass in late pregnancy in twins compared to singletons, were both still present at weaning at the same age. The decreased post-natal ADG was consistent with this reduced body weight and ST muscle mass in twins, which was in agreement with findings of McCoard et al (2010). Reduced live weight at weaning was also associated with decreased muscle mass, which was still evident when twin lambs were grown to the same live weight as their singleton counterparts. These results highlight that despite the high plane of maternal nutrition, skeletal muscle growth is compromised in twins during pregnancy. The events occurring during fetal life regulate, at least in part, post-natal muscle growth capacity, constraining twin lamb performance. This is supported by the ADG observed in Twin(wt) lambs which was not ameliorated after increasing nutrient availability between 81 and 98 days, and are consistent with findings by Freetly and Leymaster (2004).

Changes in the concentration of intracellular FAA modulate the activation of specific signalling pathways which regulate protein synthesis (e.g. mTOR) (Beugnet et al., 2003; Wullschleger et al., 2006; Sancak et al., 2008). It was therefore hypothesized that reduced muscle growth in twins compared to singletons is associated with decreased intracellular concentration of FAA, especially those associated with mTOR activation (e.g. leucine, arginine and glutamine (Wu, 2009). At P140, twins had reduced ST muscle concentrations of valine and aspartate, but a higher concentration of methionine compared to singletons. Valine has been associated with activation of downstream targets of mTOR (Xu et al., 2001). However, little effect of valine on mTOR activation compared to other FAA, such as leucine, was found in rats (Anthony et al., 2000). Lower concentrations of valine and aspartate in twin fetal muscle could also be associated with glutamine synthesis (Chang and Goldberg, 1978). Valine, together with leucine and isoleucine, are generally metabolised to generate alanine and glutamine under maternal nutrient restriction, which contribute as net gluconeogenic precursors in the liver (Liechty et al., 1987b). In the present study, muscle leucine and isoleucine concentrations did not differ between singletons and twins. This could suggest that utilisation of BCAA depends on the level of nutrient restriction. Valine is the only glucogenic BCAA (Teleni et al., 1986) and might have been the first BCAA to be used as a glucogenic precursor which may explain in part the reduced concentration of valine in twins. The increase in methionine in plasma and muscle of twins is in agreement with our previous study (Sales et al., 2013; Chapter 2). The increased plasma methionine concentration could be associated with reduced protein synthesis, as methionine plays a major role in the initiation of protein accretion (Lucas-Lenard et al. 1971) or high protein breakdown (Millward, 1970; Millward et al., 1974). A higher methionine concentration is consistent with the observed reduction in muscle mass. In this study, changes in specific muscle intracellular FAA concentrations have been observed between singletons and twins. However, there is not a link between these AA and the signalling cascade in the literature. The results suggest that other factors in addition to changes in intracellular FAA concentration must determine the difference in muscle mass between singletons and twin fetuses under a well-fed maternal regime.

A positive correlation was found between fetal muscle weight with both arginine and glutamine concentrations in muscle. The positive relationship between fetal muscle mass and intracellular concentration of arginine in the muscle was previously described in sheep in response to different planes of maternal nutrition (Sales et al., 2013; Chapter

2). However, the association with glutamine is a novel finding. Both glutamine (Neu, 2001) and arginine (Wu et al., 2000) play a key role in fetal development. Glutamine transport in the cell has a role in mTOR activation (Nicklin et al., 2009). Arginine is involved in the formation of nitric oxide, polyamine, proteins, participates in the urea cycle (Wu and Morris Jr, 1998), and is a regulator of muscle growth in non-ruminants by acting as an activator of mTOR signalling (Yao et al. 2008). The positive association at P140 of arginine and glutamine concentrations in muscle with muscle mass, reinforces the notion of the importance of both AA for fetal muscle growth.

The relationship between muscle mass and intracellular FAA at weaning contrasts with the observations at P140. At weaning, Twin(age) lambs had greater intracellular concentrations of glutamine, histidine, threonine, alanine, asparagine, glutamate, serine and taurine, compared to singletons, whilst Twin(wt) lambs displayed a FAA pattern in the muscle closer to that observed in singletons. Glutamine, glutamate, serine and alanine concentrations, which are glucogenic AA, in muscle were higher in Twin(age) lambs. These glucogenic AA are released under nutritional restriction (Wolff and Bergman, 1972; Heitmann and Bergman, 1980), which may indicate protein degradation in Twin(age) lambs to cover body energy demands. This is consistent with the reduced protein synthetic efficiency and reduced total protein in muscle in this study. In contrast, increased nutrient intake in Twin(wt) lambs, as suggested by reduced concentration of NEFA (Annison, 1960), may explain the decrease in intracellular concentration of histidine, threonine, asparagine, glutamate and serine, to support growth, compared with Twin(age) lambs. This is supported by the increased concentration of protein and higher muscle protein synthetic efficiency in Twin(wt) lambs. After accounting for rank and sex of lambs in the weaning groups, muscle weight was positively correlated with the EAA leucine, lysine, methionine, phenylalanine and threonine and the non-essential AA (NEAA) proline and tyrosine. Methionine, lysine, phenylalanine and threonine are limiting AA in the diet of sheep under different feeding regimens (Nimrick et al., 1970; Potter et al., 1972; Storm and Orskov, 1984; van E. Nolte et al., 2008). This suggests that muscle growth is associated with the level of the limiting FAA in muscle. The differences in terms of correlations between muscle weight and plasma AA at P140 and at weaning suggests that the relevance of FAA for muscle hypertrophy may differ according to development stage of the animal. Thus, arginine and glutamine may be a key regulator of

muscle growth in singleton and twin fetuses in late pregnancy, while leucine, lysine, methionine, phenylalanine and threonine may be more important near weaning.

The capacity of muscle to growth is associated with DNA and RNA content (Greenwood et al., 1999). In the present study we found that twin fetuses had lower total abundance of RNA in muscle and reduced RNA:DNA ratio, suggesting lower capacity for protein accretion. Twin(age) lambs had reduced content of RNA and a lower Protein:RNA ratio compared to singletons at weaning, which resulted in a lower muscle protein content, indicating a reduced efficiency of protein formation. In contrast, Twin(wt) lambs had similar protein and RNA concentration but a higher Protein:RNA ratio compared to singletons, indicating a higher protein synthetic efficiency. Increased protein synthetic efficiency in Twin(wt) lambs may be due to a higher food intake after removal of one twin, as shown to occur in bull calves after re-alimentation (Therkildsen, 2005). However, muscle growth was only partly recovered despite having the same RNA content, which represents ribosome number (Nader et al., 2005) as singletons. Therefore, muscle growth remained compromised in twin lambs after birth or a longer period would be needed to observe full growth recovery. The results from this study agree with previous reports which have shown that total DNA, RNA, protein and Protein:DNA ratio were lower in the muscle of twin compared to singleton fetuses during pregnancy (Rattray et al., 1975) and in lambs after birth (Greenwood et al., 2000a). This study reinforces the notion that capacity for protein synthesis depends on the regulation of ribosomal production and muscle ribosome concentration (Davis and Fiorotto, 2009). Therefore, intervention strategies to ameliorate reduced muscle mass in twins could be effective during pregnancy, to influence ribosome number and/or efficiency of protein synthesis.

A better understanding of the association of FAA profiles between the three compartments (maternal→fetal→muscle) is important when establishing strategic interventions leading to increased fetal muscle growth. Despite the lack of differences in plasma FAA concentrations between singleton- and twin-bearing ewes, twin fetuses had lower plasma concentrations of aspartate, glutamate, citrulline, and ornithine but, greater plasma concentration of glutamine, histidine and methionine compared with singleton fetuses. These observations suggest that placental AA exchange and/or fetal or placental metabolism differ between singleton and twin pregnancies. Differences in plasma FAA between singleton and twin fetuses in the present study contrast with a previous report, where twin fetuses had reduced histidine, arginine, leucine and glutamine compared to

singletons (van der Linden et al., 2012). There were two main differences between both studies. Firstly, plane of maternal nutrition, as ewes in the present study were offered twice the pasture dry matter as in the aforementioned study; secondly, the breed of the ewes, Rissington Breedline Primera® in the present study versus Romney in van der Linden et al. (2012). Both maternal nutrition and ewe breed are known to influence fetal AA plasma concentration (Kwon et al., 2004; Ashworth et al., 2011). In the present study, all FAA with lower plasma concentrations in twin fetuses compared to singletons are intermediates in the formation of arginine (Wu and Morris Jr, 1998), reinforcing the notion of the correlation of arginine with muscle weight found in the present study. The mechanisms associated with differences in plasma FAA between singletons and twins are however not clear and deserve further investigation.

Differences observed between sexes at weaning, for specific intracellular FAA in muscle, is a novel finding in this study. The increased concentration of the BCAAs, isoleucine, leucine and valine, in females may indicate an increased uptake of these BCAA to generate glutamine and alanine (Teleni et al., 1986), which is supported by the reduced concentration of both glutamine and alanine in female muscle compared to male muscle. In contrast, the lower concentration of arginine in female muscle is noteworthy when considering the large number of functions associated with this AA, such as synthesis of proteins, nitric oxide, urea, proline, glutamate, polyamines, creatine and agmatine (Wu and Morris Jr, 1998; Wu, 2009; Wu et al., 2009). Changes in FAA concentrations, coupled with reduced ST weight in females compared to males suggest that arginine and glutamine may have a role in the regulation of muscle growth in females, and suggest differences between sexes in terms of the relevance of specific FAA for muscle growth until weaning.

3.7 CONCLUSIONS

Under an unrestricted maternal pasture nutrient regime, specific plasma and muscle FAA differ between twin and singleton offspring. The results suggest that the relevance of specific FAA in the regulation of muscle growth may vary according to stage of development. While arginine and glutamine may play important roles in the regulation of muscle growth during pregnancy, FAA other than Arg and glutamine appear as relevant at weaning. Differences between males and females in the concentration of

specific FAA in muscle at weaning, give a new insight of possible factors explaining variations in muscle growth between sexes. Further studies are warranted to evaluate the mechanisms by which specific FAA may regulate muscle growth in ruminants.

Chapter 4: Effect of maternal parenteral arginine supplementation during mid-late pregnancy on twin fetal muscle mass in late pregnancy and post-weaning

General overview of the chapter:

Research presented in Chapter 2 and Chapter 3 identified an association between arginine and muscle mass. In addition, Research in Chapter 2 showed twin fetuses had reduced concentration of arginine (Arg) in muscle compared to singletons in late pregnancy. This suggested arginine could be associated with the reduced muscle mass observed in twin fetuses. The aim of this chapter was to establish if parenteral Arg supplementation of well-fed twin-bearing ewes from 100 to 140 days of pregnancy improves fetal skeletal muscle growth through the activation of mTOR and to determine the post-natal effect of maternal Arg supplementation on muscle growth.

Chapter 4 has been partially published in: McCoard, S., Sales, F., Wards, N., Sciascia, Q., Oliver, M., Koolaard, J., & van der Linden, D. (2013). Parenteral administration of twin-bearing ewes with L-arginine enhances the birth weight and brown fat stores in sheep. *SpringerPlus*, *2*(1), 1-12.

4.1 ABSTRACT

The aims of this study were firstly, to determine if parenteral arginine (Arg) supplementation of well-fed twin-bearing ewes from 100 to 140 days of pregnancy improves fetal skeletal muscle growth through the activation of mTOR. Secondly, to determine the effect of maternal Arg supplementation on lamb muscle growth from birth to 153 days of life (PN153). Twin-bearing ewes fed 100% of NRC-recommended nutrient requirements were supplemented with an *i.v.* bolus of either 345 µmol Arg-HCl/kg body weight or saline solution (control) 3 times a day. At 140 days of pregnancy (P140), 11 control and 9 Arg-treated ewes were euthanised. Maternal and fetal plasma samples were obtained and maternal and fetal plasma concentrations of amino acids (AA), hormones and metabolites were evaluated. Fetal weight and sex were recorded, and *semitendinosus* (ST), *longissimus dorsi* (LD), *biceps femoris, gastrocnemius, plantaris, semimembranosus, adductor, gluteus, quadriceps* and *psoas major* muscles were excised and weighed. A sample from LD was snap frozen for later analysis of FAA concentration, mTOR abundance and phosphorylation and biochemical indices (DNA, RNA and protein content). The remaining ewes 25 (Arg n = 13; Control n = 12) were allowed to lamb. Arginine supplementation was continued until term of pregnancy. Lambs were raised indoors with their dam for 14 days on lactation only, with concentrate feeding followed by a transition to a pasture-only grazing regime. Lambs were weaned at PN82 and maintained on grazing until PN153, when a subset of 20 lambs (n = 10 per group) was euthanised. At P140 there was no difference in fetal weight. Only *psoas major* was heavier in the Arg supplemented group compared to the control group. Females from Arg-treated ewes (Arg-F) had increased abundance of mTOR, RNA concentration and RNA: DNA ratio in LD, compared to Con-F while males did not differ. At birth, Arg-F were heavier than control females (Con-F), while males did not differ. At PN153, Arg-F were heavier than Con-F, had heavier *LD*, *plantaris* and a trend for heavier *psoas major* muscles compared to Con-F, whereas the weight of these muscles did not differ in response to treatment in male lambs. Lambs from Arg-treated ewes had heavier *semimembranosus* and tended to have heavier *biceps femoris* compared to control lambs. The RNA concentration in LD was greater in Arg-F compared to Con-F and DNA concentration was greater in Arg compared to control group. This study revealed that maternal Arg supplementation increased indices of muscle protein synthesis capacity at P140, and this could have potentially contributed to a heavier birth weight and improved

post-weaning muscle growth, but only in female lambs. These effects were associated with an increased abundance of mTOR at P140 in Arg-F compared with Con-F. The potential for Arg to improve birth weight and muscle growth in females could have important implications for sheep production.

4.2 INTRODUCTION

Reduced muscle mass observed in twin sheep fetuses in late pregnancy (McCoard et al., 1997; McCoard et al., 2000a; McCoard et al., 2001; Sales et al., 2013: Chapter 2), may impact negatively not only on vital animal functions at birth (Tygesen and Harrison, 2005), but also body composition and meat quality later in life (Rehfeldt et al., 2011). In well-fed pregnant ewes, decreased fetal muscle mass in twins compared to singletons results from reduced hypertrophy and not from a reduction in the number of fibres (McCoard et al., 1997; Greenwood et al., 2000a). Reduced fibre hypertrophy results from an inadequate supply of nutrients to the growing fetus (Gootwine et al., 2007). The reduced muscle mass in twins compared to singletons reported in Chapter 2 was observed regardless of the plane of maternal nutrition, and was associated with reduced concentration of specific intracellular free amino acids (FAA) concentrations, in particular, arginine (Arg). In addition, research presented in Chapter 2 and Chapter 3 showed a correlation between muscle mass and intracellular concentration of Arg in the muscle. These observations suggest Arg may play an important role in muscle hypertrophy in twin sheep fetuses.

Arginine is a nutritionally essential AA for fetal and neonatal growth (Wu et al., 2004), and plays multiple roles in animal metabolism (Wu et al., 2009). Arginine stimulates the secretion of insulin (Fowden, 1980; Thureen and Baron, 2002) and growth hormone (Merimee et al., 1967). Arginine increases muscle protein synthesis in neonatal pigs through the activation of the mechanistic target of rapamycin (mTOR) signalling pathway (Yao et al., 2008). mTOR is a cell nutrient sensor which regulates skeletal muscle protein accretion (Kim et al., 2002), in response to AA, hormones (e.g. insulin), and energy (Wang and Proud, 2006). There is growing evidence that Arg, especially in growing pigs, increases muscle growth (Yao et al., 2008; He et al., 2009; Tan et al., 2009). In sheep, a restricted plane of maternal nutrition during late pregnancy has been shown to reduce maternal and fetal plasma concentrations of Arg-family AA and fetal weight

(Kwon et al., 2004). Maternal Arg supplementation increases the birth weight of quadruplets but not triplet or twin sheep fetuses, when supplementation occurs from day 100 to 121 of pregnancy (Lassala et al., 2011). However, fetal muscle weight diverges between singletons and twins from day 115-120 of pregnancy (McCoard et al., 2000a). Therefore, is feasible that Arg supplementation in the study by Lassala et al. (2011) did not cover the time of highest fetal nutrient demand, and thus resulted in the lack of effect on twin growth. In contrast, parenteral supplementation with Arg in undernourished single-bearing ewes from day 60 of pregnancy to birth increases singleton birth weight (Lassala et al., 2010). These results suggest that Arg supplementation may have a positive effect on fetal growth in a crowded uterine environment or when maternal nutrition is limiting. However, the potential for maternal Arg supplementation to increase fetal and muscle growth when ewes are well fed and from 100 days pregnancy to birth and the possible mechanisms involved are unknown. Furthermore, it is not clear what the long-term effects of Arg supplementation during mid-late pregnancy are on post-natal muscle growth.

The aims of this study were to (1) determine if parenteral Arg supplementation of well-fed twin-bearing ewes from 100 to 140 days of pregnancy improves fetal skeletal muscle growth through the activation of mTOR and (2) to determine the post-natal effect of maternal Arg supplementation on lamb muscle growth at 153 days of life (near market weight). We hypothesized that maternal Arg supplementation during mid-late pregnancy would increase fetal muscle growth in late pregnancy, at least in part by enhancing muscle hypertrophy, through the activation of mTOR. Further, that post-natal skeletal muscle growth would be improved in lambs from Arg-supplemented ewes, resulting in lambs with heavier muscles compared to untreated counterparts at 153 days of life.

4.3 MATERIALS AND METHODS

This study and all animal handling procedures were approved by the University of Auckland Animal Ethics Committee, New Zealand in accordance with the 1999 Animal Welfare Act (C889).

4.3.1 Animals

Multiparous Romney ewes, with a live weight of 65-75 kg and a body condition score of 3-3.5, using a 1-5 scale (Jefferies, 1961) were synchronised with an intravaginal progesterone-containing device (CIDRs; Pharmacia and Upjohn Ltd. Co., Auckland, New Zealand) and naturally mated to Poll Dorset sires at a ratio of one ram per 10 ewes over 2 days in 2 different cohorts of 80-90 ewes. The same Polled Dorset rams were used for each breeding cohort. Ewes in cohort 1 were used for measurements during pregnancy and were mated in two sub-groups 3 weeks apart (Figure 4.1). Both sub-groups were euthanised at 140 days of pregnancy (P140). Ewes in cohort 2 were mated one month apart from Cohort 1 and allowed to lamb. At approximately P60, ewes were pregnancy scanned via transabdominal ultrasonography. Forty nine twin-bearing ewes were selected and maintained under *ad libitum* grazing conditions. From P70, all ewes were exposed to a lucerne-based pellet diet (University B mix, Camtech Nutrition, Cambridge, NZ) by providing up to 20% of daily requirements while grazed on pasture. At P80, ewes were housed indoors and acclimatised for one week in group pens and then in individual pens with open mesh sides allowing visualisation of other animals. Ewes were fed *ad libitum* with the same concentrate diet used during the grazing period and had water freely available. The diet for all ewes consisted of lucerne pellet, offered once a day between 0800 and 0900, which contained 6.69 mg/g of Arg (6% of total AA), 17% crude protein and 10.5 MJ/kg metabolisable energy. The diet was formulated to meet 100% of NRC-recommended requirements for twin-bearing ewes (NRC, 1985).

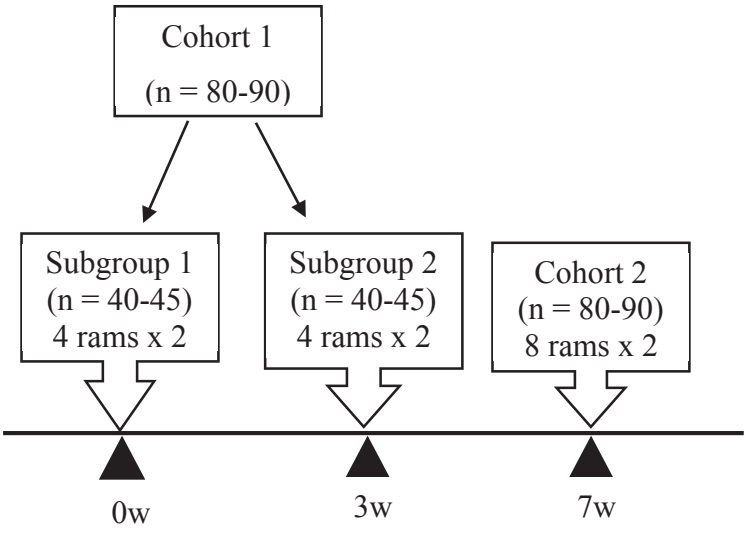

Figure 4.1. Experimental design presenting the time of mating, number of ewes and rams.

4.3.2 Experimental design

At P85, animals were randomly assigned to Arg (cohort 1, *n* = 12; cohort 2, *n* = 13) or control (cohort 1, *n* = 11; cohort 2, *n* = 13) groups. At P97, polyvinyl catheters (0.040 inch internal diameter polyvinyl tubing: Critchley Electrical Products Pty Ltd, Australia) were inserted into the tarsal vein of the hind leg under brief anaesthesia induced by injectable anaesthetic with Ketamine (10 mg kg^{-1} body weight, Parnell Laboratories, New Zealand) and Diazepam (0.5 mg kg^{-1} body weight: Pamlin, Parnell Laboratories, New Zealand). The catheter was tunnelled subcutaneously, sealed with a stopcock, secured in a plastic bag and anchored to the mid line of the ewe. The bag and catheter were further secured with tube net. This was followed by a single prophylactic intramuscular (hind leg) administration of antibiotics (0.04 ml per kg^{-1} body weight: Duplocillin LA, Intervet Ltd, Newmarket, Auckland,). Catheters were flushed regularly with heparinised saline (10 U ml^{-1} sodium heparin, 0.9% sodium chloride, Baxters Healthcare Pty Ltd, Australia).

From P100 Arg ewes received an intravenous (*i.v.*) bolus of L-Arg-mono-hydrochloride (345 µmol/kg BW L-Arg-HCL; Merck KGaA, Darmstadt, Germany) three times daily (0800, 1600, 2400 h) via the catheter. Arg doses was adjusted weekly according to the live weight of the ewe. The L-Arg-HCL solution was prepared daily; dissolved using sterile physiologic saline (0.9% sodium chloride) with a final concentration of 1.8 g Arg per 5 mL. The pH was adjusted to 7.0 with 1M NaOH and filtered-sterilised using a 0.22 µm PES syringe filter (Jet Biofil, Elgin IL, USA). The dose of L-Arg-HCL administered was based on a previous study and was calculated to be at a maximum dose-rate known to be safe for use in sheep (Lassala et al., 2011), and the administration route chosen to avoid ruminal (Chacher et al., 2012) and/or intestinal (Wu et al., 2007b) Arg degradation. The remaining ewes received the same volume of sterile saline via *i.v.* (Baxters Healthcare Pty Ltd, Australia).

Cohort 1; euthanised at P140

From the initial 23 ewes fitted with catheters, three were removed from the study (one due to a defective catheter and two ewes due to abortion, not associated with catheterisation), and therefore 20 ewes completed the study at P140 (Arg group *n* = 9; Control group *n* = 11). Ewes were euthanised by overdose of barbiturate (0.5 mL/kg BW

Pentobarb 300, Provet NZ Pty Ltd, Auckland) one-hour following their last Arg bolus administration. A blood sample was obtained immediately before euthanasia from the ewe via jugular venipuncture. A fetal blood sample was obtained via cardiac puncture immediately after removal of the fetus from the uterus. After removal of both fetuses from the uterus, the excess fetal fluid was stripped by hand and the fetuses were weighed. All blood samples were placed on ice until centrifugation at 1000 x g for 15 min at room temperature. Triplicate plasma aliquots were stored at -20ºC until analysis. The following muscles were excised and weighed from the right side of each fetus: *M. semitendinosus, M. longissimus dorsi, M. biceps femoris, M. gastrocnemius, M. plantaris, M. semimembranosus, M. adductor, M. gluteus, M. quadriceps* and *M. psoas major*. Samples from the *M. longissimus dorsi* (LD) were snap frozen in liquid nitrogen and stored at -80ºC. Determination of FAA profiling, biochemical indices (DNA, RNA, protein and their ratios), SDS-Page and Western Blotting procedures were undertaken in LD muscle samples, as phenotypic differences were detected in this muscle at PN153 and it represents a high market value muscle.

Cohort 2; euthanised at PN153

From the initial 26 ewes from cohort 2 and fitted with catheters, one ewe from the control group was removed from the study, due to abortion, not associated with the treatment regimen. The remaining 25 ewes (Arg group $n = 13$; control group $n = 12$) were allowed to lamb naturally. From 50 lambs that were born alive, 15 pairs were followed to weaning (Arg group $n = 16$; control group $n = 14$), with the other 10 pairs of lambs eliminated from the study due to maternal mammary gland problems (Arg ewes $n = 5$; control ewes $n = 5$) which could interfere with lactation and thus, normal growth of lambs. During the lambing period, ewes were monitored 24 h a day to provide assistance when required. Administration of the Arg or control treatment ceased at the first sign of parturition, which ranged between 1 and 10 h prior to birth. Within 2 h of birth, lambs were ear-tagged, sexed and weighed. Ewes and lambs were maintained on pasture from day 15 (PN15) to PN82, when lambs were weaned and maintained on pasture until PN153. Male lambs were not castrated. At PN153, a randomly selected subgroup of 20 lambs (Arg group, $n = 10$: 5 males and 5 females; Control, $n = 10$: 6 males and 4 females) were blood sampled by jugular venipuncture as previously described, and euthanised by

captive bolt and exsanguination. Muscles were rapidly dissected and weighed using the same protocol to that described for Cohort 1, and stored at -80ºC. Similar to Cohort 1, the LD muscle was used for determination of FAA and biochemical indices.

4.3.3 Intracellular FAA profiles

Amino acid concentrations in plasma and muscle were determined by ion-exchange chromatography as previously described (Chapter 3). Amino acids in muscle were determined in a randomly selected subgroup of P140 fetuses (n = 10 per group, 5 males and 5 females per group) and in all euthanised lambs (n = 10 per group) at PN153.

4.3.4 Biochemical indices: DNA, RNA and protein content

Total RNA and DNA and protein were extracted from LD muscle samples, from the same randomly selected subgroup of P140 animals used for muscle AA profile and previously described (n = 10 per group) and from all euthanised lambs at PN153 (n = 10 per group), using the protocol previously described in Chapter 3.

4.3.5 Protein immunoblot analysis: mTOR

To determine the effect of Arg supplementation on mTOR signalling, only protein muscle extracts from fetuses at P140 (n = 10 per group) were analysed. Animals sampled at PN153 were not considered in this study as Arg supplementation ceased at birth. Protein was separated by reducing SDS-PAGE. Protein targets mTOR and phosphorylated (Ser^{2448}) mTOR were separated on 3 to 8% Tris-acetate gels (Invitrogen, Auckland, New Zealand), according to manufacturer's instructions, loading 15 µg of protein per sample. Proteins were then transferred using an iBlot Gel Transfer Device to a polyvinylidene difluoride membrane (Invitrogen, Auckland, New Zealand). The membrane was then blocked for 1 h at room temperature in 5% non-fat dry milk with Tris-buffered saline-0.05% Tween (TBST). After washing three times with TBST for 5 min each, the membrane was incubated with primary antibodies (mTOR 1:10.000; mTOR-Ser^{2448} 1:5000; Cat. Nos. 2972 and 2971, respectively, Cell Signalling Technologies, Boston, MA, USA) overnight at 4ºC, followed by secondary antibody for 60 min (horseradish peroxidase-conjugated, Cell Signalling Technologies, Boston, MA, USA). The blots were visualised using SuperSignal West Pico enhanced chemiluminescence reagents (BioRad, Auckland, New Zealand) and exposed to Kodak Biomax XAR film (Rochester, NY, USA). Phosphoprotein blots were stripped (15 g

glycine, 1 g SDS, 10 ml Tween20, adjusted pH to 2.2), by incubating the membrane at room temperature twice for 5-10 min. Then, membranes were washed twice with phosphate buffered saline (PBS, GIBCO, Invitrogen) for 10 min, followed by two washes with TBST for 5 min. Absence of signal was verified, and then blots were re-probed with the total mTOR protein antibody. Blots were processed in triplicate. After exposure, films were scanned at 600 dpi with a HP Photosmart B110 scanner (Hewlett Packard, Auckland, New Zealand). ImageJ software (National Institutes of Health, Bethesda, MD, USA) was used to determine the signal intensity of individual bands by densitometry. Within each film individual peak areas were calculated as total abundance in LD muscle. The calculated values were then expressed as a ratio of the total signal from all bands corresponding to mTOR or mTOR-Ser^{2448} on the film to reduce band intensity variation between repeated films.

4.3.6 Plasma metabolites and hormones

Maternal, fetal and lamb plasma samples were analysed for insulin, IGF-I, glucose, non-esterified fatty acids (NEFA), glycerol, beta-hydroxybutyrate (BHBA), urea and triglyceride concentrations. Plasma insulin was measured by radioimmunoassay (RIA), with ovine insulin as the standard (Sigma Chemical Co., Batch no. I9254, St. Louis, MO, U.S.A.). The minimum detectable concentration was 0.02 ng/mL and the inter-assay CV was 11.6%. Plasma IGF-I concentration was measured by specific RIA using an IGFBP-blocked RIA. A Hitachi 902 autoanalyser (Hitachi High Technologies Corporation, Tokyo, Japan) using commercial kits was used to measure glucose and triglyceride (CV 1.2% and 10.8% respectively) (Roche, Mannheim, Germany); NEFA and glycerol (CV 3.7% and 5.3%, respectively) (Randox Laboratories Ltd, Ardmore, Crumlin, UK). Urea and BHBA were determined using a Roche diagnostics Kinetic UV assay performed on a Hitachi modular P800 (CV 1.8% and 1.9%, respectively). All analyses were performed by New Zealand Veterinary Pathology, Hamilton, New Zealand.

4.4 STATISTICAL ANALYSIS

Maternal weight and plasma AA concentration at P140 was analysed by ANOVA, using a linear model including the effect of treatment (control *vs.* Arg) and the random

effect of cohort, with the MIXED procedure (SAS, 2006). Fetal muscle weights, plasma and intracellular muscle AA concentration, mTOR, biochemical indices (RNA, DNA and protein contents) and plasma hormone and metabolite concentration data were analysed using a linear model which included the fixed effects of treatment, sex of fetus (female *vs.* male) and a treatment by sex of fetus interaction and the random effects of ewe to adjust for the twinning effect and cohort.

Lamb birth weight, muscle weights, plasma and intracellular muscle AA concentrations, biochemical indices (RNA, DNA and protein contents) and plasma hormone and metabolite concentration data obtained at PN153 in Cohort 2, were analysed using a linear model, which included treatment, sex of lamb, treatment by sex of lamb interaction as fixed effects and the random effects of ewe to adjust for the twinning effect. Live weight from birth to weaning for PN153 lambs was analysed using repeated measurement, considering treatment, sex of lamb, treatment by sex of lamb interaction as fixed effects and the random effects of ewe to adjust for the twinning effect. For birth weight all lambs born were considered while for live weight lambs (n = 30) from ewes without mammary problems were considered.

A separate analysis using the weight of the fetus at P140 and lamb carcass weight at PN153 respectively as a covariate was performed in order to define the proportionality of the weight of each evaluated muscle to body (P140) or carcass weight (PN153).

All data were analysed for normality of the residuals, using the UNIVARIATE procedure (SAS, 2006). The PDiff option of the LSMeans statement of SAS was used for means separation. Statistical significance was set at a probability value of $P \leq 0.05$ and $P \leq 0.10$ was considered as a trend. For FAA only those values with $P \leq 0.05$ are discussed.

4.5 RESULTS

4.5.1 Maternal, fetal and lamb body weight

Arginine supplementation of ewes from P100 to P140 had no effect on maternal live weight (85 ± 2 *vs.* 83 ± 2 kg, for control and Arg-treated ewes respectively, $P = 0.60$) or fetal weight (Table 4.1). At birth, there was a trend for a treatment by sex of lamb interaction ($P = 0.07$), where females lambs from Arg-treated ewes (Arg-F) were heavier

than control females (Con-F) (P = 0.03), but males from Arg-treated ewes (Arg-M) and control males (Con-M) were not different (P = 0.80) (Table 4.1).

There was a significant 3-way interaction between treatment, time and sex of lamb on post-natal live weight (P<0.0001), such that there was a divergence in live weight between Arg-F compare to Con-F from day PN120, while there was no effect of maternal Arg-supplementation on the live weight of the males (Figure 4.2). At 141 days, Arg-F tended (P = 0.07) to be heavier than Con-F while Arg and control males did not differ (P = 0.50). At 153 days of age, Arg-F were heavier than Con-F (P = 0.03), with no difference observed between Arg-M and Con-M (P = 0.48). The divergence in live weight between males and females varied according to treatment, where Con-M diverged from Con-F at 77 days (27.8 ± 0.7 vs. 25.4 ± 1 kg, P = 0.04) while Arg-M diverged from Arg-F at day 120 (41.0 ± 0.9 vs. 38.4 ± 0.78 kg, P = 0.02).

Table 4.1. Body weight of fetuses at day 140 of pregnancy and birth weight of fetuses/lambs born to ewes either supplemented with arginine (Arg) or saline (controls). Data shows least square means ± standard error of mean (SEM). [ab]Different superscripts within rows represent $P \leq 0.05$. Values for treatment (T), sex of the lamb/fetus (S) and the interaction between treatment and sex of the lamb/fetus (T x S) are presented. In brackets number (n) of animal.

	Control		Arginine		*P*-value		
	Male	Female	Male	Female	T	S	T x S
Fetal weight	5.7 ± 0.2 (n = 8)	5.1 ± 0.2 (n = 14)	5.5 ± 0.2 (n = 10)	5.0 ± 0.2 (n = 8)	0.51	<0.01	0.43
Birth weight	5.4 ± 0.2 [a] (n = 18)	5.0 ± 0.2 [b] (n = 6)	5.4 ± 0.2 [a] (n = 9)	5.6 ± 0.1[a] (n = 17)	0.22	0.46	0.07

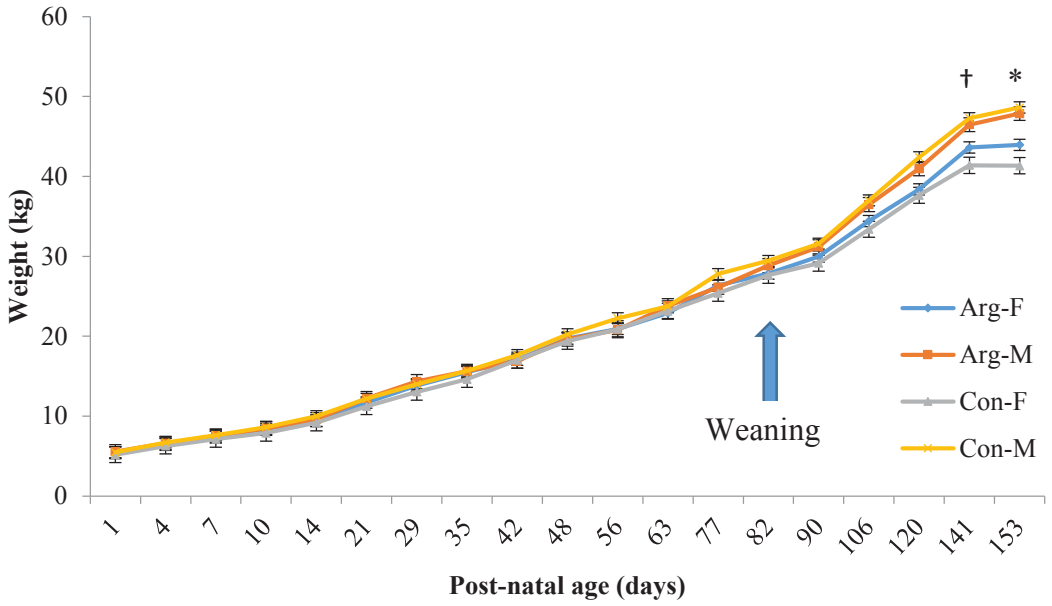

Figure 4.2. Post-natal live weight change of lambs ($n = 30$) from ewes either supplemented with arginine (Arg, n = 16, 6 male and 10 female) or saline (controls, Con, n = 14, 10 males and 4 females) from P100 to birth. Data shows least square means ± standard error of the mean (SEM) for treatment (Arg and control) by sex (Male (M) and Female (F)) by time interaction. * $P \leq 0.05$, † $P < 0.10$ for female lambs from ewes supplemented with Arg (Arg-F) *vs.* female lambs from control ewes (Con-F) comparison.

4.5.2 Skeletal muscle mass

P140. After adjustment to a common fetal weight, *M. psoas major* was heavier in the fetuses from Arg-treated ewes compared to controls (Table 4.2). Maternal Arg supplementation had no effect on the weight of other muscles in the fetuses at P140. A trend for a treatment by sex of fetus interaction was observed for the *M. adductor*, which was lighter ($P = 0.02$) in Con-M compared to Arg-M, while no difference was observed between females. A trend for an interaction was also found for the *M. semitendinosus,* which was heavier ($P = 0.07$) in Arg-M compared with Con-M, while no difference existed between female groups. Males had heavier muscles than females, with the exception of the *M. adductor* where males and females did not differ. The difference between males and females was maintained only for *M. gastrocnemius* after adjustment to a common fetal weight, with a trend for heavier *M. quadriceps* in males compared to females.

Table 4.2. Individual muscle weights (g) at 140 days of pregnancy (P140) of fetuses born to ewes either supplem from P100-P140. Table shows least square means ± standard error of the mean (SEM) of muscle weight (unadjus model (adjusted).Values for treatment (T), sex of the fetus (S) and the interaction of treatment by sex of fetus (T x S at $P \leq 0.05$ and a trend for $P \leq 0.10$.

	Unadjusted									
	Control		Arginine		*P*-value			Control		
	Male	Female	Male	Female	T	S	T x S	Male	Female	
n	14	8	10	8				14	8	
Longissimus dorsi	48.6±2.6	42.8±2.3	44.6±2.6	41.7±2.6	0.44	0.01	0.35	44.5±1.8	44.8±1.5	42
Psoas major	7.6±0.6	6.7±0.5	8.1±0.6	7.4±0.6	0.35	0.09	0.85	6.8±0.4	7.1±0.3	7.
Semitendinosus	9.8±0.6	9.3±0.4	10.2±0.5	9.0±0.5	0.95	0.06	0.39	9.1±0.3	9.7±0.3	10
Biceps femoris	29.0±1.5	26.3±1.3	28.5±1.4	25.3±1.5	0.66	0.02	0.80	27.2±0.9	27.1±0.7	27
Gastrocnemius	13.8±0.5	11.7±0.4	13.2±0.5	11.5±0.5	0.51	<0.01	0.55	13.1±0.2	12.1±0.2	12
Plantaris	5.5±0.3	4.5±0.3	5.2±0.3	4.5±0.3	0.62	<0.01	0.41	5.0±0.2	4.8±0.1	5.
Semimembranosus	25.2±1.4	21.2±1.1	24.3±1.3	22.3±1.3	0.93	0.01	0.37	23.8±0.9	21.8±0.7	23
Adductor	10.1±0.7	10.1±0.6	10.0±0.7	9.1±0.7	0.53	0.35	0.33	9.0±0.6	10.6±0.5	9.
Gluteus	15.8±0.8	13.6±0.7	15.0±0.8	13.2±0.8	0.53	<0.01	0.76	14.7±0.5	14.1±0.4	14
Quadriceps	42.8±1.8	37.3±1.6	42.8±1.7	37.3±1.8	0.98	<0.01	0.99	39.8±1.0	38.7±0.8	41
Total leg muscle	161.6±7.1	140.7±6.2	157.8±6.8	139.3±7	0.76	<0.01	0.79	148.9±3.1	146.1±2.4	151

[1]Adjusted data: muscle weights adjusted for fetal weight.

PN153. An interaction between treatment and sex of the lamb was observed for skeletal muscle mass, whereby Arg-F had heavier *M. longissimus dorsi*, *M. plantaris* and a trend for heavier *M. psoas major* muscles compared to Con-F, whereas the weight of these muscles did not differ in response to treatment in male lambs (Table 4.3). Lambs from Arg-treated ewes had heavier *M. semimembranosus* and tended to have heavier *M. biceps femoris* compared to control lambs. It is noteworthy that while not statistically significant, Arg-F lambs had absolute heavier muscle weights than Con-F for all muscles assessed. After adjustment to a common carcass weight, lambs from Arg-treated ewes tended to have heavier *M. semimembranosus* compared with control lambs. Females had heavier *M. longissimus dorsi* and *M. semimembranosus,* and tended to have heavier *M. quadriceps* and total leg muscle compared with males (Table 4.3).

Table 4.3. Individual muscle weights (g) of offspring at 153 days of age born to ewes either supplemented with arg birth. Table shows unadjusted and adjusted values for carcass weight. Data is expressed as least square means ± sta was stated at $P \leq 0.05$ and a trend for $P \leq 0.10$. Values for treatment (T), sex of the lamb (S) and the interaction betw presented.

	Unadjusted							Control	
	Control		Arginine		*P*-value			Control	
	Male	Female	Male	Female	T	S	T x S	Male	Female
n	10	4	6	10				10	4
Longissimus dorsi	582±25	558±29	559±31	648±31	0.32	0.24	0.05	559±19	618±27
Psoas major	102±4	92±5	100±5	106±5	0.35	0.68	0.06	99±4	101±5
Semitendinosus	119±6	110±7	121±7	120±7	0.42	0.46	0.53	114±4	123±6
Biceps femoris	325±12	319±15	343±14	348±14	0.10	0.96	0.69	318±12	336±17
Gastrocnemius	117±4	111±4	122±5	123±5	0.13	0.54	0.39	114±4	117±5
Plantaris	47±3	39±3	44±3	52±3	0.25	0.96	<0.01	45±3	43±3
Semimembranosus	299±11	310±13	327±13	352±13	0.02	0.15	0.55	292±10	329±14
Adductor	138±5	132±6	138±6	140±6	0.46	0.73	0.50	134±4	142±5
Gluteus	258±11	249±13	260±13	271±13	0.37	0.94	0.43	250±10	268±14
Quadriceps	421±19	427±23	424±21	445±21	0.62	0.53	0.72	409±16	458±22
Total leg muscle	1825±58	1794±69	1876±68	1959±68	0.14	0.70	0.40	1776±41	1920±57

[1]Adjusted data: muscle weights adjusted for carcass weight.

4.5.3 Concentration of FAA at P140

Maternal plasma. Ewes supplemented with Arg had greater plasma concentrations of arginine (Figure 4.3) and ornithine, resulting in higher NEAA, but reduced concentrations of methionine, glycine and serine compared with control ewes (Table 4.4).

Fetal plasma. Fetuses from Arg-treated ewes had greater plasma concentration of ornithine, while concentrations of methionine, threonine, glycine, taurine and tyrosine were reduced, resulting in lower total plasma FAA concentration compared to control fetuses (Table 4.4). A treatment by sex of fetus interaction was observed for histidine, which was reduced by 70% in Arg-F compared with Con-F (187 ± 45 *vs.* 399 ± 45, $P < 0.01$), and by 44% in Arg-M compared with Con-M (120 ± 45 *vs.* 332 ± 46, $P < 0.01$). No other treatment by sex of the fetus interaction nor other sex of fetus effect was observed for fetal plasma FAA concentration (see Appendix B: Table B.1).

Muscle. Fetuses from Arg-treated ewes had lower intracellular concentrations of histidine, lysine and methionine, but alanine was increased (Table 4.4). No treatment by sex of fetus interaction was observed for any of the FAA (see Appendix B: Table B.2). Females had lower concentration of asparagine in LD muscle compared to males (8 ± 2 *vs.* 1 ± 2 respectively, $P = 0.03$). No other sex of fetus effect was evident (see Appendix B: Table B.2).

Table 4.4. Treatment effect for free amino acid (AA) concentration in maternal plasma (μmol/L), fetal plasma concentrations in *M. longissimus dorsi* (μmol/g) at 140 days of pregnancy (P140), of fetuses born to ewes either (control) from P100-P140. Data are presented as least square means and averaged standard error of the mean (SE

	Ewe plasma (μmol/L)				Fetal plasma (μmol/L)			
	Control	**Arginine**	**SEM**	***P*-value**	**Control**	**Arginine**	**SEM**	***P*-value**
n	**6**	**6**			**10**	**10**		
Essential								
Histidine	103	91	9	0.37	366	153	40	<0.01
Isoleucine	1140	1139	11	0.88	82	88	8	0.47
Leucine	164	161	18	0.92	173	172	14	0.90
Lysine	192	212	17	0.44	192	151	25	0.24
Methionine	38	26	2	<0.01	104	57	7	<0.01
Phenylalanine	62	55	5	0.30	121	110	6	0.23
Threonine	235	148	30	0.07	763	616	47	0.04
Valine	223	227	27	0.91	342	351	62	0.82
Total EAA	1131	1033	132	0.50	2139	1641	86	<0.01
Non-essential								
Alanine	167	169	14	0.86	399	414	27	0.63
Arginine	109	992	84	<0.01	192	237	23	0.17
Asparagine	44	24	8	0.13	37	43	9	0.64
Aspartate	7	6	1	0.56	35	29	2	0.11
Citrulline	262	211	32	0.29	200	218	37	0.51
Glutamate	65	80	13	0.40	88	122	22	0.28
Glutamine	266	257	13	0.60	372	405	22	0.30
Glycine	795	516	58	<0.01	944	748	55	0.02
Ornithine	137	394	38	<0.01	213	367	50	0.03
Proline	101	93	7	0.43	203	197	27	0.87
Serine	91	66	8	0.05	567	530	38	0.49
Taurine	77	61	12	0.34	84	47	13	0.03
Tyrosine	64	57	4	0.27	162	115	15	0.04
Total NEAA	2284	2926	149	<0.01	3406	3394	181	0.94
Total	3415	3959	270	0.07	5561	5066	260	0.08

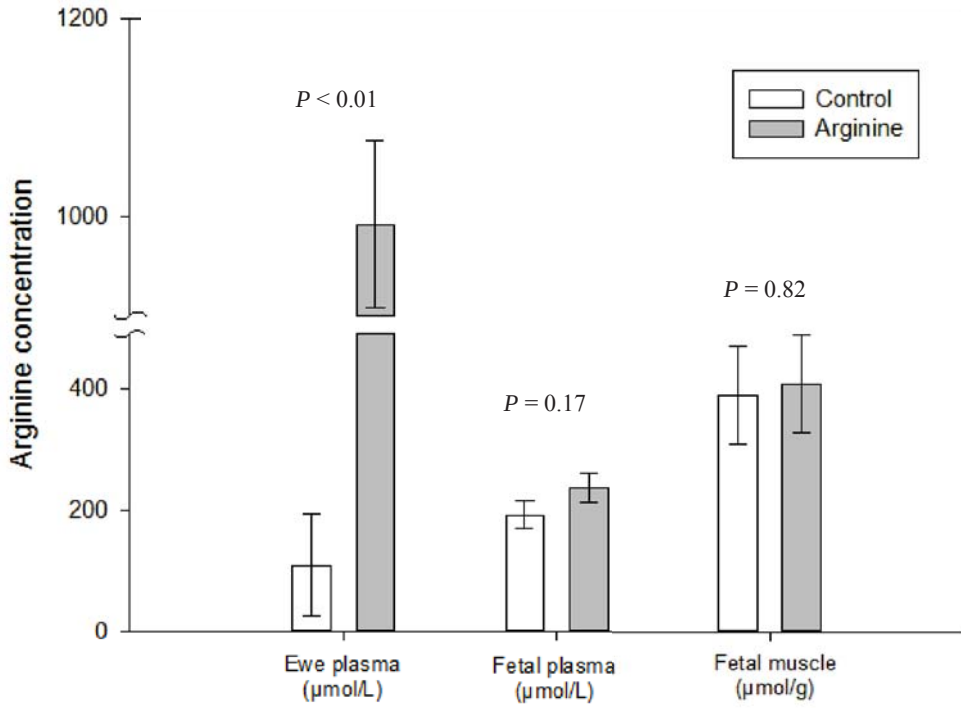

Figure 4.3. Arginine concentration in ewe plasma (µmol/L, n = 6/group), fetal plasma (µmol/L, n = 10/group), and fetal muscle (µmol/g, n = 10/group), at 140 days of gestation (P140). Data shows least square means ± standard error of the mean (SEM).

4.5.4 Concentration of AA at PN153

Plasma. A treatment by sex of lamb interaction was evident for plasma isoleucine (P = 0.005), where Arg-M had lower isoleucine concentration compared to Con-M (91 ± 4 *vs.* 110 ± 4 µmol/L, P = 0.01), while no difference was found between Arg-F and Con-F (94 ± 4 *vs.* 86 ± 5 µmol/L, P = 0.23). A treatment by sex of lamb interaction was also evident for leucine (P = 0.03), where Arg-F tended to have greater concentration of leucine compared to Con-F (154 ± 8 *vs.* 130 ± 9 µmol/L, P = 0.06), while no difference was observed between Arg and control males (131 ± 8 *vs.*145 ± 4 µmol/L, P = 0.20). Lambs from Arg-treated ewes had lower alanine concentration compared to control lambs. Females had lower concentration of lysine and citrulline compared to males (Table 4.5).

Table 4.5. Treatment and sex of lamb effect for plasma FAA concentration (μmol/L) at 153 days of life (PN153) of twin lambs born to ewes supplemented with arginine (Arg) or saline (control) from 100 days of pregnancy (P100) to birth. Data corresponds to least square means ± standard error of the mean (SEM). Values for treatment (T) and sex of the lamb (S) are presented. Significance was established at $P \leq 0.05$.

	Treatment			Sex of lamb		
	Control	**Arginine**	***P*-value**	**Male**	**Female**	***P*-value**
n	**16**	**14**		**16**	**14**	
Essential						
Histidine	95 ± 8	85 ± 8	0.46	84 ± 8	96 ± 9	0.36
Isoleucine[1]	98 ± 3	92 ± 3	0.06	101 ± 3	90 ± 3	0.03
Leucine[1]	138 ± 6	143 ± 5	0.66	138 ± 5	142 ± 6	0.59
Lysine	160 ± 9	162 ± 9	0.96	175 ± 8	147 ± 9	0.04
Methionine	31 ± 2	29 ± 2	0.46	32 ± 2	28 ± 2	0.09
Phenylalanine	54 ± 3	60 ± 3	0.16	60 ± 2	53 ± 3	0.08
Threonine	142 ± 10	146 ± 9	0.88	144 ± 9	144 ± 10	0.98
Valine	269 ± 13	283 ± 13	0.47	268 ± 12	284 ± 14	0.40
Total EAA	986 ± 35	988 ± 34	0.87	1003 ± 33	971 ± 36	0.53
Non-essential						
Alanine	218 ± 10	184 ± 10	0.02	200 ± 9	202 ± 10	0.85
Arginine[2]	185 ± 14	205 ± 13	0.32	198 ± 13	192 ± 14	0.74
Asparagine	34 ± 2	36 ± 2	0.59	37 ± 2	33 ± 2	0.23
Aspartate	12 ± 2	15 ± 2	0.31	12 ± 2	14 ± 2	0.39
Citrulline	147 ± 12	181 ± 12	0.07	187 ± 11	141 ± 12	0.01
Glutamate	76 ± 4	72 ± 4	0.49	70 ± 4	77 ± 4	0.25
Glutamine	270 ± 13	277 ± 13	0.71	281 ± 11	265 ± 11	0.19
Glycine	506 ± 28	513 ± 29	0.73	497 ± 24	522 ± 25	0.44
Ornithine	99 ± 6	112 ± 6	0.18	109 ± 6	102 ± 6	0.41
Proline	541 ± 36	550 ± 37	0.77	536 ± 32	555 ± 33	0.67
Serine	77 ± 7	94 ± 7	0.13	84 ± 7	87 ± 7	0.80
Taurine	65 ± 11	70 ± 12	0.78	62 ± 10	72 ± 10	0.38
Tyrosine	79 ± 4	87 ± 4	0.26	88 ± 4	78 ± 4	0.07
Total NEAA	2266 ± 88	2387 ± 81	0.28	2341 ± 77	2313 ± 83	0.78
Total	3258 ± 107	3375 ± 103	0.44	3350 ± 102	3283 ± 113	0.66

[1]Refer to text as a treatment by sex of lamb interaction was observed.
[2]Deemed as conditionally essential for neonate growth (Wu, 2009).

Muscle. A treatment by sex of lamb interaction (P = 0.03) was found for alanine concentration in LD muscle at PN153, where Arg-F tended to have a higher concentration compared with Con-F (1839 ± 130 *vs.* 1485 ± 115 μmol/g, $P = 0.06$) while no difference was observed between Arg and control males (1380 ± 130 *vs.* 1543 ± 104 μmol/g $P =$

0.35). Females had significant higher concentration of leucine and valine compared with males (Table 4.6). Citrulline was not resolved for muscle samples.

Table 4.6. Intracellular free amino acids (FAA) concentrations (μmol/g) in *M. longissimus dorsi* post-weaning (PN153), of twin lambs born to twin-bearing ewes either supplemented with arginine (Arg) or saline (control) from day 100 of pregnancy (P100) to birth. Treatment and sex of lamb effects are presented. Data corresponds to least square means ± standard error of the mean (SEM). Significance was established at $P \leq 0.05$.

	Treatment			Sex of lamb		
	Control	**Arginine**	***P*-value**	**Male**	**Female**	***P*-value**
n	**10**	**10**		**11**	**9**	
Essential						
Histidine	84 ± 4	82 ± 4	0.83	79 ± 4	86 ± 4	0.18
Isoleucine	47 ± 3	52 ± 3	0.24	48 ± 3	50 ± 3	0.56
Leucine	83 ± 5	90 ± 5	0.30	78 ± 5	95 ± 5	0.03
Lysine	61 ± 4	63 ± 4	0.73	60 ± 4	64 ± 5	0.49
Methionine	26 ± 2	23 ± 2	0.27	23 ± 2	26 ± 2	0.25
Phenylalanine	43 ± 2	46 ± 2	0.38	44 ± 2	45 ± 2	0.88
Threonine	205 ± 18	208 ± 17	0.97	220 ± 16	192 ± 18	0.23
Valine	146 ± 7	158 ± 7	0.23	140 ± 7	164 ± 8	0.04
Total EAA	693 ± 4	713 ± 3	0.63	678 ± 3	728 ± 4	0.29
Non-essential						
Alanine	1514 ±92	1609 ± 96	0.52	1462 ± 83	1662 ± 87	0.07
Arginine[1]	88 ± 8	105 ± 8	0.20	100 ± 8	93 ± 9	0.53
Asparagine	30 ± 2	28 ± 2	0.81	27 ± 2	31 ± 3	0.34
Aspartate	83 ± 6	81 ± 6	0.63	85 ± 6	78 ± 6	0.45
Citrulline	ND	ND	ND	ND	ND	ND
Glutamate	1045 ± 141	837 ± 140	0.30	1066 ± 125	816 ± 140	0.16
Glutamine	1717 ± 204	1870 ± 202	0.64	1970 ± 179	1617 ± 202	0.16
Glycine	590 ± 47	658 ± 45	0.28	648 ± 45	600 ± 49	0.47
Ornithine	64 ± 4	70 ± 4	0.36	69 ± 4	65 ± 4	0.56
Proline	612 ± 61	645 ± 61	0.75	680 ± 57	577 ± 61	0.22
Serine	129 ± 14	134 ± 14	0.63	130 ± 13	134 ± 15	0.94
Taurine	3048 ± 606	2878 ± 636	0.83	2588 ± 542	3338 ± 564	0.27
Tyrosine	53 ± 2	57 ± 2	0.21	56 ± 2	54 ± 2	0.32
Total NEAA	9413 ± 567	8978 ± 528	0.69	8827 ± 497	9564 ± 580	0.35
Total	10109 ± 565	9691 ± 524	0.71	9506 ± 495	10294 ± 578	0.32

[1]Deemed as conditionally essential for neonate growth (Wu, 2009). ND: Not determined.

4.5.5 Biochemical indices: DNA, RNA and protein content

At P140 a treatment by sex of fetus interaction was found for RNA concentration and RNA: DNA ratio, such that Arg-F had higher values compared with Con-F, while no difference was observed between males (Table 4.7). A treatment by sex of fetus interaction was found for Protein: RNA ratio, such that Con-F had higher ratio compared with Arg-F, while no difference was found between Arg and control males. Fetuses from Arg-treated ewes had higher protein concentration and Protein: DNA ratios, compared to control fetuses.

At PN153, a treatment by sex of lamb interaction for RNA concentration and total RNA content was found, such that Arg-F had higher values compared with Con-F, while no difference was observed between males. Arg lambs had higher total DNA compared to control lambs (Table 4.8).

Table 4.7. Biochemical indices (DNA, RNA, protein and ratios) of *M.* l*ongissimus dorsi (LD)* in control and Arg of fetuses born to ewes either supplemented with arginine (Arg) or saline (control) from P100-P140. Data are repre of the mean (SEM). Significance was established at $P \leq 0.05$ and values are presented for effect of treatment (T) treatment and sex of the fetus (T x S). [ab]Different superscripts represents statistical difference ($P \leq 0.05$).

	Control		Arginine	
	Male	**Female**	**Male**	**Female**
n	**5**	**5**	**5**	**5**
Concentration (mg/g LD)				
DNA	1.43 ± 0.12	1.45 ± 0.12	1.46 ± 0.12	1.18 ± 0.12
RNA	2.19 ± 0.14^{ab}	1.91 ± 0.14^{b}	2.17 ± 0.14^{ab}	2.50 ± 0.14^{a}
Protein	54.82 ± 3.08	51.96 ± 3.08	64.23 ± 3.08	57.52 ± 3.08
Total (mg)				
DNA	65.66 ± 5.71	64.63 ± 5.71	62.81 ± 5.71	49.53 ± 5.71
RNA	97.65 ± 8.32	85.49 ± 8.32	97.78 ± 8.32	104.57 ± 8.32
Protein	2.49 ± 0.21	2.31 ± 0.21	2.84 ± 0.21	2.42 ± 0.21
Ratios				
RNA:DNA	1.57 ± 0.21	1.35 ± 0.21	1.62 ± 0.21	2.16 ± 0.21
Protein:DNA	0.038 ± 0.004	0.036 ± 0.004	0.046 ± 0.004	0.050 ± 0.004
Protein:RNA	0.024 ± 0.003^{ab}	0.030 ± 0.003^{b}	0.030 ± 0.003^{b}	0.022 ± 0.003^{a}

Table 4.8. Biochemical indices (DNA, RNA, protein and ratios) of *M.* l*ongissimus dorsi (LD)* in control and Arg are represented as least square mean ± standard error of the mean (SEM). Significance was established at *P* treatment (T), sex of lamb (S) and the interaction between treatment and sex of the lamb (T x S). [ab]Different sup 0.05).

	Control		Arginine	
	Male	**Female**	**Male**	**Female**
n	**5**	**5**	**5**	**5**
Concentration (mg/g LD)				
DNA	0.45 ± 0.02	0.45 ± 0.03	0.48 ± 0.02	0.49 ± 0.02
RNA	0.47 ± 0.01^{ab}	0.45 ± 0.01^{b}	0.45 ± 0.01^{ab}	0.49 ± 0.01^{a}
Protein	74.66 ± 5.48	80.26 ± 6.71	75.62 ± 6.00	79.88 ± 6.00
Total (mg)				
DNA	259.80 ± 13.04	253.14 ± 15.97	269.63 ± 14.28	312.35 ± 14.28
RNA	274.09 ± 13.70^{b}	255.85 ± 16.77^{b}	251.76 ± 15.00^{ab}	313.72 ± 15.00^{a}
Protein	43.42 ± 3.91	45.86 ± 4.79	42.41 ± 4.28	51.22 ± 4.28
Ratios				
RNA:DNA	1.06 ± 0.06	1.02 ± 0.07	0.94 ± 0.06	1.01 ± 0.06
Protein:DNA	0.17 ± 0.01	0.18 ± 0.02	0.16 ± 0.02	0.16 ± 0.02
Protein:RNA	0.16 ± 0.01	0.18 ± 0.01	0.17 ± 0.01	0.16 ± 0.01

4.5.6 Effect of Arg on mTOR at P140

A treatment by sex of fetus interaction (P = 0.02) was found for total abundance of mTOR in LD muscle, where Arg-F had a higher value compared to Con-F, while no difference was observed between males (Figure 4.3). For total abundance of mTOR-Ser^{2448}, a trend (P = 0.06) for a treatment by sex of fetus interaction was found, with Con-M showing a tendency (P = 0.07) to have a higher value compared to Arg-M, while no difference was observed between females (Figure 4.4). No effect of treatment on mTOR: Ser^{2448} ratio was observed (Data not presented).

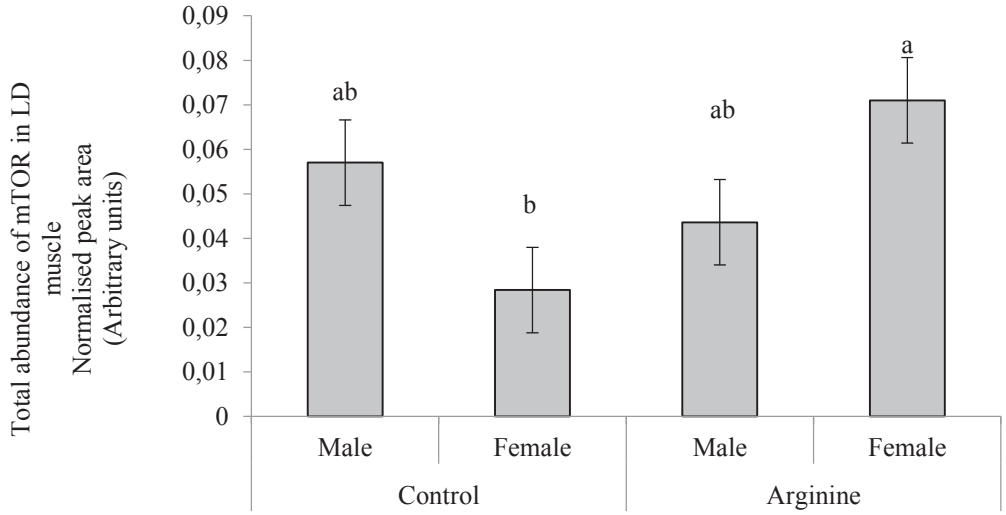

Figure 4.4. Treatment by sex of fetus interaction for total abundance of mechanistic target of rapamycin (mTOR) in *M. longissimus dorsi* from fetuses born to ewes either supplemented with arginine (Arg, n = 10) or saline (controls, n = 10) from day 100 to 140 of pregnancy (P100 to P140). The figure shows the least square mean ± standard error of the mean (SEM). ^{ab}Different superscript for each target represents statistical difference (P ≤ 0.05).

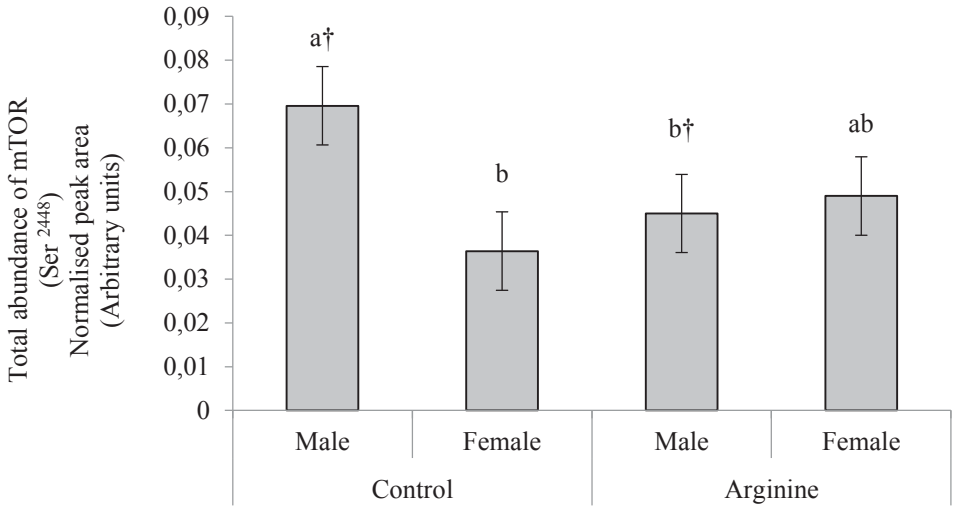

Figure 4.5. Treatment by sex of fetus interaction for total abundance of phosphorylated mTOR (Ser^{2448}) in *M. longissimus dorsi* from fetuses born to ewes either supplemented with arginine (Arg, n = 10) or saline (controls, n = 10) from day 100 to 140 of pregnancy. The figure shows the least square mean ± standard error of the mean (SEM). ^{ab}Different superscript represents statistical difference ($P \leq 0.05$; † $P < 0.10$).

4.5.7 Plasma concentration of hormones and metabolites

At P140, Arg-treated ewes had greater insulin and glucose concentrations and lower NEFA concentrations compared with control ewes (Table 4.9). No differences were found for IGF-1, glycerol, BHBA, urea or triglyceride concentrations. In the fetuses, a treatment by sex of fetus effect was observed for NEFA, where Arg-F had a higher concentration compared with Con-F, while no difference was detected between males (Table 4.9). No difference was observed in the concentrations of insulin, IGF-1, glucose, glycerol, BHBA, urea or triglycerides in fetuses.

At PN153, a treatment by sex of lamb interaction was observed for lamb plasma glucose concentration, where Arg-F lambs had greater concentration compared with Con-F, while Arg-M had lower concentration of glucose compared with Con-M (Table 4.10). Arg lambs tended to have lower NEFA, but higher triglycerides compared with control lambs. Females had lower IGF-1 but higher NEFA compared to males. No difference was observed for insulin between groups. However, in seven of the 10 lambs from Arg-treated ewes and in 4 of the 10 control lambs, the concentration of insulin was below the minimum detection limit, therefore it was not possible to estimate sex of lamb or treatment by sex of lamb effects. No difference was observed for BHBA or urea for lambs at PN153.

Table 4.9. Maternal and fetal plasma concentrations of hormones and metabolites 1 hour after final supplement supplemented with arginine (Arg) or saline (control) from P100-P140. Table shows least square means and averag for treatment (T), sex of the fetus (S) and the interaction of treatment by sex of fetus (T x S) are presented.

	Maternal plasma (P140)						
	Control	Arg		*P*-value	Control		A
			SEM	T	Male	Female	Male
n	6	6			3	3	3
Insulin, ng/mL	0.06	0.40	0.07	0.01	0.14	0.11	0.05
IGF-1, ng/mL	138.5	130.6	12.3	0.66	103.75	84.95	98.34
Glucose, mmol/L	2.92	3.74	0.18	0.01	1.78	1.23	1.35
NEFA[1], mmol/L	0.76	0.33	0.14	0.05	0.13	0.07	0.10
Glycerol, mmol/L	0.07	0.05	0.01	0.11	0.14	0.11	0.09
BHBA[2], mmol/L	0.63	0.72	0.09	0.50	0.12	0.07	0.10
Urea, mmol/L	5.98	6.70	0.35	0.18	6.35	6.77	6.97
Triglycerides, mmol/L	0.35	0.41	0.04	0.40	0.15	0.13	0.11

[1] Non-esterified fatty acids (NEFA); [2] Beta-hydroxybutyrate (BHBA).

Table 4.10. Plasma concentrations of hormones and glucose at PN153 of lambs born to ewes either supplemented day 100 of pregnancy (P100) to birth. Table shows least square means ± averaged standard error of the mean (SE (S) and the interaction of treatment by sex of lamb (T x S) are presented.

	Control		Arg		
	Male	**Female**	**Male**	**Female**	**SEM**
n	**6**	**4**	**5**	**5**	
Insulin[1], ng/mL	0.09 (4)	0.10 (2)	0.04 (1)	0.11 (2)	0.03
IGF-1, ng/mL	148.04	87.31	125.93	99.16	10.91
Glucose, mmol/L	4.13	3.89	3.87	4.18	0.10
NEFA[2], mmol/L	0.19	0.39	0.10	0.12	0.06
Glycerol, mmol/L	0.04	0.06	0.04	0.04	0.01
BHBA[3], mmol/L	0.25	0.26	0.27	0.25	0.03
Urea, mmol/L	7.57	7.35	7.16	6.98	0.41
Triglycerides, mmol/L	0.25	0.20	0.25	0.27	0.02

n.e: non-estimated. [1] The number of animals where insulin was detected are given in brackets; [2] hydroxybutyrate (BHBA).

4.6 DISCUSSION

The increase in the number of fetuses can reduce fetal muscle mass in sheep (McCoard et al., 1997). Our previous study in sheep (Sales et al., 2013: Chapter 2) and studies in pigs (Yao et al., 2008; Tan et al., 2009) suggest that Arg may play a role in twin fetal muscle growth. Therefore, it was hypothesised that maternal parenteral supplementation with Arg from day 100 of pregnancy to term would result in increased fetal muscle hypertrophy, improving fetal muscle growth during late pregnancy and after birth in twins, and that this effect would be mediated, at least in late gestation, by the mTOR signalling pathway. This study revealed that maternal Arg supplementation increased muscle protein synthesis capacity at 140 days pregnancy, potentially contributing to the increase in birth weight and improved post-weaning muscle growth, but only in female lambs. These effects were associated with increased abundance of mTOR at P140.

Arginine plays a key role during pregnancy, influencing fetal growth (Wu and Morris Jr, 1998). A previous experiment where pregnant ewes were supplemented with Arg from day 100 to day 121 of pregnancy reported an effect on birth weight only in quadruplets, but not in twin fetuses (Lassala et al., 2011), which contrasts with the results observed in the present study. One feasible explanation for the difference in Arg effect on birth weight between both studies could be the length of Arg supplementation. In the present work, dams were treated from day 100 to birth. Divergence in fetal growth between twins and singletons is evident from approximately day 115-120 of pregnancy (McCoard et al., 2000a), which is associated with increased fetal nutrient demand. The period of supplementation in the present study, contrasted with Lassala et al. (2011), and covered a longer period of higher fetal nutrient demands, which under a maternal *ad libitum* nutrient regimen, would be evident near term. This is supported by the finding of similar fetal weight observed at P140 between treatments, but an increased birth weight in ewe lambs. The heavier *M. psoas major* muscle weight at P140, suggests the beneficial effects of Arg supplementation were beginning to be evident by P140. In other study where supplementation with Arg to undernourished single-bearing ewes resulted in increased birth weight (Lassala et al., 2010), reinforces the role of Arg in situations of fetal nutrient restriction. The observations in the current study highlight the potential for Arg supplementation of twin-bearing ewes to partially ameliorate maternal constraint on fetal growth during the last month of pregnancy in female fetuses. Although the effect of

differential nutrition during pregnancy in a sex-specific manner has been previously shown in humans (Mora et al., 1979) and by periconceptional undernutrition in sheep (Jaquiery et al., 2012), the mechanisms associated with the effect of Arg observed only in females is intriguing and deserves further investigation.

The positive effect of maternal parenteral Arg supplementation from P100 to birth on ewe lamb birth weight is a novel finding. The increased growth observed in Arg-F compared to Con-F was associated with improved muscle protein synthesis capacity, despite no difference in LD muscle weight at P140. The higher capacity was supported by the increase in RNA content, coupled with a higher protein concentration, protein synthesis capacity (RNA: DNA ratio) and thus hypertrophy (Protein: DNA ratio). Over 85% of the RNA corresponds to ribosomal RNA (Iadevaia et al., 2012a), and an increase in ribosome number has been proposed as a long-term effect of mTOR activation (Wang and Proud, 2006). mTOR regulates protein formation (reviewed in Sarbassov et al., 2005), through activation of downstream targets which regulate translation initiation (Iadevaia et al., 2012b) and ribosome biogenesis (Hannan et al., 2003; Iadevaia et al., 2012a). The increase in RNA in Arg-F fetuses is consistent with the observed increase in total abundance of mTOR in that group. However, there was no difference in Ser^{2448} or Ser^{2448}: mTOR ratio between treatments indicating no divergence in the relative activation of mTOR. Increased abundance but no activation of mTOR suggests that an acute activation of mTOR was not evident at the time of sampling, but rather a chronic response to Arg supplementation was evident. The higher capacity for protein synthesis likely explains the increased birth weight of Arg-F compared with Con-F as skeletal muscle mass accounts for 30-40% of body weight (Teleni, 1993). Collectively, these results suggest that maternal Arg supplementation from day 100 of pregnancy to term improves the capacity for protein synthesis in Arg-F via increased abundance of mTOR and ribosome number. However, whether this increased capacity results in higher muscle mass at birth and therefore, increased birth weight requires further investigation.

The higher muscle translational capacity observed in Arg-F at P140, was still present at PN153, as shown by the increased RNA in Arg-F compared with Con-F individuals. In addition, increased DNA content in Arg-F lambs may be indicative of increased incorporation of satellite cells (Moss and Leblond, 1971), which contributes to muscle growth (Rosenblatt et al., 1994), as previously shown in the fetus and neonate lambs (Greenwood et al., 2000a; McCoard et al., 2001). Increased RNA and the carryover

effect on post-natal muscle growth in female offspring could be a response to a nutritional programming effect of the post-natal capacity for muscle growth in Arg-F. There is growing evidence nutritional programming events occurring during fetal development may affect the trajectory of muscle growth, exerting long-term effects on the offspring growth performance (Du et al., 2010). The effect on Arg-F resulted in a growth trajectory similar to that of Arg-M. Male growth diverges from females in some sheep breeds from day 63 after birth (Gbangboche et al., 2006). In the present study the difference in growth between males and females was delayed in Arg group compared to control animals by 43 days. Therefore Arg supplementation during gestation enhanced growth in females after birth and reduced the divergence in growth pattern normally observed between males and females, although the finding that effect was restricted to females is intriguing and requires further investigation.

The activation of mTOR occurs in response to changes in the concentration of specific FAA (Beugnet et al., 2003; Wullschleger et al., 2006; Sancak et al., 2008), hormones (e.g. insulin and IGF-1), and energy (Wang and Proud, 2006). In the present study, the 9-fold increase in ewe plasma Arg levels compared to control ewes, had no effect on fetal plasma or muscle Arg concentration. However, the numeric increase in Arg concentration in fetal plasma and the increase in ornithine levels, which was likely originated from the breakdown of Arg by the enzyme arginase in the urea pathway (Morris Jr, 2002), suggests that an increase in Arg concentration did occurred in the fetus. It is feasible that the time of sampling was either too early or too late to catch the increase in Arg concentration. Therefore, the increased abundance of mTOR measured in this study could be associated with the effect of Arg supplementation. While maternal supplementation with Arg increased insulin concentration in ewe plasma, in agreement with Davis (1972), there was no effect on the circulating levels in fetal plasma, suggesting elevated insulin levels are unlikely to have mediated the effect of Arg on mTOR. In addition, no differences were observed in IGF-1 in maternal or fetal plasma. Both insulin and IGF-1 can activate the mTOR signalling pathway in sheep fetuses (Shen et al., 2002; Shen et al., 2003). Further, the increase in maternal glucose in Arg-treated animals resulted in no increase in circulating fetal glucose concentration. As described for Arg, this could be associated with the time at which fetuses were sampled and in consequence is not possible to discard an effect of glucose on mTOR. The findings from this study suggest that increased abundance of mTOR is potentially elicited by Arg supplementation

although not through the effect of insulin or IGF-1. However, these results are consistent with the findings reported for the effect of Arg through mTOR pathway in skeletal muscle growth of piglets (Yao et al., 2008).

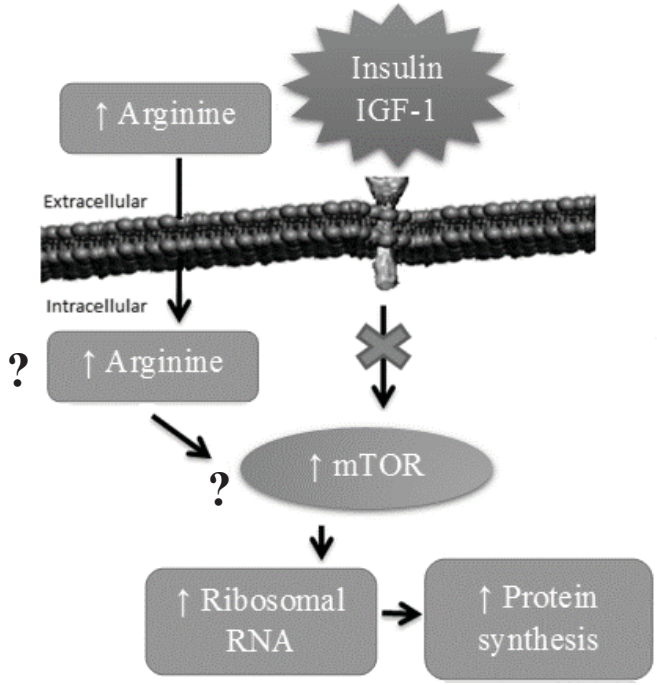

Figure 4.6. Proposed model for increased protein synthesis as an effect of Arginine (Arg) supplementation in *M. longissimus dorsi* from fetuses born to ewes supplemented from day 100 to 140 of pregnancy.

Maternal Arg supplementation affects the concentration of specific FAA of maternal (Lassala et al., 2010) and fetal (Satterfield et al., 2011) plasma. Increased concentration of ornithine and decreased concentration of methionine in the ewe are in agreement with previous reports (Lassala et al., 2010; Lassala et al., 2011; Satterfield et al., 2011). Maternal ornithine participates in the synthesis of polyamines and nitric oxide (Wu and Morris Jr, 1998), which are mediators of placental growth and angiogenesis (Wu et al., 2000) and therefore may enhance the transfer of nutrients to the fetus. Reduction in the concentrations of specific FAA in the ewe following Arg supplementation (e.g. methionine, glycine and serine), may be associated to their utilisation for tissue growth (Rius et al., 2012), AA imbalance, as demonstrated to occur in pigs when fed excess Arg (Southern and Baker, 1982) or creatine production, which involves the use of methionine and glycine (Wu and Morris Jr, 1998). In the fetus, methionine, glycine and ornithine followed the same pattern observed in maternal plasma. The lower concentration of glycine in fetal plasma could be related to lower maternal serine concentration, as fetal

glycine is produced mainly from maternal serine (Chung et al., 1998). It is unclear if changes in maternal and fetal FAA concentrations are related to AA imbalance, transport or utilisation. Further studies are necessary to better understand to the effect of maternal Arg supplementation on AA metabolism.

Maternal supplementation with Arg resulted in metabolic differences between treatments. Increased glucose concentrations in maternal plasma after Arg supplementation in association with increased levels of insulin has been previously reported in sheep (Hertelendy et al., 1970). Reduced NEFA concentration suggests an improvement in the utilisation of nutrients in Arg-treated ewes which may have supported increased fetal growth. The decrease in maternal NEFA concentration contrasts with Lassala et al. (2010; 2011), where no changes in NEFA concentration were observed. Interestingly, at PN153, ewe lambs from Arg-treated ewes had higher glucose concentration, suggesting a change in glucose metabolism, and tended to have higher triglycerides concentration compared to Con-F. In addition, Arg-treated lambs at PN153 tended to have lower NEFA and glycerol concentration. Combined, these metabolic changes suggest a lower mobilisation of reserves. The mechanisms associated with these differences are not clear, however, a programming effect during pregnancy could explain in part the observed results.

4.7 CONCLUSIONS

Maternal supplementation of well-fed twin-bearing ewes with Arg from day 100 of pregnancy to birth resulted in increased birth weight and muscle mass at PN153 in females but not males. These phenotypic differences in ewe lambs appear to be associated with a higher capacity for protein synthesis in muscle via increased abundance of mTOR and ribosome number. The potential for Arg to improve birth weight and muscle growth in females could have important implications for sheep production, in terms of lamb survival and meat production, as females have lower market weight and muscle mass compared to males. Further studies are warranted to confirm the effect of Arg supplementation observed only in females in this study. Future studies will generate a better understanding of the possible effects of maternal Arg supplementation on cellular and metabolic mechanisms underpinning the observed phenotypic shifts of improved twin growth during pregnancy and in early post-natal life, especially in females.

Chapter 5: Effect of oral supplementation of L-Arginine on skeletal muscle growth in artificially-reared twin born lambs from birth until weaning

General overview of the chapter:

Research in Chapter 3 of this thesis identified a group of intra-cellular amino acids (AA) associated with muscle mass at weaning, but did not identify which of the AA could be acting as a limiting signalling for muscle growth in twin-born lambs. Previous studies have shown an association between arginine (Arg) and muscle growth during neonatal period in monogastrics, acting through activation of mTOR pathway. Therefore, the aim of this chapter was to establish if oral Arg supplementation of twin-born lambs from birth to weaning influences skeletal muscle growth and also, the impact of Arg supplementation on mTOR signalling.

5.1 ABSTRACT

Dietary Arg supplementation increases muscle growth in pigs, an effect driven by the mTOR signalling pathway. Twin-born lambs have reduced muscle growth from birth to weaning compared to singletons. The possible role and mechanisms of Arg supplementation on muscle growth in twin lambs is not clear. Therefore, the aims of this study were firstly, to determine the effect of oral Arg supplementation on post-birth skeletal muscle growth, and secondly, the impact of Arg supplementation on mTOR signalling. Twin-born lambs were fed milk-based diets supplemented with either 500 mg Arg-HCl/kg body weight or saline solution (control) between birth and 28 days (Cohort 1) and between birth and 70 days of life (Cohort 2). Lambs of Cohort 1 were euthanised at 28 while Cohort 2 at 70 days of life, one-hour after treatment. Blood samples were collected prior to slaughter for plasma free amino acid (FAA) and insulin concentration analyses. Once euthanised, *M. semitendinosus, M. longissimus dorsi* (LD), *M. biceps femoris, M. gastrocnemius, M. plantaris, M. semimembranosus, M. adductor, M. gluteus, M. quadriceps* and *M. psoas major* were excised and weighed. A sample from the LD was snap frozen for later analysis of FAA concentration, mTOR abundance and phosphorylation and biochemical indices (DNA, RNA and protein content) in both cohorts. Arginine supplementation increased plasma Arg concentration at both time points, and increased the live weight of Arg-treated female lambs (Arg-F) at 28 days, compared to control females (Con-F). The increased live weight of Arg-F lambs was associated with an increased average daily gain (ADG) between 7 and 21 days of life. There was no difference in ADG between Arg and control male lambs. A positive effect of Arg supplementation was observed only in *M. biceps femoris* muscle weight of Arg-F at 28 days. In agreement with the lack of treatment effect on LD weight, no changes in mTOR signalling or biochemical indices were detected at 28 days. A longer supplementation period in Cohort 2 resulted in an increased ADG between 7 and 28 days of life in both males and female Arg-treated lambs. At 70 days, Arg-F had heavier muscle weights compared to Con-F, including LD. Con-F had increased abundance and activation of mTOR compared with Arg-F, coupled with a higher protein synthetic capacity. Supplementation with Arg resulted in the decreased concentration of a group of plasma and muscle AA, which may be associated with their utilisation for tissue growth. No effect of Arg supplementation was determined on insulin concentration between treatments. Results suggest Arg supplementation during the first 3 weeks of life increases

body growth, resulting in heavier muscles at weaning but only in females, and was not associated with changes in mTOR signalling at 28 or 70 days of life. Future work is warranted to evaluate the association between Arg supplementation and mTOR signalling in muscle growth during the rapid phase of growth (7-28 days post-natally) when differences in growth were observed.

5.2 INTRODUCTION

Improved prolificacy has positively impacted sheep meat production (Amer et al., 1999). However, twin-born lambs have slower growth rates (Hopkins et al., 2007), take longer to reach market weight (Nordby et al., 1987; Dimsoski et al., 1999) and can have lighter carcasses at the same age (Afolayan et al., 2007) as singletons. Skeletal muscle represents up to 45% of body weight (Teleni, 1993) and up to 70% of carcass weight, when considering only the saleable meat yield (Hopkins and Fogarty, 1998). Therefore, any attempt to improve muscle growth in twin-born lambs should have a positive impact on meat production.

After birth, skeletal muscle growth occurs through an increase in the size of the fibres (Brameld et al., 1998; Rehfeldt et al., 2000), and requires myoblast incorporation to increase genomic DNA content (Yates et al., 2012). In the neonate, this hypertrophic process is associated with a high fractional rate of protein synthesis, resulting in elevated protein deposition (Davis et al., 1989; Davis et al., 1996). However, the high protein synthesis observed during the first days of life, decreases rapidly with age (Davis et al., 1996), suggesting the neonatal stage is an important period for post-natal interventions aimed to increase muscle hypertrophy. Protein synthesis is modulated by the mechanistic target of rapamycin (mTOR), a serine/threonine kinase which integrates a series of signals, resulting in translation initiation and elongation (Proud, 2004a). Amino acids can act as signalling molecules for mTOR activation, and therefore, intracellular protein turnover (Wu, 2009). Although the mechanisms by which AA activate mTOR are not fully understood (Jewell et al., 2013), changes in the intracellular concentration of specific AA are responsible of mTOR activation (Beugnet et al., 2003; Nicklin et al., 2009). In monogastrics, supplementation with arginine (Arg) between 7 and 21 days of life increases body weight (Kim et al., 2004), and muscle growth in finishing pigs (Tan et al., 2009), an effect mediated by the activation of mTOR (Yao et al., 2008).

In ruminants, the effect of Arg supplementation on muscle hypertrophy is not well understood. The high degradability of Arg in the rumen (Chacher et al., 2012) creates a challenge when evaluating the effect of dietary Arg supplementation. Supplementation with rumen-protected Arg in weaned lambs resulted in an increase in plasma Arg concentration, however no changes in body growth were observed (Davenport et al., 1995). Considering that rapid growth occurs early in neonatal life and the magnitude of the response to nutrient intervention diminishes with development (Davis and Fiorotto, 2009), the lack of effect in the aforementioned study was potentially due to the advanced age of animals at the time of supplementation with Arg. Further, the effect of unprotected Arg supplementation pre weaning, when the rumen is not fully developed (Wardrop and Coombe, 1961), has not been evaluated and could give a practical approach for Arg supplementation.

The objectives of this study were to determine 1) the effect of oral Arg supplementation to twin-born lambs, on post-natal skeletal muscle growth up to 70 days of life and 2) the impact of oral Arg supplementation on mTOR signalling. It was hypothesised that Arg supplementation during the neonatal period in artificially-reared twin-born lambs will improve skeletal muscle growth, and that the effect will be mediated by mTOR signalling.

5.3 MATERIALS AND METHODS

This study and all animal handling procedures were approved by the University of Auckland Animal Ethics Committee, New Zealand in accordance with the 1999 Animal Welfare Act (C889) and by AgResearch Grasslands Animal Ethics Committee (12371).

5.3.1 Ewes

Multiparous Romney ewes were mated to Poll Dorset sires in two groups of 80-90 ewes at a ratio of one ram per 10 ewes over 2 days, 6 weeks apart, using estrus synchronisation with a progesterone-controlled internal drug release device (CiDR, 0.3 g progesterone, Pharmacia & UpJohn, Auckland, New Zealand). At approximately 60 days

pregnancy (P60) ewes were pregnancy scanned using trans-abdominal ultrasound and thirty twin-bearing ewes were selected, separated in two cohorts (Cohort 1; n = 16 and Cohort 2; n = 14) and maintained under a commercial grazing regimen. From P110, selected ewes were adapted to concentrate feeding while on pasture as previously described in Chapter 4. At P125, ewes were moved indoors into group pens for adaptation over a week period, fed only concentrate.

Ewes had free access to drinking water and were fed once daily (between 0800 and 0900 h) with a lucerne-based pellet diet (University C mix, Camtech Nutrition, Cambridge NZ) formulated to meet 100% of the NRC-recommended maintenance requirements for twin-bearing pregnant ewes. The pellets contained 10.5 MJ/kg dry matter of metabolisable energy and 17% crude protein.

Ewes were kept in a 12 h light–dark cycle during the trial. Ewes were monitored 24 h a day from one week before estimated lambing date. Lambs were removed from the ewe prior to suckling, to avoid any potential differences in the intake or composition of maternal colostrum that could influence the lamb growth.

5.3.2 Lambs

Cohort 1. Immediately after removal from the ewe, lambs were towel dried, ear-tagged, weighed, had their body dimensions measured and a blood sample was taken. Lambs were housed indoors in group pens (4-6 lambs per pen), and both lambs from each twin pair were randomly allocated to either control or treatment (Arg-treated) group, balanced by sex of the lamb and birth weight. Lambs born to ewe in Cohort 1 were managed as follows. Lambs were individually bottle-fed 6 times per day during the first 4 days using a commercial colostrum replacer (JumpStart®, Anlamb, Anchor NZ Dairy Board, Auckland, NZ). From day 2, colostrum was gradually blended with milk replacer (AnLamb®, Anlamb, Anchor NZ Dairy Board, Auckland, NZ), using a 80:20, 50:50 colostrum: milk ratio. From day 4 to 28, lambs were fed 5 times per day with milk replacer. Lambs were initially fed 40 g DM/kg/day of milk replacer, however, due to suspected subclinical abomasal bloat in 3 lambs, the feed allocation was decreased to 35 g DM/kg/day on day 10 of age, and the number of feeds maintained at 5 times daily until day 28. Milk intake of each individual lamb was recorded at every feed. Amino acid composition of the feeds are presented in Table 5.1.

The control group received no arginine supplementation, while supplementation of Arg was applied from birth via fortification of colostrum and milk replacer with an equivalent of 500 mg kg^{-1} live weight of Arg, using L-Arginine-HCL (Ajinomoto Inc., Tokyo, Japan), based on a previous study (Wu et al., 2007b). The L-arginine-HCl solution was prepared daily (300 mg L-arginine per ml), using sterile physiologic saline; pH adjusted to 7.0 with 1 mol/L NaOH and stored at 4ºC until used, via mixing the solution with colostrum/milk. The doses of Arg were calculated based on body weight and adjusted every 2 days.

Three lambs died during the course of the trial and post-mortem study was performed to determine the cause of death. One lamb died after birth due to trauma, the second one died at 10 days of age due to intestinal torsion. The third lamb died at 21 days of age, due to pneumonia. Therefore, in Cohort 1, a total of 29 lambs completed the study (control n = 14, Arg-treated n = 15). At day 28, lambs were euthanised by intravenous (i.v.) injection of sodium pentobarbitone (3 g *i.v.*, Pentobarb 500; Chemstock Animal Health, Christchurch, New Zealand) one-hour after being fed.

Cohort 2. Lambs born to Cohort 2 were managed as per Cohort 1, and allocated to either a control or Arg group, with the following modifications. Lambs received the same management and feeding regime from birth to day 4 to that of Cohort 1. Then they were fed 40g DM/kg/day milk replacer during the whole experiment, which contrasts with Cohort 1, with the daily allowance split into 4 feeds daily (5-34 days), 3 feeds a day (35-40 days) and then 2 feeds a day until day 70. Arg-treated lambs in Cohort 2 received from birth to sampling at day 70 the same Arg doses described for Cohort 1. From birth to 28 days of life, pasture was withheld from the diet to delay rumen development. From day 28 to 70, lambs were provided a mixed grass sward *ad libitum,* in order to stimulate rumen development and evaluate the effect of pasture feeding on Arg absorption. Amino acid composition of the grass is also presented in Table 5.1. Milk intake of each individual lamb was recorded at every feed. In Cohort 2, one animal died due to pneumonia at 40 days of life, reducing the number of lambs to n = 27 (control n = 11; Arg-treated n = 16). At 70 days of life, one-hour after being milk fed and in the Arg group, supplemented with Arg, lambs were euthanised by captive bolt, immediately followed by exsanguination.

Table 5.1 Amino acid content (mg/g DM) in colostrum, milk replacer and pasture.

	Colostrum replacer (mg/g DM)	Milk replacer (mg/g DM)	Pasture (mg/g DM)
Essential AA			
Histidine	<0.01	6.84	2.83
Isoleucine	23.54	15.78	5.34
Leucine	44.10	26.34	10.29
Lysine	26.95	18.57	8.07
Methionine	7.89	6.57	2.99
Phenylalanine	15.42	11.39	6.78
Threonine	17.73	10.45	5.42
Tryptophan	n.d	n.d	2.75
Valine	23.39	14.88	6.78
Non-essential AA			
Alanine	12.86	8.15	7.78
Arginine	13.01	8.07	6.83
Aspartic acid	28.06	18.15	12.38
Cystine	4.15	1.96	1.90
Glutamic acid	69.53	51.88	13.81
Glycine	8.63	4.77	6.70
Proline	31.03	22.95	6.08
Serine	20.44	12.10	5.25
Tyrosine	16.90	11.44	4.35
Total	373.63	250.28	116.33

n.d.: non detected

5.3.3 Animal evaluation

Cohort 1. Lamb live weight was recorded before euthanasia at 28 days. After euthanasia, the following muscles were excised from the right side of the carcass and their weight was recorded: *M. semitendinosus, M. longissimus dorsi* (LD), *M. biceps femoris, M. gastrocnemius, M. plantaris, M. semimembranosus, M. adductor, M. gluteus, M. quadriceps* and *M. psoas major*.

Cohort 2. Lambs were weighed every two days until day 28 and weekly thereafter until day 70. Lambs were subject to body composition analysis by dual X-ray absorptiometry (DEXA, XR-800, Norland Corp., Fort Atkinson, WI, USA) at 28 days of age, in order to evaluate lean muscle deposition at a similar age to lambs in Cohort 1. A previous study in lambs showed a high correlation between muscle weights obtained using DEXA and those obtained by dissection (Mercier et al., 2006). A pre-anaesthesia blood screen (New Zealand Veterinary Pathology, Ltd., Hamilton, NZ) was undertaken for each lamb one week before DEXA, to confirm general health status prior to sedation. This screen evaluated globulin, total protein, creatinine, GGT, albumin, glutamate dehydrogenase (GDH), albumin:globulin ratio (AGR) and urea (Table C.1, Appendix C). A total of 9 lambs from the control and 12 from Arg-treated group were scanned. Lambs were fasted for 12 h before the DEXA analysis and scans were performed under sedation using an equivolume mixture of diazepam (5 mg ml^{-1}) and ketamine (100 mg ml^{-1}) administered intravenously (combination of 0.5 mg/kg BW Pamlin and 10 mg/kg BW Ketamine, Parnell Technologies NZ). Fat and lean mass composition was obtained using an area defined proximally by the last two cervical vertebrae, the base of the tail distally, and from the back superiorly to the base of the humerus and femur inferiorly. In addition, images were also captured for the thorax, abdomen and rump separately (Figure C.1, Appendix C). Data was analysed using ILLUMINATUS 434S160 software (Norland Corp., Fort Atkinson, WI, USA) and expressed as a percentage of body weight. Lambs were euthanised at 70 days of life and muscles described previously for Cohort 1 were excised and weighed separately.

For Cohort 1 and Cohort 2, after euthanasia, a sample from the distal portion of the LD muscles was collected, snap frozen in liquid nitrogen and stored at -80ºC. The LD was used for free AA (FAA) profiling, biochemical indices, SDS page and Western

Blotting due to the magnitude of the effect of Arg supplementation on muscle mass at 70 days.

5.3.4 Amino acids analysis

Free AA concentrations were determined in the LD muscle from both cohorts (Cohort 1 *n* = 29; Cohort 2 *n* = 27), by ion-exchange chromatography using post-column derivatisation with ninhydrin, as previously described in Chapter 3 and by van der Linden et al. (2012).

5.3.1 Biochemical indices

Total RNA, DNA and protein from LD muscle of both cohorts (Cohort 1 *n* = 29; Cohort 2 *n* = 27) were extracted as previously described in Chapter 3.

5.3.2 mTOR

In order to process all samples on the same gel and avoid gel-to-gel variation, a subgroup of 24 lambs were selected in Cohort 1, corresponding to 6 males/treatment and 6 females/treatment. For Cohort 2, a subgroup of 23 lambs, consisting in 6 males in each treatment, 5 females in the control group, which corresponded to all females, and 6 females in Arg-treated group were studied. The protocol used for total mTOR and phosphorylated (Ser^{2448}) mTOR abundance in LD was previously described in Chapter 4.

5.3.3 Plasma insulin

Plasma samples from both cohorts (Cohort 1 *n* = 29; Cohort 2 *n* = 27) obtained at the time of euthanasia via jugular venipuncture were analysed for insulin as described in Chapter 4.

5.3.4 Statistical analysis

Muscle weight, FAA concentrations, biochemical indices, plasma insulin and mTOR data for Cohort 1 at 28 days and Cohort 2 at 70 days of life, including DEXA values from day 28, were analysed by ANOVA, with the MIXED procedure (SAS, 2006) using a linear model, which considered treatment (control *vs.* Arg), sex of lamb and their interaction as fixed effects, and ewe as a random effect to account for the twinning effect. A separate analysis using the weight of the lamb at 70 days as a covariate was performed in order to define the proportionality of the weight of each evaluated muscle to body

weight. Data for mTOR signalling is presented considering the concentration and also the total abundance in LD muscle.

Body weight data was analysed with a repeated analysis using MIXED procedure with the same parameters previously described. For Cohort 2, live weight change was analysed considering the data from birth to 28 days which corresponds to the monogastric stage (Wardrop and Coombe, 1961), and a separate analysis was carried out from day 1 to day 70. Average daily gain (ADG) was analysed at weekly periods using a similar lineal model described for other data.

Normality in the distribution of residuals for all variables was confirmed using the UNIVARIATE procedure of SAS. Differences between means were determined using the PDiff option of the LSMeans procedure of SAS. Probability (P) values ≤ 0.05 were taken to indicate significant differences and $P \leq 0.10$ was considered as a trend. For FAA only those values with $P \leq 0.05$ are described.

5.4 RESULTS

5.4.1 Effect of Arg supplementation on growth performance

Birth weights did not differ between lambs allocated to the Arg-treated and control groups in either Cohort 1 or Cohort 2 (Table 5.2). In Cohort 1 there was a trend ($P = 0.10$) for a group by time interaction for live weight, where Arg-treated lambs began to diverge from control lambs between day 7 to 14 (Figure 5.1), which is explained by a greater ADG in Arg-treated lambs from day 7 to 14 (Figure 5.2). Arg-treated lambs were 4% heavier than control lambs by day 14 and remained heavier to day 28 (Figure 5.1). No sex effect was determined.

Table 5.2. Lamb weight (kg) at birth for Cohort 1 and Cohort 2, from lambs supplemented with Arg or unsupplemented (control). Table shows least square means and average standard error of the means (SEM). The effect of treatment (T, Arginine or control), Sex of lamb (S) and treatment by sex of lamb interaction (T x S) are shown. In brackets, number of animal.

	Control		Arginine			*P*-value		
	Male	Female	Male	Female	SEM	T	S	T x S
Cohort 1	5.2 (7)	4.5 (9)	5.2 (8)	4.8 (8)	0.3	0.66	0.03	0.69
Cohort 2	5.4 (6)	5.2 (6)	5.3 (7)	5.4 (7)	0.3	0.98	0.91	0.44

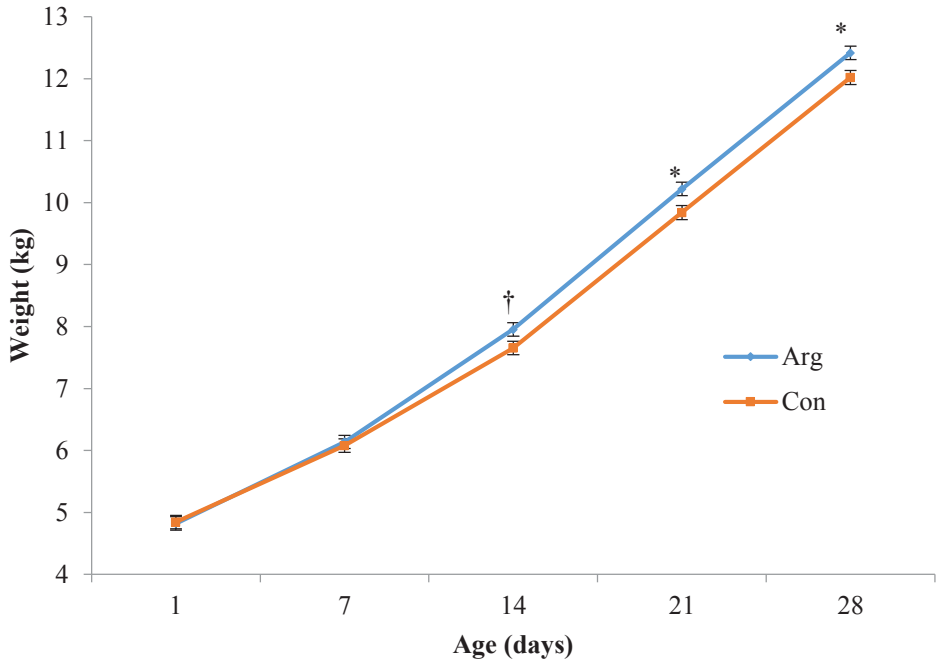

Figure 5.1. Lamb live weight for Cohort 1, either supplemented with arginine (Arg, n = 15) or unsupplemented (controls, Con, n = 14) from birth to day 28. Data shows least square means ± standard error of the means (SEM) for treatment (Arg *vs.* control) by time interaction. †*P* <0.10; **P* ≤ 0.05.

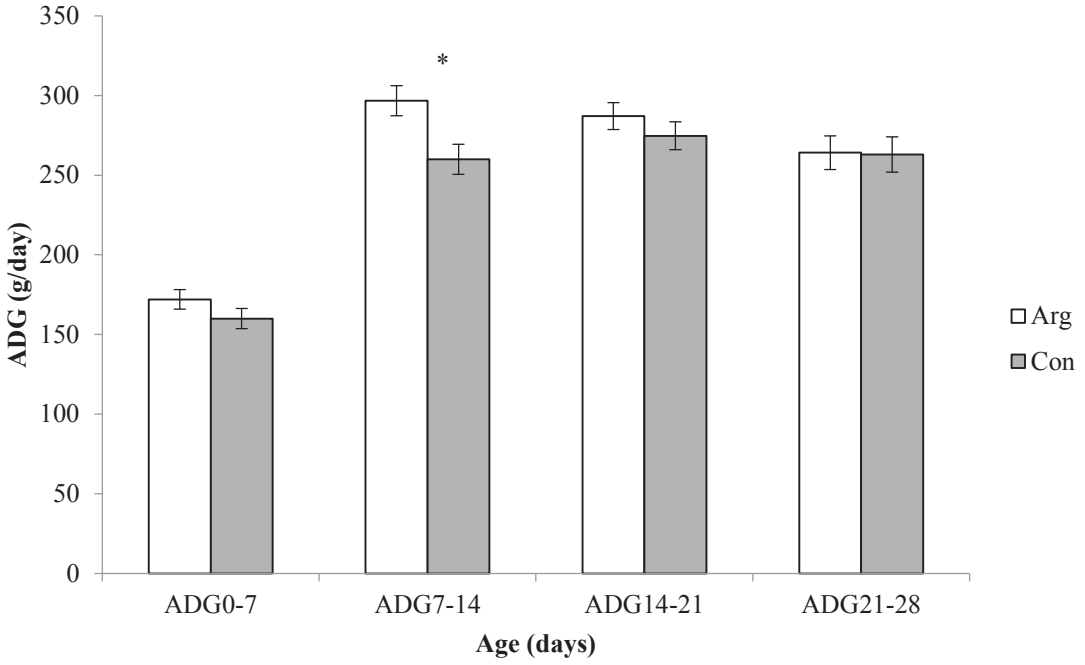

Figure 5.2. Average daily gain (kg/day) of Cohort 1 from lambs supplemented with Arginine (Arg, n = 15) or unsupplemented (control, Con, n = 14) during the first 28 days of life. Data shows least square means and standard error of the mean (SEM). *$P \leq 0.05$.

In Cohort 2, a treatment by time interaction was observed for live weight change from birth to day 28 ($P = 0.003$). Arg-treated lambs were heavier than control lambs at day 21 and 28 ($P < 0.05$) (Figure 5.3), which is explained by a higher ADG from day 7 to 14 (308 ± 6 *vs.* 281 ± 6 g day^{-1}, $P < 0.05$) and from day 14 to 21 (358 ± 8 *vs.* 325 ± 9 g day^{-1}, $P < 0.05$). No differences ($P > 0.05$) were observed between treatments when weight was analysed from day 1 to day 70 (Figure 5.4). However, group by sex of the lamb interaction ($P = 0.05$) for ADG between day 45 and day 52 was observed, where Arg-treated females (Arg-F) had a greater ADG than control females (Con-F) (330 ± 21 *vs.* 270 ± 28 g day^{-1}, $P < 0.05$), while Arg treated (Arg-M) and control (Con-M) males did not differ (249 ± 24 *vs.* 291 ± 26 g day^{-1}, $P > 0.05$).

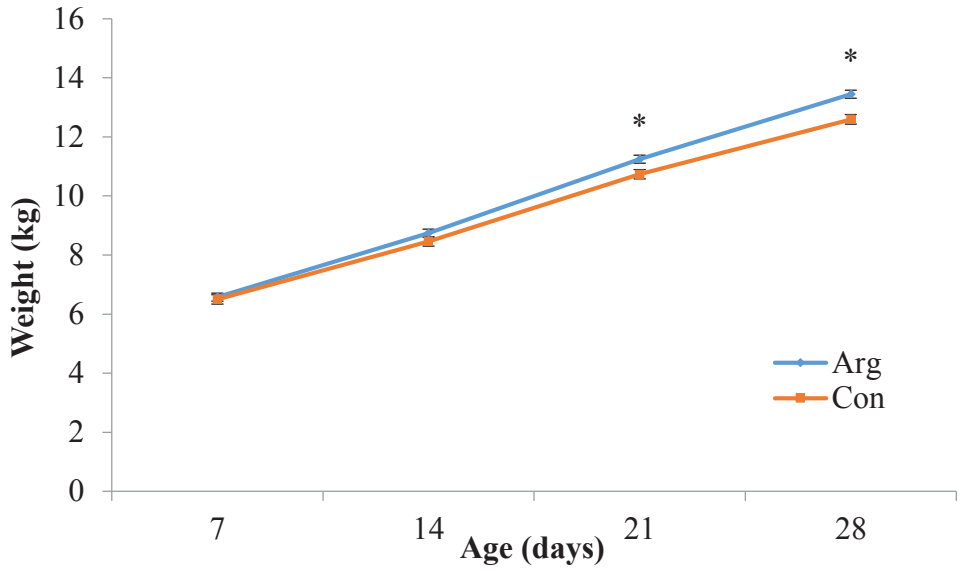

Figure 5.3. Live weight change for Cohort 2, either supplemented with arginine (Arg, n = 16) or unsupplemented (controls, Con, n = 11) from birth to day 28. Data shows least square means ± standard error of the mean (SEM) for group by time interaction. *Arg *vs.* Control $P < 0.01$.

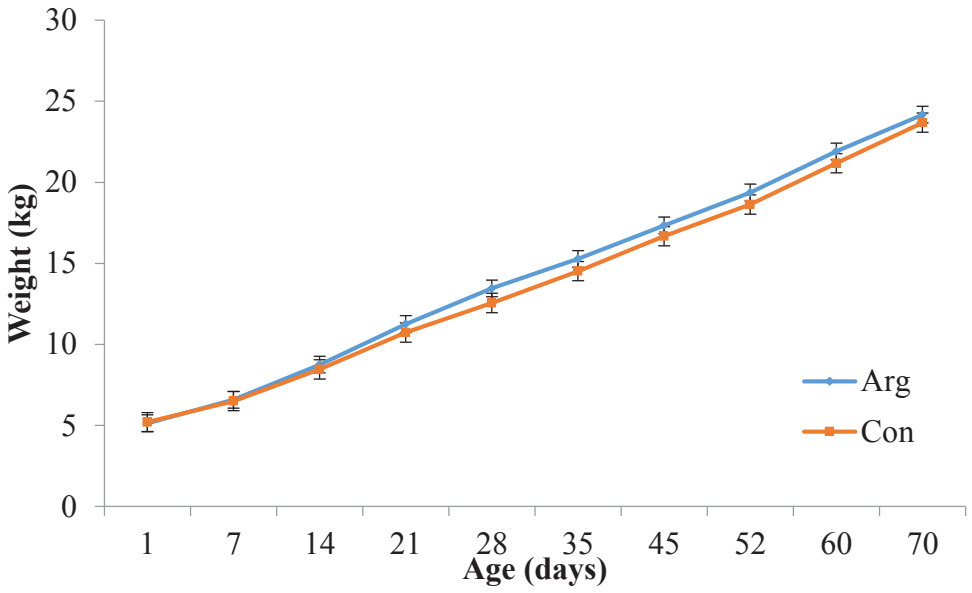

Figure 5.4. Live weight change for Cohort 2, either supplemented with arginine (Arg, n = 16) or unsupplemented (controls, Con, n = 11) from birth to day 28. Data shows least square means ± standard error of the mean (SEM) for group by time interaction.

5.4.2 Effect of Arg supplementation on muscle weight

5.4.2.1 Cohort 1

There was no effect of Arg supplementation on muscle weight compared to the control group at 28 days of life when data was not adjusted for live weight (Table 5.3). Males had heavier *M. biceps femoris*, *M. plantaris*, *adductor* and *gluteus* and tended to have heavier *M. semitendinosus* and *M. psoas major* muscles compared to females, resulting in a higher total muscle leg weight. When data was adjusted for body weight (Table 5.3), a trend for treatment by sex of lamb interaction was observed for *M. biceps femoris*, which tended to be heavier in Arg-F compared to Con-F ($P = 0.06$), while no difference was observed between males ($P = 0.96$). After adjustment for body weight, females had heavier *M. longissimus dorsi*, *M. gastrocnemius* and *M. quadriceps* than males.

Table 5.3. Cohort 1; muscle weight (g) from lambs supplemented with Arg or unsupplemented (control) at 28 day
average standard error of the means (SEM). The effect of treatment (T, Arginine and control), Sex of lamb (S) an
are shown.

	Unadjusted								Control	
	Control		Arginine			*P*-value				
	Male	Female	Male	Female	SEM	T	S	T x S	Male	Female
n	6	8	7	8					6	8
Longissimus dorsi	179	154	184	176	11	0.25	0.12	0.39	166	171
Psoas major	30	27	33	29	2	0.28	0.07	0.67	28	30
Semitendinosus	38	34	39	36	2	0.38	0.06	0.79	36	36
Biceps femoris	109	95	115	108	7	0.27	0.01	0.29	109	99
Gastrocnemius	44	41	45	42	2	0.73	0.14	0.90	42	44
Plantaris	18	15	18	16	1	0.74	0.01	0.72	17	16
Semimembranosus	106	99	112	106	6	0.40	0.16	0.93	105	104
Adductor	46	38	46	41	3	0.80	0.02	0.57	43	43
Gluteus	73	63	78	68	5	0.39	0.03	0.96	69	70
Quadriceps	136	126	139	130	7	0.61	0.12	0.88	132	135
Total leg muscle	598	540	625	577	31	0.41	0.05	0.85	579	577

[1]Adjusted data: muscle weights adjusted for lamb weight at 28 days of age.

5.4.2.2 Cohort 2

A treatment by sex of lamb interaction was observed whereby Arg-F had heavier *M. quadriceps* and *M. psoas major* compared to Con-F ($P < 0.05$), while no difference was observed between males ($P > 0.05$) (Table 5.4). A tendency was observed for a group by sex of lamb interaction where Arg-F had heavier *M. longissimus dorsi*, *M. biceps femoris*, *M. semimembranosus*, *M. gluteus* and total leg muscle weight compared to Con-F ($P < 0.10$). These effects were not observed in males ($P > 0.05$). Males tended to have heavier *M. plantaris* muscle compared to females, which tended ($P = 0.06$) to be significant after adjusting to a common body weight.

5.4.3 DEXA analysis in Cohort 2

A treatment by sex of lamb interaction ($P = 0.05$) was observed for lean content in the abdomen region, determined by DEXA scanning in lambs in Cohort 2 at 28 days of life, where Arg-M had a lower lean content compared to Con-M (25.49 ± 0.65 *vs.* 28.31 ± 0.68 %, $P = 0.01$), while no difference was observed between females (26.88 ± 0.58 *vs.* 27.31 ± 0.75%, $P = 0.65$). For the whole animal, Arg-treated lambs had a lower percentage of lean tissue compared to control lambs at 28 days of life (71.35 ± 0.40 *vs.* 73.32 ± 0.51%, $P < 0.01$) (Table C.2, Appendix C).

Table 5.4. Cohort 2; muscle weight (g) from lambs supplemented with Arg or unsupplemented (control) at 70 day average standard error of the means (SEM). The effect of treatment (T, Arginine and control), Sex of lamb (S) and are shown.

	Unadjusted								Control	
	Control		Arginine			*P*-value				
	Male	Female	Male	Female	SEM	T	S	T x S	Male	Female
n	6	5	7	9					6	5
Longissimus dorsi	341	311	335	360	15	0.23	0.83	0.06	334	329
Psoas major	55	48	56	57	3	0.20	0.14	0.05	54	51
Semitendinosus	76	66	70	71	4	0.92	0.25	0.11	74	71
Biceps femoris	200	182	193	208	12	0.50	0.88	0.10	195	196
Gastrocnemius	73	67	72	74	4	0.53	0.49	0.22	71	72
Plantaris	30	25	29	29	2	0.41	0.07	0.13	29	27
Semimembranosus	194	179	184	200	10	0.63	0.96	0.09	190	191
Adductor	80	77	80	85	3	0.32	0.87	0.19	80	80
Gluteus	150	134	143	149	7	0.65	0.44	0.09	147	143
Quadriceps	246	215	239	251	13	0.36	0.38	0.05	241	232
Total leg muscle	1090	995	1064	1122	50	0.39	0.66	0.08	1067	1060

[1]Adjusted data: muscle weights adjusted for lamb weight at 70 days of age.

5.4.4 Amino acid profiles in Cohort 1

5.4.4.1 Plasma

A treatment by sex of lamb interaction was observed for methionine and threonine concentration in plasma (Table 5.5), where Arg-F had 44% lower methionine concentration compared to Con-F, while Arg-M had 23% lower methionine concentration compared to Con-M. For threonine, while no difference was observed between treatments for males, Arg-F had lower concentration compared to Con-F. Arg-treated lambs had higher plasma concentration of arginine and ornithine, but lower concentration of glutamine, histidine, phenylalanine, alanine, asparagine, aspartate, citrulline, glycine, tyrosine, resulting in lower total FAA concentration in plasma. Females had higher plasma concentrations of asparagine and serine, while lower plasma concentration of citrulline compared to males.

5.4.4.2 Muscle

In muscle, a treatment by sex of lamb interaction was found for intracellular concentration of isoleucine and threonine (Table 5.5). Arg-M tended to have higher isoleucine compared to Con-M, while no difference was found between females. For threonine, Arg-F had lower concentration compared to Con-F and no difference was observed between males. Higher Arg and ornithine, but lower histidine, methionine and glycine concentrations were observed in Arg-treated lambs. Males had higher concentration of histidine, leucine and valine compared to females, resulting in higher total EAA.

Table 5.5. Free amino acid concentration in Cohort 1 plasma (μMol/L) and in *M. longissimus dorsi* muscle (nM birth to 28 days. The effect of treatment (T), sex of lamb (S) and treatment by sex of lamb interaction (T x S) are means and average standard error of the means (SEM).

	Plasma										
	Control		**Arginine**			***P*-value**			**Control**		
	Male	**Female**	**Male**	**Female**	**SEM**	**T**	**S**	**T x S**	**Male**	**Female**	**M**
n	**6**	**5**	**7**	**9**					**6**	**5**	
Essential AA (EAA)											
Histidine	235	229	171	154	13	<0.01	0.35	0.69	242	201	1
Isoleucine	80	77	77	77	8	0.91	0.82	0.83	20	22	
Leucine	154	157	154	148	14	0.79	0.86	0.67	74	61	
Lysine	176	177	168	164	18	0.60	0.93	0.88	103	97	1
Methionine	48	55	37	31	3	<0.01	0.81	<0.01	25	29	
Phenylalanine	79	93	65	68	7	0.01	0.26	0.45	32	45	
Threonine	256	347	222	219	19	<0.01	0.04	0.02	336	421	4
Valine	252	276	271	247	21	0.78	0.95	0.14	127	119	1
Total EAA	1237	1405	1173	1102	89	0.09	0.52	0.10	956	918	9
Non-essential AA (NEAA)											
Alanine	220	245	190	186	17	0.02	0.49	0.37	1160	1414	1
Arginine	195	172	360	386	28	<0.01	0.91	0.32	277	226	4
Asparagine	46	59	34	46	5	0.04	0.02	0.82	37	48	
Aspartate	23	21	16	15	3	0.03	0.50	0.71	178	160	1
Citrulline	190	156	146	127	9	<0.01	0.01	0.41	61	57	
Glutamate	155	136	141	136	17	0.76	0.51	0.69	1170	1000	1
Glutamine	340	365	236	240	22	<0.01	0.32	0.48	2637	2807	2
Glycine	720	692	509	514	54	0.01	0.84	0.76	2476	2263	1
Ornithine	89	98	199	194	12	<0.01	0.86	0.47	116	117	2
Proline	274	276	270	238	20	0.29	0.44	0.42	397	357	4
Serine	121	136	106	131	8	0.26	0.02	0.56	262	253	2
Taurine	26	29	25	29	2	0.89	0.14	0.74	1128	1024	1
Tyrosine	116	126	106	102	6	0.03	0.59	0.19	77	81	
Total NEAA	2502	2465	2288	2350	123	0.24	0.90	0.67	9755	9378	9
Total	3806	3948	3522	3470	171	0.05	0.79	0.54	10722	10287	10

5.4.5 Amino acid profiles in Cohort 2

5.4.5.1 Plasma

A treatment by sex of lamb interaction was observed for glycine concentration in plasma, where Arg-F had lower concentration compared to Con-F, while no difference was observed between males (Table 5.6). Arg-treated lambs had higher plasma concentration of Arg and ornithine compared to control lambs. Males had lower concentration of leucine, lysine, methionine, valine, resulting in lower total EAA concentration compared with females. In addition males had lower concentration of the NEAA proline, serine, taurine and tyrosine compared to females.

5.4.5.2 Muscle

Arg-treated lambs had higher concentrations of aspartate and ornithine in muscle, but lower total NEAA and total AA compared to control lambs (Table 5.6). Males had higher concentration of Arg, but lower concentration of isoleucine, leucine, phenylalanine, valine, taurine and tyrosine, compared to females.

Table 5.6. Free amino acid concentration in Cohort 2 plasma (μMol/L) and in *M. longissimus dorsi* (nMol/g) fro from birth to 70 days. The effect of treatment (T), sex of lamb (S) and treatment by sex of lamb interaction (T x square means and average standard error of the means (SEM).

	Plasma										
	Control		**Arginine**			***P*-value**			**Control**		
	Male	**Female**	**Male**	**Female**	**SEM**	**T**	**S**	**T x S**	**Male**	**Female**	**M**
n	**5**	**6**	**9**	**7**					**5**	**6**	
Essential AA (EAA)											
Histidine	108	104	111	101	13	0.96	0.54	0.80	129	109	
Isoleucine	98	118	88	108	15	0.62	0.17	0.98	47	55	
Leucine	154	199	125	185	21	0.48	0.01	0.66	89	115	
Lysine	141	223	155	203	22	0.98	<0.01	0.32	115	124	
Methionine	39	42	30	53	7	0.75	0.04	0.17	31	29	
Phenylalanine	70	66	64	79	7	0.55	0.22	0.17	39	44	
Threonine	212	202	193	227	31	0.90	0.60	0.45	299	273	
Valine	289	394	268	340	35	0.51	<0.01	0.52	150	191	
Total EAA	1056	1335	1021	1260	116	0.84	0.02	0.84	899	942	
Non-essential AA (NEAA)											
Alanine	202	233	205	196	18	0.47	0.72	0.26	1707	1802	1
Arginine	207	186	358	298	33	<0.01	0.17	0.53	231	183	
Asparagine	32	52	35	30	8	0.22	0.48	0.12	39	32	
Aspartate	17	22	19	20	2	0.88	0.31	0.40	115	130	
Citrulline	214	180	299	213	34	0.16	0.07	0.44	56	52	
Glutamate	121	125	114	104	9	0.19	0.64	0.46	748	694	
Glutamine	245	266	245	247	26	0.80	0.50	0.49	2326	2351	2
Glycine	556	624	605	561	26	0.79	0.94	0.04	1372	1488	1
Ornithine	79	105	145	132	11	<0.01	0.83	0.08	94	96	
Proline	128	184	133	171	16	0.92	0.01	0.55	290	394	
Serine	73	103	79	100	11	0.85	0.04	0.70	153	152	
Taurine	22	39	27	31	3	0.86	0.01	0.06	1238	1754	
Tyrosine	99	128	83	111	14	0.46	0.02	0.97	63	75	
Total NEAA	1906	2189	2304	2181	118	0.17	0.67	0.05	8433	9222	7
Total	2962	3521	3308	3454	215	0.51	0.09	0.23	9332	10164	8

5.4.6 Effect of Arg supplementation on mTOR activation

5.4.6.1 Cohort 1

There was no difference in concentration or total abundance of mTOR or mTOR-Ser^{2448} in Cohort 1 lambs (Figure 5.5 A, B).

5.4.6.2 Cohort 2

In Cohort 2, there was a treatment by sex of lamb interaction for concentration of mTOR ($P = 0.05$), where Con-F had higher concentration compared to Arg-F ($P = 0.04$), while males did not differ (Figure 5.6A). Similarly, a trend for a treatment by sex of lamb interaction was observed for concentration of Ser^{2448} ($P = 0.08$), where Con-F had higher concentration compared to Arg-F ($P = 0.03$), while no difference was observed between males (Figure 5.6B). A trend ($P = 0.08$) for treatment effect was found for mTOR:Ser^{2448} ratio, an indicator of mTOR activation, where Arg-treated lambs had a lower ratio compared to control lambs (Figure 5.6C).

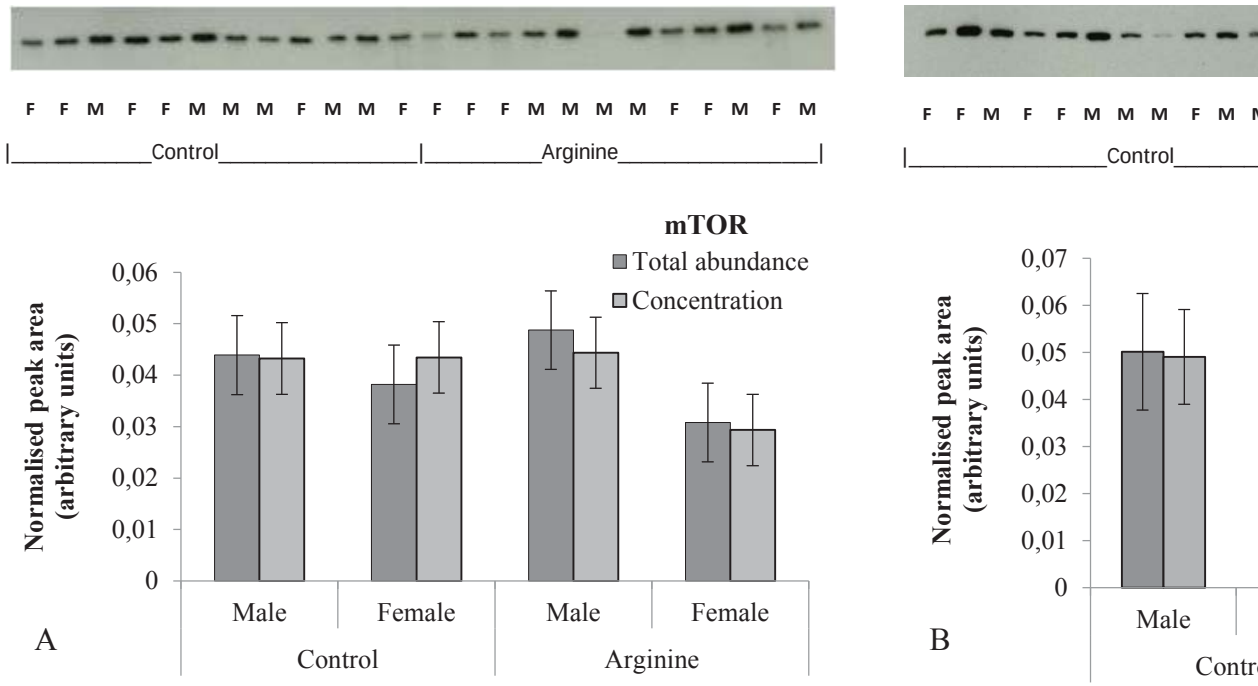

Figure 5.5. Treatment (Arginine *vs.* Control) by sex of lamb (male *vs.* female) interaction for concentration and muscle) of (A) mechanistic target of rapamycin (mTOR) and (B) phosphorylated mTOR (Ser2448) in *M. longissi* with arginine or unsupplemented (control), from birth to 28 days of life. The figure shows the least square mean

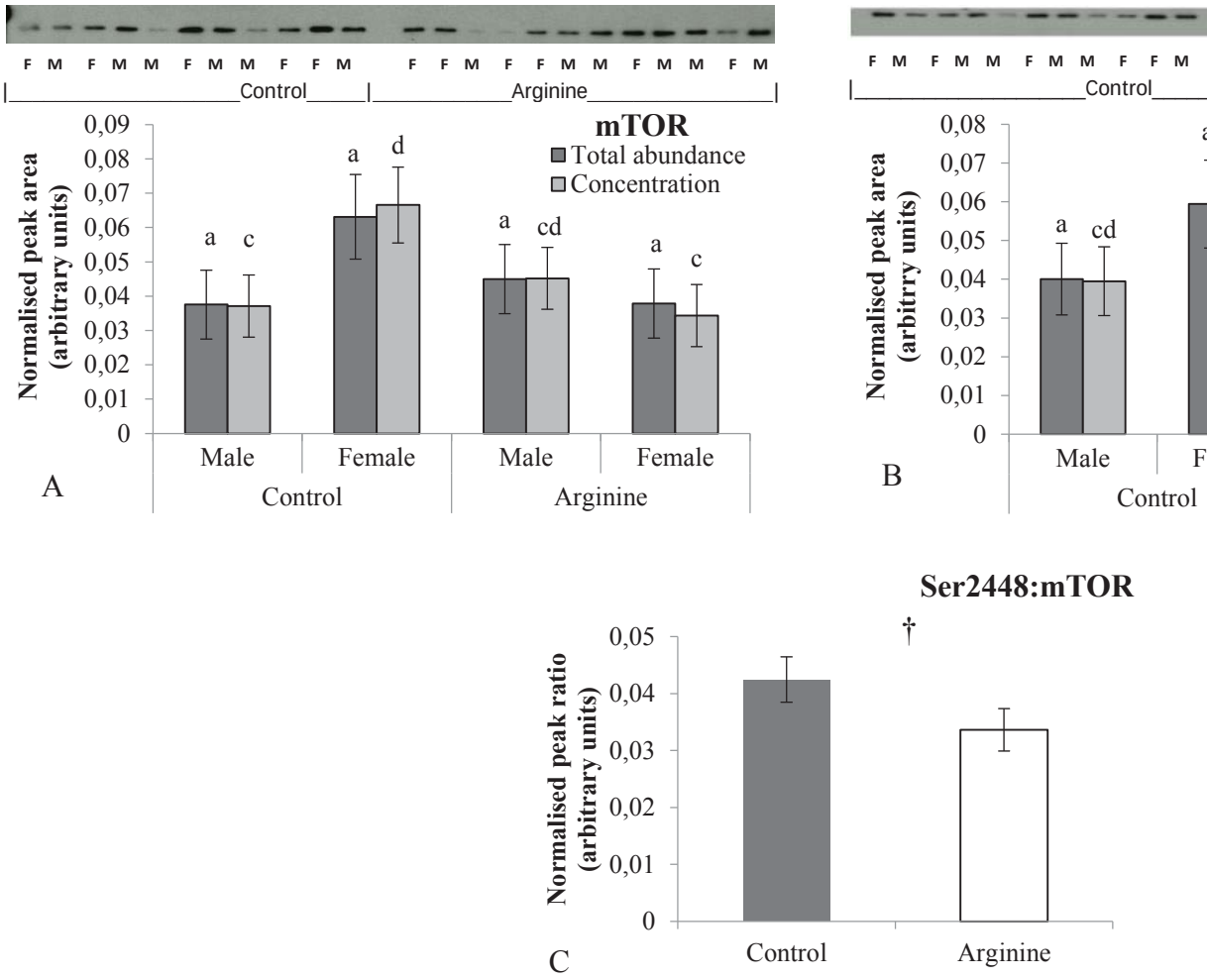

Figure 5.6. Treatment (Arginine *vs.* Control) by sex of lamb (male *vs.* female) interaction for concentration and rapamycin (mTOR), (B) phosphorylated mTOR (Ser2448) and ratio of Ser2448 :mTOR (C) in *M. longissimus d* arginine or unsupplemented (control), from birth to 70 days of life. The figure shows the least square mean ± superscript for each variable ([a,b]total abundance; [c,d]concentration) represents $P \leq 0.05$, †$P < 0.10$.

5.4.7 Biochemical indices

5.4.7.1 Cohort 1

There was little evidence of a treatment effect on concentration of DNA, RNA or protein at 28 days (Table 5.7). Males had higher DNA concentration, total DNA, but lower RNA: DNA and Protein: DNA ratios compared to females at 28 days.

5.4.7.2 Cohort 2

A treatment by sex of lamb interaction was observed for protein concentration, Protein: DNA and Protein: RNA ratios. Arg-F had lower protein concentration than Con-F while Arg-M had higher concentration than Con-M (Table 5.7). Arg-F had a lower Protein: DNA and Protein: RNA ratio compared to Con-F, while no difference was found between males. Higher DNA, total DNA but lower RNA: DNA ratio was found for Arg-treated compared to control lambs.

Table 5.7. Effect of arginine supplementation on biochemical indices of arginine treated and control lambs from C (T), sex of lamb (S) and treatment by sex interaction (T x S) are presented. Data corresponds to least square m (SEM).

	Cohort 1									
	Control		Arg			*P*-value			Control	
	Male	Female	Male	Female	SEM	T	S	T x S	Male	Female
n	**6**	**8**	**7**	**8**					**6**	**5**
Concentration (mg/g ST)										
DNA	0.95	0.79	0.87	0.69	0.07	0.26	0.01	0.85	0.49	0.48
RNA	0.97	0.99	0.96	1.02	0.04	0.74	0.25	0.56	0.64	0.61
Protein	83.75	84.12	79.38	90.02	4.46	0.81	0.21	0.26	71.70	80.37
Total (mg)										
DNA	158.46	122.83	159.90	120.39	9.62	0.94	<0.01	0.85	167.73	148.98
RNA	165.84	153.32	176.49	179.42	10.80	0.13	0.61	0.41	217.90	191.03
Protein	15.10	13.09	14.67	15.82	1.48	0.41	0.79	0.28	24.35	25.21
Ratios										
RNA:DNA	0.98	1.27	1.19	1.59	0.14	0.13	0.01	0.64	1.31	1.28
Protein:DNA	0.08	0.11	0.10	0.13	0.01	0.15	<0.01	0.59	14.73	17.14
Protein:RNA	0.09	0.09	0.08	0.09	0.01	1.00	0.55	0.63	11.21	13.30

5.4.8 Insulin concentration

No difference between Arg and control animals was observed in insulin concentration in Cohort 1 or Cohort 2 animals (Table 5.8).

Table 5.8. Insulin concentration (mMol/L) for Cohort 1 and Cohort 2. The effect of treatment (T), sex of lamb (S) and treatment by sex of lamb interaction (T x S) are presented. Data corresponds to least square means ± averaged standard error of the mean (SEM).

	Control		Arginine			*P*-value		
	Male	Female	Male	Female	SEM	T	S	T x S
Cohort 1	1.22 (6)	0.81 (8)	1.54 (7)	1.77 (8)	0.40	0.11	0.85	0.44
Cohort 2	0.46 (6)	0.37 (5)	0.24 (7)	0.42 (9)	0.12	0.52	0.62	0.29

5.5 DISCUSSION

The objective of this study was to investigate 1) the effect of oral Arg supplementation of twin-born lambs, on skeletal muscle growth as a monogastric (from birth until 28 days of life) and from birth until 70 days of life and 2) the impact of Arg supplementation on mTOR signalling. Supplementation with oral Arg resulted in an increase in Arg concentration in the plasma of Cohort 1 and Cohort 2 lambs. To my knowledge, this study is the first to demonstrate that dietary supplementation with Arg via milk to lambs from birth to weaning increases plasma Arg concentration. Oral supplementation with Arg resulted in an increase in ADG during the first 3 weeks of life. A longer period of supplementation positively impacted skeletal muscle growth in Arg-F, compared to Con-F, as evidenced at 70 days, however no effect was evidenced in mTOR abundance or activation in the supplemented group, suggesting that the Arg effect occurred previous to the day 70 sampling.

The results of the current study showed oral Arg supplementation stimulated growth between 7 and 28 days of life but not in older lambs. The early neonatal period is characterised by a rapid growth rate (Davis et al., 1989) which is reduced as age advances (Davis et al., 2000). Results from the present study are in agreement with previous reports in piglets, showing a greater growth response to food intake in 7- compared to 26-day-old individuals due to increased protein synthesis (Davis et al., 1996; Wray-Cahen et al., 1998), and in 5- compared to 16- or 28-day-old rats (Davis et al., 1993). The positive

effect of Arg supplementation on ADG in the current study from day 7 to 14 in lambs from Cohort 1 and between day 7 and 21 in Cohort 2, resulted in increased weight of Arg-treated lambs in both cohorts at 28 days of life. Increased body weight gain during the first week of life and not later on, has been described to occur in piglets from Arg-supplemented gilts (Mateo et al., 2008). However, the treatment effect on ADG from day 7 to 21 was mainly driven by a change in Arg-F growth and not Arg-M, suggesting females respond differently to Arg supplementation compared to males. Such sexual dimorphism in Arg metabolism, was previously observed during gestation in Chapter 4 and also occurs in rats (Ruzafa et al., 2003). Taken together, the results of the present study and those in other species support the idea that Arg supplementation in artificially reared neonatal lambs is effective for increasing growth during the first 3 weeks of life especially in female lambs.

The increase in ADG in Arg-F at day 28 in Cohort 1 was coupled with an increase only in *M. biceps femoris* muscle weight, but also with a numeric increase in the weight of most evaluated muscles, when compared with Con-F at 28 days of life. A previous study reported that oral Arg supplementation from 7 to 14 days of life increased muscle growth in neonate piglets (Yao et al., 2008). However, Arg-F from Cohort 2, despite having a lower lean content at 28 days as measured by DEXA compared to Con-F, had heavier weights of most of the evaluated muscle at 70 days, suggesting supplementation with Arg for 28 days may not have been long enough to elicit a significant effect on muscle growth. The positive effect in Arg-F at 70 days and not in males was unexpected and the mechanisms are not clear. Results from Chapter 4 showed that females from dams that had been supplemented with Arg during gestation had increased muscle mass compared to unsupplemented females at 153 days of life (post-weaning). In addition, in Chapter 3, females, regardless of the birth rank, had lower intracellular muscle Arg concentration than males at 85 days of age. Results from the present study and previous chapters suggest females differ from males in the metabolism of Arg and this may influence female muscle growth.

The serine/threonine kinase mTOR controls protein accretion (Proud, 2004a) through the mediation of ribosomal RNA biogenesis (Iadevaia et al., 2012a) and co-regulation of mRNA translation (Iadevaia et al., 2012b). Changes in the intracellular concentration of specific AA are responsible for mTOR activation (Beugnet et al., 2003; Nicklin et al., 2009) and its activation by Arg has been clearly demonstrated in different

tissues (Bauchart-Thevret et al., 2010; Kim et al., 2010; Kong et al., 2011; Wang et al., 2011; Zeng et al., 2013). In the present study, despite the increase in intracellular Arg concentration in muscle at 28 days in Cohort 1, there was no evidence of an effect on mTOR abundance or Ser^{2448}:mTOR ratio or other biochemical indices, which is consistent with the lack of effect of Arg on LD mass. At day 70, contrary to what was hypothesised, Con-F lambs had higher Ser^{2448}:mTOR ratio compared to Arg-F lambs, suggesting a higher activation of the mTOR signalling. The higher Ser^{2448}:mTOR ratio in Con-F was coupled with higher abundance and activation of mTOR which was coincident with a higher protein synthetic capacity, cell size and protein efficiency as determined by the biochemical indices. However, the increased DNA content in muscle, which is associated with muscle hypertrophy (Greenwood et al., 2000a; Adams, 2006), coupled with increased muscle mass in Arg-F at 70 days, suggests muscle growth in Arg-F occurred before sampling. Further, mTOR is developmentally regulated, and its content or activity decreases in muscle of older animals, as shown in 7- compared with 26-day-old pigs (Kimball et al., 2002). Altogether, the results suggest that a potential effect of Arg supplementation on mTOR signalling likely occurred before day 70 sampling, during the rapid growth phase observed early in life.

Arginine supplementation resulted in marked changes in plasma FAA of lambs from Cohort 1 at 28 days of age, with higher concentrations of Arg and ornithine, but reduced concentration of glutamine, histidine, methionine, phenylalanine, threonine, alanine, asparagine, aspartate, citrulline, glycine, and tyrosine in plasma. In contrast, at day 70, supplemented lambs from Cohort 2 had only higher concentrations of Arg and ornithine. The absence of differences in FAA concentration in plasma in Cohort 2 could potentially be explained by a buffering effect produced by pasture intake and rumen development (Merchen et al., 1986). The increased concentration of ornithine in plasma of Arg-treated lambs in both cohorts, is in agreement with previous studies of Arg supplementation in pigs (Southern and Baker, 1982; Kim et al., 2004; Yao et al., 2008) and is associated with Arg metabolism by arginase (Wu and Morris Jr, 1998). Reduction in glutamine, histidine, methionine, phenylalanine, threonine, alanine, asparagine, aspartate, citrulline, glycine, and tyrosine concentrations in plasma of Cohort 1, is similar to a previous report in growing animals, and is associated with AA removal by the peripheral tissue to support body growth (Rius et al., 2012). Similar to what was observed in plasma, there was a reduction in the concentration of histidine, methionine, threonine,

glycine and increased Arg and ornithine in muscle of Arg supplemented animals in Cohort 1, reinforcing the possibility of AA utilisation for tissue growth.

Arginine stimulates insulin secretion in growing lambs (Godden and Weekes, 1981) and the role of insulin in muscle growth has been well characterised (Davis et al., 2003). However, Arg supplementation resulted in no effect on plasma insulin concentration at any time point. This result is in agreement with a previous observation in piglets supplemented with Arg, where no response in insulin secretion was elicited, however, mTOR activation was stimulated (Yao et al., 2008) which also occurred in lambs receiving abomasal infusion of Arg (Davenport et al., 1990). Therefore, although changes in muscle mass do not appear to be associated with changes in insulin concentration. The idea that Arg may have elicited a response on insulin secretion, during the rapid phase of growth, between day 7 and 21, is a possibility that needs to be considered.

5.6 CONCLUSIONS

Arginine supplementation via milk to lambs from birth to weaning is an effective method to increase plasma circulating levels of Arg, even after rumen development has been initiated. The positive effect of Arg supplementation on muscle growth of females confirms the benefit of implementing strategic incorporation of Arg into growing lambs diets, however this could be achieved with a short period of intervention, between birth and 21 days of life. In addition, it is unknown why no effects were observed in Arg supplemented males. In the present study a relationship between Arg supplementation with mTOR and lamb growth could not be clearly identified. Whether the effect of Arg on muscle growth in females is associated with mTOR signalling needs to be determined by considering studies during the phase of high growth, which occurred during the first 3 weeks of supplementation.

Chapter 6: General discussion

6.1 OVERVIEW

The lower birth weight of twin-born lambs compared to singletons, which has implications for post-natal survival and post-natal growth, is a major constraint for the global sheep industry. However, these limitations are especially relevant for the New Zealand sheep flock due to the increased prolificacy achieved in the last 15 years. Late-gestation and early post-natal skeletal muscle growth is reduced in twins compared to singletons, leading to decreased birth weight and subsequently poorer survival and/or post-natal growth. Therefore, any attempt to improve skeletal muscle growth during pregnancy and/or after birth may have a major impact on sheep production by reducing mortality and increasing growth. Despite the importance of muscle growth in sheep meat production, there is still little understanding of the mechanisms leading to reduced muscle growth in twins. However, studies in monogastrics have shown that dietary supplementation with specific amino acids (AA) significantly increase skeletal muscle growth during pregnancy and after birth (Wu et al., 2007a). This effect is mediated by the signalling role that specific AA have on pathways that regulate protein accretion, such as the mechanistic target of rapamycin (mTOR) pathway. However, it is not fully understood which AA could play a role in the regulation of the pathways involved in muscle growth and the mechanisms associated with specific AA supplementation during pregnancy and post-natally on muscle growth in twin sheep are unclear.

The objectives of this thesis were, firstly, to identify intracellular AA which could act as regulatory factors for muscle growth in late pregnancy and post natally in twin sheep; secondly, to evaluate *in vivo* the effect of supplementing a potential regulatory AA on muscle growth, and thirdly to examine the role of mTOR signalling in mediating any observed effects on muscle growth.

The findings of the studies undertaken in this thesis reinforce the observation that twin lambs have reduced muscle growth during late pregnancy and from birth to weaning compared to singletons, regardless of the level of maternal or lamb nutrition. This confirms that factors other than plane of nutrition influence muscle growth. Reduced intracellular concentration of specific AA in muscle, namely arginine (Arg) and glutamine were associated with reduced muscle mass in twin fetuses. This suggested that Arg and glutamine may play a role in fetal muscle growth. In contrast, skeletal muscle weight at weaning was associated with changes in several intracellular AA in muscle,

with no clear indication of which AA could be driving the difference in muscle growth between singletons and twins. Based on these results and the current literature Arg was selected as a candidate regulator of muscle growth during late pregnancy and post-natally. Two *in vivo* trials were conducted in order to evaluate, firstly, the effect of maternal Arg administration (via *i.v* bolus three times daily), during pregnancy on twin fetal and post-natal muscle growth, and secondly, the effect of direct Arg supplementation of the lamb (via milk) from birth to weaning on muscle growth.

While maternal supplementation with Arg resulted in no effect on fetal weight and only increased the *M. psoas major* muscle weight in females at 140 days of pregnancy, it increased the birth weight of female lambs from supplemented ewes, compared to control females. Results suggested Arg supplementation affected the growth of female fetuses in the last two weeks of pregnancy and this effect was coupled with increased abundance of mTOR and protein synthesis capacity in muscle at 140 days of pregnancy. A developmental programming effect of maternal Arg supplementation was also described for postnatal muscle growth, whereby female lambs from ewes supplemented with Arg during pregnancy had increased muscle growth at market weight compared to control female lambs. Post-natal supplementation of artificially reared lambs with Arg also demonstrated a positive response in muscle growth only in females. This effect was associated with increased weight gain during the first 3-4 weeks of growth, resulting in a 11% to 18% increased muscle weight of lambs at weaning, compared with control females.

This thesis provides a new insight on the role of AA in sheep muscle growth and the effect of Arg supplementation both during pregnancy and post-natally. Similar to what has been observed in monogastrics, Arg supplementation during pregnancy and post-natally influences muscle growth. However, it is intriguing that the effect was observed only in female offspring. The cellular mechanisms explaining the difference in body and muscle growth between female fetuses from supplemented versus control ewes appeared to be associated at least in part with the mTOR pathway. Greater total mTOR concentration in muscle of Arg-supplemented females, leading to an increase in protein synthesis capacity, at least during late pregnancy, could be associated with heavier birth weight, while the role of mTOR during postnatal Arg supplementation was not clear. The causes of the unexpected sexual dimorphism in response to Arg supplementation requires further investigation. However, these results highlight the potential of Arg to improve

muscle growth in female lambs through supplementation, creating the baseline for future research in ruminants.

6.2 MAIN FINDINGS AND IMPLICATIONS

Skeletal muscle growth is a complex process which relies on the interaction of multiple factors. The outcome of these interactions is the result of the balance between protein synthesis and degradation (Lobley et al., 1980). In this process, AA play a major role, not only as building blocks but also as key signals, regulating both anabolic and catabolic process through the mTOR pathway (Wu, 2009). The regulatory effect of AA is mediated by changes in the intracellular concentration of specific AA (Beugnet et al., 2003; Wullschleger et al., 2006; Sancak et al., 2008), resulting in the mobilisation of mTOR to the lysosome and subsequent activation (Sancak et al., 2010). Results described in Chapter 2 and Chapter 3 of this thesis demonstrated that reduced muscle mass in twins compared to singletons during pregnancy and post-natally was associated with differences in the concentration of specific free AA (FAA) in muscle (i.e. Arg and glutamine). This had two major implications. Firstly, it showed that while intracellular FAA levels in muscle are continuously changing, and determined by the balance between AA input and removal (Munro, 1970), sampling at a single time point detected consistent differences in FAA concentration in muscle between twins and singletons. Secondly, it established the association between muscle weight and Arg and glutamine during late pregnancy (Chapter 2 and Chapter 3). In contrast, several FAA were associated with muscle mass at weaning, providing no clear insight into which FAA may be having a regulatory role (Chapter 3).

Reduced skeletal muscle weight in twins compared to singletons during late pregnancy was observed in the studies described in Chapter 2 and Chapter 3. During pregnancy, an *ad libitum* plane of nutrition on pasture (1.8 times maintenance, Chapter 2), resulting in increased body condition score in ewes (Kenyon et al., 2009), was not sufficient to ameliorate the difference in muscle weight between singletons and twins, in agreement with previous studies (Freetly and Leymaster, 2004). In contrast, a maintenance plane of maternal nutrition, designed to achieve a total increase in ewe liveweight during pregnancy that was similar to that of the expected conceptus mass (Kenyon et al., 2009), generated the biggest difference in muscle weight between twins

and singletons from heavy ewes, and also affected muscle weight of both twin and singleton fetuses from small ewes (Chapter 2). These results confirmed that maternal plane of nutrition in pasture-fed ruminants plays a major role in fetal growth during pregnancy. However, the results described in Chapter 2 and Chapter 3 reinforced the notion that specific nutrients, such as AA, may be important regulators of metabolic pathways which regulate protein synthesis and therefore, influence muscle growth in twins. Regardless of the similar concentration of AA in maternal plasma during pregnancy (Chapter 3), differences were observed in both fetal plasma and muscle concentration of specific FAA between singletons and twins. Although causality was not established, the differences found in the concentration of specific FAA in muscle of singleton and twin fetuses, coupled with the correlation found between Arg and glutamine with muscle mass in late-gestation (Chapter 2 and Chapter 3), support the hypothesis that the concentration of specific FAA differ between singletons and twins. Further, Arg and glutamine may be acting as regulators of the differential skeletal muscle growth observed between singleton and twin fetuses at late pregnancy. Both Arg and glutamine play important roles in multiple metabolic pathways, thereby regulating intracellular protein turnover (Wu, 2010) and have been shown to activate the mTOR pathway in previous studies (Rhoads and Wu, 2009). However, based on previous studies showing the effect of Arg supplementation on growth (Fligger et al., 1997; Kim et al., 2004; De Boo et al., 2005; Yao et al., 2008; Jobgen et al., 2009; Tan et al., 2009; Bérard and Bee, 2010; Lassala et al., 2010) through the activation of mTOR pathway (Yao et al., 2008), and as a precursor to nitric oxide and polyamines (Kwon et al., 2003; Wu et al., 2010b), Arg was chosen as the candidate AA to be evaluated *in vivo*.

Arginine is a nutritionally essential AA for fetal and post-natal growth (Wu et al., 2009), and has been shown to improve fetal growth and development in pigs (Wu et al., 2013). Dietary Arg improves protein synthesis and muscle growth (Kim et al., 2004; Yao et al., 2008), reduces body fat mass in growing pigs (Tan et al., 2009) and improves post-natal growth lambs from nutrient-restricted ewes supplemented with Arg during late gestation (Peine et al., 2013). Altogether, the effect on animal performance have suggested positive economic impacts for the use of Arg. The primary result of Chapter 4 showed that maternal administration of Arg from P100 to birth increases birth weight in females. This result contrasts with the study by Lassala et al (2011) which reported no effect on the growth of the fetuses from twin-bearing ewes supplemented with Arg from

60 to 120 days of gestation, compared to twin fetuses from control ewes. Divergence in fetal growth between singleton and twins is known to occur after 110-120 days of pregnancy (McCoard et al., 2000a), which is the period of high nutritional demands (Mellor, 1983). When nutritionally-restricted singleton-bearing ewes are supplemented with Arg from 60 days of pregnancy to parturition, an increase in birth weight has been observed (Lassala et al., 2010), supporting the notion that supplementation in the last 3-4 weeks of gestation is required to elicit an effect on birth weight. Therefore, the results described in Chapter 4 indicate that supplementation beyond 120 days gestation is required to elicit a phenotypic response in twins.

In addition to the effect on birth weight in females, maternal supplementation with Arg increased muscle growth at 153 days post-natally in female lambs (Chapter 4). The long-term effect on female lambs due to maternal supplementation, suggests a potential carry-over effect on muscle growth. A higher capacity for protein synthesis in muscle, as defined by the higher RNA content, observed in female fetuses from Arg-supplemented ewes compared with control females at 140 days gestation, was also evident at 153 days of life. Greater content of RNA has been associated with heavier muscle weight in fetal sheep (Greenwood et al., 1999), while results described in Chapter 3 showed that twins have reduced RNA content at 140 days gestation compared to singletons, supporting the notion that twins have reduced capacity for protein synthesis. Results described in Chapter 4 indicate that maternal supplementation with Arg improves the capacity for protein synthesis in twin female fetuses. While previous studies have shown that the accumulation of myonuclei by the fetus is the most important developmental factor for regulation of postnatal growth potential of ovine muscle (Greenwood et al., 2000a; McCoard et al., 2001), the present results suggest that post-natal muscle growth can also be modulated by increasing protein synthesis machinery during gestation. That the increase in RNA content was still evident at market weight, suggests a developmental programming effect, opening a new possibility for strategic nutritional interventions in order to improve post-natal growth. However, with the existing data it is not possible to explain the mechanisms associated with the observed sexual dimorphism and further studies are required.

Post-natal Arg supplementation resulted in an increase in muscle weight in artificially-reared female but not male lambs supplemented from birth to 70 days of life (Chapter 5). The heavier muscle weight in Arg-treated females versus control at 70 days

of life, was associated with an increased live weight gain which occurred during the first 3 weeks of life only. Therefore, results described in Chapter 5 likely indicate that the intervention window to elicit a positive response is the first 3-4 weeks of life. The potential for a "window of opportunity" is also supported by the results of Chapter 3, where the largest difference in weight gain between singleton and twins occurred during the first 28 days of life. Studies in sheep (Attaix et al., 1988), pigs (Davis et al., 1996; Wray-Cahen et al., 1998) and rats (Davis et al., 1993) have shown that rapid growth occurs during the early post-natal period and this rate is reduced as the animal ages (Davis et al., 2000). This reduction is concomitant with a decrease in the activation and abundance of mTOR (Kimball et al., 2002; Suryawan et al., 2007), reinforcing the present results. Collectively, these findings suggest that early post-natal life (first three to four weeks of life) is a critical time window for supplementation with specific AA (e.g. Arg).

Results described in Chapter 3 suggest protein synthesis capacity in late gestation is reduced in the muscle of twin fetuses compared with singletons. The results from this study agree with previous reports which have shown that total DNA, RNA, protein and Protein:DNA ratio were lower in the muscle of twin compared with singleton fetuses during pregnancy (Rattray et al., 1975) and in lambs after birth (Greenwood et al., 2000a). The capacity for protein synthesis is associated with, amongst other factors, the production and number of ribosomes (Davis and Fiorotto, 2009). The protein kinase mTOR regulates ribosome biogenesis, protein synthesis and cell growth (Bodine et al., 2001; Pallafacchina et al., 2002; Sakamoto et al., 2003). Results from Chapter 4 indicate that it is possible to increase the abundance of mTOR in the fetal muscle of females from ewes supplemented with Arg from 100 to 140 days of pregnancy. The positive effect on mTOR abundance results in a higher protein synthesis capacity in muscle of female fetuses at 140 days gestation, through the increase in RNA content. That mTOR plays a role in the difference observed in muscle growth between twin and singleton fetuses was previously described by Sciascia et al. (2010). Comparing the same singleton versus twin fetuses used by Sciascia et al (2010) or a subsequent study (Chapter 2), it was shown that twin sheep fetuses at late pregnancy have reduced abundance of downstream targets of mTOR. This suggests this signalling pathway is down-regulated in twins at this age, resulting in lower ribosome number and translational machinery. In contrast to the effect of maternal Arg supplementation on mTOR abundance, postnatal supplementation with Arg from birth to 28 or 70 days of life (Chapter 5) resulted in no effect on mTOR

abundance or activation. The lack of a significant effect on mTOR during postnatal supplementation with Arg (Chapter 5), contrasts with results observed in piglets supplemented with Arg (Yao et al., 2008). The main difference between the studies is the age at which animals were evaluated. In the work of Yao et al. (2008), piglets were evaluated at 14 days of age, a period in which stimulation of protein synthesis is elevated (Davis et al., 1996), while in the present study, the evaluation was performed at 28 and 70 days. Considering that the greatest difference in live weight gain of lambs resulting from Arg supplementation occurred between 14 and 21 days of life, it is plausible to suggest that possible changes in abundance and/or activation of mTOR occurred before sampling. Therefore, with the existing data it is difficult to establish the association between Arg supplementation and the mTOR signalling pathway in the post-natal period. However, the increase in the protein synthesis capacity in control females at weaning was in agreement with increased growth and activation of mTOR, suggesting mTOR may play a role in post-natal muscle growth.

The fact that Arg elicited a response only in female offspring when supplemented to dams or early post-natally is intriguing. A potential hypothesis to be tested is associated with the different response females have to stress, compared with males. Female lambs have a greater cortisol response to stress produced by restraint than males (Turner et al., 2002) and cortisol is negatively correlated with growth rate in lambs (Sharpe et al., 1986). In addition, cortisol down-regulates the phosphorylation of the mTOR signalling pathway in sheep fetuses (Jellyman et al., 2012). Supplementation with Arg has been shown to prevent the negative effect of corticoids on bone growth in rats (Pennisi et al., 2005) and reduced cortisol secretion during transportation in pigs (Srinongkote et al., 2003). Combined, these reports provide plausible mechanisms by which Arg supplementation could ameliorate the negative effects of cortisol in females and have a positive effect on growth in this group and not in males. This hypothesis deserves further studies.

Differences in the intracellular concentration of other FAA in muscle between singletons and twins, such as glutamine and methionine (Chapter 2 and Chapter 3) warrant mention. Glutamine was correlated with muscle mass during gestation (Chapter 3) and was reduced in the more severe nutrient-restricted fetuses at P140 (Chapter 2). Glutamine regulates key metabolic pathways which improve health, survival, growth, development, lactation, and reproduction of organisms (Wu, 2010). In addition, glutamine participates in the activation of mTOR (Nicklin et al., 2009). A marked

reduction in the concentration of glutamine in ovine fetal plasma at both mid- and late-gestation occur under maternal nutrient restriction (Kwon et al., 2004). Therefore, based on existing information, it is possible that glutamine may play a role in fetal growth/muscle growth in nutrient-restricted models, however more research is warranted. Methionine, contrasting with glutamine, was increased in muscle of twins compared to singleton fetuses (Chapter 2 and Chapter 3). Interestingly, Arg supplementation reduced the concentration of methionine in twins from supplemented ewes at 140 days gestation (Chapter 4) and at 28 days of life (Chapter 5). Methionine is an EAA involved in the initiation of protein synthesis (Kozak, 1983); participates in oxidative processes (Hoshi and Heinemann, 2001) and has other metabolic functions (Brosnan and Brosnan, 2006). Methionine is also involved in DNA methylation (Rees et al., 2006; Waterland, 2006). The increased concentration of methionine in twin fetuses could be associated with less methionine utilisation due to the lower protein synthesis. Although protein synthesis was not determined in the present studies, it was predicted by the reduced protein synthesis capacity (RNA: DNA ratio). The increase in ribosome number and protein synthesis capacity as a result of Arg supplementation during gestation could have resulted in the lesser methionine concentration compared to control animals, as a result of methionine incorporation into protein. Studies in pregnant gilts have shown that that both restricted and excessive dietary protein during pregnancy alter the offspring's epigenetic marks and influence gene expression in muscle (Altmann et al., 2012). Whether differences in methionine concentration observed between singletons and twins influence methylation profiles in muscles deserves further study.

The present thesis has given promising insights into the role of AA on muscle growth in sheep. The determination of Arg as a regulatory AA for muscle growth in late pregnancy and post-natally in twin fetuses/lambs, opens up new opportunities to ameliorate productive constraints clearly identified in twin lambs when compared to singletons. The positive effect of Arg supplementation for increasing birth weight and post-natal body and muscle growth albeit only in female twins, is intriguing, but should lead to future implementation of targeted strategies for improving female growth. The study has also given an insight into the mechanisms by which Arg supplementation results in phenotypic changes in muscle, and although further work is necessary, the mTOR signalling pathway appears a key component of the cascade of process leading to muscle growth through Arg supplementation.

6.3 POTENTIAL LIMITATIONS

Supplementation with Arg in Chapter 4 and Chapter 5 was not compared against an isonitrogenous control counterpart. Although interpretation of results could potentially have been difficult in both animal studies, previous studies of Arg supplementation of underfed ewes have shown that the effects of supplemental Arg on ewes were not likely due to a non-specific action of increased nitrogen provision (Lassala et al., 2010). However, the use of an isonitrogenous control like alanine (Yao et al., 2008; Tan et al., 2009) should be considered in future studies.

In order to estimate the effect of Arg supplementation on muscle FAA profile in the fetus (Chapter 4) and in the growing lamb (Chapter 5), animals were evaluated one-hour after receiving Arg supplementation via bolus injection in dams or via fortified milk in the lambs. Previous studies had shown sampling one-hour after Arg supplementation was sufficient to detect significant changes in the concentration of Arg in maternal and fetal plasma (Lassala et al., 2009; Lassala et al., 2011). Similar results were observed in plasma of post-natal pigs, resulting in the activation of mTOR (Yao et al., 2008). Sampling one-hour after oral Arg supplementation in lambs at 28 days of life, resulted in increased Arg concentration in muscle (Chapter 5). In contrast, despite the numerical increase in Arg concentration in fetal plasma observed one-hour after maternal bolus application, Arg supplementation resulted in no increases in intracellular Arg concentration in fetal muscle (Chapter 4). The absence of an increase in intracellular Arg concentration, suggests the time between supplementation and measurement may not have been appropriate to identify changes in the concentration of free Arg in fetal muscle. This could potentially explain the lack of an effect on Arg concentration in the muscle and the lack of difference in the activation of mTOR, even though total mTOR was increased in females from treated ewes. However, the higher concentration of ornithine in plasma and muscle of Arg-supplemented animals, a product from Arg degradation (Wu et al., 2009), suggests that an increase in Arg concentration occurred. Interestingly, while the study by Lassala et al. (2011) reported an increase in fetal Arg concentration in singleton-bearing ewes, there was no clear evidence of the effect of Arg supplementation in twin-bearing ewes. Results from Chapter 3 clearly demonstrated that, despite similar maternal plasma AA concentration, twin fetuses differ in specific AA compared with singletons. This is supported by the van der Linden study (2012) which showed changes in plasma AA concentration between singletons and twin fetuses at term. Based on this

information, it should be considered that, at least in the maternal supplementation approach, further studies are needed to better understand the pharmacokinetics of Arg in twin pregnancies.

6.4 FUTURE WORK

Based on the results of the research presented in this thesis it is concluded that Arg supplementation is a potential strategy to increase fetal and postnatal growth and ultimately muscle mass in lambs, which has important implications for sheep production. In order to generate a more precise model for the strategic supplementation with Arg, further investigation considering different supplementation lengths is needed to determine the best window in which to initiate Arg supplementation in the ewes. Similarly, the positive effect on live weight observed during the first 3-4 weeks of post-natal supplementation, in agreement with studies in pigs (Mateo et al., 2008), suggested that a more limited period of supplementation with Arg could be sufficient to elicit a growth response in post-natal lambs.

The intracellular FAA levels in muscle are determined by the rates of protein synthesis and degradation, and by the rates of uptake and release. In order to get a better understanding of the changes in Arg concentration in maternal plasma, fetal plasma and muscle after Arg supplementation, the use of arterio-venous approach and labelled AA as previously described by Lobley et al (1996) and Biolo et al. (1995), could provide complementary information to that generated in this thesis. This type of research, in addition to the evaluation of different levels of Arg supplementation, could lead to the development of more accurate recommendations in terms of AA doses required to elicit a desired response in muscle growth.

Further investigation into the practical ways to supply Arg (or a precursor) in pregnant ewes carrying multiple fetuses and growing lambs could help to extend the work of this thesis as the rapid metabolism of Arg in the rumen decreases the feasibility of a simply dietary supplementation. The use of protected AA has been previously explored and the methods and effectiveness have been reviewed elsewhere (Wallace, 1994). In addition, a recent study using protected Arg to supplement pregnant ewes has shown to have post-natal effects on lamb growth (Peine et al., 2013). Another feasible option is to supplement with specific compounds which result in increased Arg in the plasma.

Previous studies have shown citrulline may increase circulating Arg concentration in the fetus (Lassala et al., 2009). The use of Arg precursors such as N-carbamoylglutamate (NCG) has become an option in recent years and its use has been previously reviewed (Chacher et al., 2013). NCG increases circulating Arg concentrations and has a low rumen degradation rate (Chacher et al., 2012). Studies in piglets have shown oral NCG increases protein synthesis in skeletal muscle (Frank et al., 2007). Use of the above mentioned possibilities must be explored in order to identify the most appropriate way to increase Arg in plasma, from a technical and economic point of view.

The role of Arg supplementation during gestation and post-natally on mTOR activation was evaluated in Chapter 4 and Chapter 5 respectively. The objective was to better understand the signalling mechanism associated with the potential effect of Arg supplementation on muscle growth. Although during late gestation there was evidence of an effect on total mTOR abundance, which can be considered a chronic response, the opposite occurred in lamb supplementation at day 28 or day 70 of life. Evaluation of mTOR downstream targets, such as S6K1, which amongst other functions is associated with ribosome biogenesis and 4EBP1 and eIF4G–eIF4E, would give a better understanding of possible long-term effects of Arg on the modulation of the mTOR signalling pathway. In addition, the study of mTOR in the phase of rapid lamb growth, between 3 and 4 weeks of life should be considered as an approach for estimating a more acute effect of Arg on mTOR signalling.

Extensive management systems for sheep production are common in most sheep producing countries, where nutrition can frequently be suboptimal. Nutritional supplementation under these circumstances can be a common practice to enhance lamb growth and development. The evaluation of post-natal Arg supplementation via milk fortification in a more restricted nutrient regimen, compared to that utilised in the thesis, could be of benefit.

The search for increased productivity has led farmers to select animals for increased prolificacy in many countries. As ewe litter size increases, the proportion of triplet-bearing ewes in the flock increases (Amer, 2006). In addition to reduced weight at birth (Scales et al., 1986), mortality before weaning is higher in triplets compared to twins (Kenyon et al., 2006). Therefore, determination of the effect of Arg supplementation in triplet-bearing ewes should be considered as a mean to alleviate this constraint.

6.5 CONCLUDING REMARKS

This thesis is one of the first studies to identify that the difference in muscle weight between singleton and twins during late pregnancy and post-natally is associated with changes in the intracellular concentration of specific FAA in muscle. Secondly, it determined that muscle mass is associated with the concentration of Arg and glutamine during late pregnancy, and this association varies during the post-natal period. Arginine plays both metabolic and regulatory functions (Jobgen et al., 2006), signalling specific pathways which regulate protein accretion such as mTOR (Yao et al., 2008). The role of Arg in muscle growth was confirmed with the evaluation of Arg supplementation *in vivo* during gestation and from birth to weaning. The main results of maternal Arg supplementation during gestation were the increase of birth weight, potentially mediated by the mTOR pathway, and a carryover effect associated with an increase in muscle mass, in females only. Post-natal supplementation improved daily weight gain and muscle growth in females, compared to untreated counterparts. However, the role of mTOR during post-natal Arg supplementation was not established. The potential productivity benefits of Arg supplementation include a reduction in post-natal mortality and an increase of female growth.

The implementation of any dietary intervention by producers relies on practical applicability and the economic impact generated. Based on the results of the present thesis, it is feasible to suggest that there are "windows of intervention" where Arg could be supplemented. The last 3 weeks of the dam's gestation and first 3 to 4 weeks of life appear as the most promising periods for supplementation, although future research is required. The analysis of shorter periods of supplementation during gestation and post-natal phase are required to maximise the effective use of Arg.

Chapter 7: Appendices

7.1 APPENDIX A

Table A.1. Partial correlation between *M semitendinosus* weight and muscle free amino acid concentration for singleton and twin fetuses at weaning.

	r	*P*-value
Essential		
Histidine	-0.18	0.19
Isoleucine	0.22	0.12
Valine	0.12	0.41
Non-essential		
Alanine	-0.03	0.85
Arginine[1]	-0.10	0.47
Asparagine	0.14	0.42
Aspartate	-0.02	0.92
Carnosine	0.09	0.52
Glutamate	-0.24	0.11
Glutamine	-0.12	0.41
Glycine	0.00	0.98
Ornithine	0.07	0.63
Serine	-0.05	0.75
Taurine	-0.01	0.96

7.2 APPENDIX B

Table B.1. Treatment by sex of fetus and sex effect for fetal plasma FAA (μmol/L) in fetuses at P140, from ewes saline (control) from P100-P140. Data are presented as least square means and average SEM. Values for the interac (T x S) and sex of the fetus (S) are presented. Significant difference at $P \leq 0.05$.

	Control		Arg			*P*-value	Sex of
	Male	Female	Male	Female	SEM	T x S	Male
Essential							
Histidine	332	399	120	187	45	0.04	260
Isoleucine	90	74	86	91	10	0.46	91
Leucine	185	162	165	178	19	0.78	181
Lysine	203	181	172	131	34	0.36	167
Methionine	103	104	55	60	9	0.71	81
Phenylalanine	121	122	100	120	9	0.24	121
Threonine	802	723	622	610	62	0.44	706
Valine	370	315	388	314	65	0.03	342
Non-essential							
Alanine	404	394	431	398	36	0.53	401
Arginine[1]	192	193	258	216	34	0.58	204
Asparagine	39	35	38	48	13	0.80	44
Aspartate	35	35	31	28	3	0.61	31
Carnosine	16	16	40	9	18	0.38	13
Citrulline	199	201	226	210	38	0.66	204
Glutamate	100	76	83	162	31	0.39	131
Glutamine	376	368	432	378	31	0.32	377
Glycine	926	962	737	758	77	0.71	842
Ornithine	217	209	379	354	59	0.71	286
Proline	190	216	182	213	34	0.33	201
Serine	570	563	524	537	53	0.96	553
Taurine	86	81	46	47	17	0.88	67
Tyrosine	173	151	111	119	21	0.76	146
Total	5742	5618	5217	5180	315	0.64	5461

[1]**Deemed as conditionally essential for fetal growth (Wu, 2009).**

Table B.2. Intracellular FAA concentrations (μmol/g) in *M. longissimus dorsi* fetuses at P140, from ewes either supplemented with arginine (Arg) or saline (control) from P100-P140. Data are presented as least square means and average standard error of the mean (SEM). Values sex of the fetus (S) and the interaction between treatment and sex of the lamb (T x S) are presented. Significant difference at $P \leq 0.05$.

	Control		Arg		*P*-value	
	Male	**Female**	**Male**	**Female**	**S**	**T x S**
Essential						
Histidine	582 ± 70	310 ± 74	413 ± 69	479 ± 70	0.18	0.63
Isoleucine	19 ± 4	13 ± 5	19 ± 4	13 ± 5	0.09	0.98
Leucine	67 ± 11	53 ± 13	67 ± 12	53 ± 12	0.25	0.50
Lysine	176 ± 30	84 ± 31	136 ± 30	125 ± 30	0.45	0.56
Methionine	114 ± 11	66 ± 12	93 ± 10	87 ± 11	0.71	0.56
Phenylalanine	57 ± 4	46 ± 4	56 ± 4	46 ± 4	0.13	0.50
Threonine	1720 ± 243	1499 ± 270	1367 ± 238	1852 ± 247	0.06	0.96
Valine	79 ± 6	65 ± 7	68 ± 6	76 ± 6	0.35	0.74
Non-essential						
Alanine	1227 ± 107	1523 ± 109	1387 ± 107	1363 ± 109	0.84	0.25
Arginine[1]	389 ± 77	408 ± 84	377 ± 77	419 ± 83	0.54	0.95
Asparagine	3 ± 2	7 ± 2	8 ± 2	1 ± 2	0.03	0.10
Aspartate	106 ± 10	119 ± 10	126 ± 10	99 ± 10	0.06	0.23
Carnosine	2001 ± 139	2186 ± 168	2045 ± 141	2142 ± 167	0.57	0.67
Citrulline	161 ± 32	135 ± 34	147 ± 32	149 ± 33	0.93	0.92
Glutamate	2562 ± 94	2534 ± 121	2463 ± 94	2633 ± 108	0.23	0.72
Glutamine	4048 ± 554	3753 ± 583	3678 ± 546	4123 ± 550	0.17	0.56
Glycine	2420 ± 139	2293 ± 143	2355 ± 139	2358 ± 143	0.99	0.46
Ornithine	207 ± 30	289 ± 35	234 ± 30	262 ± 31	0.51	0.16
Proline	2410 ± 156	2343 ± 160	2399 ± 156	2354 ± 160	0.83	0.51
Serine	1104 ± 165	798 ± 189	1074 ± 170	827 ± 184	0.34	0.59
Taurine	4766 ± 617	3799 ± 651	4601 ± 621	3965 ± 654	0.18	0.49
Tyrosine	79 ± 6	65 ± 7	68 ± 6	76 ± 6	0.35	0.74
Total	24348 ± 743	23081 ± 831	23412 ± 743	24018 ± 831	0.60	0.19

[1]Deemed as conditionally essential for fetal growth (Wu, 2009).

7.3 APPENDIX C

Table C.1. Pre-anaesthesia blood screen (New Zealand Veterinary Pathology, Ltd., Hamilton, NZ) from all animals previous DEXA. Globulin, total protein, creatinine, GGT, albumin, glutamate dehydrogenase (GDH), albumin:globulin ratio (AGR) and urea are presented. In brackets, adult values for sheep were used as reference values.

Lamb ID	GROUP	Globulin g/L (35-47)	Total Protein g/L (56-88)	Creatinine umol/L (73-150)	GGT IU/L (32-70)	Albumin g/L (21-41)	GLDH IU/L (0-20)	AGR Ratio (0.48-0.90)	UREA mmol/L (5.1-15.6)
B1	CON	25	54	47	107	29	9	1.16	5.2
B2	CON	20	48	53	69	28	12	1.4	3.5
B3	CON	18	45	55	49	27	7	1.5	3.5
B4	CON	25	50	49	95	25	9	1	3
B5	CON	19	44	53	60	25	9	1.32	4.1
B6	CON	17	42	63	53	25	9	1.47	4.7
B7	CON	22	47	51	55	25	7	1.14	5
B8	CON	19	45	47	56	26	11	1.37	3.8
B9	CON	20	48	51	37	28	3	1.4	4.1
B10	CON	29	52	50	78	23	4	0.79	5.5
B13	CON	22	49	51	44	27	2	1.23	4.8
B14	CON	19	45	50	58	26	3	1.37	3.9
Y1	ARG	16	43	48	62	27	9	1.69	4.5
Y2	ARG	18	46	55	66	28	6	1.56	4.8
Y3	ARG	17	46	44	90	29	11	1.71	5.2
Y4	ARG	18	44	49	52	26	7	1.44	4.5
Y5	ARG	17	45	49	72	28	6	1.65	4.7
Y6	ARG	16	44	52	65	28	22	1.75	6.9
Y7	ARG	21	49	46	72	28	7	1.33	5.6
Y8	ARG	16	44	51	57	28	9	1.75	5.5
Y9	ARG	19	43	45	58	24	2	1.26	4.1
Y10	ARG	17	44	52	56	27	3	1.59	5.8
Y11	ARG	41	64	52	26	23	2	0.56	5.8
Y12	ARG	17	42	53	85	25	9	1.47	5.3
Y13	ARG	19	46	56	64	27	13	1.42	6.5
Y14	ARG	17	45	54	42	28	11	1.65	6.6
Y15	ARG	19	42	61	44	23	7	1.21	6.3
Y16	ARG	19	44	56	47	25	5	1.32	5.2

Table C.2. Cohort 2; dual-energy x-ray absorptiometry (DEXA) scan data from lambs at 28 days of life, expressed as a percentage of body weight. Table shows least square means and average standard error of the means (SEM). The effect of treatment (T, Arginine and control), sex of lamb (S) and treatment by sex of lamb interaction (T x S) are shown.

	Control		**Arginine**			***P*-value**		
	Male	**Female**	**Male**	**Female**	**SEM**	**T**	**S**	**T x S**
n	5	4	6	9				
Abdomen								
Lean %	28.31	27.31	25.49	26.88	0.67	0.06	0.41	0.05
Fat %	0.63	0.87	0.61	0.91	0.22	0.81	0.23	0.89
Rump								
Lean %	17.45	16.50	16.61	16.32	0.77	0.48	0.48	0.66
Fat %	0.41	0.53	0.39	0.54	0.13	0.94	0.31	0.92
Thorax								
Lean %	27.91	28.11	29.24	28.14	0.56	0.32	0.28	0.25
Fat %	0.64	0.88	0.69	0.96	0.24	0.67	0.29	0.95
Whole								
Lean %	73.55	73.10	71.30	71.40	0.64	0.01	0.86	0.68
Fat %	1.66	2.30	1.68	2.41	0.60	0.78	0.27	0.95

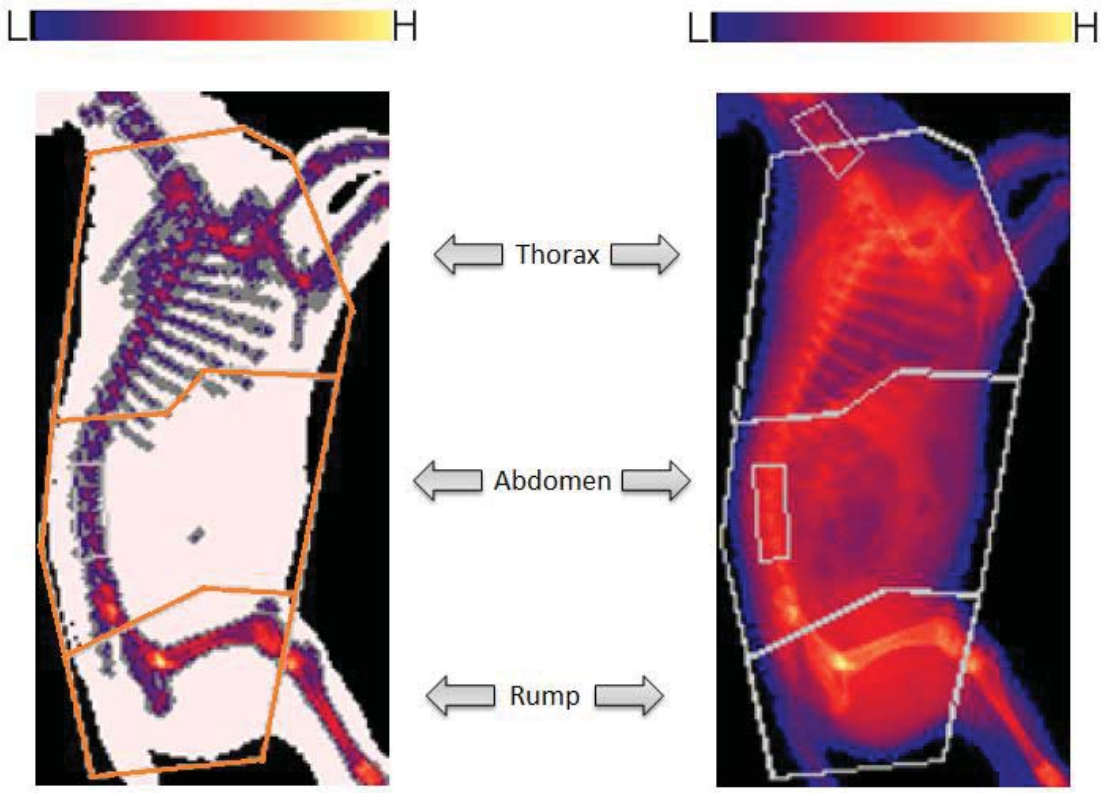

Figure C.1. Areas defined for DEXA study.

7.4 APPENDIX D: Statement of contribution to doctoral thesis containing publications

STATEMENT OF CONTRIBUTION TO PUBLICATIONS

Name of Published research output and full reference:

McCoard, S., Sales, F., Wards, N., Sciascia, Q., Oliver, M., Koolaard, J., & van der Linden, D. (2013). Parenteral administration of twin-bearing ewes with L-arginine enhances the birth weight and brown fat stores in sheep. *SpringerPlus*, *2*(1), 1-12.

Chapter: Chapter 4

Contribution of Sales, F.:

F.Sales contributed to the design, participated in the practical work such as catheterisation, animal handling and feeding, measurements at sampling, samples processing and measurement. Also participated in the analysis and discussion, and contributed to the writing and peer-review of the manuscript.

DRC 16

MASSEY UNIVERSITY
GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION
TO DOCTORAL THESIS CONTAINING PUBLICATIONS

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Francisco Sales Z.

Name/Title of Principal Supervisor: Professor Hugh Blair

Name of Published Research Output and full reference:

Sales F, Pacheco D, Blair H, Kenyon P, McCoard S (2013) Muscle free amino acid profiles are related to differences in skeletal muscle growth between single and twin ovine fetuses near term. SpringerPlus 2 (1):1-9

In which Chapter is the Published Work: Chapter 2

Please indicate either:

- The percentage of the Published Work that was contributed by the candidate: 80%

  and / or

- Describe the contribution that the candidate has made to the Published Work:

  The paper was based on a study using archived samples provided by Hugh Blair and Paul Kenyon. All laboratory work in order to obtain the data, was done by Francisco Sales. The data was analysed by Francisco Sales, Sue McCoard and David Pacheco. The paper was written by Francisco Sales with contribution of all other co-authors.

Francisco Andres Sales Zlatar — Candidate's Signature

31-3-2014 — Date

Hugh Blair — Principal Supervisor's signature

1 April 2014 — Date

GRS Version 3– 16 September 2011

Chapter 8: References

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