THE UNIVERSITY OF SOUTHAMPTON

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The Effect of Maternal Nutrition and Body Condition on the Structure and Function of Skeletal Muscle in the Offspring

by

Paula Mary Costello

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Abstract

Doctor of Philosophy

THE EFFECT OF MATERNAL NUTRITION AND BODY CONDITION ON THE STRUCTURE AND FUNCTION OF SKELETAL MUSCLE IN THE OFFSPRING

by Paula Mary Costello

Epidemiological and animal studies link reduced maternal nutrient intake during pregnancy and early postnatal life with an increased risk of metabolic disease in offspring in later life. Skeletal muscle is a strong candidate mechanism underlying these associations since it is the primary tissue for glucose utilisation, and insulin resistance in skeletal muscle is the earliest identifiable abnormality in pre-diabetic patients. The aim of this thesis was to investigate the concept that adaptations in skeletal muscle structure and function to undernutrition during fetal life may establish a phenotype that pre-disposes to metabolic disease.

In the first study, pregnant ewes received either 100 % of total nutrient requirements throughout gestation, 50 % 1-31 days gestation (dGA) or 40 % from 104 – 127 dGA. Skeletal muscle morphology, components of the insulin signalling pathway and brachial arterial vascular properties were then studied in late gestation. In the second study, ewes were established, by dietary manipulation, at a body condition score of 2 or $\geq$ 3 before and during pregnancy and skeletal morphology, insulin signalling pathways and glucose tolerance were studied in mature adult life.

In both studies maternal nutrient restriction reduced myofibre and capillary density in the offspring in a muscle bed-specific manner. The effect on myofibre type in the fetal offspring was dependent on the timing of the challenge (early or late gestation). The early gestation undernutrition was associated with altered vascular properties of the brachial artery in the fetus. Altered muscle morphology was associated with changes in molecular markers of glucose uptake and insulin signalling pathways, as well as mediators of growth. However, altered muscle composition did not affect glucose handling in mature adulthood.

Reduced myofibre density is consistent with a redistribution of resources at the expense of specific peripheral tissues by undernutrition and may be mediated by a decrease in capillary density and altered growth factors. Altered muscle composition did not affect glucose handling in mature adulthood and this may have been due to compensatory upregulation of glucose uptake with the aim of maintaining glucose homeostasis in the face of fewer myofibres. These findings may be important in informing on dietary advice given to pregnant women and have implications for future interventions aimed at reducing type-II diabetes.

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Declaration of the Author

I, Paula Mary Costello declare that the thesis entitled:

The Effect of Maternal Nutrition and Body Condition on the Structure and Function of Skeletal Muscle in the Offspring

and the work presented in it are my own. I confirm that:

- this work was done wholly or mainly while in candidature for a research degree at this University;
- where any part of this thesis has previously been submitted for a degree or any other qualification at this university or any other institution, this has been clearly stated;
- where I have consulted the published work of others, this has always been clearly attributed;
- where I have quoted the work of others, the source is always given. With the exception of such quotations this thesis is entirely my own work;
- I have acknowledged all main sources of help;
- where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
- parts of this work have been published as:

Costello, PM, Rowlerson A, Astaman NA, Anthony FA, Aihie Sayer A, Cooper C, Hanson MA and Green LR (2008). Peri-implantation and late gestation maternal undernutrition differentially affect fetal sheep skeletal muscle development. *The Journal of Physiology* **586**, 2371-2379.

For further published presentations at scientific meetings please see Appendices.

Signed:……

Date:……

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Abbreviations

ACh	acetylcholine
ACTH	adrenocorticotrophic hormone
ADP	adenosine diphosphate
AFRC	Agriculture and Food Research Council
ANOVA	analysis of variance
ATP	adenosine triphosphate
AUC	area under the curve
BCS	body condition score
BMI	body mass index
BSA	bovine serum albumin
C	control diet
Ca^{2+}	calcium ion
CI	confidence intervals
cDNA	complimentary DNA
cGMP	cyclic guanosine monophosphate
CHD	coronary heart disease
CPT-1	carnitine palmitoyltransferase
CSA	cross sectional area
CVO	combined ventricular output
DAB	diaminobenzidine
dGA	days gestational age
DMEM	Dulbecco's modified eagle's medium
DNA	deoxyribose nucleic acid
DOHaD	developmental origins of health and disease
EC_{50}	concentration required to produce a half-maximal response
EDHF	endothelium derived hyperpolarising factor
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme linked immunoadsorbant assay
eNOS	endothelium derived nitric oxide synthase
FFA	free fatty acids
FGF	fibroblast growth factor
G	gauge
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GC	guanylate cyclise
GH	growth hormone
GLUT	glucose transporter
GSK-3β	glycogen synthase kinase-3 beta
GTT	glucose tolerance test
HBCS	higher body condition score
i.m.	intramuscular
i.v.	intravenous
ICM	inner cell mass
IGF	insulin-like growth factor
IGF-IR	IGF-I receptor
IGFBP	IGF binding protein
InsR	insulin receptor
IRS	insulin receptor substrate
IUGR	intrauterine growth restriction
L	late gestation dietary restriction
LBCS	lower body condition score
LiHep	lithium heparin
MHC	myosin heavy chain
mmHg	millimetres of mercury
MRF	myogenic regulatory factor
mRNA	messenger ribonucleic acid
mTOR	mammalian target of rapamycin
NA	noradrenaline
NO	nitric oxide
OCT	optimal cutting temperature compound
p110	catlytic subunit of PI 3-kinase
p85	regulatory subunit of PI 3-kinase
PAR	predictive adaptive response
PBS	phosphate buffered saline
pCO_2	partial pressure of carbon dioxide
PCR	polymerase chain reaction
PEPCK	phosphoenolpyruvate carboxykinase
PGI_2	prostacyclin
PI	peri-implantation dietary restriction
PI 3-kinase	phosphatidylinositol 3-kinase
PKCζ	protein kinase C zeta
PM	post mortem
pO_2	oxygen partial pressure
RNA	ribonucleic acid
RT-PCR	reverse transcriptase PCR
RVC	Royal Veterinary College
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
SNP	sodium nitroprusside
SR	sarcoplasmic reticulum
TBS	tris-buffered saline
TGF-β	transforming growth factor beta
UPE	uteroplacental embolisation
VEGF	vascular endothelium growth factor

1 General Introduction

1.1 Adult health is affected by size at birth

Epidemiological studies from populations worldwide have shown that size at birth is a strong determinant of the subsequent health of the offspring. Babies who are born small or disproportionately thin or short at birth have a higher risk of developing cardiovascular disease and diabetes in later life. The initial data came from a study of British subjects that showed an inverse correlation between birth weight and adult systolic blood pressure (Barker *et al.*, 1989a). This was followed by data from a cohort of men in Hertfordshire that showed death rates from ischemic heart disease were highest in those with the lowest birth weight (Barker *et al.*, 1989b) and in both men and women low birth weight was related to coronary heart disease (CHD, Osmond *et al.*, 1993, Figure 1.1). From the Hertfordshire cohort it was also shown that birth weight was inversely related to the chance of developing type II diabetes at 64 years of age (Hales *et al.*, 1991) and thinness at birth was associated with impaired glucose tolerance in adult life (Phillips *et al.*, 1994). Similar trends occurred for the related disorders of stroke, hypertension, hyperlipidaemia and insulin resistance (Barker, 1998). There have also been replications of the UK findings in other countries including India (Stein *et al.*, 1996), Sweden (Leon *et al.*, 1998) and the United States (Rich-Edwards *et al.*, 1997).

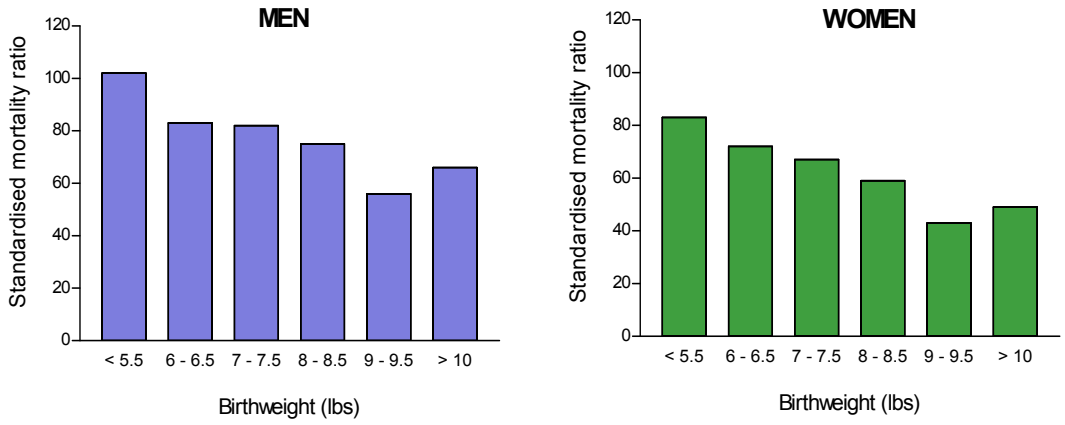


Figure 1-1: Standardised mortality ratios for coronary heart disease in men and women according to weight at birth (adapted from Osmond *et al.*, 1993).

Hand grip strength is a simple measurement of skeletal muscle function and has been used in several prospective epidemiological studies to relate fetal growth and later health. A cohort of men and women, aged 53 years, had a decreased grip strength in relation to low birth weight (Kuh *et al.*, 2002). This relationship remained after adjustment for adult height and weight and additionally for childhood height and weight. The relationship between birth weight and hand grip strength has also been shown in an older cohort of men and women aged 64-73 years (Sayer *et al.*, 2004) and also remained significant after adjustment for adult height and weight, age, sex and social class. Therefore, retarded fetal growth, as shown by reduced birth weight, can affect skeletal muscle growth so that muscle strength is impaired in postnatal life. The Kuh study is interesting since the relationship between birth weight and hand grip strength was shown in a younger cohort and grip strength has been associated with long-term mortality risk in middle aged men, independent of BMI (Rantanen *et al.*, 2000). Low birth-weight has also been associated with defects in the expression of insulin-signalling intermediates in young adult skeletal muscle which could be a precursor of longer term altered glucose handling (Ozanne *et al.*, 2005). Impaired *in utero* growth can therefore affect both muscle strength and its metabolic function.

1.2 The Developmental Origins of Health and Disease hypothesis

These observations have led to the Developmental Origins of Health and Disease (DOHaD) hypothesis. This proposes that the relationship of low birth weight and adult disease is caused by adaptive responses that the fetus makes in the face of an altered *in utero* environment. Although the fetal genome may determine the growth potential of an offspring *in utero*, the growth that is actually achieved is primarily determined by environmental effects, such as the availability of nutrients to the fetus (Godfrey & Barker, 2000). The fetus can adapt its development and physiology in times of malnutrition or stress in order to promote immediate survival, but these adaptations can have detrimental effects in later life.

This fetal 'programming' is thought to occur during critical periods of developmental plasticity, where the growth and development of an organ system is especially sensitive to its environment. If adverse stimuli occur during these critical windows it may alter the physiology of the fetus and subsequently affect adult health.

There are several types of response to the early environment that may occur. There are those with no adaptive value e.g. effects of environmental teratogens (Finnell *et al.*, 2002). There are those that confer immediate survival advantage but may have subsequent postnatal costs e.g. alteration in blood flow to protect growth of the brain during reduced fetal oxygen supply (Gluckman & Hanson, 2004b). These adaptive responses are thought to represent a short term survival strategy for the fetus, at the expense of long term function. Finally, there are the predictive adaptive responses, those which have potential adaptive potential.

1.2.1 Predictive adaptive response

A predictive adaptive response (PAR) is proposed as a response to the early environment which appears to have mainly future adaptive value. An example is the coat of the meadow vole which is thicker at birth when the offspring is born at a time of decreasing day length, therefore preparing the offspring for winter (Gluckman & Hanson, 2004a). PARs are believed to allow the developing fetus, through developmentally plastic processes, to set its postnatal physiological phenotype to that which it *predicts* will give it optimal chance of survival. Where a mismatch occurs between the predicted and actual postnatal environment this adaptive response may lead to an increased risk of disease (Gluckman *et al.*, 2007).

1.2.2 Timing of developmental insult

Nutrient deprivation at the time of fetal growth may be detrimental to later health but the timing of the insult will dictate the specific effect. The Dutch Winter Famine offers an assessment to the effects of altered maternal nutrition at varying periods of gestation. This famine was caused by a German military blockade in Holland between October 1944 and May 1945 which resulted in a severe food shortage. The health of those exposed to the famine whilst *in utero* was then later investigated. Analysis of the cohort in adult life revealed that famine exposure led to reduced glucose tolerance, atherogenic lipid profiles, obesity and an increased incidence of coronary heart disease

(Painter *et al.*, 2005a), yet it was the timing of the famine which determined the specific effect upon the exposed generation. Those individuals exposed to famine in early gestation were more obese (Ravelli *et al.*, 1999), had a more atherogenic lipid profile (Roseboom *et al.*, 2000b) and a higher risk of CHD (Roseboom *et al.*, 2000a). Exposure in mid gestation was associated with obstructive airways disease and microalbuminuria (Painter *et al.*, 2005b) and late gestation exposure was associated with decreased glucose tolerance (Ravelli *et al.*, 1998). It therefore appears that nutrient restriction during early pregnancy, when the nutrient demands of the conceptus are low, can still have specific long-term effects. The different responses, dependent upon the timing of undernutrition, may reflect the differing maturation rates between organ-systems and this will differ according to species.

1.2.3 Animal models of nutrient restriction

Animal models have been developed to investigate the underlying cellular and molecular mechanisms that link altered fetal growth to disease in later life. Animal models are useful as they are free from confounding socio-economic factors, can have their diets experimentally moderated and allow the use of invasive measurements.

The rat is widely used in such studies as it has a short gestation period, allowing the study of multiple generations. However, this short gestation is partnered with pups born at a relatively immature state, as compared to humans, and makes the use of nutrient manipulation and investigation of developmental windows of susceptibility difficult.

The sheep is an excellent model for the investigation of fetal development as it has a similar rate of pre- and postnatal growth to that of humans. The sheep, like humans, is born with a full complement of nephrons and cardiomyocytes and with a gestation of ~147 days enables the study of more finite times of nutrient restriction than the rat. Sheep have a high incidence of singleton births making them comparable to human pregnancies. The sheep also allows the direct study of fetal growth due to its size and high tolerance to surgery and chronic instrumentation. However, it is important to remember the physiological differences of the animal model and care should be taken in the interpretation of results from animal studies and extrapolating these to human scenarios. For example, sheep are ruminants which are mammals that digest their food

in two steps, first by eating the raw material and then regurgitating a semi-digested form known as cud from within their first stomach (rumen). Ruminants derive little glucose from their diet due to fermentation of carbohydrates in the rumen and so have to synthesise most of their glucose requirements by gluconeogenesis (Leng, 1970).

1.3 Growth and development

The gestation period varies according to species, in humans it is ~266 days (38 weeks) and in sheep ~147 days. In humans the period up until 8 weeks constitutes the embryonic period, during which most of the major organ systems are formed. The remaining weeks of gestation constitute the fetal period and this is mainly devoted to the maturation of the pre-formed organ systems and their growth. Postnatal growth facilitates the further maturation of organs, and following puberty growth rate declines until the final adult status is reached.

1.3.1 Birth weight as a measure of fetal growth

Birth weight is used routinely as a measurement of fetal growth and yet it is an inadequate measurement since it fails to distinguish between a short fat baby and a tall skinny one (both can have the same birth weight despite a huge difference in body composition). Subsequently, not all population studies have found a correlation between birth weight and adult disease. The Dutch Winter Famine actually had little impact upon birth weight (Painter *et al.*, 2005a), despite the many adverse health outcomes in adult life. Although fetal growth may be affected by a nutritional insult, birth weight alone may not detect this since the same weight can be attained from differing paths of fetal growth e.g. asymmetrical organ growth where certain organs will maintain their normal growth trajectory at the expense of others. Birth weight is therefore a *proxy* measure of fetal development; fetuses can be of the same weight and yet vastly differ in their body measurements or composition. A better indicator of fetal growth is the ponderal index (birth weight/length^3) which can highlight asymmetrical intrauterine growth retardation. A low ponderal index is associated with increased risk of coronary heart disease (Eriksson *et al.*, 2001).

1.3.2 Development of the embryo

The basic sequence of events in early development is the same for all mammals with only the timings being vastly different (Gilbert, 2006). The timings described in the following section are for human development, unless otherwise stated.

1.3.2.1 Pre-implantation

After fusion of the oocyte and spermatozoon the newly formed zygote undergoes a series of cell divisions called cleavage as it travels down the oviduct towards the uterus. Following a series of mitotic divisions a ball of smaller cells known as the morula is formed. Compaction occurs at the 8 cell stage with a hollow forming inside the cell ball and the position of cells at this stage determining their tissue type destiny. The relatively small inner cell mass (ICM) is destined to become the fetus and the outer trophoblast layer the placenta. The trophoblast creates a sealed environment around the developing ICM into which it transports growth factors and nutrients. In most species the blastocyst will only spend a few days in the uterus before implanting in the endometrium and in humans this occurs 8 to 10 days after ovulation (Figure 1.2). Sheep and other ungulates have a much longer period of pre-implantation, lasting around 15-17 days after fertilisation. The sheep embryo enters the uterus on day 4 at the morula stage of development and then develops into a blastocyst by day 6. The blastocyst hatches from the zona pellucida by day 8 and through fluid accumulation develops from a spherical to tubular form by day 11. It then elongates, marking the beginning of implantation, with transient attachment by days 12-15 and firm adhesion by day 16 (Spencer *et al.*, 2004). Until implantation is complete nutrients are obtained by the embryo from the uterine fluid which is secreted by the endometrium (Guillomot, 1995).

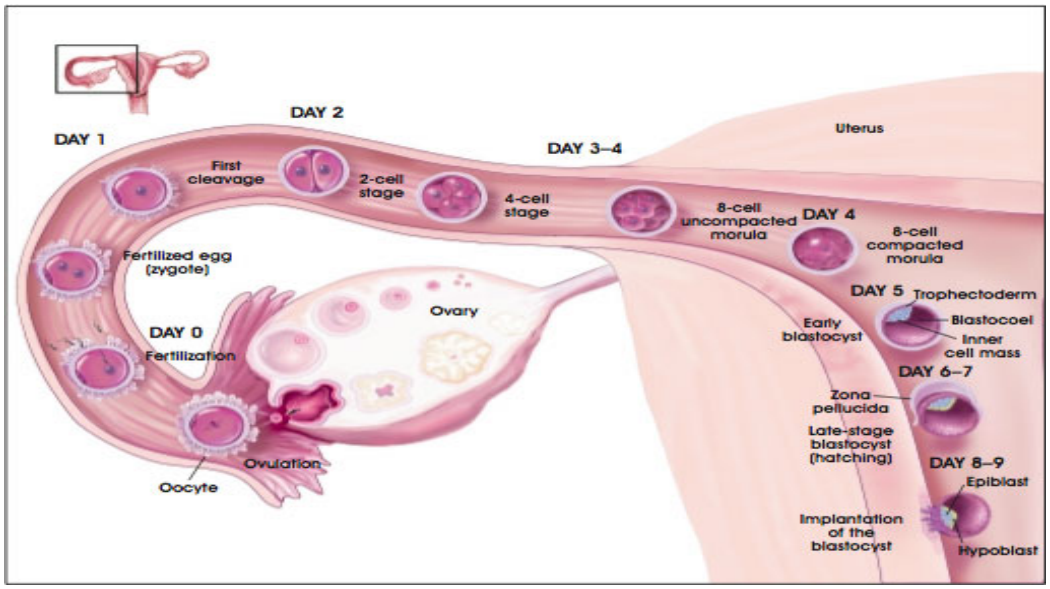


Figure 1-2: Early stages of development in the human. Although timings of events are different within species the sequence of events is similar in all mammals (from Thibodeau & Patton, 2003).

Maintenance of pregnancy during the pre-implantation period is dependent upon progesterone production from the corpus luteum and maternal progesterone levels are essential for normal development to the blastocyst stage (Spencer *et al.*, 2007; Lea & Sandra, 2007). The environment of the oviduct is also of importance during this period and is created by fluids secreted by the endometrium and this is dependent upon the production of steroids (Lea & Sandra, 2007). Embryo development is also under the control of various growth factors such as insulin-like growth factors (IGFs), epidermal growth factor (EGF), transforming growth factors alpha and beta (TGF-α -β) and fibroblast growth factors (FGFs; Harvey *et al.*, 1995).

1.3.2.2 Implantation

Implantation begins at the end of the first week and is completed by the end of the second week in humans. The blastocyst attaches to the endometrial epithelium, and during the second week the trophoblast begins to differentiate into the syncytiotrophoblast and the cytotrophoblast. The syncytiotrophoblast produce proteolytic enzymes and penetrate the endometrium. This induces the decidual reaction in the maternal endometrium, where the endometrium becomes a nutrient packed, highly vascularised tissue called the decidua.

1.3.2.3 Formation of the germ layers

During embryonic life the primary germ layers develop and give rise to all the major organs. This begins in the second week of development for humans and prior to implantation at 9-10 days gestation (dGA) in the sheep (Guillomot *et al.*, 2004). The cells of the ICM become the fetal mesoderm (the origin of muscle, bone and heart) the endoderm (gut, lungs and liver) and ectoderm (nervous system and epidermis). The trophoblast gives rise to the syncytiotrophoblast which invade the endometrium and begin to form the placenta. The tissue layers then differentiate to form the primordial of most of the major organ systems of the body.

1.3.2.4 Placenta

The fetus is dependent upon the placenta for its supply of nutrients and oxygen from the mother. The placenta also has heavy demands for its own growth and maintenance and in early to mid gestation placental growth rates exceed those of the fetus (Schneider, 1996). Development of the placenta begins with blastocyst implantation into the maternal endometrium and induction of the decidual reaction. The syncytiotrophoblast penetrate the endometrium and fluid-filled spaces called trophoblastic lacunae appear in the rapidly enlarging trophoblastic mass. The uteroplacental circulation begins to develop when the lacunae are infiltrated with expanding maternal capillaries. Cytotrophoblast extensions penetrate the blood-filled lacunae, carrying with them a covering of syncytiotrophoblast, resulting in primary stem villi. These gradually develop mesenchymal cell cores to form the secondary villi. The mesenchymal cells within the villi differentiate into blood capillaries and form the tertiary villi which soon become connected with the developing vessels of the embryo and thus establishing a functioning uteroplacental circulation. The maternal blood enters the intervillous space through endometrial or spiral arteries. As the artery enters it is under high pressure, forcing the blood deep into the intervillous space and bathing the villi, it then flows back and enters the spiral veins. Fetal blood passes through the umbilical arteries to the placenta which branch to form chorionic arteries. The fetal blood in the villus vessel exchanges nutrients with maternal blood across the villus wall.

Placentae differ according to species. In humans, rats and mice the placenta is discoid and the chorionic villi organised into a circular plate. Ruminants have a cotyledonary placenta, where instead of having a single large area of contact between maternal and fetal vascular systems the villi are arranged within numerous (70-100) separate compartments called placentomes, a term which encompasses both the cotyledon (fetal side of placenta) and the caruncle (maternal side of placenta) (Wooding, 2006). Placentae are also classified according to the number of tissue layers between maternal and fetal blood. Sheep have an epitheliochorial placenta (three maternal layers and three fetal layers), which is where the chorion is merely in contact with and does not erode the endometrium. Humans have a haemochorial placenta (no maternal layers and three fetal layers) and the chorion comes in direct contact with the maternal blood (Gilbert, 2006).

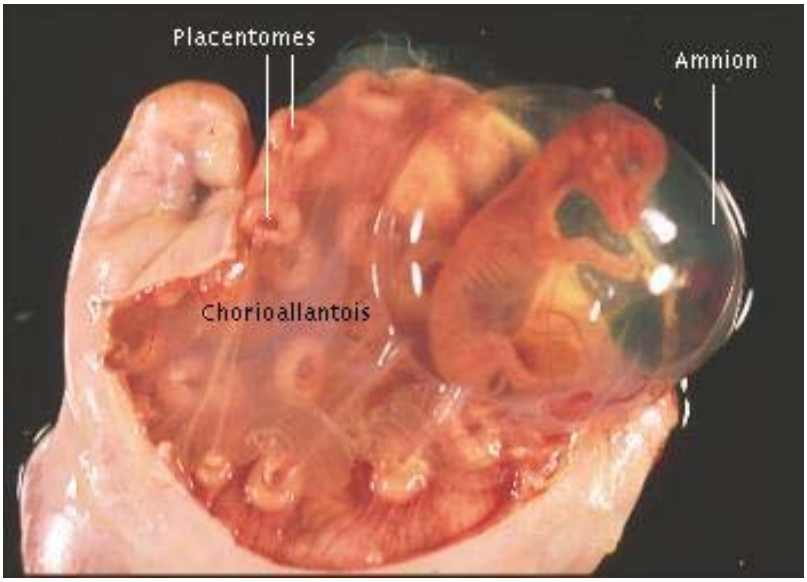


Figure 1-3: An incised uterus from a pregnant sheep at 50 dGA. The numerous button-shaped structures are the placentomes and the surface in view are the cotyledons, the fetal side of the placentome (from Colorado State University Hypertextbook, 2000).

The growth of the fetus depends on maternal glucose and amino acids being actively transported across the placenta by a number of transporters (Jones *et al.*, 2007) and in turn the transfer of nutrients is dependent upon the uteroplacental blood flow and maternal metabolic status. Maternal undernutrition during pregnancy can attenuate the uteroplacental blood flow in the rat (Rosso & Kava, 1980) and sheep (Chandler *et al.*, 1985). In order to adapt to a changing nutrient environment the fetus must be able to 'sense it' and it has recently been suggested that the placenta might act as a nutrient sensor (Jansson & Powell, 2006).

1.3.3 Fetal growth

The fetal period is a phase of organ maturation and rapid growth. In humans and sheep fetal weight follows a sigmoid growth curve, it is slow for the first few weeks and then increases exponentially until near term (Schneider, 1996), with the human growing from 8 g at 8 weeks to ~3,400 g at birth (Marsál *et al.*, 1996). The growth and development of the fetus is determined by two factors i) the genetic make up of the fetus, passed on from the parents and ii) the intrauterine environment provided by the mother. Thus the genetic make up of the fetus dictates the potential growth and the rate limiting factor to this growth is the *in utero* environment.

1.3.3.1 Maternal constraints on fetal growth

1.3.3.1.1 Maternal size

Maternal size is associated with birth weight (Kirchengast & Hartmann, 2003; Sacks, 2004), with birth weight following egg donation being more closely related to maternal weight of the recipient rather than the donor (Brooks *et al.*, 1995). The concept that maternal size rather than fetal genotype is the primary determinant of birth size has also been observed in animal studies. In crosses between Shire horses and Shetland ponies the birth size of the foals matched that of pure-bred foals of the breed of mare (Walton & Hammond, 1938) and the same observation has been noted in crosses between ponies and thoroughbred horses (Giussani *et al.*, 2003).

1.3.3.1.2 Maternal body composition

Maternal body composition can affect birth outcome since the mother will provide for the fetus from both her dietary intake and own body reserves. Maternal fat free mass in late gestation has been positively linked to birth weight in humans (Mardones-Santander *et al.*, 1998; Larciprete *et al.*, 2003) and the offspring of mothers with a low body mass index have been shown at increased risk of insulin resistance as adults (Fall *et al.*, 1998;Mi *et al.*, 2000;Shiell *et al.*, 2000).

1.3.3.1.3 Parity

In humans the first born infant is often born smaller than the second or third (Ong *et al.*, 2002) but the mechanisms underlying this effect of parity are not fully understood. It has been proposed that there is an altered capacity for spiral arteries to fully dilate and be invaded by trophoblast in a second pregnancy (Hafner *et al.*, 2000).

1.3.3.1.4 Maternal age

In both animals and humans an adolescent mother will give birth to smaller offspring (Kirchengast & Hartmann, 2003). This may be due to altered nutrient partitioning, with an adolescent mother retaining nutrients for her own growth at the expense of her fetus. In the adolescent pregnant ewe, overfeeding actually causes a greater proportion of the nutrients to be partitioned to the mother to support maternal growth, leading to restriction of placental and fetal growth (Wallace *et al.*, 2001).

1.3.3.2 Critical periods of fetal growth

Although all the organ systems are present by eight weeks of gestation in the human, few of them are functional (the heart being the exception). Fetal growth therefore involves the growth and maturation of these organ systems. In gross weight terms, the majority of fetal growth occurs in the last third of gestation. However, as each tissue has its own timetable for growth and maturation these will be more susceptible to the effects of an adverse environment when their metabolic demands are greatest. The timings described in the following section are for human development, unless otherwise stated.

In humans the first organ system to start developing is the nervous system which begins to form around 18 dGA. The development order is similar in humans and rats, although their relative timings and durations are different (Morgane *et al.*, 1993) but comprehensive information is not available in the sheep as they are not a model through which brain neurogenesis is studied (studies are costly and access to the brain difficult compared to rats). The primitive heart tube is formed early in the fourth week of gestation and soon after begins to beat and produce weak circulatory movements (Larsen, 2001). A functional circulatory system is necessary for growth beyond this stage as simple diffusion of oxygen is unable to support cell metabolism. During the third week of gestation lung buds begin developing and by six weeks the two lungs can be distinguished as separate organs in the thorax (Haworth & Hislop, 2003). Liver buds arise by week four and rapid mitosis of hepatocytes increases liver size so that by the end of the embryonic period the liver represents 10 % of body weight (Kinoshita & Miyajima, 2002). Kidney development is similar for the sheep and human, with nephrogenesis completed by full-term (Lumbers, 1995). The earliest structure to form is the pronephros at around week four (Hinchliffe *et al.*, 1992). The pancreas arises as

two separate and distinct buds which subsequently meet and fuse to form a single organ. The dorsal pancreatic bud is the first to appear at around 26 dGA, followed by the ventral pancreatic bud less than a day later (Hales *et al.*, 1996). Final maturation of the ovine pancreas occurs at around 2 months of postnatal age (Titlbach *et al.*, 1985).

Skeletal muscle development is discussed in section 1.5.6

1.3.3.3 Regulation of growth

Each organ system relies on a variety of external factors such as hormones to modify growth rates and trigger maturational changes. Hormones can also translate external factors, such as changes in nutrition, into growth changes. Maternal hormones have varying abilities to cross the placenta, which acts as a barrier to the passage of some molecules from the mother to fetus, including insulin (Alexander *et al.*, 1972), catecholamines (Bzoskie *et al.*, 1997) and ACTH (Dupouy *et al.*, 1980). The placenta also produces numerous hormones itself, including oestrogen and progesterone, human chorionic gonadotropin, placental growth hormone, and placental lactogen, some of which play a role in the regulation of fetal growth. Placental lactogen is thought to exert its influence on the fetus by stimulating production of other hormones such as IGF-I and insulin (Handwerger & Freemark, 2000). The IGFs are also regulators of fetal growth and these are discussed in section 1.5.8.2. Fetal insulin promotes growth of the fetus, acting as a signal of nutrient availability (Fowden & Forhead, 2004). Insulin deficiency will reduce fetal growth, with fetal tissues decreasing their uptake and utilisation of nutrients (Fowden, 1993).

1.3.4 Nutritional requirements during pregnancy

1.3.4.1 Oxygen

Oxygen crosses the placenta by passive diffusion down the concentration gradient from the maternal to fetal blood, which is relatively hypoxic. A significant amount is consumed by the uteroplacental tissues resulting in an arterial fetal pO_2 of ~ 25 mmHg. The supply of oxygen is of great importance as it is the only nutrient which the fetus is unable to store or synthesise.

1.3.4.2 Glucose

Glucose is the main source of energy for the human fetus and placenta. The fetus produces minimal amounts of glucose and is dependent upon transport from the mother. Placental transport is by facilitated diffusion through two non-insulin dependent glucose transporters, GLUT-1 (Das *et al.*, 1998) and GLUT-3 (Das *et al.*, 2000). The gradient for transport of glucose is determined by maternal and fetal blood glucose concentrations and transport is not affected by maternal insulin concentrations (Hay, 1994). The uteroplacental tissues consume a significant proportion of the glucose before it reaches the fetus, for oxidation or non-oxidation purposes that supply the fetus with products such as amino acids and lactate (Hay, Jr. *et al.*, 1984). In humans, fetal glucose levels are > 4.0 mmol/l and in ruminants these are lower at 2.5 – 3.5 mmol/l (Hay, Jr. *et al.*, 1984). In mid- and late gestation the rate of facilitated glucose transport across the placenta increases in both humans and sheep (Hay, 1991). The fetus is not solely reliant on maternal supplies of glucose since the placenta stores glycogen and is capable of gluconeogenesis (Sengupta *et al.*, 1986). The fetus can also produce its own glucose in the liver and kidneys (Fowden, 1993). Under basal conditions fetal gluconeogenesis makes a negligible contribution to overall requirements (Hay, Jr. *et al.*, 1984) but becomes significant during adverse intrauterine conditions such as undernutrition (Hay, 1991).

1.3.4.3 Other nutrients

Lactate is produced by uteroplacental tissues in both sheep (Carter *et al.*, 1993) and humans (Toschi *et al.*, 2002). The fetus will also produce its own lactate during late gestation (Sparks *et al.*, 1982) and it is the major precursor of hepatic glycogen and lipid synthesis (Fowden, 1995).

Amino acids are essential for protein synthesis as well as being a source for other substrates and oxidation. Levels are far greater in the fetus (Velázquez A *et al.*, 1976) and so have to be supplied from the maternal circulation and across the placenta via energy dependent transporters, or by synthesis in fetoplacental tissues (Battaglia & Regnault, 2001). Amino acid transporters exist within the fetal and maternal facing syncytiotrophoblast plasma membranes (Cetin, 2003). Non-essential amino acids can be derived from either route but the essential amino acids are maternal in origin.

There are a number of different forms of lipid found in the fetus including triglycerides, phospholipids and free fatty acids (FFA). They are important for not only fat accretion but also as cell membrane components and are vital for fetal growth. They are also potential substrates for oxidative metabolism and hormone synthesis. FFA can be derived from the mother, from synthesis in the fetus or from triglyceride and phospholipid break down (Duyne *et al.*, 1960). FFA may be transferred across the placenta via passive diffusion as well as by fatty acid binding proteins and fatty acid transfer proteins in the placental membranes (Haggarty, 2002).

1.3.5 Undernutrition effects on growth

Maternal nutrient restriction can reduce the nutrient supply to the fetus. Fetal glucose uptake is reduced (Hay, Jr. *et al.*, 1983) and blood glucose lower (Edwards *et al.*, 2001) following maternal nutrient restriction in the sheep. One fetal response to lack of nutrients is to reduce nutrient expenditure, by decreasing the rate of cell division and therefore reducing fetal growth. Studies of maternal nutrient restriction in sheep (Mellor & Murray, 1981) and rats (Ozaki *et al.*, 2001) have produced fetal growth restriction relative to controls. Tissues vary in their susceptibility to nutrient restriction and it is those tissues which are undergoing critical periods of growth at the time of the restriction or those of which function is not as crucial to survival (e.g. skeletal muscle in comparison to the brain) which may be most affected. This can be demonstrated in protein restricted chick embryos that had asymmetric organ growth with relative sparing of the heart, brain, lungs and kidneys (Miller *et al.*, 2002). In the sheep a 3 week nutrient restriction in late gestation also produced asymmetric fetal growth, with the fetuses of restricted ewes having an increased brain weight relative to body weight (Charlton & Johengen, 1987). Skeletal muscle has a lower priority for nutrients compared to the brain and heart (Bauman *et al.*, 1982) and so may be more vulnerable to a nutrient restriction.

Maintaining brain and heart development is of the upmost importance to the fetus and this is well demonstrated by the fetal response to hypoxia, whereby fetal blood flow to the brain and heart is maintained at the expense of other tissues (Cohn *et al.*, 1974;Sheldon *et al.*, 1979). Kidney development is an important focus of DOHaD research since the kidney contributes to blood pressure control. The effects of twinning and late-gestational uteroplacental embolisation (UPE) on nephron number have been

investigated in the sheep model. Both twin and UPE fetuses showed a similar reduction in birth weight and relative kidney weights were unchanged between the two groups. Yet only the twin fetuses showed a reduction in their nephron number (Mitchell *et al.*, 2004) suggesting that an earlier challenge (competing twins) during the period of cell division affects nephrogenesis but that a later challenge (UPE) has no effect. Fetuses from rat dams that received a mid-gestation administration of corticosteroids suffered a 30 % reduction in nephron number (Ortiz *et al.*, 2001) and nephron number is also reduced, along with postnatal hypertension, in pups exposed to a low-protein diet throughout gestation (Langley-Evans *et al.*, 1999). The Dutch Winter Famine found microalbuminuria (presence of albumen in the urine and indicative of abnormal renal filtration) in those exposed mid-gestation, a period of rapid increase in nephron number. A low protein diet (8 %) reduced pancreatic weight in the offspring at birth, along with reduced ß cell mass and islet size (Snoeck *et al.*, 1990). Islets isolated in late fetal life, following the low protein diet, had lower basal insulin release and a blunted insulin release in response to glucose (Cherif *et al.*, 1998).

The pre-implantation embryo is very sensitive to changes in its environment. Maternal undernutrition during the pre/peri-implantation period causes postnatal hypertension in both rats (Kwong *et al.*, 2000) and sheep (Edwards & McMillen, 2002). Manipulation during this period was also shown to reduce cell numbers in both the inner cell mass and trophectoderm of the developing blastocyst (Kwong *et al.*, 2000). Early maternal undernutrition may therefore have an affect through the restriction of early embryonic proliferation and generation of appropriately sized stem-cell lineages (Kwong *et al.*, 2000). Imprinting of genes also occurs in the early embryo, during which one of a pair of genes (maternal or paternal) is silenced by DNA methylation e.g. IGF-II which regulates both placental growth and nutrient transporter abundance (Constancia *et al.*, 2002). Epigenetic modification of imprinted genes may provide a mechanism linking environmental cues to changes in growth and development (Fowden *et al.*, 2006).

Postnatal growth patterns can also influence later health. A reduced size at birth followed by accelerated childhood growth can increase the risk of insulin resistance (Crowther *et al.*, 1998;Eriksson *et al.*, 2002) and CHD (Barker *et al.*, 2005). In contrast, the growth limiting effects of breast feeding compared to formula feed reduces risk of adult diabetes and obesity (Pettitt *et al.*, 1997;von Kries *et al.*, 1999).

It has been suggested that the response to changes in maternal nutrition may be immediately beneficial to the fetus but the long-term effects of these changes detrimental if the postnatal nutrition does not match that predicted by the fetus on the basis of its prenatal environment (Gluckman & Hanson, 2004b).

1.4 Cardiovascular system development and function

The cardiovascular system is vital for transporting nutrients to the tissues, taking waste products away and conducting hormones from one part of the body to the other. Blood pumped by the heart flows from the aorta into the major arteries, each of which supplies blood to an organ or body region. These arteries then divide and subdivide to give rise to the arterioles and it is these which provide the most resistance to and control of blood-flow. The walls of the arterioles and arteries have layers of smooth muscle and the lumen of the entire vascular system is lined by a monolayer of endothelial cells. The endothelial cells secrete substances involved in the maintenance of vascular tone (Lüscher & Tanner, 1993). The smallest arteries and arterioles play a crucial role in the regulation of tissue blood flow via vasodilatation and constriction (Pohl *et al.*, 2000). This function is regulated by the sympathetic nervous system and factors generated locally in tissues. These vessels are referred to as resistance arteries because their constriction resists the flow of blood.

1.4.1 Structural development of the heart

Heart development is highly conserved in mammalian species, reflecting the uniform structure of the organ (Icardo, 1996). In the human the primitive heart tube is formed early in the fourth week of gestation from the fusion of a pair of vascular elements called the endocardial tubes (Moorman *et al.*, 2003). Folding of the embryo causes the tubes to meet in the midline and fuse forming a single primitive heart tube and soon after this stage the primitive heart begins to function. Between the fifth and eighth weeks the heart undergoes looping, remodelling and septation to form the four chambers of the definitive heart (Anderson *et al.*, 2003).

1.4.2 The fetal circulation

The fetal circulation differs from the adult in that blood largely bypasses the lungs and perfuses the placenta and this is achieved through several shunts within the fetal cardiovascular system (Figure 1.4). The umbilical vein transports blood rich in nutrients and oxygen from the placenta (in turn from the maternal circulation) to the fetus. About 30–40 % of the blood from the umbilical vein joins the vena cava via the ductus venosus, thus bypassing the hepatic circulation (Borrell, 2004). The lungs of the fetus do not function as gas exchange organs and so blood is shunted away from the pulmonary circulation by the ductus arteriosus and foramen ovale (Rudolph, 1979).

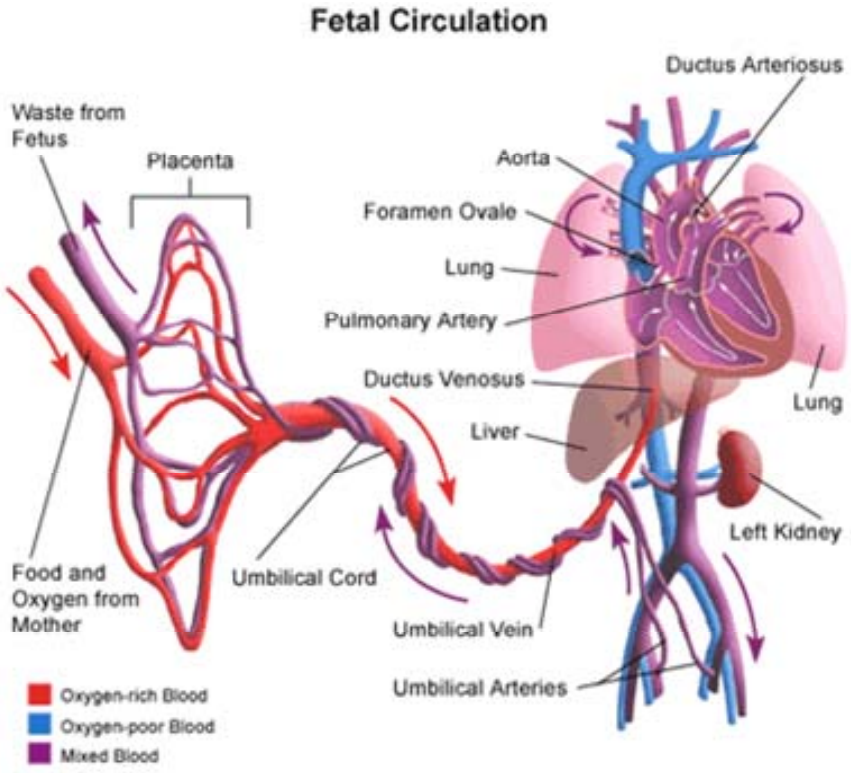


Figure 1-4: The fetal circulation (from UAB Health System, 2008).

1.4.3 Role of the endothelium

The endothelium is an unbroken layer throughout the vascular system that forms a single unit with the vascular smooth muscle via gap junctions (Davies *et al.*, 1988). It was initially thought to be a passive barrier against blood circulation but is now known to have many critical roles including the modulation of cell growth, maintenance of blood flow and blood pressure regulation.

The endothelium controls vascular tone through the synthesis and release of various relaxing and constricting factors which are released in response to neuronal, humoral and mechanical stimulation. These factors include nitric oxide (NO), prostacyclin (PGI_2), endothelium derived hyperpolarising factor (EDHF), and endothelin. Nitric oxide (NO) is synthesised from L-arginine in a reaction catalysed by nitric oxide synthase (NOS; Palmer *et al.*, 1988). Three isoforms of NOS have been isolated from activated macrophages (Stuehr *et al.*, 1991), endothelial cells (Marsden *et al.*, 1992) and neurons (Schmidt *et al.*, 1992), of which the endothelial derived form (eNOS) is of interest here. The release of NO from the endothelium is stimulated by an increased intracellular calcium ion (Ca^{2+}) concentration (Zheng *et al.*, 1994) by vasodilators such as acetylcholine (Wang *et al.*, 1996) and bradykinin (Schilling *et al.*, 1989). This can also occur independently of agonist stimulation due to shear stress or flow (Lamontagne *et al.*, 1992). Nitric oxide produced by the endothelium rapidly diffuses to the smooth muscle where its primary target is soluble guanylate cyclase (GC; Ignarro *et al.*, 1986). The activation of GC leads to production and elevation of cyclic guanosine monophosphate (cGMP) levels (Gruetter *et al.*, 1981) resulting in smooth muscle relaxation via the activation of protein kinase G (Lincoln, 1989) which phosophorylates a variety of proteins, inducing the relaxation of the smooth muscle. When various derivatives of arginine, which inhibit NO synthase, are administered to experimental animals there is a prompt rise in blood pressure suggesting that the tonic release of NO is necessary to maintain normal blood pressure (Vallance *et al.*, 1989). Vasodilatation can also occur in the absence of the endothelium as a result of direct action upon the vessel smooth muscle by either neuronal or humoral mediators. This direct action can be the stimulation of the NO signalling pathway (activation of soluble GC) through NO donors such as sodium nitroprusside (SNP).

Vascular tone is therefore maintained by a complex balance of vasoconstrictor and vasodilator (see Figure 1.5) factors that act on both the endothelium and vascular smooth muscle. Alterations in any component parts can upset this balance and thus alter regulation of blood flow and blood pressure maintenance. The importance of the endothelium is highlighted by the observed endothelial dysfunction in diseases such as atherosclerosis (Yang & Ming, 2006) hypertension (Taddei *et al.*, 2001) and type II diabetes (De Vriese *et al.*, 2000).

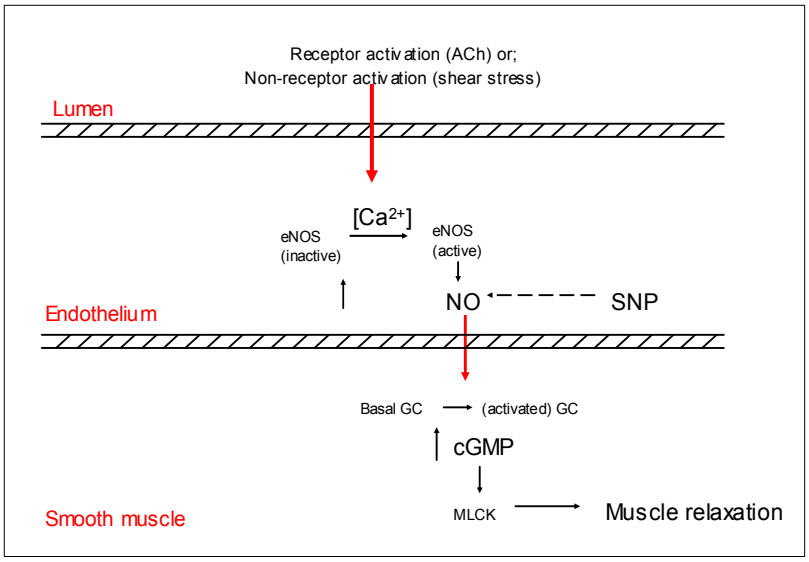


Figure 1-5: A simplified pathway of NO-mediated vasodilatation.

1.4.4 Fetal cardiovascular responses to hypoxia

Studies conducted in chronically instrumented fetal sheep have shown that in times of hypoxemia there is a pronounced redistribution of blood throughout the fetal and placental vasculature. Hypoxemia in the late-gestation sheep results in a rapid initial bradycardia (slowing of heart rate) with a slower increase in blood pressure (Boddy *et al.*, 1974; Cohn *et al.*, 1974). This is accompanied by a redistribution of combined ventricular output towards the heart, brain, adrenals and placenta and away from the carcass (Cohn *et al.*, 1974). These studies provide evidence for protective sparing of the brain and other organs by the maintenance of sufficient oxygen delivery at the expense of peripheral tissues. The redistribution of blood flow is partly mediated by peripheral vasoconstriction (Cohn *et al.*, 1974; Reuss *et al.*, 1982).

1.4.5 Fetal cardiovascular responses to undernutrition

In comparison to the hypoxemia model, relatively little is known directly about fetal cardiovascular adaptations during nutrient restriction. Since hypoxia is a nutrient restriction of sorts it has been hypothesised that similar blood flow redistribution could occur during a period of nutrient restriction and indirect measurements have supported this.

Ewe weight gain, during a nutrient challenge, can be used as a proxy measurement of nutrient delivery to the fetus. A decrease in ewe weight gain, following a 50 % global nutrient reduction in late gestation, was correlated with increased fetal adrenal and decreased liver size along with a change in brain-to-liver ratio and was consistent with that observed in hypoxic models (Burrage *et al.*, 2008). So although blood flow was not directly measured in this study it appears that a similar redistribution of blood flow did occur. An acute hypoglycaemic challenge in the late gestation sheep fetus reduced femoral artery and skin blood flow (Burrage *et al.*, 2005). These responses suggested a redistribution of blood flow away from the periphery and were reminiscent of the fetal response to hypoxia. Fetal liver sparing has been linked to a mother's slimness and diet; fetuses of slimmer mothers who had less body fat stores and were eating an unbalanced diet shunted less blood away from the liver through the ductus venosus and had a greater liver blood flow (Haugen *et al.*, 2005). Chronically hypoglycaemic late gestation sheep fetuses were observed to have significantly lower basal femoral arterial blood flow than controls (Gardner *et al.*, 2002). A 15 % global nutrient reduction for the first half of gestation did not alter fetal organ or body weight but decreased blood pressure and increased femoral vascular resistance in the late gestation fetus, followed by an elevated blood pressure in postnatal life, suggesting that development of the cardiovascular system can be altered by a mild challenge (Hawkins *et al.*, 2000a;Hawkins *et al.*, 2000b). The same challenge led to impaired *in vitro* vascular function (Ozaki *et al.*, 2000). In the sheep, a 50 % global nutrient reduction during the last month of gestation, caused an increase in mean arterial pressure (Edwards & McMillen, 2001). In the rat, a global reduction in energy intake (Woodall *et al.*, 1996) or a low protein diet (Langley & Jackson, 2005) resulted in hypertensive and growth restricted offspring.

Changes in the vasoconstrictor/dilator balance may play a role in the observed hypertension in nutrient restricted offspring. It has been demonstrated in different vascular beds in rats that undernutrition can lead to impaired endothelium-dependent and -independent relaxation. Both pathways were impaired in the rat femoral arteries following a 30 % global restriction from 0-18 dGA (Ozaki *et al.*, 2001) and in rat mesenteric arteries exposed to a 50 % protein restriction throughout gestation (Brawley *et al.*, 2003). Impaired endothelial function in the adult rat is associated with a reduced activity of eNOS and NO bioavailability (Franco *et al.*, 2004). In sheep, a 50 % global restriction between 0-70 dGA attenuated endothelium-dependent and -independent vasodilatation in isolated fetal femoral vessels but a milder reduction of 15 % had no effect on vessel function (Ozaki *et al.*, 2000). A 30 % global restriction from -12 to 70 dGA attenuated endothelium-dependent vasodilatation whereas a 30 % protein restriction in the same period attenuated both endothelium-dependent and -independent vasodilatation (Nishina *et al.*, 2003). Therefore, the type and severity of the restriction can have varying effects on vascular function.

1.4.6 Blood flow and tissue growth

Cells rely on blood flow to deliver nutrients and remove waste products and blood flow is essential for sustained tissue growth. This can clearly be seen in tumour biology; a tumour can only grow beyond a certain size if it is able to create its own blood supply and this is achieved by release of angiogenic factors and/or inhibition of anti-angiogenic factors by the tumour cells (Hanahan & Folkman, 1996). Blood flow through a vessel can be altered by vasoconstriction - a smaller diameter will create more resistance and therefore less blood will flow through the vessel. Vasoconstriction can therefore be used to divert blood away from tissues such as skeletal muscle when nutrient supply is low, maintaining adequate nutrient delivery to more vital organs such as the brain. This redistribution of blood flow has been shown during both hypoxia (see Section 1.4.4) and undernutrition (see Section 1.4.5). If blood flow is a limiting factor to tissue growth then there may be a link between local blood flow and tissue growth in the nutrient restricted model.

1.4.6.1 Angiogenesis

Angiogenesis is the growth of new blood vessels and is an essential process during wound healing and restoration of blood flow to injured tissues (Tonnesen *et al.*, 2000). It refers to the formation of new capillaries from existing capillaries and is different from the process of vasculogenesis, which describes the *de novo* formation of the vasculature from precursor cells during embryo development (Risau & Flamme, 1995). Hypoxia is an important stimulus for expansion of the vascular bed. Cells are initially oxygenated by simple diffusion but once the tissue has grown beyond a certain size hypoxia will trigger vessel growth. During angiogenesis, endothelial cells are stimulated to migrate, proliferate, and invade surrounding tissue to form capillaries. Angiogenesis can occur by two primary mechanisms - intussusception and sprouting (see Figure 1.6). Sprouting angiogenesis refers to the process in which activated endothelial cells branch out from an existing capillary. Intussusception refers to the process by which a single capillary splits into two capillaries from within, by the formation of a longitudinal divide on the luminal side of the capillary. This is thought to be a more efficient process of capillary multiplication than sprouting angiogenesis and may be the primary method of capillary formation during development (Djonov *et al.*, 2003).

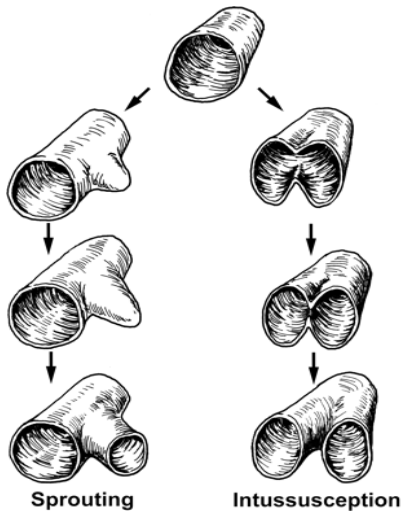


Figure 1-6: The process of sprouting and intussusception angiogenesis (from Prior *et al.*, 2004).

It has long been recognised that exercise training stimulates capillary growth in adult skeletal muscle (Carrow *et al.*, 1967;Andersen & Henriksson, 1977). An increase in muscle capillaries will enhance blood-tissue exchange properties, since a greater capillary network will increase the surface area, shorten the distance and increase the length of time for diffusive exchange between blood and tissue. Endothelial cells can sense changes in blood flow and pressure and shear stress stimulation (through elevated blood flow) causes capillary growth through intussusception (Egginton *et al.*, 2001).

Angiogenesis is regulated by many angiogenic growth factors and inhibitors such as vascular endothelial growth factor (VEGF) and FGFs (Yancopoulos *et al.*, 2000). VEGF is a general activator of endothelial cell proliferation and mobility and is the most potent factor that induces vasodilatation of the existing vessels and increases permeability of the vessel wall (Ferrara & vis-Smyth, 1997). It also increases the expression of matrix metalloproteinases for the degradation of the extracellular matrix and subsequently endothelial cell migration. Upregulation of angiogenic growth factors can occur following a single bout of exercise (Breen *et al.*, 1996).

An imbalance in angiogenic processes can lead to disease, such as rheumatoid arthritis (Paleolog, 2002) or diabetic retinopathy (Aiello, 2005). In these conditions new blood vessels feed diseased tissues and destroy normal tissues, and in the case of cancer they allow tumour cells to enter the circulation and lodge in other organs (tumour metastases; Zetter *et al*., 1998). Anti-angiogenic therapies, aimed at halting new blood vessel growth, are being developed to treat these conditions (Albo *et al.*, 2004). Conversely, insufficient angiogenesis underlies conditions such as coronary heart disease (Ware & Simons, 1997) and pre-eclampsia (Levine *et al.*, 2004). Therapeutic angiogenesis is being developed to treat these conditions and aims to stimulate new blood vessel growth (Vartanian & Sarkar, 2007).

1.5 Skeletal muscle development and function

Movement of the skeletal system is achieved through the contraction of the skeletal muscles. These are attached to the bones via tendons which pull on the bone when the muscle contracts. Muscle contraction was once thought achieved through the shortening of the muscle proteins until the work of Hanson and Huxley in 1954 who proposed that the contractile proteins do not change their dimensions but instead simply slide past each other to change their relative positions and bring about muscle contraction. Their model is known as the sliding filament mechanism. Skeletal muscle is also an important source of heat, as a skeletal muscle contracts it produces vast amounts of heat and much of this is used to maintain normal body temperature.

1.5.1 Basic structure

Skeletal muscle contains many long cylindrical cells called myofibres, which arise from the fusion of myoblast cells (Miller & Stockdale, 1986). Each fibre therefore contains multiple nuclei which lie at the periphery of the cell and the myofibres lie parallel to one another along the length of the muscle (see Figure 1.7). The plasma membrane of a muscle cell is termed the sarcolemma and this surrounds the muscle fibre cytoplasm or sarcoplasm. Mitochondria are plentiful and lie close to the muscle proteins which require ATP for contraction. The sarcoplasm contains many myofibrils, extending lengthwise within the fibre and these are the contractile elements; when the myofibrils contract, the muscle contracts. The myofibril contains the thin and thick myofilaments which overlap each other and are linked via cross-bridges. The filaments do not actually change length during muscle contraction but simply slide past each other increasing the area of overlap. Myofibre hypertrophy is achieved by the production of more myofilament proteins (Rennie *et al.*, 2004). A fluid-filled system of cisterns called the sarcoplasmic reticulum (SR) encircles each myofibril and is similar to the smooth endoplasmic reticulum found in non-muscle cells. In a relaxed myofibre the SR stores calcium ions which can be released into the sarcoplasm to trigger muscle contraction (Franzini-Armstrong, 1999).

Connective tissue surrounds the muscle fibres (Borg & Caulfield, 1980) and this is arranged in layers and supports and protects the myofibres as well as providing pathways for the passage of blood vessels and nerves. The outermost layer, encircling the whole muscle, is the epimysium. Portions of the epimysium project inward to divide the muscle into bundles of myofibres called fascicles and each fascicle is surrounded by a layer of connective tissue called the perimysium. This then divides further to surround each individual myofibre and is called the endomysium. The epimysium extends past the muscle as a tendon, a cord of dense connective tissue that attaches muscle to bone (Benjamin *et al.*, 2008).

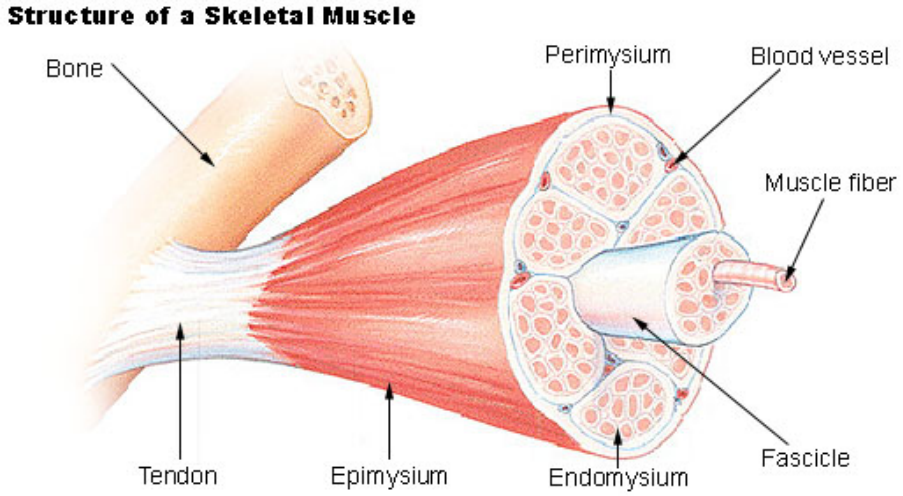


Figure 1-7: The basic structure of skeletal muscle (from Thibodeau & Patton, 2003).

1.5.1.1 Nerve supply

Skeletal muscles are well supplied with nerves and those neurons which stimulate muscle to contract are called motor neurons. The motor neuron plus all the skeletal muscle fibres it stimulates is called a motor unit. A single motor neuron can make contact with many myofibres, especially in muscles responsible for powerful gross movements such as the gastrocnemius in the leg. Muscles that control more precise movements, such as those in the retina, will have many small motor units (McComas, 1995). The activation of the neuron will cause the simultaneous contraction of all myofibres within the unit. The force that a muscle produces will depend on the percentage of its motor units that are active at a given time (Clamann, 1993).

1.5.1.2 Muscle circulation

When muscle contracts it requires large amounts of adenosine triphosphate (ATP) and a large supply of nutrients and oxygen for this ATP production. Skeletal muscle is therefore highly vascularised to cope with these high energy demands and to enable quick elimination of the resultant waste products.

Large arteries penetrate the epimysium and divide into small branches which run into the perimysium. These arterioles then branch into a vast capillary network through the endomysium. Capillaries usually run parallel to the myofibres but can have transverse extensions to form a three-dimensional lattice. Each myofibre is served by several capillary vessels, slow-twitch fibres having more than the fast-twitch (Green *et al.*, 1981;Andersen, 1975).

1.5.2 Contractile proteins

The two main contractile proteins found in myofibres are myosin (thick filaments) and actin (thin filaments; Figure 1.8). Myosin molecules consist of two identical heavy chains surrounded by four light chains (essential and regulatory; Rayment *et al.*, 1993). The heavy chains can exist in numerous isoforms and combine differently to determine the contractile properties of the fibre (Bottinelli *et al.*, 1994). Myosin molecules have long rod-shaped tails with globular heads and these heads form the cross bridges between the thick and thin filaments. Thin filaments contain the protein actin whose molecules join to form an actin filament helix (Kabsch *et al.*, 1990). Thin filaments also contain smaller amounts of the two regulatory proteins, tropomyosin and troponin (el-Saleh *et al.*, 1986). On each actin molecule is a myosin-binding site which is a location where a myosin head can attach to form the cross bridge.

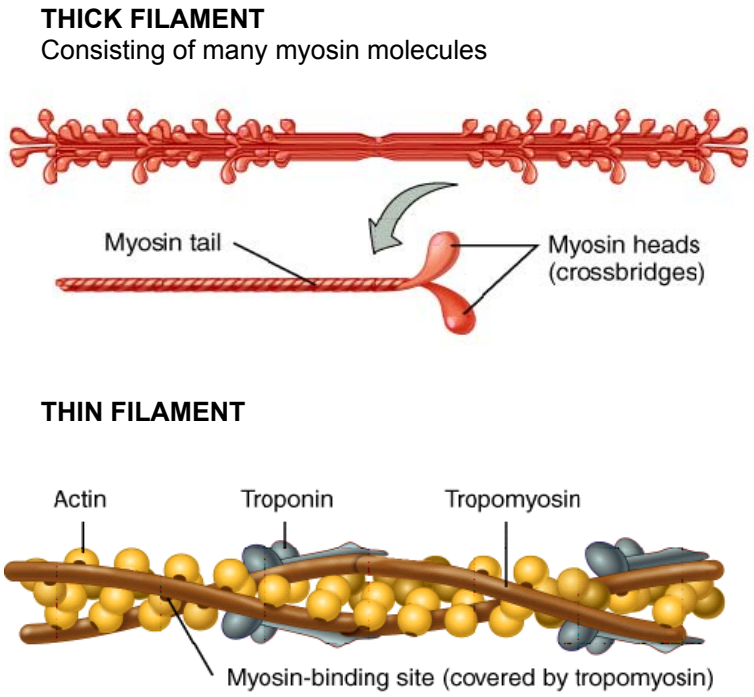


Figure 1-8: The thick and thin filaments of skeletal muscle. Actin molecules join together like a string of beads, intertwined at regular intervals to form the thin filaments. These also contain other regulatory proteins such as tropomyosin. Myosin molecules have long rod shaped tails with globular heads and the thick filaments contain many of these. The myosin heads form the cross bridges between thick and thin filaments (adapted from Purves *et al.*, 2003).

1.5.2.1 Sarcomeres

Skeletal muscle is also called striated muscle because of its appearance under the microscope (see Figure 1.9). Depending on whether the myofibre is contracted or relaxed the thick and thin filaments overlap one another to a greater or lesser extent, creating the striated appearance. The myofilaments are arranged in compartments called sarcomeres (for review see Au, 2004). Narrow regions of dense material called Z-discs separate one sarcomere from the next. Within each sarcomere is a darker area, called the A-band which consists mostly of the thick filaments and portions of the thin filaments where they overlap. A lighter less dense area called the I-band contains the rest of the thin filaments and the Z-disc passes through the centre of each I-band. A narrow H-zone in the centre of each A-band contains thick but not thin filaments. Dividing the H-zone is the M-line formed by protein molecules that connect adjacent thick filaments.

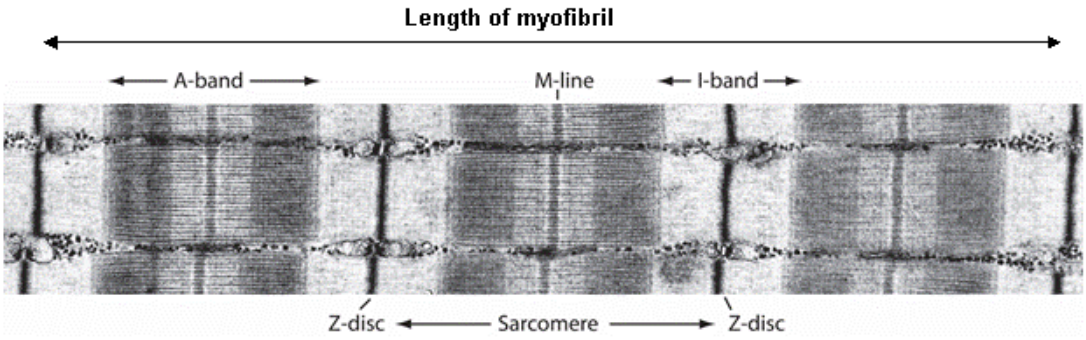


Figure 1-9: The skeletal muscle sarcomere (adapted from Purves *et al.*, 2003).

1.5.3 Muscle contraction

When a muscle fibre is relaxed the concentration of Ca^{2+} in the sarcoplasm is low due to Ca^{2+} active transport pumps in the membrane of the SR which actively transport Ca^{2+} from the sarcoplasm into the SR (Franzini-Armstrong, 1999). Once the myofibre is stimulated (by acetylcholine released from the motor neuron), the resultant action potential travels along the sarcolemma and into the transverse tubule system, activating Ca^{2+} release channels in the SR membrane and Ca^{2+} floods into the sarcoplasm. In a relaxed muscle the protein tropomyosin covers the myosin-binding sites and blocks attachment of myosin heads to actin (Craig & Lehman, 2001). An increase in Ca^{2+} will release this protein and the two filaments can then attach to bring about contraction (Lehman *et al.*, 2001). Muscle contraction also requires adenosine triphosphate (ATP)

and this is produced by the many mitochondria lying near to the contractile proteins. While the muscle is relaxed, ATP attaches to ATP-binding sites on the myosin cross bridges. A portion of each myosin bridge acts as an ATPase which splits ATP into ADP + P through a hydrolysis reaction (Rayment, 1996). This reaction transfers energy from ATP to the myosin head before contraction begins and the myosin cross bridges are thus in an activated state. When tropomyosin moves away from its blocking position the activated myosin heads spontaneously bind to the myosin-binding sites on actin (Kad *et al.*, 2005). This head then undergoes a conformational change which propels the actin along (Cooke, 1989) and as the myosin heads swivel, they release ADP. Once this is complete ATP again combines with the ATP-binding sites and the myosin head detaches. The myosin ATPase again splits ATP, transferring its energy to the myosin head so that it is ready to combine with another myosin-binding site further along the thin filament. By repeating the cycle many times these small movements add up to a coordinated movement and the steps repeat themselves as long as ATP and calcium levels remain high (see Figure 1.10).

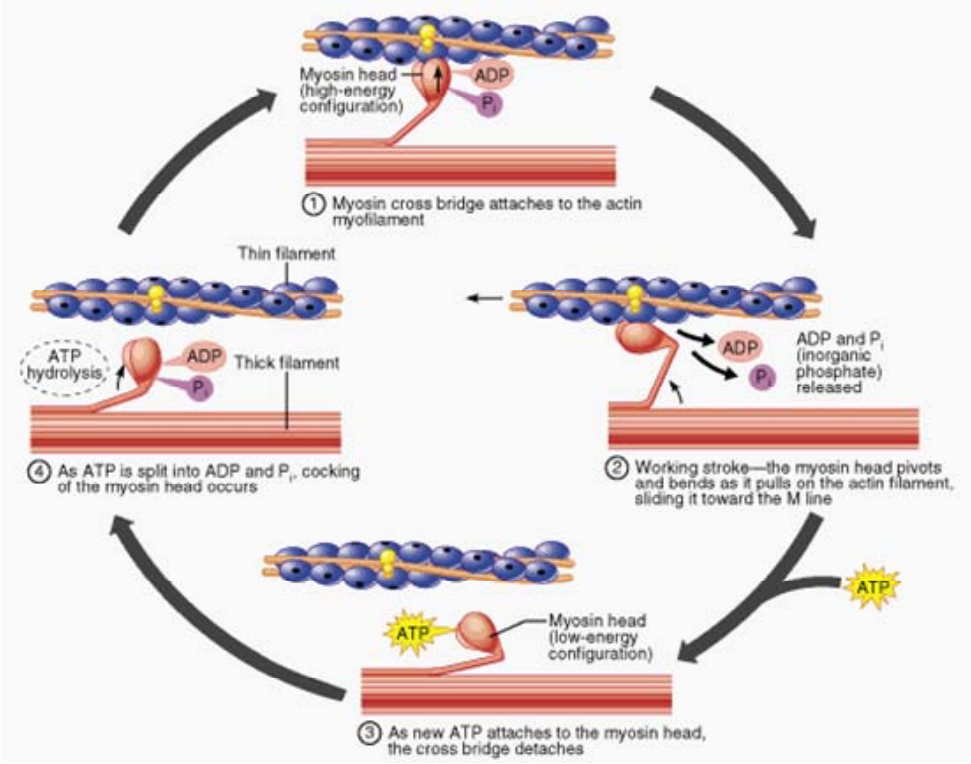


Figure 1-10: The ATP-mediated cycle of skeletal muscle contraction (from Waugh & Grant, 2006).

1.5.3.1 Sliding filament mechanism

The sliding filament mechanism states that contractile proteins do not change their dimensions but instead simply slide past each other to change their relative positions and bring about muscle contraction (Hanson & Huxley, 1954). During muscle contraction, myosin heads pull on the thin filaments causing them to slide inward toward the H zone at the centre of the sarcomere. As the thin filaments slide inward the Z discs move towards each other, and the sarcomere shortens. The lengths of the thick and thin filaments do not change, it is the sliding of the filaments and shortening of the sarcomeres that cause shortening of the myofibrils of the muscle fibre and ultimately the entire muscle (see Figure 1.11).

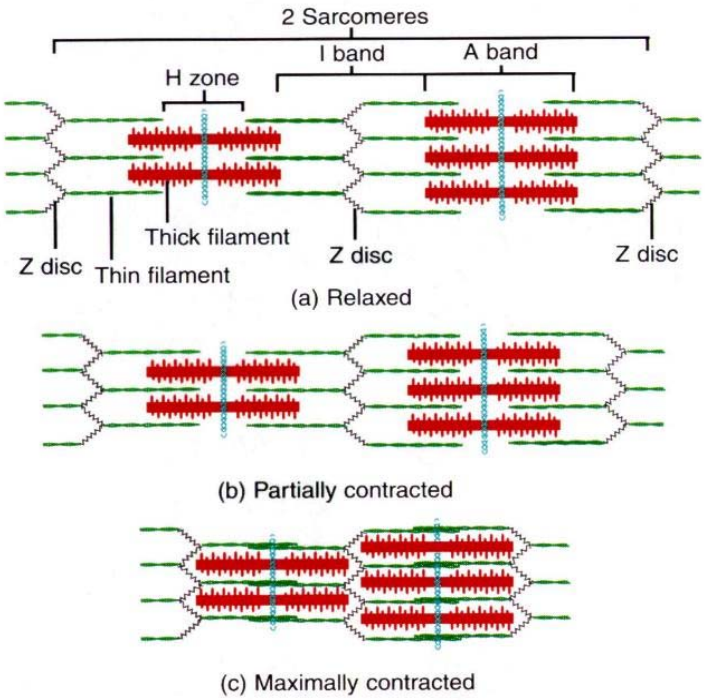


Figure 1-11: The sliding filament mechanism of skeletal muscle contraction. The position of the filaments in relaxed and contracted muscle - thick filaments in red and thin filaments in green (adapted from Tortora & Grabowski, 1996).

1.5.4 Types of skeletal muscle fibres

Muscle fibres differ in their colour, size, fatigability and contraction velocity. Myofibre colour is dependent on myoglobin content, a red-coloured protein that is similar to haemoglobin and acts as an intrinsic oxygen store (Salathe & Chen, 1993). Those fibres with high myoglobin content are termed red fibres and those with low content white fibres. Red fibres also have more mitochondria and blood capillaries than do white (Leary *et al.*, 2003;Andersen, 1975). The most important physiological characteristic used in classifying muscle fibres is their speed of contraction. The biochemical basis for the difference in speed resides principally in the rate at which myosin transduces energy from ATP (Maxwell *et al.*, 1982). Myosin exists in many distinct isoforms and fibres can be classified as either fast- or slow-twitch depending on their myosin expression. The fibres also vary in metabolic reactions used to generate ATP (Punkt, 2002), their fatigability (Kugelberg & Lindegren, 1979) and insulin sensitivity (James *et al.*, 1985). Based on these structural and functional characteristics, skeletal muscle fibres can be classified into three main types.

1.5.4.1 Slow-twitch oxidative (type-I) fibres

- Contain large amounts of myoglobin, mitochondria and blood capillaries making them red in appearance.
- Have a high capacity to generate ATP by the aerobic system.
- Spilt ATP at a slow rate and have a slow contraction velocity making them very resistant to fatigue.
- Are the most sensitive to insulin

1.5.4.2 Fast-twitch oxidative (type-IIa) fibres

- Also contain large amounts of myoglobin, many mitochondria and many blood capillaries.
- Have a large capacity for generating ATP by oxidative processes but they spilt ATP at a more rapid rate than type-I fibres and so their contraction velocity is faster.
- Are somewhat resistant to fatigue, although not as much as the slow type fibres.
- Have an intermediate sensitivity to insulin.

1.5.4.3 Fast-twitch glycolytic (type-IIb) fibres

- They have a low myoglobin content, relatively few mitochondria and blood capillaries giving them a white appearance.
- Lower blood supply to fast-fibres complements their greater dependence upon anaerobic metabolism.
- Split ATP at the fastest rate and so their contraction is rapid and strong but they are the most fatigable.
- Are relatively insensitive to insulin action.

The isoforms of the myosin heavy chain (MHC) represent the best markers of muscle fibre diversity (Pette & Staron, 1990) and although different combinations of MHC isoforms may occur within the same fibre the predominant isoform is the main determinant of the fibres functional properties such as contraction speed and fatigue resistance (Schiaffino & Reggiani, 1996). Important differences in myosin diversity related to body size have been reported. In the muscle of small species such as rat and mouse three fast MHC isoforms have been identified – IIa, IIx and IIb (Bar & Pette, 1988;Warnotte *et al.*, 1994). The relative abundance of MHC-IIb isoform decreases among mammals with increasing body size and is not expressed in humans (Smerdu *et al.*, 1994) or ruminants (Tanabe *et al.*, 1998). However, some studies in pigs (Lefaucheur *et al.*, 1998) and llamas (Graziotti *et al.*, 2001) have shown evidence for the existence of all three fast MHC isoforms and so the lack of expression of MHC-IIb may not just be a matter of body size. Those fibres termed IIb in large mammals actually express the MHC-IIx isoform (Smerdu *et al.*, 1994) and therefore much of the literature refers to IIb fibres when more accurately they are type IIx. The IIx fibres appear to be an intermediate type between IIa and IIb, in respect to their contraction velocity and resistance to fatigue (Picard *et al.*, 2002). Embryonic and fetal isoforms of myosin also exist but these are only expressed transiently during development and regeneration (Sartore *et al.*, 1982).

1.5.4.4 Composition of myofibres within a muscle

Most skeletal muscles of the body are a mixture of all three fibre types, their proportion depending on the usual action of the muscle; fast muscles develop force and relax more rapidly than do the slow muscles. Postural muscles of the neck, back and legs such as the soleus therefore have a high proportion of slow-twitch (I) oxidative myofibres as these are resistant to fatigue and are suited to their purpose (Ijkema-Paassen & Gramsbergen, 2005). Muscles of the shoulders and arms are used intermittently to produce large amounts of tension such as in lifting and so have a high proportion of fast-twitch glycolytic (IIb) fibres. The leg muscles are not only used to support the body but for walking and running and so these muscles have large numbers of both slow and fast-twitch oxidative (IIa) fibres (Jones *et al.*, 2004).

1.5.5 Muscle metabolism

Contraction of muscle requires a lot of energy but little ATP is actually present inside the myofibres and so must be produced if activity is to continue for more than a few seconds. There are three main ways which the muscle can produce energy and these will depend on the fibre type.

1.5.5.1 Phosphogen system

Muscle fibres contain a unique molecule called creatine phosphate which can donate phosphate to ADP to form ATP, itself becoming creatine (see Figure 1.12). This system provides energy to contract maximally for about 15 seconds and so is used for short bursts of activity (Jones *et al.*, 2004). Basal creatine phosphate is higher in fast-twitch than in slow-twitch fibres (Tesch *et al.*, 1989).

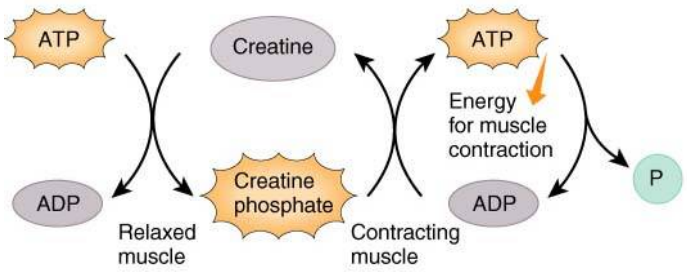


Figure 1-12: ATP derived from creatine phosphate in skeletal muscle.
This system provides energy for short bursts of activity (from Purves *et al.*, 2003).

1.5.5.2 Glycogen-lactic acid system

Once the supply of creatine phosphate is depleted ATP can be generated through glucose catabolism, which can be obtained from the blood or from glycogen breakdown within the cell. The glycolysis pathway splits each glucose muscle for a net gain of two molecules of ATP and two molecules of pyruvic acid (see Figure 1.13). This pathway does not require oxygen and so is known as anaerobic respiration. The pyruvic acid will enter the mitochondria to produce large amounts of ATP or can be converted into lactic acid which then diffuses out of the myofibre into the bloodstream. The lactic acid can be converted back into glucose by the Cori cycle in the liver, producing new glucose molecules for use in the glycolytic pathway and reducing acidity. The glycogen-lactic acid system can provide enough energy for about 30-40 seconds of maximal muscle activity (Jones *et al.*, 2004)

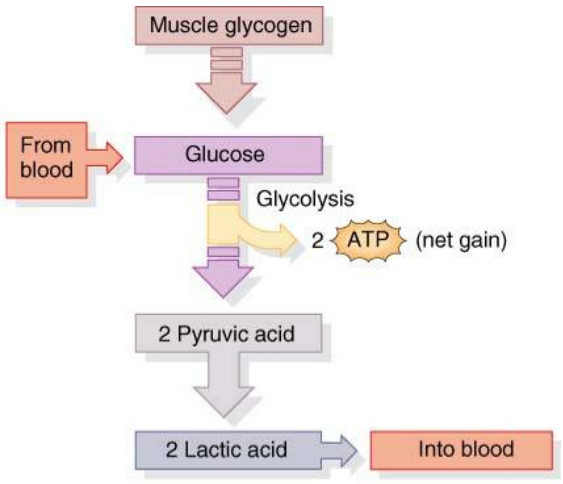


Figure 1-13: ATP derived from anaerobic respiration in skeletal muscle. This system provides enough energy for around 30 – 40 seconds of maximal muscle activity (from Purves *et al.*, 2003).

1.5.5.3 Aerobic system

Any muscular activity that lasts longer than half a minute will depend increasingly on aerobic respiration. If sufficient oxygen is present enzymes in the mitochondria can completely oxidise the pyruvic acid to produce carbon dioxide, water, ATP and heat, for a net yield of 26 molecules of ATP from each glucose molecule (see Figure 1.14). The aerobic system can also catabolise fatty acids and proteins. Muscle fibres have two

sources of oxygen, that which diffuses from the blood from haemoglobin and that which is released by myoglobin inside the muscle fibres. Both myoglobin and haemoglobin are oxygen-binding proteins; they bind oxygen when it is plentiful and release it in times of scarcity (Wilson & Reeder, 2008). If sufficient oxygen and nutrients are available then the aerobic system will provide enough ATP for prolonged activity.

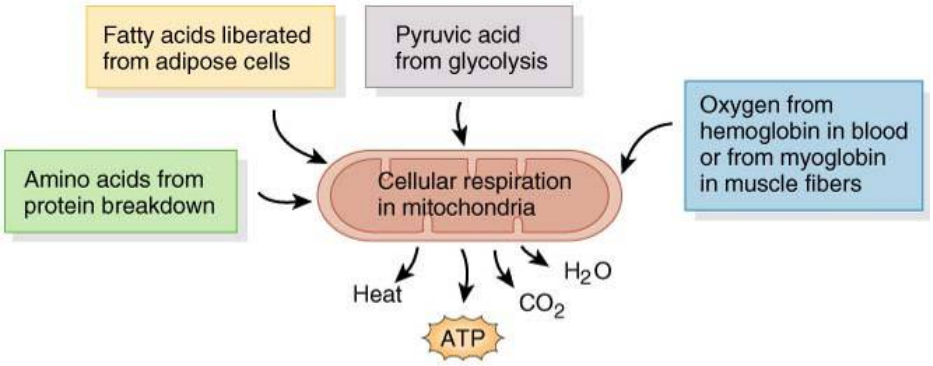


Figure 1-14: ATP derived from aerobic respiration in skeletal muscle. This system provides energy for prolonged activity (from Purves *et al.*, 2003).

1.5.6 Myogenesis

The formation of skeletal muscle is known as myogenesis and involves the fusion of myoblasts, small mononucleated cells. Myoblasts originate in the embryo from the dermomyotome, part of the segmented structures called somites (Brand-Saberi & Christ, 1999). The mesodermal progenitor cells are first specified to the myogenic lineage and the committed myoblasts then proliferate to form a large pool of cells and begin migrating (Pourquie *et al.*, 1995). As long as particular growth factors (particularly fibroblast growth factor) are present, the myoblasts will proliferate without differentiating. When these factors are depleted the myoblasts will exit the cell cycle and terminally differentiate, they then start synthesising myofibrillar proteins and prepare for fusion (Lassar *et al.*, 1994).The myoblasts align together and fuse to form the multinucleated myotubes, a step mediated by cell membrane glycoproteins (Knudsen, 1985). Myoblasts with distinct properties have been isolated at different stages of development and have been named embryonic, fetal and adult (or satellite) myoblasts and it is believed that these form different muscle fibre types (Pin *et al.*, 2002).

Myofibre formation occurs in two or three temporally distinct phases. The first wave of myofibres are derived from embryonic myoblasts and the second wave from fetal myoblasts. In all species studied the myofibres of the primary generation account for a small proportion of the future fibres whilst the secondary generation are thought to use the primary fibres as a scaffold to build up a larger population. The formation of primary myotubes is independent of innervation, whereas the critical step leading to fusion of myoblasts into secondary myotubes is though to be dependent on electrical activity (Ross *et al.*, 1987). In larger species, such as sheep (Wilson *et al.*, 1992), pigs (Mascarello *et al.*, 1992) and humans (Draeger *et al.*, 1987) a third generation of fibres has also been described (see Figure 1.15). Studies in smaller mammals may therefore be an inaccurate representation of human muscle development. The adult myoblast or satellite cell forms a quiescent pool of cells that can be called upon for regeneration in postnatal life, with proliferation and fusion to existing myofibres (Seale & Rudnicki, 2000).

1.5.6.1 Ovine myogenesis

In sheep, primary myofibres are first seen by 32 dGA and are thought to reach their maximum number by 38 dGA (Wilson *et al.*, 1992). These extend the full length of the muscle limits and define its shape, origins and insertions. Small numbers of secondary myofibres first appear at 38 dGA and use primary myotubes as a framework to guide their growth and these continue to form until the number of secondary fibres becomes far greater than that of the primary fibres (Wilson *et al.*, 1992). By 62 dGA some of the secondary myofibres are then found to be supporting a third generation of myofibres (Maier *et al.*, 1992). This third generation is thought to be the reason for the far greater muscle mass found in larger mammals.

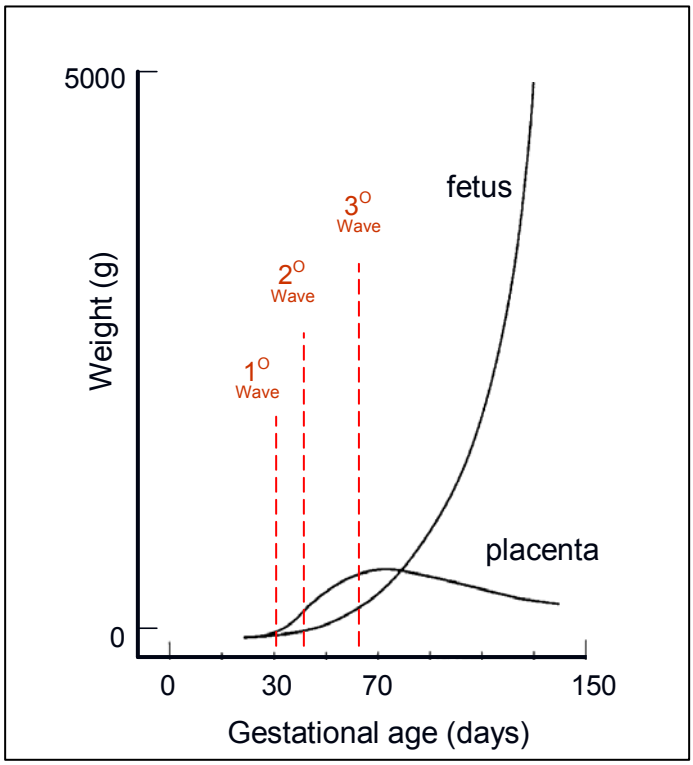


Figure 1-15: The growth trajectory of the ovine fetus showing the three distinct waves of myofibre formation (adapted from Schneider, 1996).

1.5.7 Postnatal muscle growth

For a muscle to grow there must be a net gain in protein, either from an increase in the rate of protein synthesis, a decrease in the rate of degradation, or both (Rennie *et al.*, 2004). During childhood the increase in the size of muscle fibres is partially controlled by human growth hormone, produced by the anterior pituitary gland. A further increase in size is due to the hormone testosterone (Forbes, 1985). Muscle is very sensitive to the mechanical loads which are placed upon it. If these loads are removed, such as through prolonged bed rest, a muscle will become weaker and atrophy (Bloomfield, 1997). In contrast, an increased demand placed upon skeletal muscle such as high resistance strength training will increase its size and strength through hypertrophy of the existing myofibres (McDonagh & Davies, 1984). Protein formation initially depends upon the transcription of DNA into mRNA, followed by the translation of mRNA into protein. Nutrition, exercise and hormones affect both transcription and translation in muscle (Wackerhage & Rennie, 2006). An essential requirement of hypertrophying muscle fibres is the maintenance of the myonuclear domain – the ratio of DNA to protein. The number of nuclei must increase so as to maintain the myonuclear domain (Kadi *et al.*, 1999b) and these nuclei come from the satellite cells.

1.5.7.1 Satellite cells

The muscle satellite cell fulfils the basic definition of a stem cell in that it can give rise to a differentiated cell type and maintain itself by self-renewal (Zammit *et al.*, 2006). They are anatomically distinct from the myonuclei, being located between the basal lamina and the sarcolemma of a muscle fibre (Sanes, 2003).

Satellite cells play a role in postnatal skeletal muscle growth, damage repair and maintenance of adult skeletal muscle fibres (Seale & Rudnicki, 2000). After puberty they are mitotically quiescent until a stimulus, such as exercise or injury, induces the release of growth factors which stimulate the cells to re-enter the cell cycle (Wozniak *et al.*, 2004). Upon activation satellite cells go through a series of stages where they proliferate, differentiate into myoblasts and ultimately fuse with existing myofibres to repair damaged muscle and/or facilitate an increase in its size (Hawke & Garry, 2001). Fibres that have regenerated in adult life can be detected by histological examination as they commonly contain centrally placed nuclei (Montarras *et al.*, 2005).

A proportion of the progeny of the activated satellite cells do not differentiate, but withdraw from the cell cycle to replace the activated satellite cell in a process of satellite cell renewal (Zammit *et al.*, 2006). Individuals who have been strength training for several years have a higher number of satellite cells in their muscles, compared with the muscles of control populations (Kadi *et al.*, 1999b). Satellite cell number can also be increased by strength training in previously untrained populations (Kadi *et al.*, 2004). The concept of the myonuclear domain (region of sarcoplasm associated with an individual nucleus) has been demonstrated in myofibres from weightlifting athletes, where even extremely large fibres appear to maintain a fixed ratio of fibre size to number of nuclei (Kadi *et al.*, 1999a). It has been suggested that the myonuclear domain is ~ 2000 μm^2 (Petrella *et al.*, 2006) and that beyond this a fibre will not be able to hypertrophy unless it recruits more myonuclei.

1.5.7.2 Ageing and muscle function

Muscle mass naturally declines in later life and this is particularly notable in women (Goodpaster *et al.*, 2006). It is estimated that approximately 5–8 % of muscle mass is lost per decade of life after age 30, with this rate of decline accelerating after age 65 (Greenlund & Nair, 2003). Many studies have reported an overall decrease in skeletal protein synthesis with age (Balagopal *et al.*, 2001;Yarasheski *et al.*, 2002;Rooyackers *et al.*, 1996). The loss of mass is greater than can be accounted for by atrophy of the myofibres and so numbers are also thought to be reduced (Grimby & Saltin, 1983).

1.5.8 Control of muscle growth

1.5.8.1 Growth hormone

Growth hormone (GH) is the most abundant anterior pituitary hormone and is also known as somatotropin. It stimulates cell growth but also has many effects on metabolism (Holt, 2004). Most effects of GH are indirect but the one direct effect is to promote synthesis and secretion of small protein hormones called insulin-like growth factors (IGFs). Growth hormone can be detected in the fetus by the eighth to tenth week of gestation, soon after the pituitary has formed (Costa *et al.*, 1993). Although growth hormone appears early in gestation it is not essential for fetal growth and the role of pituitary growth hormone is largely filled in fetal life by human placental lactogen (hPL; Underwood & D'Ercole, 1984). As with growth hormone, hPL acts via the insulin like growth factors (Karabulut *et al.*, 2001).

1.5.8.2 Insulin-like growth factors

The insulin-like growth factors (previously called somatomedins) are structurally and functionally similar to insulin, but have more potent growth-promoting effects (Duclos *et al.*, 1991). IGFs promote cell growth by increasing amino acid transport and protein synthesis and are the factors through which GH exerts its anabolic actions. In response to GH, cells in the liver secrete IGFs into the bloodstream (Sjogren *et al.*, 1999b). In addition it is now well known that other tissues can produce IGF-I for autocrine and paracrine enhancement of growth, and some may also contribute to circulating IGF-I (Brahm *et al.*, 1997). The IGF system is comprised of IGF-I, IGF-II, the IGF receptors and IGF binding proteins (IGFBPs) which regulate bioavailability (Fowden, 2003). IGFs are the dominant growth promoting hormones in the fetus and higher plasma concentrations are related to higher birth weight in all studied species (Gluckman & Butler, 1983;Ludwig *et al.*, 1996;Ong *et al.*, 2000). During fetal life, IGF-I production appears to be local and independent of GH, despite high GH levels. There are few GH receptors in fetal tissues and manipulation of fetal GH concentrations has little effect on the circulating IGF levels (Fowden *et al.*, 2005). The IGFs are known to bind to the IGF-I, IGF-II and insulin receptor. The IGF-I receptor binds both IGF-I and IGF-II with high affinity and nearly all of the biological activities of the IGFs result from binding to the type 1 receptor. The IGF-II receptor binds IGF-II with high affinity and IGF-I with low affinity and is thought primarily to be involved in the clearance and degradation of IGF-II. The insulin receptor binds IGF-I with a far lower affinity than insulin but high concentrations of IGF are thought to stimulate insulin signalling through this receptor (for review see Nakae *et al.*, 2001).

IGF-I regulates myofibre formation and postnatal muscle growth. Evidence for this has come largely from transgenic mice, with those lacking IGF-I having severely underdeveloped muscles (Powell-Braxton *et al.*, 1993) and the up-regulation of the IGF-I gene correlated to increased muscle growth and hypertrophy (Goldspink *et al.*, 1995;Yang *et al.*, 1997). IGFs will also increase myoblast proliferation and differentiation *in vitro* (Napier *et al.*, 1999;Rosenthal *et al.*, 1994). IGF-I is unique amongst the muscle growth factors in that it causes both proliferation and differentiation of myoblasts (Stewart & Rotwein, 1996). The mechanism behind this is not completely understood but may be associated with the IGF ability to induce expression of the myogenic regulatory factor myogenin (Stewart & Rotwein, 1996).

An increase in circulating IGF-I levels (through recombinant GH administration) does not increase hypertrophy any more than intensive exercise does and so there is obviously an important local effect of IGF-I. This was first proposed by DeVol who showed that even in the absence of circulating GH and IGF-I muscles still hypertrophied in response to mechanical overload, with the affected muscles having a threefold increase in IGF-I mRNA levels (DeVol *et al.*, 1990). In human exercise studies IGF-I mRNA expression has been found increased after a single bout of exercise (Bamman *et al.*, 2001;Hameed *et al.*, 2003). The mechanism by which IGF-I induces muscle-fibre hypertrophy involves a combination of satellite-cell activation and protein synthesis (Barton-Davis *et al.*, 1999).

Expression of the IGFs, IGFBPs and their receptors is controlled by the nutritional and hormonal environments *in utero*. Fetal undernutrition induced by maternal dietary manipulation, placental insufficiency and restriction of uterine blood flow reduced circulating levels and tissue expression of IGF-I (Fowden, 2003) and maternal nutrient restriction altered the expression of IGFs in fetal sheep liver and skeletal muscle (Brameld *et al.*, 2000). Muscle IGF-I mRNA has been found to decrease with increasing gestational age, in parallel with the prepartum rise in plasma cortisol (Li *et al.*, 2002). Abolition of the cortisol surge by fetal adrenalectomy prevents the prepartum fall in muscle IGF-I and raising the cortisol levels, by exogenous infusion, prematurely lowers muscle IGF-I levels (Li *et al.*, 2002). Cortisol is therefore a developmental regulator of IGF-I expression. If intrauterine cortisol exposure is altered, through times of stress, this may therefore affect muscle development.

1.5.8.3 Testosterone

Testosterone is a steroid hormone synthesised from cholesterol in the testes of males and the ovaries of females. Males have far higher circulating levels and this contributes to the increased muscle bulk seen in males from the onset of puberty. Endogenous testosterone increases with strength training exercise (Kraemer *et al.*, 1990) and strength training also increases the number of androgen receptors (Bamman *et al.*, 2001). Administration of testosterone to elderly men increased muscle strength and rates of muscle protein synthesis (Urban *et al.*, 1995). This was alongside increased mRNA concentration of IGF-I leading the authors to conclude that the testosterone-induced increase in protein synthesis may be mediated by the IGF-I system. Androgen receptors have also been found on satellite cells (Sinha-Hikim *et al.*, 2003).

1.5.8.4 Myostatin

Myostatin is part of the transforming growth factor-β (TGF-ß) superfamily and in contrast to IGF-I and testosterone, is a negative regulator of muscle growth. It was first identified following studies on the Belgian Blue and Piedmontese breeds of cattle which have a markedly hypertrophied or 'double muscled' phenotype and were found to have a mutation in their myostatin gene (McPherron & Lee, 1997). Knockout mice confirmed that the double muscle phenotype is a response to a myostatin gene mutation (McPherron *et al.*, 1997). Myostatin *in vitro* will inhibit myoblast proliferation (Thomas *et al.*, 2000). Human exercise studies have shown that myostatin is down-regulated with strength-training exercise (Roth *et al.*, 2003;Kim *et al.*, 2005).

1.5.8.5 Muscle specific transcription factors (myogenic regulatory factors)

The myogenic regulatory factor (MRF) family of nuclear proteins includes MyoD, Myf5, myogenin and MRF4 (see Figure 1.16). They belong to a superfamily of basic helix-loop-helix transcription factors. In early embryonic development mesenchymal cells differentiate into committed myogenic precursor cells (myoblasts) and this is through the expression of MyoD or Myf-5 (Rudnicki *et al.*, 1993). Myoblast cell differentiation into myotubes depends on myogenin (Hasty *et al.*, 1993) a positive regulator that induces myoblasts to exit from the cell cycle. Many growth factors such as FGF and TGF-β act antagonistically to myoblast differentiation by preventing cell cycle arrest and repressing myogenic gene expression (Brennan *et al.*, 1991;Vaidya *et al.*, 1989). MRF4 and myogenin are important for terminal differentiation and maturation (Rawls *et al.*, 1995).

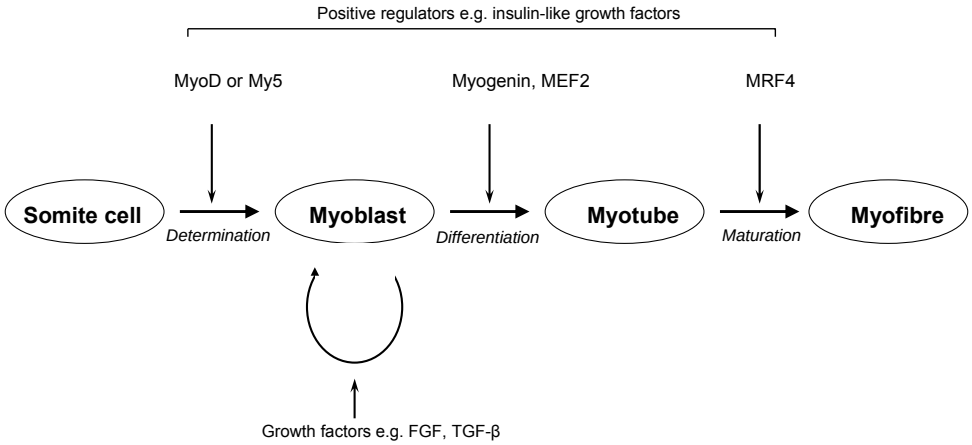


Figure 1-16: Outline of main events and regulatory factors in myogenesis and differentiation (adapted from Dauncey & Gilmour, 1996).

1.5.8.6 Akt (protein kinase B)

The Akt protein kinase family consist of Akt1, Akt2 and Akt3 (Barnett *et al.*, 2005). Akt2 is an important signalling molecule in the insulin signalling pathway and is required for glucose uptake (Cho *et al.*, 2001a). The role of Akt3 is less clear, though it appears to be predominantly expressed by the brain (Easton *et al.*, 2005).

Akt1 is an inhibitor of apoptotic processes (Zhou *et al.*, 2000) and also induces protein synthesis, it is a key signalling protein in skeletal muscle hypertrophy and general tissue growth. Since it can block apoptosis it has been implicated as a major factor in many types of cancer (Hennessy *et al.*, 2005). Akt1 is thought to elicit its effects on cell growth through phosphorylation and the activation of mammalian target of rapamycin (mTOR, Scott *et al.*, 1998) which leads to the subsequent activation of 70-kDa ribosomal protein S6 kinase (p70s6K), a potent stimulator of protein synthesis (Baar & Esser, 1999). Activation of the Akt/mTOR pathway may also promote muscle growth by inhibiting glycogen synthase kinase (GSK)-3β, an inhibitor of protein synthesis (Hardt & Sadoshima, 2002). GSK-3β activity can be inhibited by Akt phosphorylation (Cross *et al.*, 1995) and this may provide a mechanism for Akt to promote muscle growth. Inhibition of GSK-3β induced myotube hypertrophy *in vitro* (Vyas *et al.*, 2002). Akt1 has also been implicated in angiogenesis (Somanath *et al.*, 2006) and is the predominant isoform of Akt in endothelial cells (Chen *et al.*, 2005). A lack of Akt1 in mice causes enhanced angiogenesis (Chen *et al.*, 2005) suggesting that the overall effect of Akt1 on angiogenesis is suppressive, but in contrast another study has reported impaired angiogenesis in Akt1 knockout mice (Byzova *et al.*, 2002).

1.5.9 Myofibre plasticity

It has been established that fibre type composition can undergo changes if the appropriate stimuli are applied (Pette & Staron, 1997). Muscle fibres maintain some plasticity in postnatal life and exercise can induce changes in the fibres of a skeletal muscle. Endurance exercise results in cardiovascular and respiratory changes and so a better supply of oxygen and nutrients to the muscle. Exercise can also cause changes in fibre composition and metabolism (Gollnick *et al.*, 1972;Abernethy *et al.*, 1990) and different types of training can lead to different adaptations so that the muscles of sprint- and endurance-trained athletes have different fibre-type characteristics (Costill *et al.*, 1976). Sprint training will decrease the slow-twitch type-I fibres, increasing the proportions of fast-twitch type IIa. In contrast, endurance training will cause a gradual transformation of some fast glycolytic type IIb fibres into fast oxidative type IIa fibres, along with an increase in capillary supply (Andersen & Henriksson, 1977). The transformed muscles also show a slight increase in their mitochondria and strength but with no increase in muscle mass. Exercise that requires great strength for short periods of time, such as weight lifting, will produce muscle enlargement via an increase in the size and strength of type IIa fibres (Jackson *et al.*, 1990;Campos *et al.*, 2002).

Innervation is important in determining the histochemical and contractile properties of muscle fibres, with these data coming from classic cross-innervation studies in cats. A nerve from a fast-twitch muscle was transferred to a slow-twitch muscle and the muscle then took on fast characteristics, this also worked in reverse when transferring a slow-twitch muscle nerve to a fast-twitch muscle (Buller *et al.*, 1960). Motor neurons innervating slow muscle fibres are tonically active, delivering low-frequency stimulation in a sustained manner. The motor neurons innervating fast fibres are phasic, tending to fire infrequently but at a higher frequency (Grottel & Celichowski, 1999). Changing the mechanical load has also been shown to cause a fast to slow transition (Gregory *et al.*, 1986;Tsika *et al.*, 1987). Transition of fibres do not proceed from one extreme to another but occur in a sequential manner, in the order of type I ↔ IIa ↔ IIx ↔ IIb (Pette & Staron, 1997). However, it has been suggested that muscle fibre heterogeneity has its origin during development and stems from distinct populations of myoblasts which are committed to form myotubes within a fixed phenotypic range (Stockdale, 1992). Nerve impulses will still play a role in modifying

myofibre phenotype, but only within the range determined during myogenesis (Hoh, 1991). From studies on monozygotic and dizygotic twins it was determined that fibre type composition is determined firstly by the gene (Komi *et al.*, 1977). More recent data has shown that 40 % of variance in fibre type proportion is explained by environmental factors (Simoneau & Bouchard, 1995).

1.5.10 Fetal muscle responses to undernutrition

Skeletal muscle has a lower priority in nutrient partitioning compared with the brain and heart and this makes it particularly vulnerable to nutrient deficiency (Bauman *et al.*, 1982). In most mammalian species (except rodents) skeletal muscle fibres form prenatally and the total number of fibres in a muscle is fixed at the time of birth (Brameld *et al.*, 1998). Postnatal growth in the pig is correlated with myofibre number at birth and since more myofibres correlate with an increased postnatal growth, maternal nutrition can have a major influence on the growth potential of an animal (Dwyer *et al.*, 1994). In some studies the number of muscle nuclei rather than the number of fibres correlate with postnatal growth (Greenwood *et al.*, 1999) and this is probably a reflection of a larger pool of satellite cells.

Numerous studies carried out in a range of mammalian species have shown that altered maternal nutrition during gestation or weaning can significantly affect the number of both myofibres and nuclei in the offspring. In the rat myofibre (Bedi *et al.*, 1982) and nuclei (Beermann *et al.*, 1983) number are reduced following global nutrient restriction in gestation. Nuclei number and not myofibres were reduced following a 40-50 % reduction during gestation (Bayol *et al.*, 2004). Protein restriction throughout gestation and weaning in the rat also reduced muscle mass (Desai *et al.*, 1996). However, imposing a protein restriction immediately after birth by cross-fostering rat pups had almost as great an effect on muscle mass as protein restriction from conception to weaning. This suggested that there is an early postnatal period in the rat during which muscle development is highly sensitive to nutritional insults. Since myofibre number is not set at birth in rodents this period may be comparable to late gestation in other species. A cafeteria diet (*ad libitum* choice of processed foods with high fat or high sugar) during gestation and/or weaning reduced myofibre number (Bayol *et al.*, 2005). In the pig a doubling of maternal intake between 25 -50 dGA increased myofibre number (Dwyer *et al.*, 1994) and in the guinea pig a 40 % global

restriction throughout gestation reduced myofibre number (Dwyer *et al.*, 1995). In the sheep, a reduction in myofibre number has been observed following a 50 % restriction in the peri-conceptual period (Quigley *et al.*, 2005) and a 50 % restriction from 28-78 dGA (Zhu *et al.*, 2006). Temporary exposure of embryos to an advanced uterine environment increased total myofibre number in late gestation (Maxfield *et al.*, 1998). Studies indicate that primary myofibres are resistant to manipulation and secondary fibres are preferentially affected (Ward & Stickland, 1991;Dwyer & Stickland, 1992). Fetal growth retardation due to placental insufficiency resulted in a reduced number of secondary fibres in the newborn pig (Aberle, 1984). Similarly, where the number of myofibres are increased this is through changes in the secondary myofibre population (Dwyer *et al.*, 1994).

Changes in myofibre composition may also occur following maternal dietary manipulation. The offspring (8 months old) of ewes fed a 50 % diet from 28-78 dGA had a decreased number of myofibres along with increased ratio of type-IIb fibres, indicating a decrease in the type-I fibres (Zhu *et al.*, 2006). Lambs (2 weeks old) exposed to a 50 % reduction 30-70 dGA had significantly fewer fast fibres and an apparent increase in slow fibres (Fahey *et al.*, 2005b). However, when the same restriction was applied and the muscle fibres analysed in older lambs (24 weeks old) fast fibre number was increased with no difference in slow fibre number suggesting that the lambs had adapted to the change in myofibre composition previously seen at 2 weeks of age (Daniel *et al.*, 2007).

Dietary manipulation can also affect key signalling pathways in skeletal muscle. A reduction in myofibre number in nutrient restricted 78 dGA fetuses was associated with a down-regulation of mTOR signalling (Zhu *et al.*, 2004) and the content of GLUT-4 was lower in skeletal muscle of 8 month old lambs (Zhu *et al.*, 2006). A low protein diet during pregnancy and lactation reduced skeletal muscle expression of protein kinase C zeta (PKCζ) in rat offspring (Ozanne *et al.*, 2003). Activity of carnitine palmitoyltransferase-1 (CPT-1) a key enzyme in controlling fatty acid oxidation was decreased in muscles of nutrient restricted lambs (Zhu *et al.*, 2006). CPT-1 is located on the mitochondrial membrane and the reduction was thought to be due to a reduction in mitochondrial density. In rats, surgically induced IUGR in late gestation impairs mitochondrial function and oxidative capacity of skeletal muscle (Selak *et al.*, 2003). A reduction in fatty acid oxidation may lead to an accumulation

of intramuscular triglycerides and this was found in the nutrient restricted offspring (Zhu *et al.*, 2006). Skeletal muscle of IUGR rat offspring also had higher intramuscular triglycerides but this was associated with an increase in CPT-1 activity (Lane *et al.*, 2001) and nutrient restricted lambs had increased intramuscular fat (Daniel *et al.*, 2007). Accumulation of intracellular lipids leads to suppression of insulin signalling (Lowell & Shulman, 2005) and insulin resistance (Krebs & Roden, 2004) and so altered fatty acid oxidation may be a mechanism through which nutrient restriction affects glucose tolerance.

1.6 Glucose uptake and metabolism

Glucose is an important source of energy for cells as well as being a metabolic intermediate. Normal fasting blood glucose in the human ranges from 3.9 to 5.5 mmol/L but this will rise after a meal. A fasting level of $\geq$7 mmol/L is considered hyperglycaemic and indicative of diabetes (Alberti & Zimmet, 1998). Glucose levels are controlled by the hormone insulin.

1.6.1 Insulin

Insulin is the most potent anabolic hormone and is essential for appropriate tissue development, growth and maintenance of whole-body glucose homeostasis. It is secreted by the β-cells of the pancreatic islets of Langerhans in response to increased circulating levels of glucose and amino acids. It increases glucose uptake into muscle and fat and reduces hepatic glucose production (via decreased gluconeogenesis and glycogenolysis) and is therefore the primary regulator of blood glucose. Insulin also affects lipid metabolism, increasing lipid synthesis in liver and fat cells, and attenuating fatty acid release from triglycerides in fat and muscle

1.6.2 Insulin synthesis

Insulin is produced and secreted by pancreatic β-cells, located in the pancreatic islets. Insulin is a small protein consisting of 51 amino acid residues and has a molecular weight of 5808 Dalton (Kaarsholm & Ludvigsen, 1995). Insulin mRNA is translated as a single chain precursor called preproinsulin, with removal of its signal peptide during insertion into the endoplasmic reticulum generating proinsulin (Eskridge & Shields, 1983). Proinsulin consists of three domains, including a connecting peptide in the middle known as the C peptide (Snell & Smyth, 1975). Within the endoplasmic

reticulum the C peptide is proteolytically removed, generating the mature form of insulin (Davidson *et al.*, 1988). Insulin and free C peptide are packaged into secretory granules and accumulate in the cytoplasm and when the beta cell is appropriately stimulated insulin is secreted from the cell by exocytosis and diffuses into islet capillaries (Koster *et al.*, 2005). The C peptide is also secreted into the blood stream and is often used for the diagnosis of type I diabetes.

1.6.3 Insulin release

Insulin is secreted in response to elevated blood concentrations of glucose (Toschi *et al.*, 2002), neural stimuli e.g. sight and taste of food (Benthem *et al.*, 2000) and increased blood concentrations of other fuel molecules including amino acids (Floyd *et al.*, 1966) and fatty acids (Warnotte *et al.*, 1994). Type 2 glucose transporter (GLUT-2) mediates the entry of glucose into pancreatic beta cells (Thorens, 1992) which is then phosphorylated by glucokinase, creating ATP. The increased ATP : ADP ratio closes the ATP-gated potassium channels in the cell membrane, preventing movement of potassium ions out of the cell. The increased concentration of potassium ions leads to depolarisation of the cell, activating the voltage-gated calcium channels. The increased intracellular calcium triggers exocytosis of insulin-containing secretory granules, releasing insulin into the blood stream (Koster *et al.*, 2005). Insulin release is a biphasic process (Curry *et al.*, 1968), with first-phase insulin release occurring within the first few minutes after exposure to elevated glucose levels, followed by a second phase of insulin release. The first-phase is dependent upon the amounts of stored insulin, and this phase is impaired in patients with type-II diabetes (Gerich, 2002). Once the stored insulin is depleted a second phase of insulin release is initiated. The second phase is prolonged since insulin is synthesised, processed, and secreted for the duration of the increase of blood glucose. Between meals a low continuous level of basal insulin serves ongoing metabolic needs (Lang *et al.*, 1979). The enzyme insulinase (found in liver and kidneys) breaks down circulating insulin resulting in a short half-life for the hormone (Duckworth, 1998). This degradative process ensures that levels of circulating insulin are controlled and that blood glucose levels do not get dangerously low.

1.6.4 Insulin actions and signalling pathways

The binding of insulin to its associated receptor initiates a signalling cascade which has several different outcomes (see Figure 1.17). Insulin increases the uptake of amino acids and protein synthesis, whilst inhibiting protein degradation (O'Connor *et al.*, 2003) increases the uptake of fatty acids and lipid synthesis, whilst inhibiting lipolysis (Saltiel & Kahn, 2001) and it also has mitogenic effects (Conejo & Lorenzo, 2001) although these are not as potent as IGF-I and insulin plays a more important role in metabolic processes. Target tissues of insulin include skeletal muscle (main site of action and utilises glucose for energy), adipose (stores glucose as triglycerides) and liver (stores glucose as glycogen).

Insulin action is initiated through the binding to and activation of its cell-surface receptor. The insulin receptor belongs to a subfamily of receptor tyrosine kinases which include Type-I insulin-like growth factor receptor (Patti & Kahn, 1998). These receptors consist of two α- and two β-subunits and in the unactivated state the α-subunit inhibits the tyrosine kinase activity of the β-subunit (White *et al.*, 1988). Insulin binds to the extracellular α-subunits and activates the intracellular tyrosine kinase domain of the β subunit, resulting in a rapid autophosphorylation of the receptor (Kasuga *et al.*, 1982). The receptor tyrosine kinase activity also promotes the phosphorylation of insulin receptor substrate (IRS) -1 (Sun *et al.*, 1991). The phosphorylation of IRS-1 enables it to interact with a number of additional proteins including phosphatidylinositol (PI) 3-kinase (Backer *et al.*, 1992). PI 3-kinase consists of a p85 regulatory subunit which allows it to associate with phosphorylated IRS-1 (Skolnik *et al.*, 1991) and a p110 subunit, which contains the enzymes catalytic activity (Hiles *et al.*, 1992). The p110 subunit catalyses the formation of phosphoinositol lipids such as $PI(3,4,5)P_3$ which in turn activate other kinases such as PKCζ and Akt. There are 3 closely related isoforms of Akt (see Section 1.5.8.6) of which Akt2 is important for insulin signalling. Once active, Akt2 enters the cytoplasm where it leads to the phosphorylation and inactivation of glycogen synthase kinase-3 beta (GSK-3β) (Hardt & Sadoshima, 2002) which is an inhibiter of glycogen synthase, an enzyme that catalyses the final step in glycogen synthesis. Phosphorylation of glycogen synthase by GSK-3β decreases glycogen synthesis and therefore the inactivation of GSK-3β by Akt2 promotes glucose storage as glycogen. In addition to

promoting glucose storage insulin inhibits the production and release of glucose by the liver by blocking gluconeogenesis and glycogenolysis (Saltiel & Kahn, 2001). Another key action of Akt2 is to stimulate glucose uptake into cells by inducing translocation of GLUT-4 from intracellular storage to the plasma membrane (Kohn *et al.*, 1996) and PKCζ has also been implicated in this translocation process (Liu *et al.*, 2007).

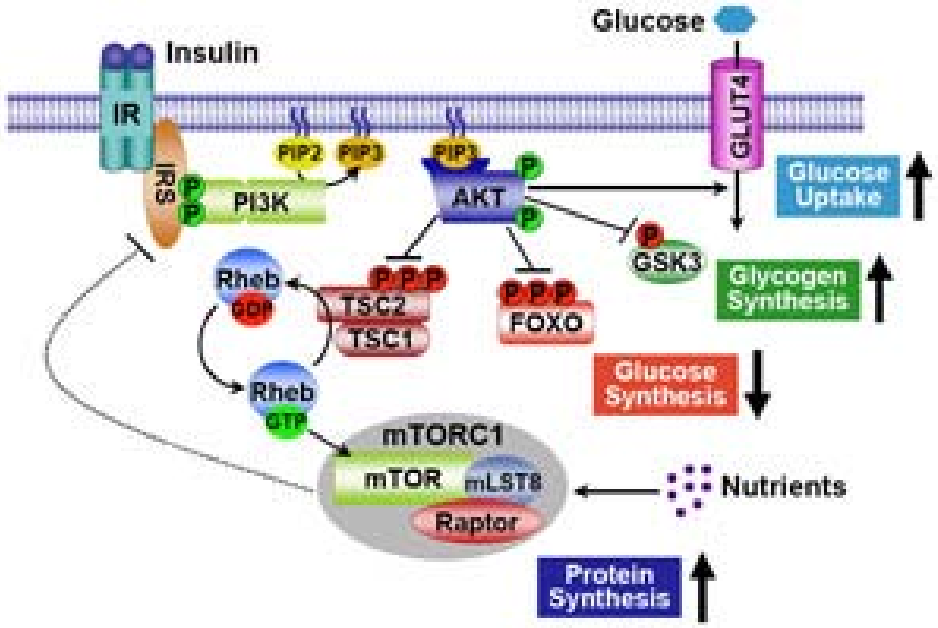


Figure 1-17: Insulin signalling pathways in skeletal muscle. The binding of insulin to the insulin receptor (IR) initiates a signalling cascade which includes insulin receptor substrate (IRS), phosphatidylinositol 3-kinase (PI3K), Akt / protein kinase B, phosphoinositol lipids (PIP3), glycogen synthase kinase-3 (GSK-3) and the mammalian target of rapamycin (mTOR). These pathways lead to an increase in glucose uptake, glycogen and protein synthesis and a decrease in glucose synthesis (from Helmreich, 2001).

Besides regulating insulin action, the insulin receptor also plays a minor role in mediating insulin-like growth factor-II (IGF-II) growth promoting actions in early development (Louvi *et al.*, 1997). The IGF-I and insulin receptor account for the entirety of the growth promoting effects of IGF-1 and IGF-II during development (Rother & Accili, 2000) which makes them excellent targets when investigating growth and metabolic responses to nutrient challenges.

1.6.5 Glucose Transporter-4

The sarcolemma and other cell membranes are relatively impermeable to glucose and so specific transporters are needed for the transport of glucose across the membrane. The glucose transporters (GLUTs) are a family of proteins expressed in a tissue specific manner and which transport glucose across the cell membrane by facilitated diffusion (Gould & Holman, 1993). There have been five glucose transporters identified, GLUT-1 to GLUT-5 (Shepherd & Kahn, 1999). In skeletal muscle the most important transporter is GLUT-4 with GLUT-1 also widely distributed in fetal tissues (Henriksen *et al.*, 1990). In the absence of insulin, GLUT-4 resides predominantly in intracellular vesicles that continuously cycle from intracellular stores to the plasma membrane (Ploug *et al.*, 1998). Insulin and IGF-I increases glucose uptake in muscle cells by increasing the rate of GLUT-4 vesicle exocytosis and by decreasing the rate of internalisation (Pessin *et al.*, 1999). A number of other stimuli are known to activate the translocation of GLUT-4 and these include contraction (Lund *et al.*, 1995) and hypoxia (Cartee *et al.*, 1991).

Levels of muscle GLUT-4 are known to alter glucose tolerance, with over-expression of the GLUT-4 gene improving peripheral glucose utilisation in mice (Treadway *et al.*, 1994) and a more efficient translocation of GLUT-4 was associated with normal glucose tolerance in rat pups of nutrient restricted dams, although total GLUT-4 content was not affected (Gavete *et al.*, 2005). Data have indicated that the insulin resistance in skeletal muscle of type II diabetes is a result of impaired translocation of intracellular GLUT-4 to the sarcolemma (Garvey *et al.*, 1998;Zierath *et al.*, 1996).

1.6.6 Diabetes

Diabetes mellitus is a metabolic disorder characterised by inappropriately high blood glucose (hyperglycaemia) resulting from either low levels of insulin secretion or from resistance to insulin effects coupled with inadequate levels of insulin secretion to compensate. There are three forms of diabetes: type I, type II and gestational diabetes and these have different causes and population distributions. Type I diabetes is usually due to autoimmune destruction of the pancreatic β cells, leading to inadequate insulin secretion. Type II diabetes is characterised by insulin resistance at the tissue level. Gestational diabetes is also caused by an insulin resistance, with pregnancy hormones causing resistance in woman genetically predisposed to developing the condition.

The pathophysiology of type II diabetes involves defects in tissue sensitivity to insulin as well as decreased insulin secretion. Under normal conditions insulin will bind to its receptor to promote intracellular glucose transport and metabolism. Insulin resistance is the inability of target tissues to respond properly to normal circulating insulin concentration and this resistance is caused by defects in signal transduction. The insulin resistance of obesity and type II diabetes are characterised by defects at many levels in the signalling pathway. Decreases have been found in insulin receptor concentration, kinase activity, concentration and phosphorylation of IRS-I, PI(3)K activity, glucose transporter translocation and activity of intracellular enzymes (Saltiel & Kahn, 2001). To maintain normal plasma glucose levels the pancreas will compensate by secreting increased amounts of insulin. However, impaired glucose tolerance will eventually develop despite the increased insulin concentrations due to increased insulin resistance. Eventually the β-cells will fail, resulting in decreased insulin secretion (Steppel & Horton, 2004). When these two defects (insulin resistance and impaired β-cell function) occur simultaneously it is described as overt clinical type II diabetes and blood glucose levels can become dangerously high. Diabetes is now one of the most common non-communicable diseases globally. There are currently around 194 million people with diabetes worldwide, and it is estimated that this will increase to 333 million by 2025 (International Diabetes Federation, 2008).

1.6.6.1 Diabetes and muscle

Impaired insulin sensitivity of skeletal muscle is an early sign in the pathogenesis of type II diabetes and is sometimes observed years before the onset of disease (Koistinen & Zierath, 2002). Type II diabetes is associated with a shift in muscle fibre composition towards a higher proportion of the relatively insulin-resistant fast-twitch fibres at the expense of the insulin-sensitive slow-twitch fibres (Marin *et al.*, 1994) and obese patients have been shown to have a higher proportion of type IIb fibres (Tanner *et al.*, 2002). Glucose uptake has been shown to be positively correlated with percentage of type I fibres and negatively correlated with percentage of type IIb fibres in adult men (Lillioja *et al.*, 1987). In parallel with fibre type alterations, insulin-resistant or glucose-intolerant subjects may have reduced capillary density (Lillioja *et al.*, 1987) and abnormalities of capillary structure, which could reduce the rate of insulin diffusion to its receptor, thereby also contributing to insulin resistance.

1.7 Hypothesis

The DOHaD hypothesis states that a fetus can adapt to changes in its environment and that these adaptations can lead to permanent physiological changes in the offspring, predisposing to disease in later life. Skeletal muscle is a crucial element when investigating the association between impaired fetal development and later insulin resistance due to it being the primary site of glucose utilisation. Through separate studies nutrient restriction in the sheep has been shown to reduce both myofibre number and glucose tolerance in the offspring, but to date these two elements have not been brought together.

Thus, the overarching hypothesis of this thesis was that:

Reduced maternal nutrient intake and/or body condition would impair skeletal muscle growth and alter its morphology (i.e. reduced myofibre/capillary density and altered fibre type composition) with ramifications for the regulation of glucose metabolism in the offspring.

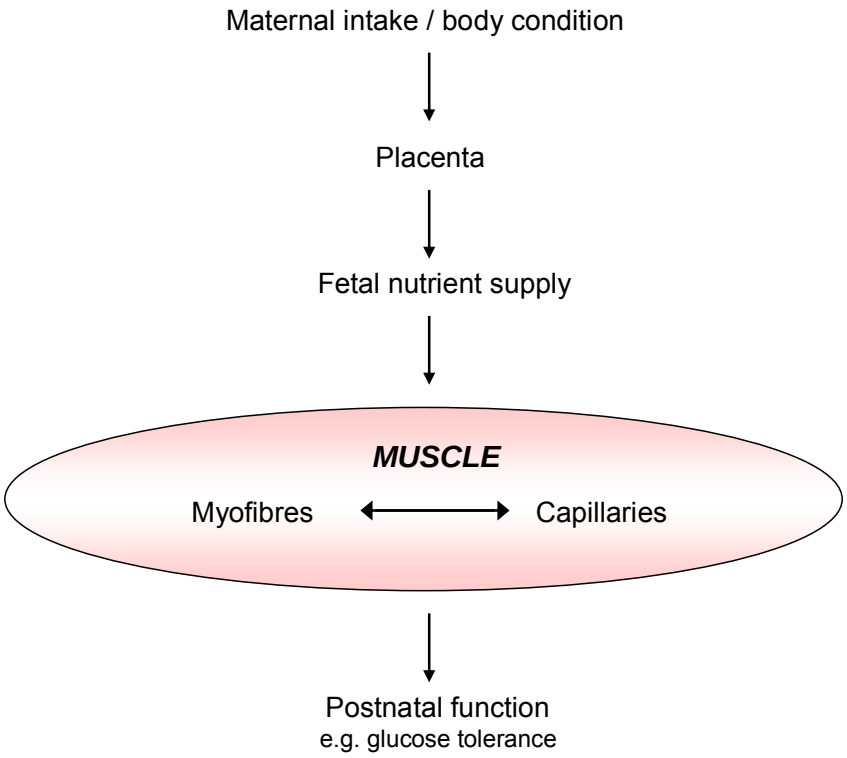


Figure 1-18: Hypothesis overview. While maternal diet has been associated with changes in skeletal muscle morphology and postnatal function one model is needed to bring these two concepts together.

1.8 Aims

The overall aim of this thesis was to elucidate the effects of decreased maternal nutrient intake and/or body condition on skeletal muscle morphology and to investigate the effects of a change in skeletal muscle morphology (i.e. reduced myofibre/capillary density and altered fibre type composition) upon glucose tolerance in the offspring.

Chapter 3

Maternal undernutrition impairs skeletal muscle development in the offspring (Bayol *et al.*, 2004; Zhu *et al.*, 2006) with the outcome dependent upon the timing of the challenge (Fahey *et al.*, 2005b; Dwyer *et al.*, 1995). Moreover, because of the different purpose of muscles (locomotion / postural), different myofibre proportions and the fact that type I myofibres are thought to be mainly from primary myofibres and more resistant to undernutrition, some muscles are more susceptible than others to a nutrient challenge (Dwyer & Stickland, 1992). Previous studies have focused on early and/or mid-gestation in the sheep or on whole gestation in the rat and have not investigated the effect of a late gestation challenge upon skeletal muscle morphology. However, since the inception of this thesis a study has investigated the impact of reduced maternal protein during three distinct periods of gestation (early, mid and late) in the muscles of young rats and found that all three periods reduced total myofibre density (Mallinson *et al.*, 2007). Although the mechanisms underlying altered muscle morphology with maternal undernutrition are likely to involve altered expression of a myriad of genes (Bryson-Richardson & Currie, 2008) the changes may also be mediated in part by a reduced blood flow to the tissue due to decreased capillary density. Indeed, Type II diabetes has been associated with changes in both fibre type composition and reduced capillary density (Marin *et al.*, 1994).

- **I hypothesise that muscle morphology will be impaired by late and early gestation undernutrition with differential effects on the proportion of myofibre types, and to a variable extent in two distinct muscle beds. These anticipated changes will be mediated in part by reduced capillarisation and will impact on muscle insulin sensitivity and glucose uptake mechanisms.**

The aim of Chapter 3 was therefore:

To determine the effect of peri-implantation and late gestation maternal undernutrition on the density of fast and slow myofibres and capillaries in two distinct skeletal muscle beds (mixed fibre type and slow twitch type) and on their expression of components in the insulin signalling and glucose uptake pathway in the late gestation fetus.

Chapter 4

In Chapter 3 it was shown that reduced total myofibre density in the fetal triceps brachii muscle was associated with reduced capillary density following an early or late gestation maternal undernutrition. It has already been established that the offspring of nutrient-restricted mothers have altered vascular reactivity of the small arteries during fetal life (Ozaki *et al.*, 2000;Nishina *et al.*, 2003) indicative of a peripheral redistribution of blood flow (an increased vasoconstriction in the peripheral blood vessels will reduced flow to the peripheral tissues such as skeletal muscle). Since angiogenesis can be a flow-mediated process (Prior *et al.*, 2004), a reduction in the upstream blood flow to the developing muscle (in this case the brachial artery) may therefore attenuate capillary formation.

- **I hypothesise that reduced blood flow to the developing skeletal muscle may mediate the observed reduction in myofibre and capillary density following periods of undernutrition and that the reduced blood flow may be reflected in changes in the vasoactive properties of the vessel.**

The aim of chapter 4 was therefore:

To assess whether the specific periods of peri-implantation and late gestation maternal nutrient restriction have an impact on the vasoactive properties of the isolated fetal femoral artery in late gestation which may indicate a reduction in blood flow through the vessel.

Chapter 5

In Chapter 3 it was established that maternal undernutrition reduced skeletal muscle myofibre and capillary density in fetal offspring along with an increase in insulin receptor, GLUT-4 and IGF-I receptor (late gestation restricted group only), components of the insulin signalling pathway. The majority of myofibres are formed

prenatally and, while there is some scope for hypertrophy and altered myofibre type in postnatal life, it is possible that those changes seen in skeletal muscle morphology in late gestation would have persisted into adult life and affected the glucose tolerance of the offspring. Previously, a low maternal body condition score during pregnancy was shown to reduce glucose tolerance in 1.5 year old sheep offspring (Cripps *et al.*, 2008). This same cohort became available to study at 4 years of age and provided the perfect opportunity to assess the effects of maternal nutrition on skeletal muscle morphology in a much older cohort, and to investigate its impact on glucose tolerance.

- **I hypothesise that a reduction in maternal body condition, through dietary manipulation, will reduce skeletal muscle myofibre density and alter fibre type composition in the mature adult offspring. These anticipated changes in myofibre density and composition will be associated with a reduced glucose tolerance in mature adulthood.**

The aim of chapter 5 was therefore:
To investigate the effects of maternal body condition on skeletal muscle morphology (fibre density and type) and to relate this directly to isolated skeletal muscle glucose uptake, and whole body glucose tolerance in mature adult offspring.

2 General Methods

All animal procedures performed were in accordance with the regulations of the British Home Office Animals (Scientific Procedures) Act, 1986.

2.1 Sheep husbandry and diet

2.1.1 Fetal study

Welsh Mountain sheep, of uniform body weight (body condition score 2.0 - 3.0), age and in their first parity were individually penned and maintained according to normal sheep husbandry at the Royal Veterinary College (RVC; North Mymms, Hertfordshire). All ewes in the study were weighed, body condition scored and their back muscle and fat depth measured from ultrasound images by John Thompson, RVC (see Figure 2.5).

Figure 2-1: The Welsh Mountain sheep. These are a breed that have adapted to a harsh highland environment and produce a high proportion of singleton fetuses.

Nutritional requirements were calculated according to ewe start weight (measured at -16 dGA) and adjusted for fetal gestational age according to Agriculture and Food Research Council (AFRC, 1993) nutritional guidelines. Ewes were randomised to control or dietary restricted groups, housed individually on wheat straw and fed a complete pelleted diet with free access to water from -16 dGA. As fed the diet provided 9.57 MJ/kg metabolisable energy and 14.75 g crude protein per 100 g (Charnwood Milling Co. Ltd, Suffolk, UK; see Appendix 1). Oestrous was synchronised by removal of a vaginal medoxyprogesterone acetate impregnated

sponge (Veramix, Upjohn Ltd., Crawley, UK) 14 days after insertion. One of two twin rams (randomly assigned) were introduced for 2 days, and 0 dGA taken as the first day which an obvious raddle mark was observed. An ultrasound scan was performed to confirm pregnancy at 55 dGA and those not pregnant or carrying twins were removed from the study.

Control animals (C, *n* = 8: female (f) = 4, male (m) = 4) were fed 100 % of nutrient requirements throughout gestation. Peri-implantation nutrient restricted animals (PI, *n* = 9: f = 4, m = 5) were fed 40 % of nutrient requirements from 1 to 31 dGA and 100 % at all other times. Late gestation nutrient restricted animals (L, *n* = 6: f = 2, m = 4) were fed 50 % of nutrient requirements from 104 dGA and 100 % at all other times (Figure 2.2). The animals were transported to Southampton at 111 dGA and kept in rooms at 17.5 to 18 ºC with a 12 hour light/dark cycle.

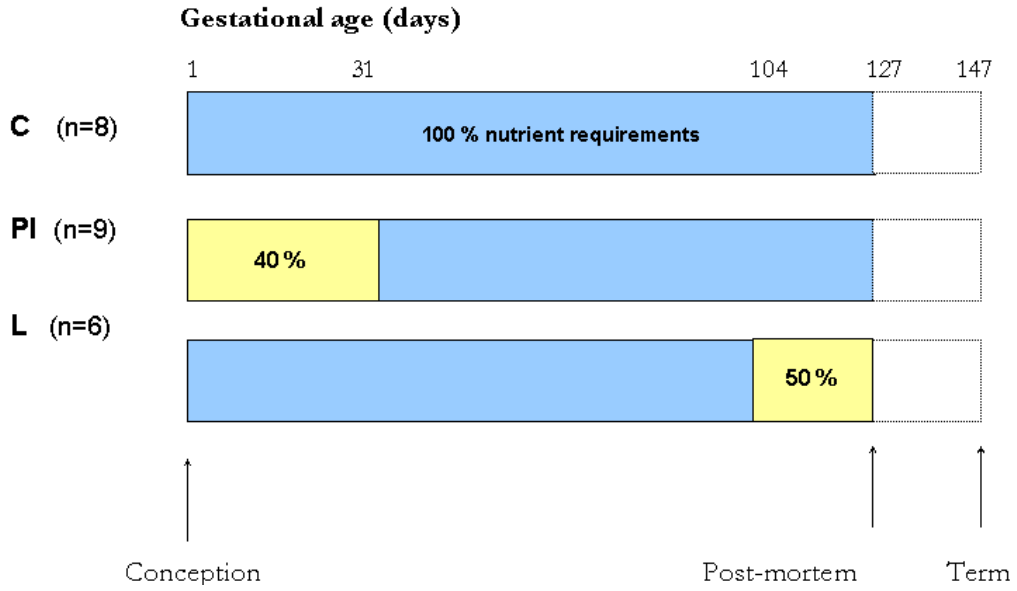


Figure 2-2: Nutritional protocol of the fetal sheep study. Yellow blocks represent periods of nutrient restriction, with 100 % of nutrient requirements at all other times. Skeletal muscle was collected at 127 dGA.

2.1.1.1 Surgery

The animals underwent surgery to catheterise the fetus for the purposes of other studies which do not concern this thesis. All nutritional groups were subject to the same procedure. Blood samples used in this thesis, for blood gas, nutrient and metabolite analysis were taken ~ 15 minutes before post mortem using a femoral artery catheter inserted at the time of surgery.

2.1.2 Adult study

Sixty 2nd parity Welsh Mountain ewes were housed individually and established at a BCS of 2 (LBCS) or equal or greater than 3 (HBCS) by adjusting the daily ration of energy intake. Ewes were fed on wheat straw and a complete pelleted diet with free access to water. As fed the pelleted diet provided 9.57 MJ/kg metabolisable energy and 14.75 g crude protein per 100 g (Charnwood Milling Co. Ltd; see Appendix 1). Once the designated stable body condition had been reached, oestrous was synchronised by removal of a vaginal medroxyprogesterone acetate-impregnated sponge (Veramix, Pharmacia and Upjohn, UK) 14 days after insertion. One of two twin rams (randomly assigned) were introduced for 2 days, and 0 dGA taken as the first day which an obvious raddle mark was observed. Ewe body weight and BCS were then measured weekly and daily pelleted food ration adjusted accordingly. In addition, gestational increases in energy intake were applied to both groups according to AFRC guidelines (AFRC, 1993). To compensate for the increase in diet ration, pelleted feed was switched to a higher energy formula at 91 dGA (Charnwood Milling Co. Ltd; see Appendix 2) which as fed provided 10.94 MJ/kg metabolisable energy and 14.56 g crude protein per 100 g. This allowed the ewes to consume enough food to give the desired energy intake.

Ewes were allowed to deliver spontaneously and female offspring removed from the study. Ewes and male offspring were housed in two groups according to the maternal BCS; HBCS (n = 17) or LBCS (n = 12). The lambs were weaned at 12 weeks and given free access to hay and water and a standardised ration of creep pellets (AFRC, 1993). As fed, these provided 10.59 MJ/kg metabolisable energy and 18 g crude protein per 100 g (Prestige Lamb Pellets + Decox, BOCM Pauls Ltd., Loughborough, UK; see Appendix 3). From 32 weeks of age adult pelleted feed was given which provided 10.38 MJ/kg and 18 g crude protein per 100 g (Ewbol 18, BOCM Pauls Ltd; see Appendix 4). The offspring were then housed as a single flock.

At birth, the lambs were weighed, and shoulder height, femur length, abdominal circumference and crown rump length were measured. At 4, 8, 12, 16, 26, 48 weeks and then every 6 months until 4 years of age BCS and body weight were measured. Fat and muscle depth were also evaluated at these ages (excluding 2, 3 and 3.5 years) by ultrasound scanning (see Figure 2.5). Additionally, at 26 and 48 weeks of age offspring

shoulder height, femur length, abdominal circumference and crown rump length were measured. At 1.5 years (72 ± 0.7 weeks) a glucose tolerance test was performed. At 4 years (210 ± 4 weeks) the rams were transported to Southampton and studies at this time are reported by this thesis.

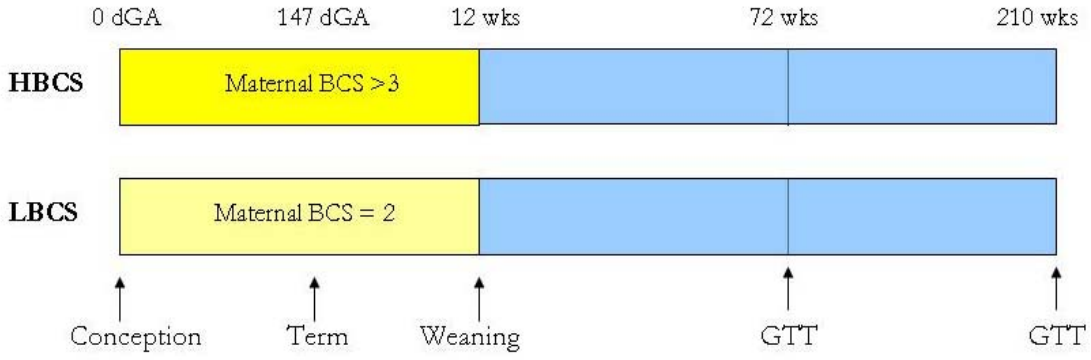


Figure 2-3: Nutritional and experimental protocol of the adult sheep study. Yellow blocks represent gestation and suckling, during which ewe BCS was established at 2 (LBCS) or ≥ 3 (HBCS) through the adjustment of energy intake. A glucose tolerance test was performed on the adult offspring at 1.5 years (72 weeks) and 4 years (210 weeks). Skeletal muscle was collected at 4 years.

2.2 Body condition score

The body condition scoring system is used by farmers to assess the condition of their animals. Scores range from 1 (emaciated) to 5 (obese) (Russell, 1991) but scores of 2 (thin), 3 (average) and 4 (fat) are most common. Half scores are often used between these levels and the study in chapter 5 uses quarter scores. The third lumbar region is felt by hand to assess BCS (see Figure 2.4). BCS were assessed by John Thompson (RVC) using the standard scoring system as described by the Department for Environment, Food and Rural Affairs (see Figure 2.6).

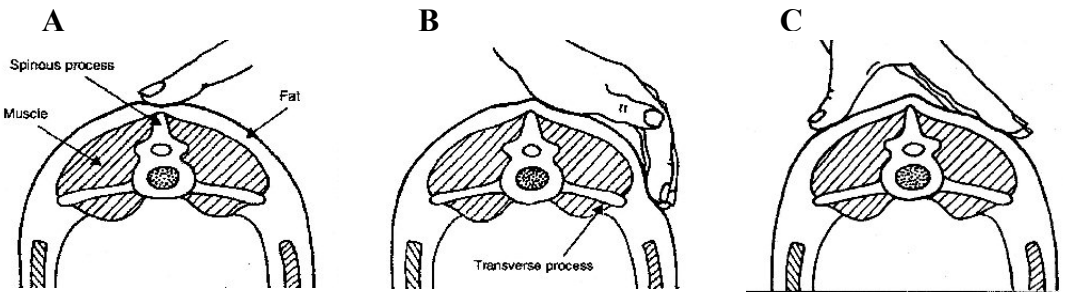


Figure 2-4: Areas on the back of the ewe that were assessed for body condition scoring. (a) spine between last rib and hip bone, b) tips of transverse processes and c) fullness of back fat and muscle cover.

2.2.1 Back fat and muscle depth

Ultrasound images of the 3rd lumbar region were also collected by John Thompson using a real-time ultrasound unit (Aloka SSD 210 DX11, BCF Technology, Livingstone, UK) with a 7.5 MHz linear array transducer. After freezing the digitised image on the screen, subcutaneous fat and muscle depth were measured using the internal callipers of the machine (see Figure 2.5).

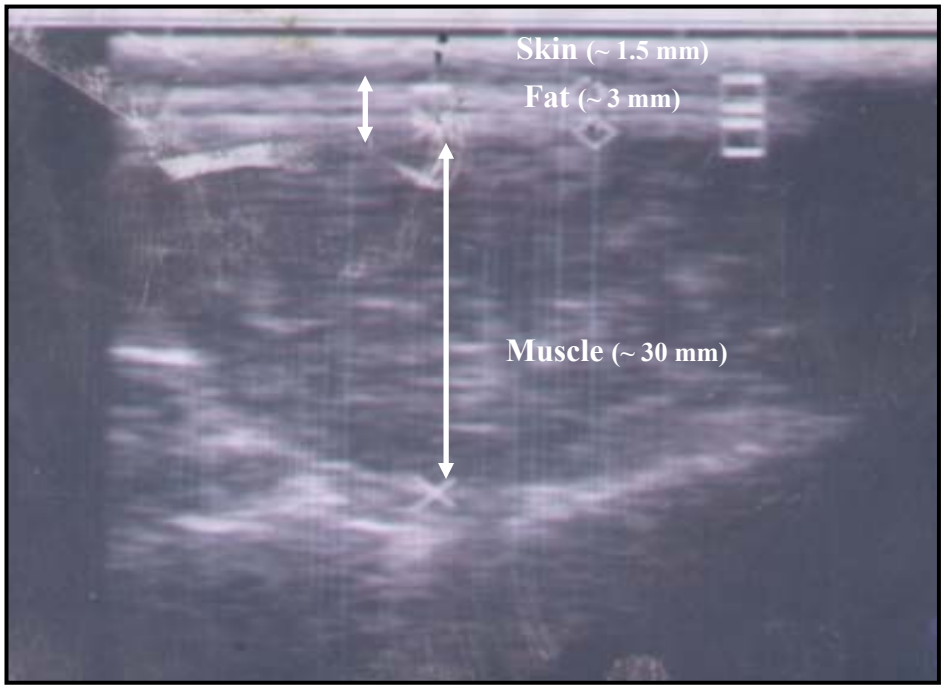
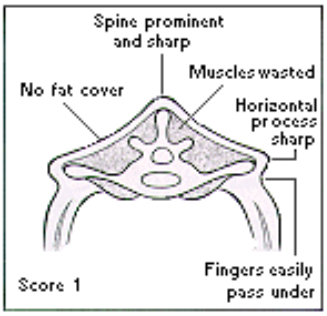
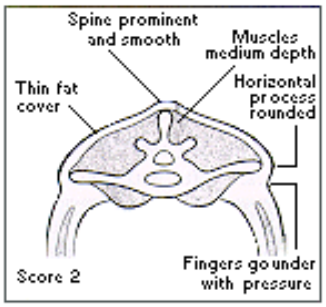


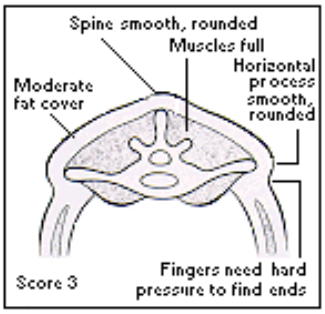
Figure 2-5: An ultrasonic image of the 3rd lumbar region of a sheep. This allows the measurement of back fat and muscle depth using the internal callipers of the machine.



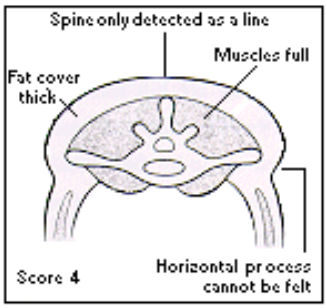
SCORE 1
The vertical and horizontal processes are prominent and sharp. The finger can be pushed easily below the horizontals and each process can be felt. The loin muscle is thin and with no fat cover



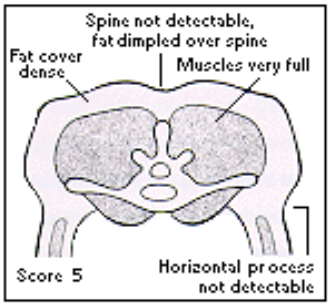
SCORE 2
The vertical processes are prominent but smooth, individual processes being felt only as corrugations. The horizontal processes are smooth and rounded, but it is still possible to press the fingers under. The loin muscle is of moderate depth but with little fat cover.



SCORE 3
The vertical processes are smooth and rounded; the bone is only felt with pressure. The horizontal processes are also smooth and well covered; hard pressure with the fingers is needed to find the ends. The loin muscle is full, with a moderate fat cover.



SCORE 4
The vertical processes are only felt as a line; the ends of the horizontal processes cannot be felt. The loin muscles are full and have a thick covering of fat.



SCORE 5
The vertical processes cannot be detected even with pressure; there is a dimple in the fat layers where the processes should be. The horizontal processes cannot be detected. The loin muscles are very full and covered with very thick fat

Figure 2-6: Diagrammatic representation of standard Body Condition Score system. Adapted from the Department for Environment, Food and Rural Affairs, Publication No. 1875.

2.3 Post-mortem procedure

Prior to the post-mortem, drapes, trays and surgical instruments were sterilised by autoclave.

2.3.1 Fetal study

Fetal blood was sampled from the femoral artery before post-mortem and blood gas glucose and lactate levels assessed with a blood gas analyser (ABL735 Blood gas analyser, Radiometer Limited, Crawley, UK).

All ewes and fetuses were killed with an overdose of sodium pentobarbitone (*i.v.*, 145 mg/kg) given to the ewe at 127 ± 1 dGA (term ~147 dGA). Fetal body weight, crown rump length, abdominal circumference, femur length and biparietal diameter were recorded. Under sterile conditions the fetal brain, heart, lungs, liver, kidneys and adrenals were removed and weighed. Samples of fetal tissues were frozen or fixed (see Appendix 5).

The brachial artery was collected, cleaned of connective tissue and mounted onto a wire myograph to assess vascular reactivity.

The placentomes were removed, weighed and typed according to A,B,C,D by visual determination of amounts and distribution of dark fetal tissue and paler maternal tissue (Figure 2.7; Vatnick *et al.*, 1991).

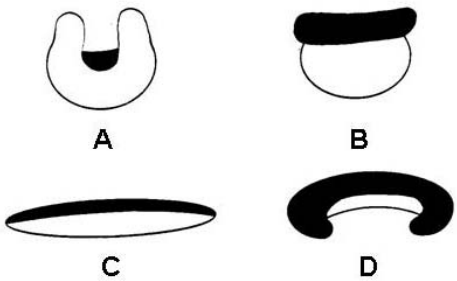


Figure 2-7: The morphology of the four types of sheep placentome. Fetal tissue is shown in black and maternal tissue in white (from Vatnick *et al.*, 1991).

2.3.2 Adult study

Feeding time and food removal were staggered on the day before the PM so that the last pelleted feed was given consistently 27 hours before, and hay removed 19 hours prior to PM. Water was provided *ad libitum.* This was to ensure that all animals were in the same state of starvation since components of the insulin signalling pathway were to be measured at a later date.

Animals were killed with an overdose of sodium pentobarbitone (*i.v.* 145 mg/kg) at 210 ± 4 weeks. Body weight, crown-rump length, abdominal circumference, biparietal diameter, femur length and shoulder height were recorded. Under sterile conditions the pancreas, adrenals, kidneys, liver, heart and lung were removed and weighed (see Appendix 7). A 1 g sample of pancreas was placed into 10 ml of 180 mmol HCl on ice and the tissue minced with scissors before being placed in a sonication bath for 30 seconds. The sample was then left overnight at 4 ºC before being centrifuged at 3000 rpm for 20 minutes and the supernatant collected and frozen.

2.3.3 Muscle collection

2.3.3.1 Isopentane-freezing

Muscle samples were frozen in isopentane to maintain their structure and allow subsequent histological examination. This rapidly freezes the sample but has an advantage over using liquid nitrogen because it better maintains the tissue morphology.

The isopentane (2-methylbutane, Sigma) was chilled on dry ice 20-30 minutes prior to use. A small amount of optimal cutting temperature compound (OCT, VWR International) was placed on a piece of corkboard. A 2 cm muscle sample was gently picked up with forceps, introduced to the OCT, and maintaining the muscle in an upright position this was lowered into the isopentane (see Figure 2.8). The sample was left in the isopentane until the bubbling had ceased (OCT will freeze around the muscle and cork, joining them together and the cork allows the sample to be mounted to a cryostat chuck). The sample was then stored at -80 ºC.

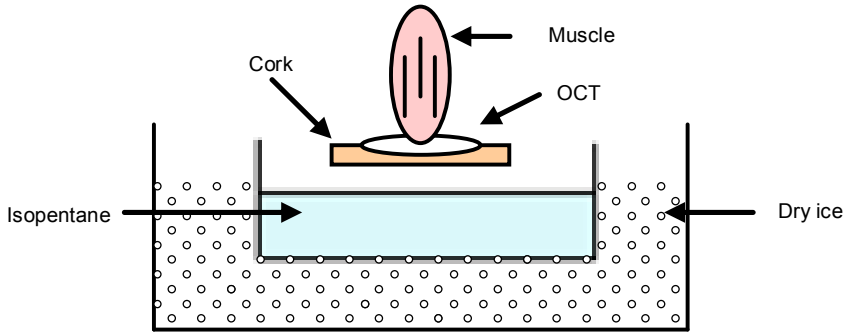


Figure 2-8: The method of slow freezing skeletal muscle samples. A 2cm sample of muscle was gently picked up with forceps and introduced to a small amount of OCT on a piece of cork, with care taken to maintain the muscle in an upright position. This was then lowered into isopentane which had been frozen over dry ice. This method maintains the morphology of the muscle and allows subsequent histological examination of the sample.

2.3.3.2 Fetal study

A mid-belly sample (see Figure 2.12a) from the long head of the triceps brachii in the right forelimb was removed and frozen in liquid nitrogen and a mid-belly sample from the left limb was frozen in chilled isopentane. A mid-belly sample from the soleus muscle in the right hindlimb was frozen by isopentane, with the remainder frozen in liquid nitrogen. For each muscle type, one large sample was frozen for future analysis of the variance between myofibres from the top, middle and bottom of the muscle.

2.3.3.3 Adult study

The soleus and gastrocnemius (lateral head) muscles from the left hindlimb were removed, cleaned of connective tissue, weighed and the circumference measured. Mid-belly samples were then taken and frozen in liquid nitrogen and isopentane. Mid-belly sections from the vastus lateralis were taken *in situ* and frozen by both methods. Small strips (~ 50 mg) were taken from all three muscles for use in glucose uptake incubations.

2.3.3.4 Sampled muscles

Triceps brachii

A mixed fibre muscle found on the posterior of the forelimb and is clearly divisible into the long, lateral and medial head (see Figure 2.9). Function is to extend the stifle joint (knee), straightening the limb (equivalent of extending the elbow in humans).

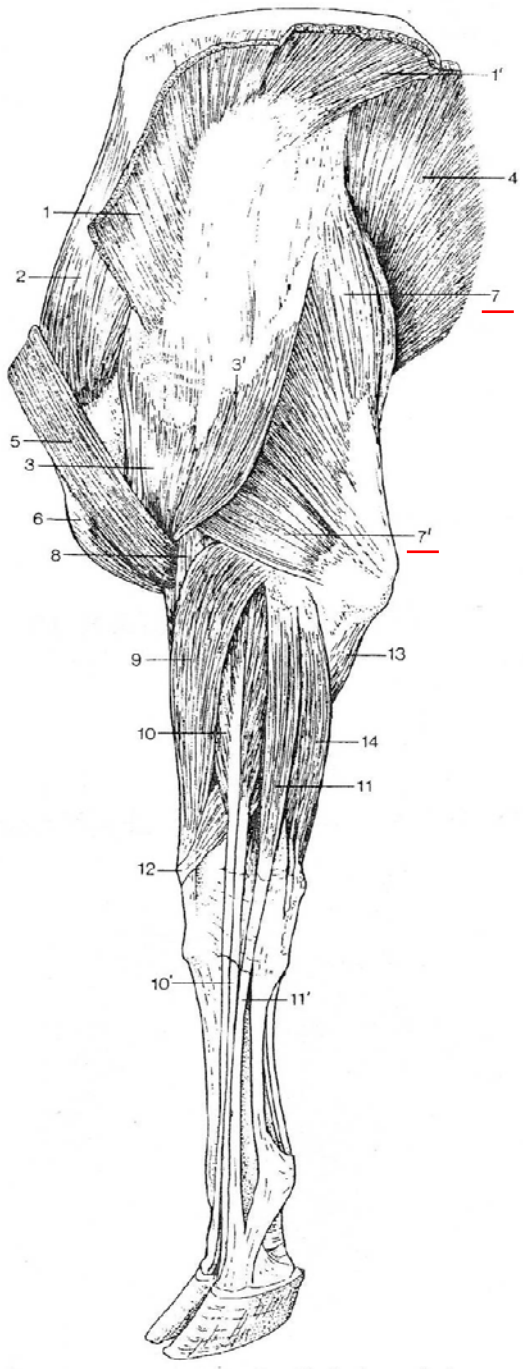


Figure 2-9: The triceps brachii muscle. Lateral view of the forelimb with the the triceps long head denoted as No. 7 and the triceps lateral head as No. 7 (from Dyce *et al*., 1987).

Gastrocnemius

A mixed fibre muscle of the hind-limb which arises from the distal end of the femur and has two heads, the lateral and medial (see Figure 2.10). Function is to extend the hock (ankle) and to flex the stifle joint, bending the limb.

Soleus

A small, slow-twitch postural muscle of the hind-limb which lies along the lateral border of the gastrocnemius (see Figure 2.10) and which assists in the extension of the hock joint, as well as maintaining posture when in an upright position.

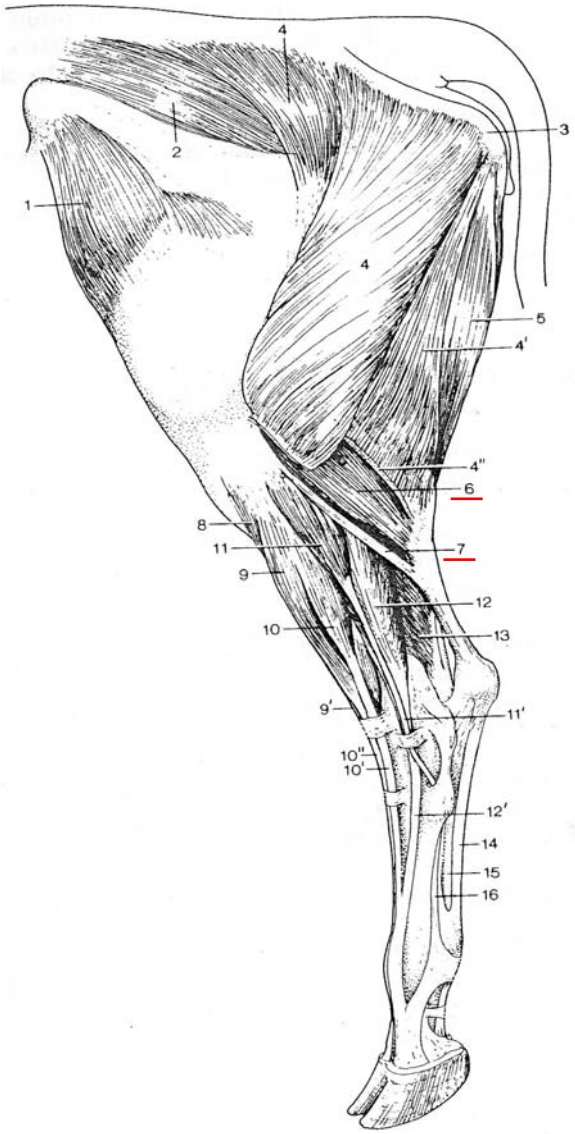


Figure 2-10: The soleus and gastrocnemius muscles. Lateral view of the hindlimb with the lateral head of the gastrocnemius muscle denoted as No. 6 and the soleus which lies behind as No. 7 (from Dyce *et al*., 1987).

Vastus

A mixed fibre muscle which lies on the lateral surface of the femur and is divided into three parts, the lateralis, medialis and intermedius (see Figure 2.11). In human studies, the vastus lateralis is the muscle of choice for biopsies due to its mixed fibre type composition, trainability, and accessibility. Function is to extend the stifle joint, straightening the limb.

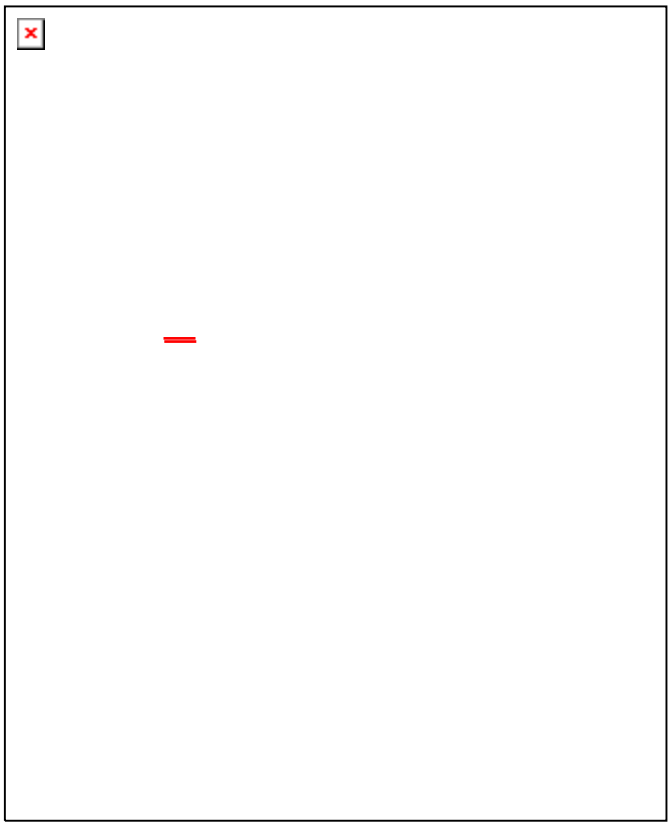


Figure 2-11: The vastus lateralis muscle. Deep dissection of the lateral thigh region with the vastus lateralis denoted as No.3 (from Neil, 1964).

2.4 Muscle immunohistochemistry

2.4.1 Muscle sectioning

The muscle sample was frozen onto a chuck of a cryostat microtome using OCT and the temperature of the cryostat chamber set to -20 ºC. The sample was trimmed and a few rough cuts were made until an even surface was produced. Sections of 10 µm thickness were then cut and transferred onto a microscope slide. The slides were viewed through a light microscope to ensure that the orientation of the muscle was in cross-section. The slides were then stored at -20 ºC until staining.

2.4.2 Staining procedure

Cryosections (10 µm) from the mid-belly sample of the fetal skeletal muscle were fixed in water-free acetone at room temperature for 15 minutes, and endogenous peroxidase activity was inhibited by incubation in 0.5 % hydrogen peroxide in methanol for 30 minutes. Non-specific protein interactions were blocked with DMEM containing 20 % fetal calf serum and 1 % BSA for 30 minutes and then incubated with either anti-skeletal fast myosin antibody (1:1000) or anti-human von Willebrand factor antibody (1:300) at 4 ºC overnight. All antibodies were diluted in Tris buffered saline (TBS). After rinsing with TBS, sections were incubated for 30 minutes with biotinylated anti-mouse (1:400) or anti-rabbit (1:400) antibody. Sections were washed and treated for 15 minutes with streptavidin biotin-peroxidase complex (1 + 1:200) and then for 10 minutes in amino ethyl carbazole. Finally, sections were counterstained with Mayers haematoxylin and baked with crystal mount (AbD Serotec, Kidlington, UK) before being mounted in Pertex (Surgipath, Peterborough, UK). A negative control section was processed simultaneously (methodology as above), replacing the primary antibody with buffer (TBS). All chemicals were from Sigma, USA unless otherwise stated.

2.4.2.1 Muscle fibre antibody

To distinguish between the fast- and slow-twitch fibres the muscle sections were stained with Monoclonal Anti-Skeletal Fast Myosin (Sigma, USA) at 1:1000 dilution. This positively stained (in red) the fast-twitch myofibres (Figure 3.1a). The nuclei were stained with Mayers haematoxylin.

2.4.2.2 Capillary antibody

Sections were stained with Polyclonal Rabbit Anti-Human Von Willebrand Factor (DakoCytomation, Denmark) at 1:300. Von Willebrand Factor is a large glycoprotein, tissue specific to the endothelium and so this antibody specifically stained (in red) the capillaries (see Figure 3.1b).

2.4.3 Acquisition of fields and counting principles

The myofibre density and size and capillaries in the section were assessed by using a photomicroscope (Zeiss, Axcorkep II) and the KS-400 image analysing system (Image Associates, Bicester, UK).

Fields were randomly captured from across the entire muscle cross-section, at x40 objective. The first of the images was captured by picking a random starting point in the top left corner of the section, and then the rest of the fields captured by moving the microscope slide plate in a snaking motion down the section to ensure no overlap between fields (see Figure 2.12b). These images were then imported into the KS-400 image analysing system. In each field of view the total countable area (μm^2) was calculated (excluding any large blood vessels which may have distorted the density results, or sections of the field where the morphology was poor and therefore difficult to count from), and using a non-biased counting frame (only those fibres entirely within the frame, or overlapping the bottom and left hand side of the frame were counted, Figure 2.12c) all myofibres, fast-twitch myofibres (identified as the positively stained fibres) and slow myofibres were counted. Using the known countable area of the field (which sometimes varied due to large blood vessels etc.) the number of myofibres per unit area (μm^2) was calculated (myofibre number / countable area) and this was multiplied by 1000 to express myofibre density as the number of fibres per mm^2 since this is the common unit found in the literature. The same principles were repeated for determining capillary density. Myofibre cross-sectional area was determined by manually drawing around the individual fast- and slow-twitch myofibres with the cursor. See the method sections of Chapters 3 and 5 for further details.

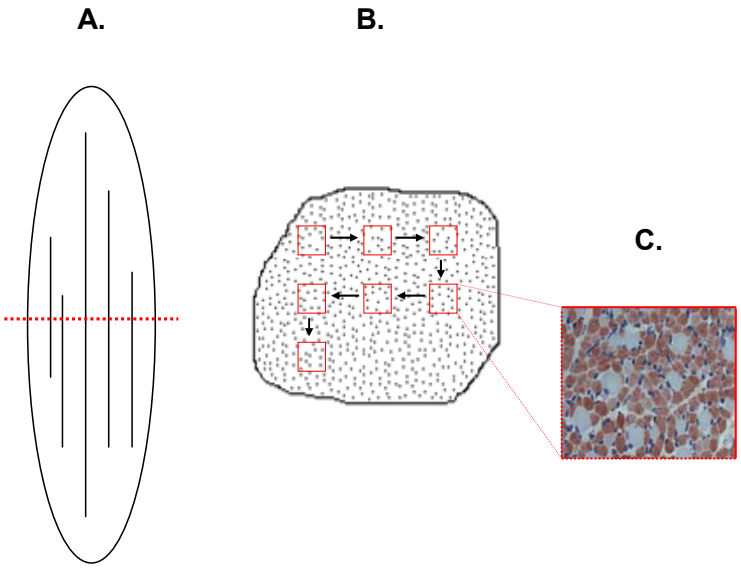


Figure 2-12: Capturing of muscle fields for image analysis. (a) location of mid-belly section of the muscle from where the sample was taken, (b) images from the section were captured in a 'snaking' pattern to prevent overlap of the fields and, (c) non-biased counting frame which was used for determining the number of myofibres in the field (only those fibres entirely within this frame, or overlapping the dotted lines, were counted).

2.5 Analysis of muscle mRNA expression

2.5.1 RNA isolation

Sections of skeletal muscle were snap frozen in liquid nitrogen and stored at -80 ºC until required for analysis. Frozen ground tissue was then homogenised in 500 µl of TRIzol® reagent, a mono-phase solution of guanidine isothiocyanate and phenol, to maintain the integrity of the RNA while the cell components were dissolved. Following homogenisation, chloroform (100 µl) was added and the tube shaken and left at room temperature for 5 minutes. The mixture was then centrifuged at 12,000 g for 10 minutes to separate the aqueous and organic phase. The RNA containing aqueous phase (~200 µl) was isolated and 20 µl of 2 mg/ml glycogen solution (Roche Diagnostics UK) added and mixed thoroughly to aid precipitation. Isopropyl alcohol was added (200 µl) and left overnight at -20 ºC to increase precipitation of the RNA. Following centrifugation at 12,000 g for 10 minutes the supernatant was removed and the precipitate washed in 500 µl of 75 % ethanol before vortexing and centrifuging again at 12,000 g for 10 minutes. The excess ethanol was removed and the RNA pellet allowed to dry before being resuspended in 20 µl of ultra pure water. To facilitate dissolution the solution was mixed by repeat pipetting at 55 ºC for 10-15 minutes. The resultant RNA stock was then stored at -80 ºC.

2.5.2 Determination of RNA yield and quality

The quantity and quality of RNA in each sample was measured using multi-wavelength spectrophotometry. 2 µl of sample RNA was added to 500 µl ultra pure water in a quartz cuvette and the absorbance at 260 nm (peak absorbance of RNA) and 280 nm (peak absorbance of protein) was recorded. The purity was estimated using the ratio A_{260}/A_{280}. Theoretically, a pure sample of RNA is expected to give an absorbance ratio of 2.0. However, this ratio can be affected by the solvent which the RNA is dissolved in and therefore ratios can vary between 1.5 and 2.0. The yield of total RNA was determined at 260nm, where 1 absorbance unit (A_{260}) equals 40µg of single stranded RNA/ml:

$$\mu g/\mu l = [(OD_{260} \text{ X } 40\ \mu g/ml/OD\ unit) \text{ X } 0.5\ ml] / 2\ \mu l$$
$$= OD_{260} \text{ X } 2$$

The integrity of the RNA was also determined using gel electrophoresis. A 50 ml agarose gel was made (500 mg agarose (1 %), 50 ml 1 X Tris-Acetate-EDTA (TAE) buffer, 1.5 µl ethidium bromide) and 10 µl of the following mix was added to each gel well: 5 µl ultra-pure water, 2 µl deionised formamide, 1 µl Northern loading buffer and 2 µl sample RNA. The gel was set to run for 30 minutes at 90 V and the visualisation of intact 18S and 28S ribosomal RNA bands determined under ultraviolet light.

2.5.3 Reverse transcription

Samples of RNA were amplified and converted to cDNA by a reverse transcriptase polymerase chain (RT-PCR) reaction. A 2 µl sample of RNA solution was added to 0.8 µl (500 ng/µl) of random primers (Promega, C1181) and 12.2 µl ultra-pure water. This was then heated to 70 ºC for 5 minutes before being cooled on ice.

A master mix was made containing the following amounts of each reagent: 5 µl of M-MLV Reverse Transcriptase 5X Reaction Buffer, 1.25 µl PCR Nucleotide Mix (Promega, C1141), 0.625 µl Recombinant RNasin Ribonuclease Inhibitor (40 u/µl, Promega, N2511), 1 µl MMLV Reverse Transcriptase (200 u/µl, Promega, M1701) and 2.125 µl ultra-pure water per sample. 10 µl of this was added to the samples, mixed and heated to 37 ºC for 1 hour, 42 ºC for 10 minutes and 72 ºC for 10 minutes. The newly formed cDNA was then stored at -80 ºC.

2.5.4 Real time PCR

Insulin receptor (InsR), glucose transporter 4 (GLUT-4) and insulin-like growth factor 1 receptor (IGF-IR) mRNA levels were analysed using Real-time PCR (7500 real-time PCR system, Applied Biosystems, Warrington). This is a method used to amplify the number of copies of a specific strand of DNA. This replication can be measured as it occurs and using fluorescence markers to quantify the DNA produced allows the calculation of the initial expression of the desired gene. The DNA was first heated to 95 ºC for 10 minutes in order to separate the strands. The DNA was then taken through 40 cycles of 95 ºC for 10 seconds and 60 ºC for 1 minute during which annealing of primers and extension of copy strands occurred (allowing the polymerase enzyme to extend the primers and make identical copies of the desired segment of DNA).

In this study two different methods of real-time PCR were utilised; SYBR Green PCR (for housekeeping gene) and real-time probe PCR (for genes of interest). The real-time probe PCR method involves a probe containing a fluorescent reporter dye at the 5' end and a quencher dye at the 3' end, which suppresses the fluorescence signal released off the reporter dye when in close contact. During polymerization the primers extend by the addition of nucleotides and when the polymerase reaches the probe it cleaves it, releasing the reporter dye which generates a fluorescent signal. SYBR Green PCR was used to assess the expression of the housekeeper gene GAPDH. This method utilises the SYBR Green fluorescent dye which when binding to any double-stranded DNA increases in fluorescence. As the primers start to anneal and extend, thus producing more double stranded DNA, the SYBR Green dye signal will increase and this increase in fluorescence is then measured.

The accumulation of PCR products within every PCR cycle is detected by the increase in fluorescence. The relative amount of cDNA (representative of RNA) is characterized by the number of cycles of PCR it takes for the fluorescence to exceed a defined fixed threshold. The higher the starting amount of cDNA, the sooner it reaches the threshold level.

Primers and probes (Eurogentec, Belgium) were designed using Primer Express Software (Applied Biosystems, USA) with reference to published sequences (see Table 2.1). GAPDH expression was measured using SYBR Green, primers and

reagents as designed and supplied in kit form by Primer Design, UK (www.primerdesign.co.uk). InsR, GLUT-4 and IGF1-R were expressed relative to total RNA concentration and GAPDH expression and the pattern of the resultant gene expression was similar in both cases. The GAPDH ratios were adopted as the SEMs were smaller for group gene expression and the GAPDH acted as an internal control which was in keeping with use of a housekeeping gene for this type of analysis.

Target Gene	Primers / Probe	Sequence	Accession No.
Insulin Receptor	Forward Primer	ACC GCC AAG GGC AAG AC	AJ844652
	Reverse Primer	AGC ACC GCT CCA CAA ACT G	
	Probe	AAC TGC CCT GCC ACT GTC ATC AAC G	
GLUT-4	Forward Primer	CCG TGG CAG GAC ATT TGA C	AY949177
	Reverse Primer	TTC CTG CTC CAG AAG AGA AGG T	
	Probe	ATC TCA GCC GTC TTC CGC CGG	
IGF-1 Receptor	Forward Primer	CTG CAG CGC CTC TAA CTT TGT	AY162434.1
	Reverse Primer	CAC TGG CCC AGG AAT GTC A	
	Probe	CAA GAA CCA TGC CTG CAG AAG GAG CA	

Table 2-1: Primer and probe sequences (5' to 3') used in the measurement of mRNA levels by Real-time polymerase chain reaction.

2.6 Intravenous glucose tolerance test

2.6.1 Fasting

At a mean age of 210 ± 4 weeks adult male offspring were transported to Southampton, kept in rooms at 17.5 to 18 ºC with a 12 hour light/dark cycle and allowed 5 days acclimatisation to the metabolic carts.

Feeds and food removal were staggered on the day before the intravenous glucose tolerance test (*iv*GTT). This was to ensure that all animals were in the same state of starvation. Pelleted food was given 27 hours before the administration of the glucose bolus, and hay was removed 19 hours before. Water was given *ad libitum.*

2.6.2 Implantation of jugular catheter

A catheter was implanted in the left jugular vein for blood sampling during the *iv*GTT. Sterile technique was used and catheters sterilised using industrial methylated spirit (70 %, Adams Healthcare, Leeds, UK). Under local anaesthesia (2 ml Lignol, Arnolds Veterinary Products Ltd, Shrewsbury, UK) a small skin incision was made and a Radiopaque FEP IV Catheter (14G X 140 mm, Abbott Laboratories Ltd) was inserted into the left jugular vein and sutured to the skin. Patency was confirmed by withdrawing blood with a syringe and a heparinised extension catheter was attached (100 U/ml heparin sodium, Leo Pharmaceuticals, UK; saline 0.9 %, 3S-Healthcare, UK). Suture and incision sites were sprayed with oxytetracycline hydrochloride (Terramycin, Pfizer, UK). Surgifix netting (Colorline surgifix size 7, FRA, Italy) was placed over the neck and incision site, and the extension catheter secured along the back of the sheep by tying it to the wool. A long acting antibiotic (1 ml/10 Kg, Betamox LA, Amoxycillin, Norbrook Laboratories Ltd., UK) was given intramuscularly at the time of catheterisation.

2.6.3 Extension catheters

Extension catheters were made out of lengths of polyvinyl tubing (I.D. 2.0 mm, O.D. 3.0 mm, Portex Ltd. Kent, UK). A 14-gauge blunt (VetTech, UK) was inserted into each end of the catheter and a male/male luer connector (Sims Portex Ltd., UK) fitted to allow connection to the vascular catheter and a 3-way tap (Vygon, UK).

2.6.4 Glucose bolus and sampling

Following catheterisation, a 2 hour recovery period was allowed prior to the *iv*GTT. Blood samples (7 ml) were collected before (-15, -7, 0 minutes) and after (5, 10, 15, 20, 30, 45, 60, 90, 120, 150, 210 minutes) an intravenous glucose bolus (0.5 g/kg body weight) of 2 minute duration. Blood samples were taken (7 ml into chilled EDTA, fluoride and LiHep plasma collection tubes; Teklab Ltd, Durham, UK). These were centrifuged and the plasma frozen in aliquots and stored at – 80 °C. Extra aliquots were taken for the -15 minute sample (11 ml blood) for subsequent basal analysis. Once the procedure was completed all food was returned.

2.6.5 Post-operative care

Patency of catheters was maintained by a continuous infusion (syringe pump PHD2000, Harvard Apparatus Ltd., Kent, UK) of saline (100 U/ml at 1 ml/h) and catheters were flushed with saline the next morning.

2.6.6 Biochemical analysis

Plasma glucose and lactate were measured in 50 µl plasma (collected into chilled fluoride tubes) on the Dade-Behring Dimension RXL analyser. Insulin concentrations in extracted pancreatic tissue and plasma (25 µl, collected into chilled EDTA tubes) were measured by Enzyme-Linked ImmunoSorbent Assay (ELISA; DRG Sheep Insulin ELISA; ImmunoDiagnostic Systems, Tyne and Wear, UK). The range of the assay was 0.1 to 2.5 µg/l. All samples were assayed in duplicate and the inter-assay coefficient of variance was 7.0 % and intra-assay was 4.4 %. These analyses were performed as part of routine assays carried out at National Health Services, Clinical Biochemistry Department, Addenbrookes Hospital, Cambridge.

Plasma cortisol (µg/dl) was measured in duplicate using an Immulite analyser (DPC, UK) in 10 µl of plasma (collected onto chilled EDTA tubes) by a solid-phase, competitive chemiluminescent enzyme immunoassay, with an incubation cycle of 30 minutes. This analysis was performed at the University of Southampton.

2.7 Glucose uptake into isolated muscle strips

2.7.1 Incubation of muscle strips

To determine glucose uptake in muscle tissue an isolated muscle strip technique was used as described by Ozanne *et al*. (1995). Small strips (~50 mg) were taken from the vastus, soleus and gastrocnemius muscles and placed in chilled Tyrodes solution until commencement of uptake experiment. There was a 45 minute delay between the time of death and commencement of the experiment and this was kept consistent between animals. All incubations were carried out at 38 ºC in a shaking water bath. The strips were placed in 5 ml of warmed Tyrodes solution containing 2 mM pyruvate, 38 mM mannitol and 0.1 % bovine serum albumin (Sigma, USA) for 10 minutes and then incubated for a further 20 minutes in an identical medium and the presence or absence of insulin (16 nM, Novo Nordisk, UK). The strips were blotted on filter paper and incubated for 10 minutes in 3 ml Tyrodes solution containing 8 mM [^{3}H]methyl glucose (437 μCi/mmol) and 32 mM [^{14}C]mannitol (8 μCi/mmol; GE Healthcare, UK). After incubation the muscle strips were blotted on filter paper to remove any radioactive buffer and then frozen in liquid nitrogen.

A concentration of 16 nM is reported to result in maximal stimulation of glucose transport in rat glucose uptake experiments (Ozanne *et al.*, 1996;Nolte *et al.*, 1995) but a further concentration of 32 nM insulin was used in this study to ensure that maximal stimulation was reached in the sheep muscle. Time course incubation was also performed, with muscle strips from the same muscle incubated for 5, 10 or 15 minutes.

2.7.2 Analysis of glucose uptake into muscle strips

Frozen muscle strips were homogenised in 400 μl of water and centrifuged at 10,000 g for 5 minutes. Radioactivity in the supernatant was determined by liquid scintillation counting, with channels for simultaneous quantitation of ^{3}H and ^{14}C (PerkinElmer, Tri-carb 2100TR). Briefly, 300 μl was put into a scintillation vial along with 6 ml of Optiphase 'Hisafe' II scintillation fluid (PerkinElmer, Massachusetts, USA) and each vial was ran on the counter for 5 minutes. Protein levels (from 20 μl of the supernatant) were measured using Coomassie blue protein assay (Pierce Biotechnology, Rockford, IL, USA) and the glucose uptake corrected for amount of protein. Extracellular space was corrected for by subtracting the mannitol levels away from the glucose, leaving actual glucose uptake.

2.8 Western Blotting

These were performed by Dr Roselle Cripps at University of Cambridge.

Total protein was extracted by homogenising 100 mg tissue in 1 ml of lysis buffer on ice (50 mM HEPES pH 8, containing 150 mM sodium chloride, 1 % Triton X-100, 1 mM Na_3VO_4, 30 mM sodium fluoride, 10 mM sodium pyrophosphate, 10 mM EDTA with a protease inhibitor cocktail from Calbiochem Novabiochem Bioscience, Nottingham, UK). The homogenate was centrifuged at 9600 g in a microcentrifuge at 4 ºC, and the cleared supernatant removed for analysis. Protein content of tissue lysates was determined using 1:50 dilution of copper sulphate in bicinchonic acid solution (200 µl acid solution added to 50 µl of diluted sample lysates, 1:20) and reading absorbance at 595 nm. Samples were measured in duplicate by comparison with suitable bovine serum albumin standards. The cleared lysates were standardised to a final concentration of 1 mg/ml in Laemmli's sample buffer (62.5 mM Tris pH 6.8, 2 % sodium dodecyl sulphate (SDS), 10 % glycerol, 0.02 % bromophenol blue and 150 mM dithiothreitol).

The protein samples were heated to 100 ºC for 5 minutes to ensure complete denaturisation, and then 20 µg of the sample was loaded onto a 10 % SDS polyacrylamide gel along with a protein marker (High-Range molecular weight marker, Amersham Biosciences, Buckingham, UK), for 3-4 hours until the bands were sufficiently separated by electrophoresis. The separated proteins were transferred onto polyvinylidene difluoride membrane (Immobilon P, Millipore, Billerica, MA) for 90 minutes at a current of 200 mA using a semi-dry transfer protocol and Western blotting was carried out.

Membranes were incubated overnight at 4 °C in blocking buffer (1 gl^{-1} Marvel milk powder and 0.25 % Tween 20 in TBS). The primary antibodies used in this study were to insulin receptor β subunit (InsR, 1:200), PI3-kinase p85α regulatory subunit (p85, 1:1000, Upstate Biotech, NY, USA) PKCζ (1:200), insulin-like growth factor-I receptor β-subunit (IGF-IRβ, 1:200), glucose transporter-4 (GLUT-4, 1:5000, Abcam, Cambridge, UK) and Akt1 (1:2000). All antibodies were rabbit polyclonal, except Akt1 (monoclonal mouse) and were obtained from Santa Cruz Biotechnologies (Santa Cruz, CA, USA) unless stated otherwise. Membranes were incubated with the primary

antibody at the above dilutions in phosphate-buffered saline (PBS) wash buffer (1 PBS tablet/200 ml, 1 gl^{-1} Marvel milk powder and 0.1 % Tween 20 in dH_2O) for 1 hour and then washed for 35 minutes in TBSE wash buffer (0.1 % SDS, 0.5 μM EDTA, 1 % Triton X-100 and 24 mM Sodium deoxycholate in TBS) followed by two 5-minute washes in PBS wash buffer. Horseradish peroxidase-conjugated secondary antibodies to rabbit or mouse immunoglobulin were obtained from DakoCytomation (Glostrup, Denmark) and the membranes incubated for 1 hour in a secondary antibody solution of 1:5000 in PBS wash buffer. The washes detailed above were then repeated. Antibody binding was detected using the enhanced chemiluminescence kit from Amersham Biosciences (Buckinghamshire, UK).

Autoradiographs of Western blots were imaged and the optical densities of the immunoreactive protein bands were measured once using AlphaEaseFC v 4.0. Linearity was confirmed on each gel by loading one sample at both 10 and 20 μg. This confirms that the measurements of optical densities were in the dynamic range of the assay.

2.9 Statistical Analysis

In all cases statistical analysis were performed with GraphPad Prism (version 3, GraphPad Software Inc., San Diego, CA, USA) or SPSS (version 12, SPSS Inc., Chicago, USA).

Shapes of histograms were analysed for normality or skewness of data. Non-parametric data were log transformed prior to testing to achieve normality and are shown as the geometric mean (95 % confidence intervals). Parametric data are shown as the mean ± standard error of the mean (SEM).

For all comparisons, statistical significance was accepted when $P < 0.05$. Trends are discussed when $0.05 < P < 0.1$.

2.9.1 Myofibre and capillary density

In Chapters 3 there were three nutritional groups and so differences between means in myofibre and capillary density were calculated using a one-way analysis of variance (ANOVA), with Bonferroni *post hoc* correction. Male and female fetuses were analysed together due to small sample sizes. In Chapter 5 differences between means of the two nutritional groups were calculated using Student's t-test. Linear relationships between variables were investigated by linear regression analysis.

2.9.2 Wire myography

All results are expressed as mean ± SEM. The raw data displayed is expressed in mN/mm and is presented purely to illustrate data collection. Multiple arterial samples were mounted for each animal and the values represent the mean arterial response per sheep. Contraction in response to KPSS is expressed as the change in pressure (kPa). Contraction in response to NA is expressed as a percentage of the maximal constriction in response to KPSS, to correct for differences in vessel diameter. Responses to the vasodilators ACh and SNP are expressed as the percentage relaxation of tone induced by a submaximal dose of NA (EC_{80}).

Concentration-response curves were analysed by transforming the data, using the non-linear regression equation with variable slope $y = (x^n) / (x^n + k^n)$ where k is a location

parameter of the x-axis. From the curve fitted equation the maximum response and the pEC_{50} (log of molar concentration of agonist that produces 50% of the maximum response) were obtained. Differences between the means were calculated using a one-way ANOVA, for comparison between more than two groups, with Bonferroni *post hoc* correction. Male and female fetuses were analysed together due to small sample sizes. The sensitivity of the individual vessels (pEC_{50}) was correlated with fetal circulating nutrients and metabolites by linear regression.

2.9.3 Gene expression

All genes of interest were normalised to RNA concentration by dividing by the housekeeping gene GAPDH for the same sample. Values represent the mean of 2 replicate measurements and are given as arbitrary units. Data are expressed as mean ± SEM for each group. Differences between the means of each group were calculated using using a one-way ANOVA with Bonferroni *post hoc* correction.

2.9.4 Glucose tolerance test

For each GTT experiment, the area under the glucose and insulin response curve (AUC) was calculated [integrated plasma concentrations following glucose administration (5–210 min) above the mean pre-GTT (–15 to 0 min) concentrations]. AUC was also calculated with baseline remaining to allow comparisons to data collected at 1.5 years when the baseline was not removed.

Comparisons in glucose tolerance between the ages of 1.5 and 4 years were made with repeated measures ANOVA, as a standard ANOVA assumes that each observation is independent of the last. Where a significant interaction was found between age and nutritional group a t-test was used to compare age within group. Linear relationships between variables were investigated by linear regression analysis.

2.9.5 Glucose uptake into isolated muscle strips

Glucose uptake into isolated muscle strips was corrected for amount of protein and the extracellular space was corrected for by subtracting the mannitol levels away from the glucose, leaving actual glucose uptake. Differences in uptake between nutritional groups were analysed by an unpaired Student's t-test.

2.9.6 Power calculations

Power calculations were performed to estimate the sample size needed to detect differences between parameters with adequate statistical power using SigmaStat (version 3.5, Systat Software Inc., San Jose, CA, USA). The primary outcome was taken to be myofibre density using data from a previous study (Dwyer *et al.*, 1994). A difference of approximately 32870 myofibres from the pig semitendinosus muscle was previously observed as significant. The average standard deviation was 18329. Therefore, to achieve a power of 80 % at significance level of 0.05, 6 animals are required per group.

3 The effect of two distinct periods of nutrient restriction on muscle morphology and capillary density

3.1 Introduction

A poor fetal environment and low birth weight have been associated with reduced muscle strength and increased risks of metabolic disease in adult life (see Section 1.1). The Dutch Winter Famine revealed that the effects on fetal development and the occurrence of disease in later life are dependent on timing of the undernutrition, with exposure in late gestation leading to decreased glucose tolerance in later life (Ravelli *et al.*, 1998). In accordance, a late gestational challenge from 110 dGA to term resulted in glucose intolerant offspring at 1 year of age in the sheep (Gardner *et al.*, 2005). Recently, low birth-weight has been associated with defects in the expression of insulin-signalling intermediates in young adult skeletal muscle, which could be a precursor of longer term altered glucose handling (Ozanne *et al.*, 2005). Muscle can therefore be affected by changes in the fetal environment and these changes may predispose individuals to metabolic disease.

Skeletal muscle is the primary tissue for the utilisation of glucose, and insulin resistance in muscle is the earliest identifiable abnormality in pre-diabetic patients (Shulman, 2000). It is therefore a logical candidate when investigating the mechanisms linking a poor fetal environment to later metabolic disease outcomes. The impact of any period of nutrient restriction is dependent on the timing of the insult and the organs and systems developing during that critical window. In the sheep, as in humans, there are three distinct waves of myofibre development (Maier *et al.*, 1992). Primary fibres develop between 32 and 38 dGA and are used as a scaffold by developing secondary fibres from ~ 38 dGA, and these in turn support a third wave of myofibre formation from 62 dGA (Wilson *et al.*, 1992;Maier *et al.*, 1992). These waves of fibres have differing susceptibilities to undernutrition (Ward & Stickland, 1991) and muscles not only consist of different fibre types (fast and slow-twitch) but can have different proportions of primary: secondary myofibres. Thus, it is likely that the effect of

undernutrition on skeletal muscle may not only depend on the timing of the challenge in relation to the waves of myofibre development, but may produce specific effects on different muscles. Periods of undernutrition which have so far been investigated in sheep muscle include peri-conception where a 50 % nutrient restriction from -18 to 6 dGA decreased total myofibre number in the mid-gestation fetus (Quigley *et al.*, 2005) and the temporary exposure of ovine embryos to an advanced uterine environment increased myofibre number in the late gestation fetus (Maxfield *et al.*, 1998). A 50 % restriction from 28-78 dGA decreased myofibre number in the 8 month old offspring, along with an increased ratio of type-IIb fibres, indicative of a decrease in type I fibres (Zhu *et al.*, 2006). Another study which looked at early to mid-gestation was a 50 % reduction from 30-70 dGA which reduced fast fibre number in 2 week old lambs (Fahey *et al.*, 2005b). However, these studies have largely focused on early and/or mid-gestation and not investigated the effect of a late gestation challenge upon skeletal muscle morphology. Since the late gestation period has been shown to be important in terms of later metabolic disease (Ravelli *et al.*, 1998;Gardner *et al.*, 2005) the effect of a late gestation challenge upon skeletal muscle morphology is of great interest. Dwyer *et al*. (1995) found that maternal nutrient restriction during the first half of gestation reduced myofibre number in the guinea pig neonate, whereas maternal undernutrition during late gestation had no effect, but since the inception of this thesis a study investigating the impact of reduced maternal protein in rats during three distinct periods of gestation (early, mid and late) found that total myofibre density was reduced by all three periods of nutrient restriction (Mallinson *et al.*, 2007). **However, to date no one has investigated the effect of two different periods of nutrient restriction, peri-implantation or late gestation, on two distinct muscle types in the sheep model.**

Skeletal muscle is a highly active tissue and so is vascularised accordingly to cope with the high oxygen demands. Capillaries are plentiful within the endomysium and each muscle fibre is in close contact with one or more capillaries, the number differing according to fibre type with type I myofibres having more than type II to support their higher oxidative capacity (Andersen, 1975). In men and women with type-II diabetes, a reduced capillary density was associated with a decrease in type I fibres (Marin *et al.*, 1994). Therefore, since both myofibre number and capillary density are susceptible to change it may be that the two are linked when nutrient availability is reduced. One

possible link may be a decreased capillary formation reducing the availability of nutrients to the developing tissue and causing a subsequent decrease in myofibre formation. The decrease in capillary formation may be caused by a reduction in the upstream flow (through a peripheral redistribution of blood flow), since the process of angiogenesis is somewhat dependent upon blood flow (Prior *et al.*, 2004). Peripheral blood flow is reduced during hypoxia in the late gestation fetus and there is now evidence to suggest that the same process may occur in response to a period of nutrient restriction (Burrage *et al.*, 2005). **The next step of this thesis was therefore the investigation of muscle capillary density, in relation to myofibre density, following the peri-implantation and late gestation periods of nutrient restriction.**

3.2 Specific hypotheses

1. Muscle morphology will be impaired by late and early gestation undernutrition with differential effects on the proportion of myofibre types and to a variable extent in two distinct muscle beds.

2. The reduction in myofibre density will be mediated in part by reduced capillarisation.

3. These anticipated changes will impact on muscle insulin sensitivity and glucose uptake mechanisms.

3.3 Aims and Objectives

1. To determine the effect of an early or late gestation nutrient restriction on fetal skeletal muscle fibre density and composition in a slow-twitch myofibre (soleus) and mixed (triceps brachii) muscle of the late gestation sheep.

2. To determine the effect of these challenges on capillary density in the same muscle beds.

3. To measure expression of components in the insulin signalling and glucose uptake pathway in the triceps brachii muscle.

3.4 Methods

3.4.1 Animals and study design

3.4.1.1 Diet

Pregnant Welsh Mountain ewes were penned individually on straw and fed either 100 % nutrient requirements (C, *n* = 8) or a restricted diet peri-implantation (PI, *n* = 9. 40 %, 1-31 dGA (term ~147)) or in late gestation (L, *n* = 6. 50 %, 104 dGA - postmortem), with 100 % at all other times. For full details see section 2.1.1.

3.4.1.2 Tissue collection

At post-mortem (see section 2.3.1) mid-belly samples of the soleus and triceps brachii muscle were immediately frozen by immersion into freezing isopentane for histological analysis (see section 2.3.3.1) and liquid nitrogen for molecular analysis.

3.4.1.3 Gene expression measurement by real-time PCR

The molecular biology in this chapter was undertaken by Nur Aida Astaman.

Total RNA was extracted from the triceps brachii muscle using TRIzol® reagent (Invitrogen, UK) method. Quality and quantity of RNA was assessed by spectrophotometry (A260/A280 nm), and the integrity of the RNA determined by gel electrophoresis. Total RNA from each sample was reverse transcribed using standard protocols with random primers, RNase inhibitor and reverse transcriptase (Promega, UK). Real-time PCR (7500 real-time PCR system, Applied Biosystems, Warrington) was then used to evaluate the mRNA levels of InsR, GLUT-4 and IGF1-R with primers and probes designed using Primer Express Software (Applied Biosystems, USA). GAPDH expression (internal housekeeping gene) was measured using SYBR Green and primers and reagents as designed and supplied in kit form by Primer Design, UK (www.primerdesign.co.uk). InsR, GLUT-4 and IGF1-R were expressed relative to GAPDH expression. For full details see section 2.5.

3.4.2 Skeletal muscle immunohistochemistry

Transverse sections (10 μM) were cut on a cryostat and kept frozen at -80 ºC until staining. The presence of fast skeletal myosin was revealed by incubation with Monoclonal Anti-Skeletal Fast Myosin (Sigma, USA, see 2.4.2.1). Capillaries were identified with Anti-Human von Willebrand Factor (DakoCytomation, Denmark, see section 2.4.2.2).

3.4.3 Skeltal muscle image analysis

Fields from across the entire muscle cross-section were randomly captured on a photomicroscope at x40 objective and analysed using the KS-400 image analysing system, as described in section 2.4.3.

3.4.3.1 Myofibre density

3.4.3.1.1 Intra-observer error

All measurements were made by one observer and the intra-observer variability tested by reproducing the counts from the same field, at different times. The intra-observer variability was less than 1.2 % (Table 3.1).

	Same section counted		
	All fibres per mm²	*Fast fibres per mm²*	*Slow fibres per mm²*
Count 1	3203	2994	209
Count 2	3230	3023	207
Count 3	3238	3029	209
Count 4	3251	3038	213
Count 5	3241	3029	212
CV	**0.6**	**0.6**	**1.2**

Table 3-1: The intra-observer error associated with repeated myofibre counts of the same fetal muscle field. Counting of myofibres from the same muscle field was repeated on different days, by the same observer, in the triceps brachii muscle and the error between these counts was then calculated.

3.4.3.1.2 Number of counting fields

From one cross-section of muscle the average myofibre density was calculated from 10 fields of view and this was considered the 'gold standard'. The percentage error of calculating density from random combinations of fields was then compared to this gold standard. The error dropped and plateud by 5 fields (Table 3.2) and so it was concluded that 5 fields were sufficient to obtain an accurate myofibre density.

TRICEPS BRACHII	
No. of fields	**Percentage error**
1	49.1
2	34.9
3	22.6
4	13.8
5	**4.8**
6	4.2
7	4.1
8	3.1
9	3.1

SOLEUS	
No. of fields	**Percentage error**
1	39.9
2	27.7
3	14.6
4	9.1
5	**4.0**
6	4.0
7	3.2
8	3.7
9	3.0

Table 3-2: Determining the minimum number of counting fields needed to obtain an accurate averge myofibre density from fetal muscle. These tables show the percentage error that is associated with calculations of myofibre density from varying numbers of fields, as compared to the gold standard of 10, for the triceps brachii and soleus muscle. For both muscles counting from 5 fields of view was sufficient to obtain an accurate myofibre density.

3.4.3.1.3 Sampling site variability

The average density from 5 fields was obtained from three distinct sections at the top, middle and bottom of one representative muscle sample. There was little difference in myofibre density between top, middle and bottom sections for the triceps (Table 3.3a) and soleus (Table 3.3b) muscles. This is consistent with the muscle fibres running the length of a muscle and suggests that, while muscle was routinely sampled from the mid-belly region, sampling site variation is unlikely to account for variation in myofibre density.

A	**TRICEPS BRACHII**								
	Top section			**Middle section**			**Bottom section**		
	Fibres per mm^2	**Fasts per mm^2**	**Slows per mm^2**	**Fibres per mm^2**	**Fasts per mm^2**	**Slows per mm^2**	**Fibre per mm^2**	**Fasts per mm^2**	**Slows per mm^2**
Field view 1	2013	1473	540	2526	1944	582	2438	1876	562
Field view 2	2649	2056	593	2246	1706	540	2405	1865	541
Field view 3	3168	2554	615	2416	1822	593	2978	2473	541
Field view 4	3243	2776	466	2872	2384	488	2596	2056	540
Field view 5	2109	1695	413	2649	2193	456	2639	2119	519
Average	**2636**	**2111**	**525**	**2542**	**2010**	**532**	**2611**	**2078**	**541**
CoV between section site for; all fibres, 1.9; fast fibres, 2.5 and slow fibres, 3									

B	**SOLEUS**		
	Slow myofibres per mm^2		
	Top Section	**Middle section**	**Bottom section**
Field view 1	1102	1144	1028
Field view 2	1240	1293	1324
Field view 3	1038	1399	1049
Field view 4	1176	1240	1229
Field view 5	1282	1059	1388
Average	**1168**	**1227**	**1204**
CoV between section site for slow fibres, 2.5			

Table 3-3: Comparisons of myofibre density obtained from three distinct sampling sites in the fetal muscle. The average myofibre density was obtained from the top, middle and bottom of one representative sample for (a) triceps brachii and (b) soleus muscle. There was little difference in myofibre density between these three sections.

3.4.3.1.4 Myofibre cross-sectional area

The average cross-sectional area of the myofibres was defined by manually drawing around them with the cursor. Myofibres were numbered and their size re-calculated on different days, with the intra-observer error between 0.15 – 2.93 % (Table 3.4). The average cross-sectional area was then obtained from 300 fast-twitch and 150 slow-twitch fibres, from across the 5 random fields.

Fibre No.	First count (μm^2)	Second count (μm^2)	Third count (μm^2)	CV
1	222.86	214.21	213.69	**2.4**
2	238.51	236.55	235.99	**0.6**
3	142.36	149.39	150.22	**2.9**
4	137.90	136.22	137.40	**0.6**
5	182.26	180.60	183.20	**0.7**
6	160.71	160.21	161.02	**0.3**
7	163.91	170.99	171.26	**2.5**
8	127.57	128.22	127.20	**0.4**
9	283.45	283.10	282.60	**0.2**
10	216.18	215.11	214.10	**0.5**
11	177.05	175.00	177.96	**0.9**
12	107.59	105.04	106.25	**1.2**
13	171.73	172.30	174.12	**0.7**
14	189.44	190.40	188.60	**0.5**
15	245.33	246.60	245.15	**0.3**
16	182.44	189.20	187.10	**1.9**
17	400.14	398.20	397.90	**0.3**
18	133.19	132.60	131.80	**0.5**
19	259.77	258.70	257.50	**0.4**
20	306.77	305.40	305.10	**0.3**
21	168.32	162.30	163.18	**2.0**
22	198.40	199.40	200.03	**0.4**
23	179.06	178.90	180.90	**0.6**
24	252.59	253.93	254.02	**0.3**
25	245.62	246.20	247.02	**0.3**

Table 3-4: The intra-observer error associated with repeated calculations of myofibre cross-sectional area, in fetal muscle. A total of 25 individual myofibres were numbered (so as to allow identification of the same fibre) and the CSA of these were determined on different days, by the same observer, and the error between these areas was then calculated.

3.4.3.2 Capillary density

The image analysis validation which was performed for myofibre density was then repeated for capillary density.

3.4.3.2.1 Intra-observer error

Same section counted		
	Capillaries per mm^2	*Capillary: myofibre*
Count 1	2879	1.63
Count 2	2702	1.78
Count 3	2931	1.66
Count 4	2874	1.59
Count 5	2861	1.64
CoV	**3.0**	**4.3**

Table 3-5: The intra-observer error associated with repeated capillary counts of the same fetal muscle field. Counting of capillary density from the same muscle field was repeated on different days, by the same observer, in the triceps brachii muscle and the error between these counts was then calculated.

3.4.3.2.2 Number of counting frames

TRICEPS BRACHII	
No. of fields	**Percentage error**
1	41.0
2	22.4
3	16.9
4	11.0
5	**5.7**
6	5.0
7	4.4
8	5.0
9	2.9

SOLEUS	
No. of fields	**Percentage error**
1	33.2
2	21.7
3	9.6
4	5.1
5	**3.4**
6	3.0
7	2.8
8	2.9
9	1.1

Table 3-6: Determining the minimum number of counting fields needed to obtain an accurate average capillary density from fetal muscle. These tables show the percentage error that is associated with calculations of capillary density from varying numbers of fields, as compared to the gold standard of 10, for the triceps brachii and soleus muscle. For both muscles counting from 5 fields of view was sufficient to obtain an accurate capillary density.

3.4.3.3 Sampling site variability

A	TRICEPS BRACHII					
	Top section		Middle section		Bottom section	
	Capillaries per mm^2	Capillary: Myofibre	Capillaries per mm^2	Capillary: myofibre	Capillaries per mm^2	Capillary: myofibre
Field view 1	1884	1.56	1659	1.49	1793	1.57
Field view 2	1569	1.46	1569	1.37	1889	1.69
Field view 3	1652	1.47	1756	1.55	1545	1.49
Field view 4	1869	1.59	1876	1.59	1694	1.56
Field view 5	1987	1.55	1700	1.51	1688	1.52
Average	**1792**	**1.53**	**1712**	**1.50**	**1722**	**1.57**
CoV between section site for; Capillaries, 2.5 and Capillary:myofibres, 2.3						

B	SOLEUS					
	Top section		Middle section		Bottom section	
	Capillaries per mm^2	Capillary: Myofibre	Capillaries per mm^2	Capillary: myofibre	Capillaries per mm^2	Capillary: myofibre
Field view 1	2128	1.85	2326	1.97	1889	2.07
Field view 2	2321	2.0	2391	2.56	2569	2.49
Field view 3	2476	2.08	1989	1.77	2989	1.56
Field view 4	2658	1.95	2569	1.23	1803	1.98
Field view 5	2225	1.83	2429	1.56	1963	2.36
Average	**2362**	**1.94**	**2341**	**1.82**	**2243**	**2.03**
CoV between section site for; Capillaries, 2.8 and Capillary:myofibres, 5.4						

Table 3-7: Comparisons of capillary density obtained from three distinct sampling sites in the fetal muscle. The average capillary density was obtained from the top, middle and bottom of one representative sample for (a) triceps brachii and (b) soleus muscle. There was little difference in capillary density between these three sections.

3.4.4 Analysis and statistics

For a general description of the analysis strategy and statistics packages used, see 2.9.

- The intra-user error in determining myofibre/capillary density and fibre cross-sectional area and the variation with muscle sample site was calculated using a coefficient of variation.

- Male and female fetuses were analysed together due to the small size of groups when divided according to sex.

- Data were tested for normal distribution and are expressed as mean ± SEM.

- Differences in myofibre and capillary density and mRNA expression between nutritional groups were analysed by one-way ANOVA with Bonferroni *post-hoc* correction.

- Linear relationships between variables e.g. myofibre density and IGF-IR expression were investigated by linear regression analysis.

- Statistical significance was accepted when $P < 0.05$ and were considered as trends when $0.05 < P < 0.1$.

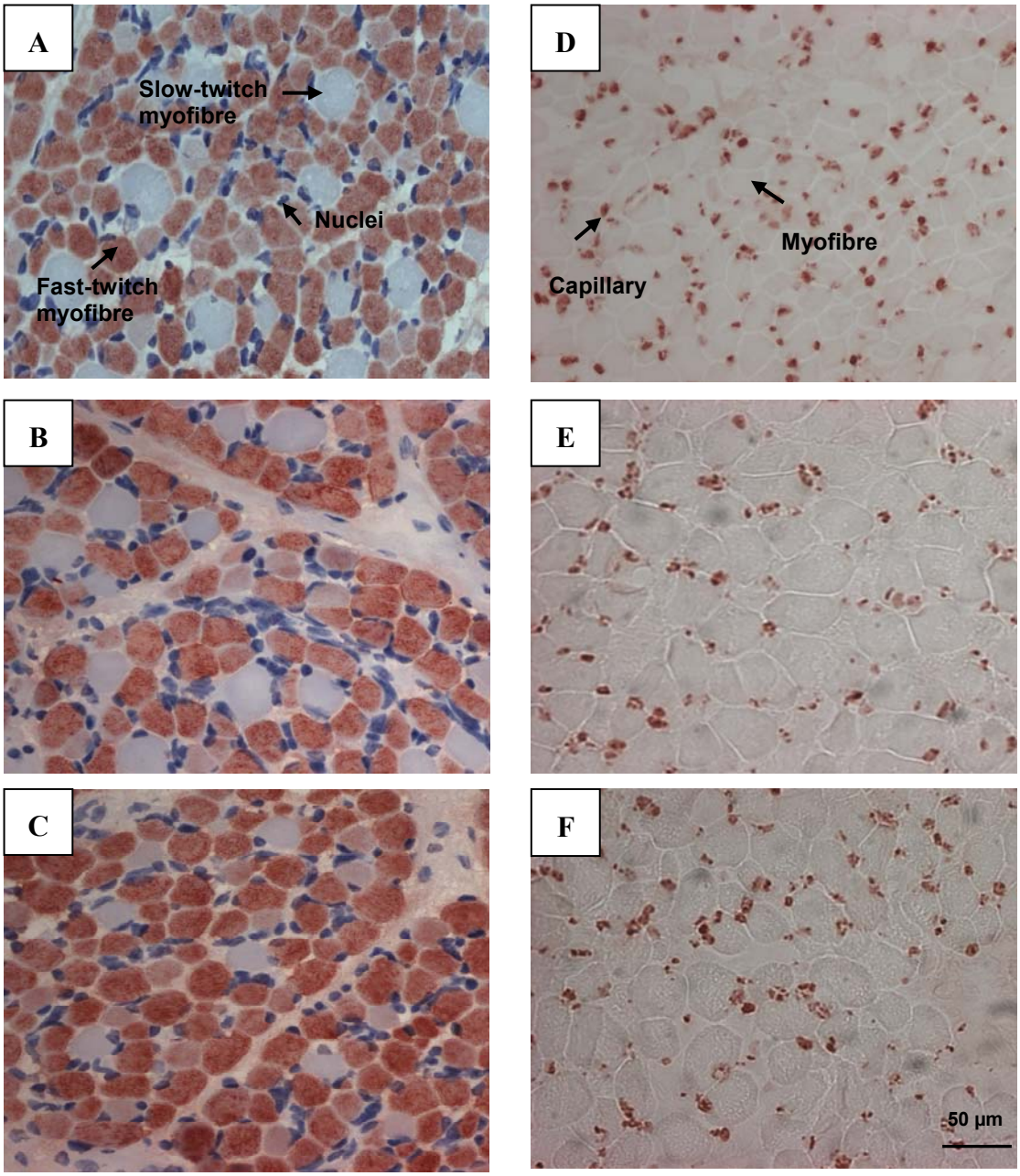


Figure 3-1: Representative counting fields of the triceps brachii muscle in late gestation sheep fetuses from each nutritional group. Slides were immunostained to visualise myofibres in (a) control animals, (b) peri-implantation nutrient restricted and (c) late gestation nutrient restricted fetuses and capillaries in (d) control, (e) peri-implantation nutrient restricted and (f) late gestation nutrient restricted fetuses at x 40 objective.

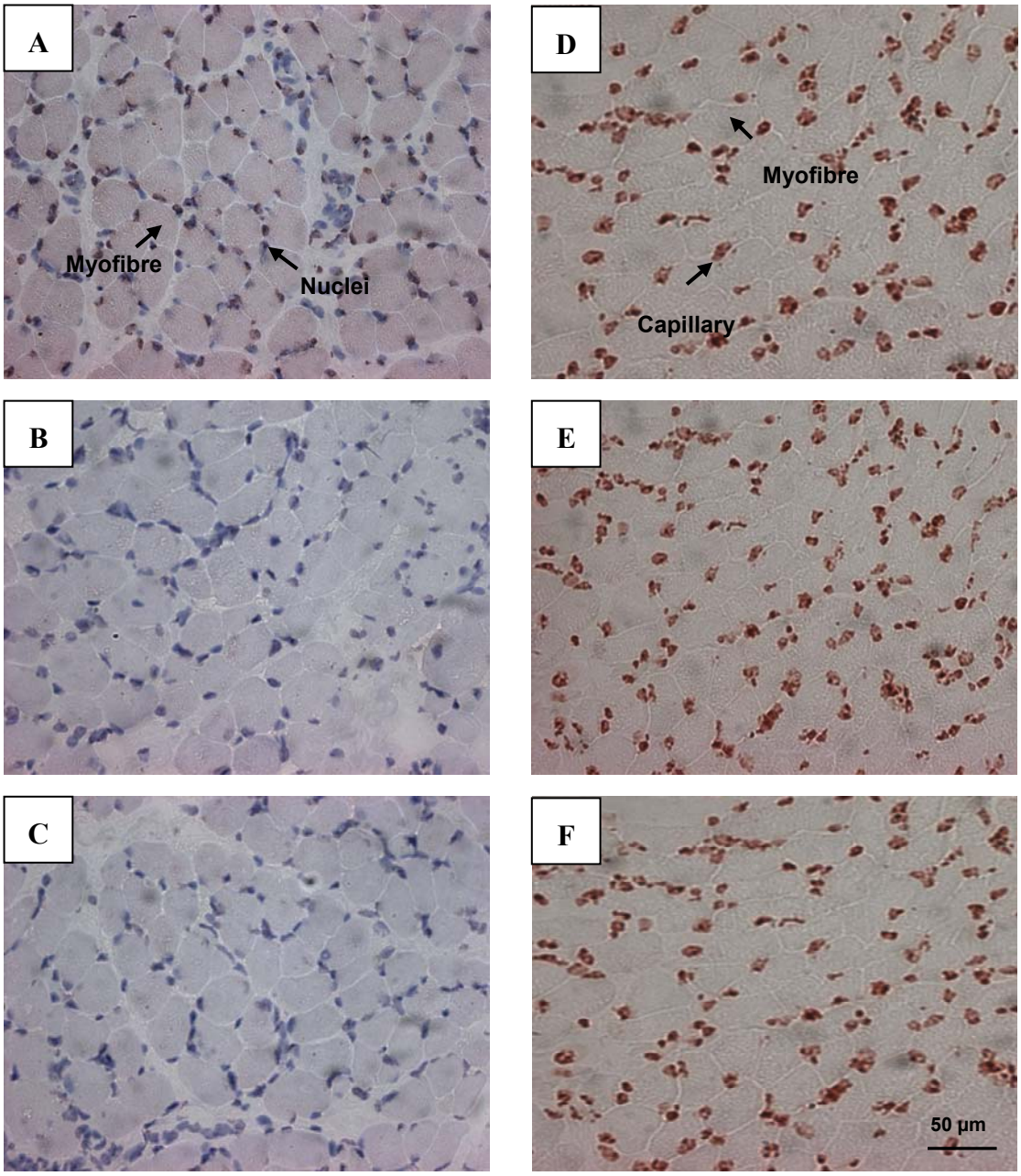


Figure 3-2: Representative counting fields of the soleus muscle in late gestation sheep fetuses from each nutritional group. Slides were immunostained to visualise myofibres in (a) control animals, (b) peri-implantation nutrient restricted and (c) late gestation nutrient restricted fetuses and capillaries in (d) control, (e) peri-implantation nutrient restricted and (f) late gestation nutrient restricted fetuses at x 40 objective.

3.5 Results

3.5.1 Myofibre density

3.5.1.1 Triceps brachii

There was a reduction in total myofibre density in the PI ($p < 0.01$) and L ($p < 0.05$) compared to C fetuses (Figure 3.2a). Fast fibre density tended to be lower in PI ($p < 0.1$), but not L, compared to C fetuses (Figure 3.2b). Slow fibre density was significantly lower in L ($p < 0.05$), but not PI, compared to C fetuses (Figure 3.2c).

There was no difference in myofibre cross-sectional area between the groups for fast and slow fibre density (Figure 3.3).

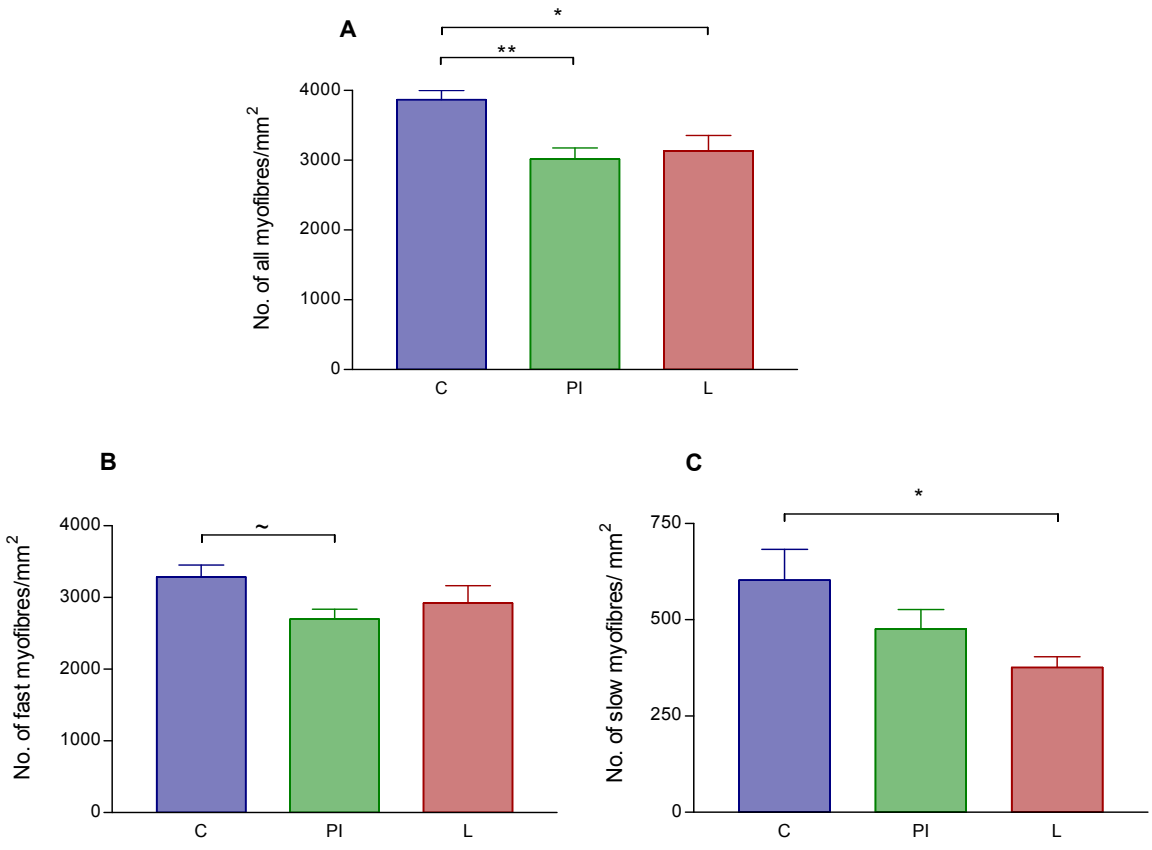


Figure 3-3: Myofibre density in the triceps brachii muscle of late gestation sheep fetuses. Density of (a) all myofibres, (b) fast-twitch fibres and (c) slow-twitch fibres in control (C, $n = 6$), peri-implantation nutrient restricted (PI, $n = 9$) and late gestation nutrient restricted (L, $n = 6$) fetuses at 127 ± 1 dGA, ~ $p < 0.1$, *$p < 0.05$, **$p < 0.01$. Data are shown as the mean ± SEM and ANOVA with Bonferroni *post-hoc* test was used.

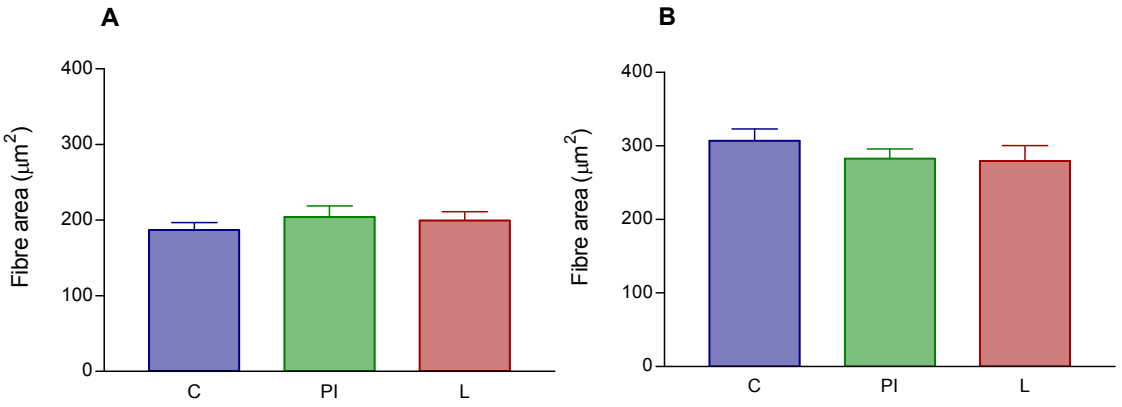


Figure 3-4: Cross-sectional area of myofibres in the triceps brachii muscle of late gestation sheep fetuses. Average CSA of (a) fast-twitch myofibres and (b) slow-twitch myofibres in control (C, *n* = 6), peri-implantation nutrient restricted (PI, *n* = 9) and late gestation nutrient restricted (L, *n* = 6) fetuses at 127 ± 1 dGA. Data are shown as the mean ± SEM and ANOVA with Bonferroni *post-hoc* test was used.

3.5.1.2 Soleus

There was no difference between the groups for total myofibre density (Figure 3.4a) or myofibre cross-sectional area (Figure 3.4b).

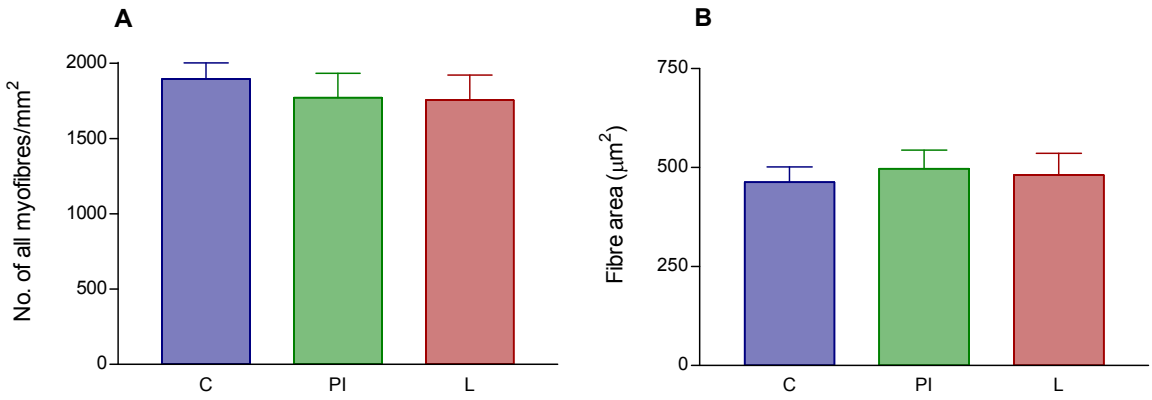


Figure 3-5: Myofibre density & cross-sectional area of myofibres in the soleus muscle of late gestation sheep fetuses. (a) myofibre density and (b) average CSA in control (C, *n* = 8), peri-implantation nutrient restricted (PI, *n* = 9) and late gestation nutrient restricted (L, *n* = 6) fetuses at 127 ± 1 dGA. Data are shown as the mean ± SEM and ANOVA with Bonferroni *post-hoc* test was used.

3.5.2 Capillary density

3.5.2.1 Triceps brachii

There was a reduction in capillary density in both the PI ($p < 0.05$) and L ($p < 0.05$) compared to C fetuses (Figure 3.5a). There was a reduction in capillary : myofibre ratio in both the PI ($p < 0.05$) and L ($p < 0.05$) compared to C fetuses (Figure 3.5b).

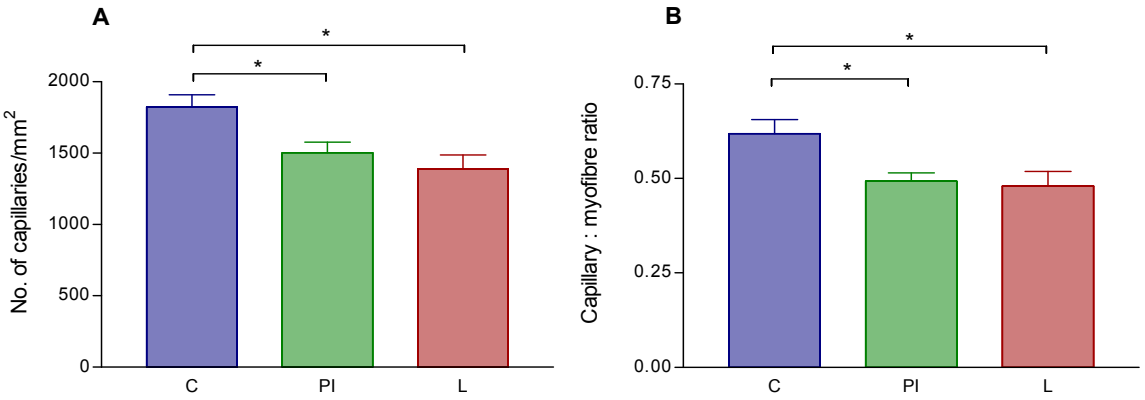


Figure 3-6: Capillary density & capillary : myofibre ratio in the triceps brachii muscle of late gestation sheep fetuses. (a) capillary density and (b) capillary : myofibre ratio in control (C, *n* = 6), peri-implantation nutrient restricted (PI, *n* = 9) and late gestation nutrient restricted (L, *n* = 6) fetuses at 127 ± 1 dGA, *p < 0.05. Data are shown as the mean ± SEM and ANOVA with Bonferroni *post-hoc* test was used.

3.5.2.2 Soleus

There was no difference between the groups for capillary density (Figure 3.6a) or capillary : myofibre ratio (Figure 3.6b).

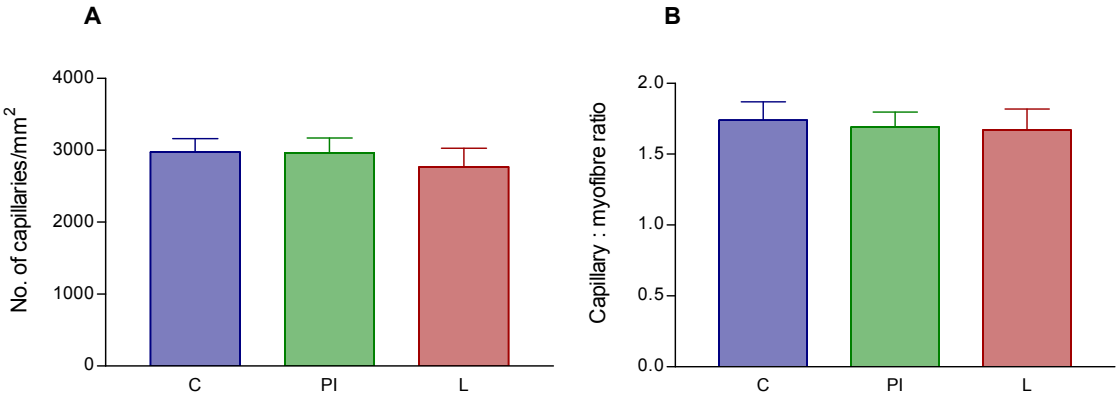


Figure 3-7: Capillary density & capillary : myofibre ratio in the soleus muscle of late gestation sheep fetuses. (a) capillary density and (b) capillary : myofibre ratio in control (C, *n* = 8), peri-implantation nutrient restricted (PI, *n* = 9) and late gestation nutrient restricted (L, *n* = 6) fetuses at 127 ± 1 dGA. Data are shown as the mean ± SEM and ANOVA with Bonferroni *post-hoc* test was used.

3.5.3 Capillary and myofibre density correlations

Capillary density was positively related to myofibre density (Figure 3.7a, $p < 0.001$) and fast fibre density (Figure 3.7b, $p < 0.001$) in the triceps brachii muscle. Capillary density was not related to slow fibre density (Figure 3.7c). When data were split into the nutritional groups only fast fibre density was related to capillary density in the L group (Table 3.8).

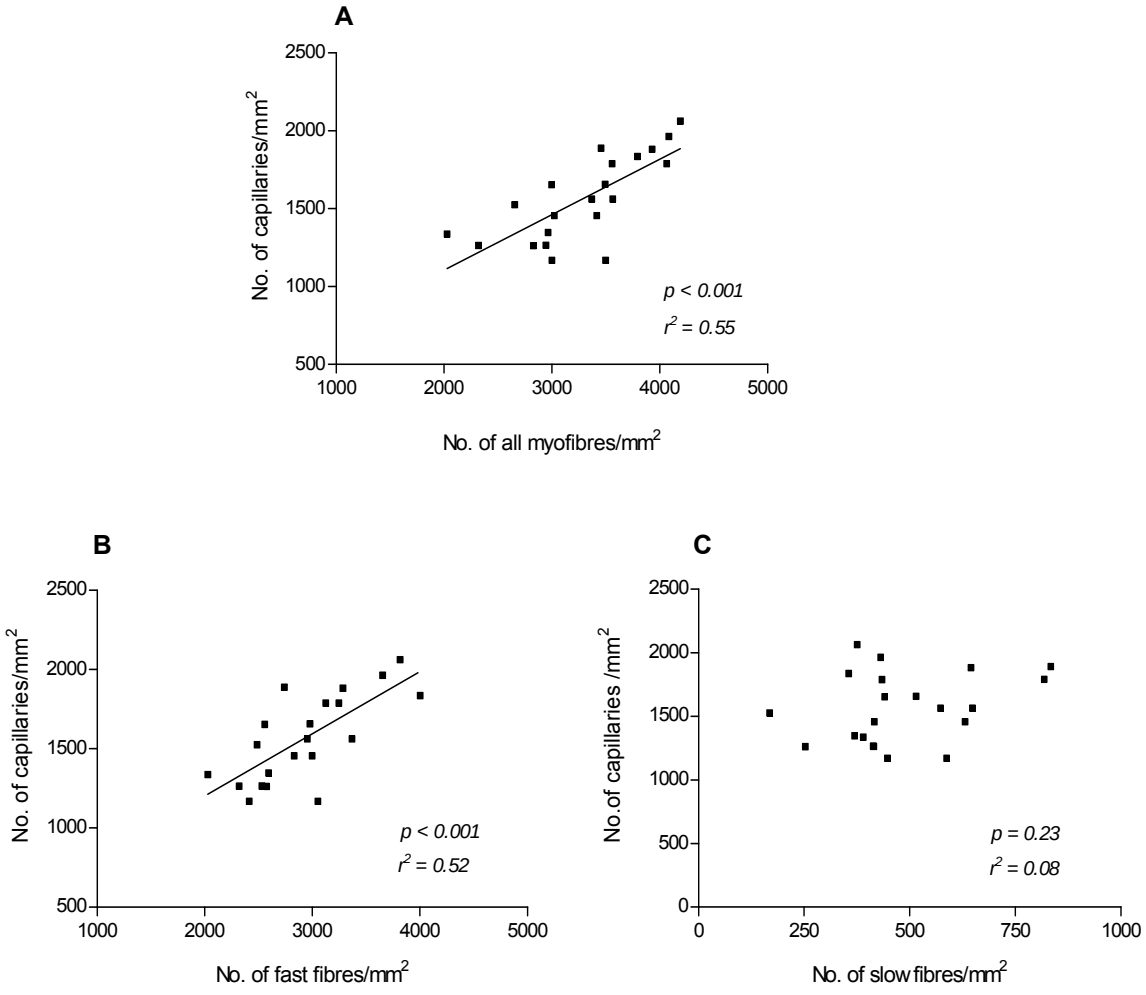


Figure 3-8: The correlation of capillary to myofibre density in the triceps brachii muscle of late gestation sheep fetuses. The capillary density of the triceps muscle from fetuses at 127 ± 1 dGA was correlated against a) all myofibre (b) fast myofibre and (c) slow myofibre density.

	C	PI	L
	Capillary density	**Capillary density**	**Capillary density**
All myofibres	0.392	0.264	0.355
Fast myofibres	0.477	0.375 ~	0.690 *
Slow myofibres	0.153	0.009	0.018

Table 3-8: The correlation of capillary to myofibre density in the triceps brachii muscle of late gestation sheep fetuses according to nutritional group. The capillary density of the triceps muscle from control (C, *n* = 6), peri-implantation nutrient restricted (PI, *n* = 9) and late gestation nutrient restricted (L, *n* = 6) fetuses at 127 ± 1 dGA was correlated against all myofibre, fast myofibre and slow myofibre density, * $p < 0.05$, ~ $p < 0.1$. Data are correlation coefficients r^2.

3.5.4 Correlations between biometric and metabolic data

Biometric and metabolic data were taken at the time of post-mortem (see Appendix 6 for full data set). There were no differences between groups in glucose and blood gases just prior to post-mortem (for all fetuses, pH, 7.38 ± 0.01; $pC0_2$, 42.02 ± 0.81 mmHg; $p0_2$, 15.96 ± 0.57 mmHg and glucose, 0.77 ± 0.03 mmol/L) or in body biometry. These data were correlated against the muscle morphology results (Table 3.9). There were no significant correlations between muscle morphology and blood data except plasma lactate, which tended to be negatively related to fast fibre and capillary density. Indices of fetal growth were not related to muscle morphology.

	Total fibre Density	Fast fibre Density	Slow fibre Density	Capillary density	Capillary : myofibre ratio
Fetal body weight	0.039	0.092	0.003	0.14	0.011
Abdominal circumference	0.013	0.001	0.014	0.001	0.047
Crown-rump length	0.028	0.077	0.059	0.120	0.111
Femur length	0.016	0.07	0.012	0.110	0.108
Shoulder height	0.029	0.011	0.136	0.056	0.017
Biparietal diameter	0.148	0.134	0.016	0.087	0.000
$p0_2$	0.081	0.033	0.067	0.096	0.002
pCO_2	0.007	0.018	0.197	0.000	0.009
Plasma glucose	0.086	0.099	0.040	0.002	0.003
Plasma lactate	0.178	-0.256 *	0.076	-0.240 *	0.073

Table 3-9: The correlation of capillary and myofibre density in the triceps brachii muscle to fetal growth and metabolic data in the late gestation sheep fetus. Muscle morphology of the triceps muscle was correlated against growth and metabolic data taken from the fetus at 127 ± 1 dGA, ~ $p < 0.1$, * $p < 0.05$. Data are correlation coefficients r^2.

3.5.5 Gene expression in triceps brachii muscle

Insulin receptor (InsR) mRNA levels were greater in the triceps brachii muscle of L compared to C ($p < 0.001$) and PI ($p < 0.001$) fetuses (Figure 3.8a). GLUT-4 mRNA levels tended to be higher in L compared to C fetuses ($p < 0.1$), and were significantly higher compared to PI fetuses ($p < 0.05$, Figure 3.8b). Type-1 insulin like growth factor receptor (IGF1-R) mRNA levels were significantly higher in L compared to C ($p < 0.001$) and PI ($p < 0.001$) fetuses (Figure 3.8c).

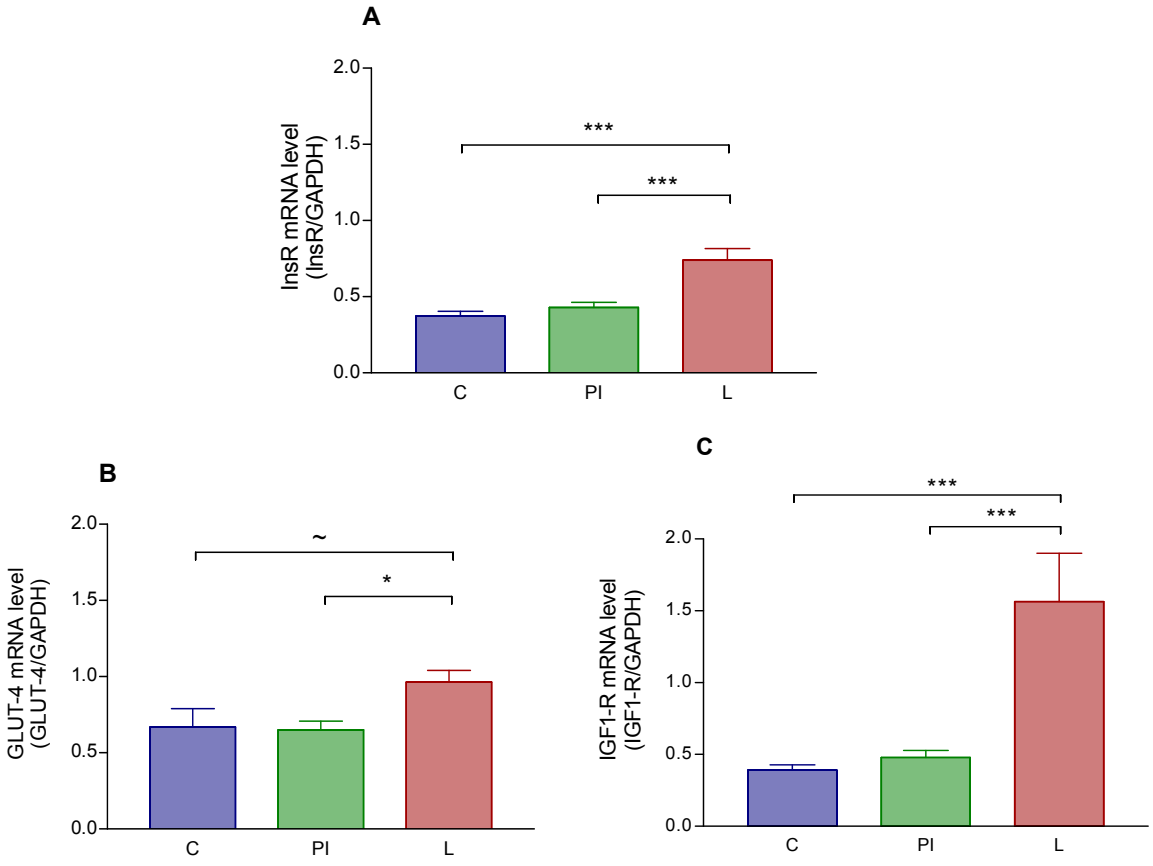


Figure 3-9: Insulin receptor, glucose transporter 4 and type 1 insulin like growth factor receptor mRNA levels of the triceps brachii muscle in late gestation sheep fetuses. mRNA levels of (a) InsR, (b) GLUT-4 and (c) IGF1-R in control (C, *n* = 8), peri-implantation nutrient restricted (PI, *n* = 9) and late gestation nutrient restricted (L, *n* = 6) fetuses at 127 ± 1 dGA, *** $p < 0.001$, * $p < 0.05$, ~ $p < 0.1$. Data are shown as the mean ± SEM and ANOVA with Bonferroni *post-hoc* test was used.

3.5.6 Relationship between muscle morphology and molecular indices of function and growth

The mRNA data were correlated against muscle morphology, fetal biometry and blood gas data and no relationships were found (Table 3.10).

	InsR	GLUT-4	IGF-1R
Total fibre density	0.041	0.009	0.116
Fast fibre density	0.000	0.017	0.025
Slow fibre density	0.135	0.027	0.093
Capillary density	0.132	0.029	0.133
Capillary: fibre ratio	0.145	0.035	0.033
Fetal weight	0.004	0.053	0.099
Abdominal circumference	0.027	0.000	0.000
Crown-rump length	0.055	0.102	0.004
Femur length	0.003	0.089	0.010
Shoulder height	0.000	0.040	0.017
Biparietal diameter	0.003	0.004	0.023
pO_2	0.056	0.003	0.054
pCO_2	0.040	0.170	0.109
Blood glucose	0.001	0.085	0.005
Blood lactate	0.064	0.023	0.072

Table 3-10: The correlation of muscle morphology and fetal growth and metabolic data to mRNA levels of insulin receptor, glucose transporter 4 and type 1 insulin like growth factor receptor in the triceps brachii muscle of the late gestation sheep fetus. Muscle morphology of the triceps muscle and growth and metabolic data taken from the fetus at 127 ± 1 dGA were correlated against mRNA levels of InsR, GLUT-4 and IGF-IR. Data are correlation coefficients r^2.

3.5.7 Placentomes

There were no differences between the groups in placentome weight and type or in total placental weight (Table 3.11).

	C	PI	L
Type A placentome (g)	99.00 ± 11.19	95.00 ± 27.60	155.08 ± 45.76
Type B placentome (g)	149.45 ± 16.43	109.46 ± 19.68	143.05 ± 28.25
Type C placentome (g)	78.28 ± 27.34	68.11 ± 23.94	30.08 ± 11.87
Type D placentome (g)	18.25 ± 7.94	35.17 ± 15.50	17.28 ± 15.84
Total placental weight (g)	344.97 ± 20.79	307.73 ± 14.50	337.59 ± 28.51

Table 3-11: Placentome weight according to type. The placentomes were collected and typed into the 4 groups (A, B, C and D) and the weight of these types was then measured. Data are shown as the mean ± SEM and ANOVA with Bonferroni *post-hoc* test was used.

3.6 Discussion

This chapter has shown that both the peri-implantation and late gestation maternal nutrient restriction reduced skeletal muscle myofibre density in the late gestation fetus and that the reduction was muscle bed specific. The reduction in myofibre density of the triceps brachii muscle was associated with a reduction in capillary density and capillary : myofibre ratio. The late gestation nutrient challenge also increased key mediators of growth and metabolism in the triceps muscle.

3.6.1 Timing of the nutritional challenge

3.6.1.1 Peri-implantation undernutrition and skeletal muscle morphology

The peri-implantation nutrient challenge (1-31 dGA) reduced myofibre density in the triceps brachii muscle. These findings are consistent with other studies which highlight the period of peri-implantation as an important determinant of later muscle growth. Temporary exposure of ovine embryos to an advanced uterine environment increased myofibre number in the late gestation fetus (Maxfield *et al.*, 1998) and a 50 % nutrient restriction from -18 to 6 dGA decreased total myofibre number by mid-gestation in the fetal semitendinosus muscle (Quigley *et al.*, 2005). Although the PI group had a reduction in total fibre density the significance disappeared when split into fibre types; the slow-twitch fibres were unaltered and the fast-twitch fibres only tended to be reduced. Changes in myofibre composition have also been seen following early periods of nutrient restriction. A 50 % maternal restriction from 28 to 70 dGA in sheep, decreased total myofibre number and increased fast myosin type IIb isoform levels in the longissumus dorsi muscle of 8 month old offspring (Zhu *et al.*, 2006), whereas in other studies a similar challenge decreased the proportion of fast-twitch myofibres in the vastus lateralis of 14 day old sheep (Fahey *et al.*, 2005b). There was no change in the cross-sectional area of the myofibres, therefore it is not likely that the observed reduction in myofibre density was due to larger myofibres distorting the results (larger myofibres resulting in fewer counts). Live muscle weights were not taken in this study, but it has previously been shown that an increase or decrease in myofibre number can occur without a change in muscle weight (Quigley *et al.*, 2005;Maxfield *et al.*, 1998). Thus, while not assessed here it can be speculated that any space created by the decrease in myofibre density, in absence of myofibre

hypertrophy, may have been filled by connective tissue such as the endomysium which surrounds each individual myofibre. Indeed, greater volume of tissue between the myofibre (intermyofibre volume density) has been found in bovine fetuses from embryos produced *in vitro* (Crosier *et al.*, 2002).

The environment of the peri-implantation embryo plays a significant role in establishing myogenic potential because this is the time of myoblast proliferation and differentiation. The PI nutrient challenge coincided with the formation of the myogenic cells and so is likely to have decreased myoblast proliferation during this period, reducing the available pool of myoblasts and subsequent number of myofibres. Asynchronous embryo transfer (temporary transfer of a day 3 embryo to an advanced day 6 environment) has been shown to increase myofibre number (Maxfield *et al.*, 1998) but with little impact on fetal and muscle weight. The switch back to control nutrition in these animals at 32 dGA and the subsequent period of nutritional 'catch-up' may also have encroached on the period during which primary (32 dGA) and secondary (38 dGA) myotubes emerge and so myofibre numbers may have been reduced by attenuation of this process. A direct target of this period, with 50 % reduction from 30-70 dGA, resulted in fewer fast fibers and more slow fibres in the 2-week old lamb (Fahey *et al.*, 2005b). However, it was suggested by the authors that slow fibre numbers were not increased but that they appeared proportionally more because of the decrease in fast fibres. When the same restriction was applied and the muscle fibres analysed in older lambs fast fibre number was increased with no difference in slow fibre number (Daniel *et al.*, 2007). These data suggest that the lambs adapted to changes in muscle fibre composition previously observed at 2 weeks. Therefore, although myofibre number may be set at birth, changes in composition can be reversed since the myofibres are known to maintain their plasticity throughout life (Fluck, 2006).

Apart from a direct effect upon myofibre development, the PI nutrient challenge may also have had an indirect effect on later muscle growth via placental adaptations. Placental growth is maximal during the first half of gestation (Schneider, 1996) and so the challenge may have had additional effects on myofibre development through altered placental structure and function. Treatment with somatotropin from 10 to 127 dGA has been shown to improve placental function (Rehfeldt *et al.*, 2001b) and the

same treatment increases total myofibre number in the neonatal pig semitendinosus muscle (Rehfeldt *et al.*, 2001a), with the effect of the early treatment on later myofibre number believed to be through improved placental function. In this study there were no significant differences in total placentome weight or between the different types. Similarly, a 50 % nutrient challenge from -18 to 6 dGA decreased myofibre number but no changes were found in placental weight or placentome number (Quigley *et al.*, 2005). However, weight and type is not a conclusive indication of placental function and changes in nutrient transfer may still have occurred. These early nutrient challenges are in some way affecting the environment of the embryo. One of these may be through circulating progesterone levels which are known to vary with feed intake (McEvoy *et al.*, 1995;Parr, 1992). Elevated progesterone concentration in the peri-implantation period is known to increase fetal size, along with altered placental morphology (Kleemann *et al.*, 1994;Kleemann *et al.*, 2001). A 40 % nutrient restriction in the guinea pig reduced fetal and placental weights and placental efficiency was significantly impaired in early restriction, although this was equivalent to controls by late gestation (Dwyer *et al.*, 1992). The reduction in myofibre density seen in the current study may therefore have resulted from a progesterone-mediated alteration of placental-function, although neither progesterone concentrations nor functional capacity of the placenta were determined.

The nutrient restriction may have had an effect on muscle morphology through interactions with growth factors. In the fetus the insulin-like growth factors are the dominant mediators of growth. IGF-I also has an important role in muscle, regulating both myofibre formation and postnatal growth, with transgenic mice lacking IGF-I having severely underdeveloped muscles (Powell-Braxton *et al.*, 1993). In addition, over-expression of IGF-I in skeletal muscle induces both muscle hypertrophy (Coleman *et al.*, 1995) and hyperplasia (Mitchell *et al.*, 2002). Nutritional status has been shown to affect circulating concentrations of IGF-I in humans, rats and ruminants (McGuire *et al.*, 1992). A peri-conceptual (-60 to 30 dGA) nutrient restriction did not change circulating levels in the 104 day fetus but it did alter the IGF response to a further nutrient challenge, with those exposed to the earlier challenge having a greater reduction in circulating IGF-I and IGFBP-3 at 115 dGA (Gallaher *et al.*, 1998). Studies on the effect of nutrient restriction on the IGF system focus largely on circulating or expressed levels of IGFs but recent studies have demonstrated that normal systemic

IGF-I levels are not required for normal postnatal growth and that the local IGF system may actually be more important (Sjogren *et al.*, 1999a;Yakar *et al.*, 1999). This study looked at mRNA levels of the IGFI-R in skeletal muscle but found no changes following the PI challenge. It is possible that any changes in the IGF system, in response to the challenge, were no longer detectable from just the one muscle sample in late-gestation. However, the peri-implantation challenge may have caused longer term effects on the IGF system, as in the Gallaher (1998) study, through epigenetic modification of the IGF gene. The term epigenetics refers to the ways in which the developmental environment can influence the mature phenotype, through structural changes to genes that do not alter the nucleotide sequence (Godfrey *et al.*, 2007). This can be through methylation of specific gene promoters (Lillycrop *et al.*, 2005) or modification of the chromatin histone core around which DNA wraps (Rice & Allis, 2001). DNA methylation is a common modification in mammalian genomes and methylation of promoter regions of genes is associated with transcriptional repression and hypo-methylation is associated with transcriptional activity (Razin, 1998). DNA methylation patterns are largely established *in utero* and so the fetal environment may alter DNA methylation to induce stable changes in gene expression.

Levels of other growth factors may also have been changed by the PI nutrient restriction. Differentiation of myofibres is coordinated by the myogenic regulatory factors (MRFs) consisting of the transcription factors MyoD, Myf-5, myogenin, and MRF4 (Dauncey & Gilmour, 1996). Asyncronious embryo transfer has been shown to increase protein levels of Myf5, along with an increase in myofibre number, suggestive of a prolongation of myoblast hyperplasia (Maxfield *et al.*, 1998). In addition, myostatin expression, which is not considered to be a member of the MRF family but acts as an inhibitor of muscle development by limiting myofibre number (McPherron & Lee, 1997) can be altered by manipulation of the embryo environment, with mRNA expression decreased in skeletal muscle from *in vitro* fertilised fetuses, along with a greater secondary-to-primary myofibre number (Crosier *et al.*, 2002). In the same study there was no effect of treatment on the expression of mRNA for Myf-5, MyoD, or myogenin. Expression of these growth factors were not determined in the current study but should be considered for future work.

3.6.1.2 Late gestation undernutrition and skeletal muscle morphology

The late gestation nutrient challenge (104-127 dGA) reduced myofibre density and had a significant impact upon the slow-twitch myofibres. This is the first study which has looked at the effects of a distinct late gestation challenge on skeletal muscle morphology in the fetus.

A big part of development during the late restriction challenge would potentially have been hypertrophy and maturation of the existing myofibres, as well as development of tertiary myofibres. The absence of an effect on myofibre CSA suggests there were no changes in fibre hypertrophy and that it was the formation of new fibres which were reduced. The absence of effects on CSA also highlights that the reduction in density is true and not due to changes in the fibre size. The challenge coincided with the third wave of myogenesis, which would have begun around 62 dGA (continuing to parturition), and so it is likely that the reduction in myofibre density was through a reduction in the formation of these tertiary myofibres. Alternatively, the challenge may have caused apoptosis of existing myofibres, although this was not investigated. When the myofibre density was split into fibre types a significant reduction in the slow twitch (insulin sensitive) fibres was seen. Another study saw changes in fibre type composition following maternal nutrition, with a 50 % global reduction much earlier in gestation (28–78 dGA) reducing myofibre number and increasing the ratio of myosin IIb in 8 month old-lambs (Zhu *et al.*, 2006). This chapter did not distinguish between the fast myosin isoforms and this is something which should be addressed in the future, since any changes in the fast fibre population are likely to have a bigger impact upon postnatal function as they make up a larger proportion of total fibres. Energy consumption due to movement is obviously going to be lower in the fetus than the adult, but the observed changes in myofibre composition could still impact on fetal energy requirements in the face of a poor *in utero* nutrient supply since slow-twitch fibres have a higher oxidative capacity, slower rate of ATP utilization and slower contraction velocity compared to fast-twitch fibres. The changes in myofibre composition i.e. reduced slow-twitch fibre density following the late-gestation challenge may therefore be an adaptive mechanism, aimed at conserving energy in the face of reduced nutrient delivery. However, since energy consumption is far lower in the fetus than the adult it is more likely that the change in myofibre composition is a predictive adaptive response, with the aim being to conserve energy in the *predicted* poor postnatal environment (Gluckman & Hanson, 2004b).

Insulin receptor and GLUT-4 receptor mRNA levels were greater in the triceps brachii muscle from the L group compared to C and PI fetuses. Type I fibres contain higher amounts of GLUT-4 (Henriksen *et al.*, 1990) and insulin receptor (James *et al.*, 1986), in line with their higher insulin sensitivity and the increase in the density of two key components of glucose utilisation may therefore constitute a compensatory mechanism (increase in mRNA per myofibre) to conserve metabolic function in the face of the decrease in slow-twitch myofibres in the L group. It was beyond the scope of the study to determine the impact of such adaptations on postnatal glucose handling of the offspring, but it is interesting that an increased expression of InsR and GLUT-4 translocation in skeletal muscle of suckling rats from maternal protein restriction dams is associated with normal glucose tolerance (Gavete *et al.*, 2005). Insulin receptor and GLUT-4 translocation was also increased in rat offspring following a protein restriction (Ozanne *et al.*, 1996). Similarly, an increase in InsR was seen following maternal restriction but alongside a decrease in both GLUT-4 protein and mRNA expression, although GLUT-4 was again more efficiently translocated (Agote *et al.*, 2001). Protein levels were not measured in this study and so it is not known whether the altered mRNA levels were reflected in protein expression, but the Agote (2001) study did show a decrease in both mRNA and protein GLUT-4 expression. Since previous studies in humans and sheep have implicated the importance of late gestation with glucose tolerance in adulthood (Gardner *et al.*, 2005;Ravelli *et al.*, 1998) it is unclear whether the late challenge in this study would have reduced glucose tolerance in postnatal life or whether the adaptations in mRNA expression would have been sufficient in maintaining function.

In the L group triceps brachii muscle there was an increase in IGF type I receptor mRNA and nearly all of the biological activities of the IGFs result from binding to the type I receptor (Nakae *et al.*, 2001). IGF-I is a well-characterised regulator of fetal growth (Florini *et al.*, 1996) and up-regulation of the IGF-I gene is correlated to both increased muscle growth and hypertrophy (Goldspink *et al.*, 1995;Yang *et al.*, 1997). It is thought that IGF-I during the later stages of gestation is acting on muscle fibre maturation rather than proliferation (Gerrard *et al.*, 1998). While this appears to be inconsistent with the findings of fewer myofibres it may reflect a compensatory increase in the expression of this gene in response to decreased growth. IGF levels naturally fall in late gestation, with higher levels of IGF I mRNA found in fetal sheep

muscle at 84 dGA compared with 134 dGA (Dickson *et al.*, 1991) and levels peaked at 100 dGA (Fahey *et al.*, 2005a). I therefore speculate that the increase in IGF-IR seen in the L group is a reflection of an earlier fall in IGF levels (although these were not measured). The increase in IGF-I may also have been part of the same compensatory mechanism adopted by the insulin-signalling pathway (see earlier discussion), as IGF-I is known to induce the same signalling cascade to stimulate glucose uptake via GLUT-4 (Fernandez *et al.*, 2001). The increased mRNA expression for InsR, IGF-IR and GLUT-4 in the L group were not correlated with reductions in myofibre density on an individual animal basis, yet the pattern of the changes between groups was similar to that of slow-myofibres (both mRNA levels increased and slow-fibre density reduced in the L group).

3.6.2 The effect of maternal undernutrition on different skeletal muscle beds

There was no change in myofibre density or size in the slow-twitch soleus muscle. This is interesting since it is made up completely of slow-twitch myofibres and it was these that were significantly hit by the late-gestation challenge in the triceps brachii. However, this resistance of the soleus muscle to an undernutrition challenge is in agreement with previous studies using 40 % maternal restriction throughout gestation in the guinea pig (Ward & Stickland, 1991) where reductions in myofibre density were seen in the neonatal biceps muscle but not in the soleus. It is thought that the soleus muscle is less susceptible to prenatal undernutrition due to its higher proportion of primary myofibres and it has been shown in studies that the primary myofibres are resistant to manipulation and secondary myofibres are preferentially affected (Ward & Stickland, 1991;Dwyer *et al.*, 1994). From studies on polytocous species it has been suggested that the number of primary fibres is genetically determined (hence variations between litters) whereas the secondary fibres, which vary between littermates, can be determined by environmental factors such as nutrition (Dwyer *et al.*, 1993;Dwyer & Stickland, 1991). Primary myofibres have been found to be smaller in small fetuses compared to large fetuses, and so it is hypothesised that the small size of the primary myofibres may restrict the available surface area for secondary myofibre formation (Wigmore & Stickland, 1983). The decrease in slow-twitch fibres in the triceps brachii of L fetuses is therefore likely to be through a reduction in the secondary population of slow-twitch fibres and not the primary population.

3.6.3 Maternal undernutrition and skeletal muscle capillary density

Capillary density was reduced in the triceps brachii of PI and L fetuses, along with the reduction in myofibre density. There were no changes in capillary density of the soleus muscle, along with no changes in myofibre density, and so this suggests that capillary density is linked to muscle development during a nutrient restriction.

Each myofibre is served by several capillaries, slow-twitch fibres having more than the fast-twitch to sustain their higher oxidative function (Andersen, 1975;Green *et al.*, 1981). Thus, a decrease in capillary density in the L group is consistent with the reduction in slow-twitch myofibre density. However, capillary density reduction was also seen in the PI group. The decrease in capillary density may therefore have been due to overall decreased myofibre density i.e. fewer fibres need fewer capillaries. Alternatively, capillary density may drive myofibre density. There was a reduction in capillary : myofibre ratio – so while there are fewer capillaries and fewer fibres, the remaining fibres are supplied by *fewer* capillaries. Thus, it is possible that a fall in blood flow to the developing tissue decreased capillary formation, in turn decreasing myofibre formation and reducing capillary density further. Previous studies in sheep indicate that early gestation undernutrition (Nishina *et al.*, 2003;Hawkins *et al.*, 2000b), and reduced substrate availability (oxygen and other nutrients) in late gestation (Giussani *et al.*, 1993;Burrage *et al.*, 2005) causes a decrease in fetal peripheral blood flow. Thus, a reduced flow-mediated angiogenesis may in part have caused the reduction in capillary density. It is possible that the late restricted group had a redistribution of blood flow in response to the challenge. Indeed, femoral blood reduced was reduced during an acute hypoglycaemic challenge in the same group of fetuses (Burrage *et al.*, 2005). Peripheral blood flow may have been reduced in the PI group through adaptations of the placenta, since the cardiovascular system was not fully formed at the time of the challenge. Other early nutrient restriction challenges studies have seen effects on the cardiovascular system such as impaired arterial function (Nishina *et al.*, 2003;Ozaki *et al.*, 2000). As expected there was a positive correlation between myofibre and capillary density but this relationship disappeared when divided into the different fibre types; fast-fibre density was still positively correlated to capillary density but slow-fibre density was not. This was unexpected in light of slow-twitch fibres being more vascularised and aerobically active than fast-twitch fibres and so perhaps more dependent upon number of capillaries for their

function. I speculate that the slow-twitch fibres were already fed by multiple capillaries and so an increase in their population was sustainable with existing capillary number.

3.6.4 Fetal biometric and metabolic data

There were no differences between the groups in blood gas or glucose levels prior to post-mortem. Arterial pO_2 levels appeared to be low (normoxia ~ 24 mmHg; Giussani *et al.*, 1993) but can be explained since the blood sample was taken from the femoral artery as apposed to the carotid. Plasma lactate was negatively related to fast-twitch myofibre and capillary density. Higher lactate may be indicative of increased anaerobic respiration and so fewer capillaries (reduced oxygen supply) could result in higher levels of lactate. The negative relationship with the fast fibres and lactate levels is surprising considering their greater dependence upon anaerobic metabolism (which would produce more lactic acid).

3.6.5 Postnatal consequences of changes in skeletal muscle

3.6.5.1 Muscle mass

Reduced birth weight has been correlated to impaired muscle strength in adult life (Kuh *et al.*, 2002;Sayer *et al.*, 2004) and decreased muscle mass is a known factor of low birth weight (Hediger *et al.*, 1998). Postnatal muscle growth is due to the elongation and hypertrophy of myofibres, and so a muscle may compensate for decreased number by the hypertrophy of existing fibres. Although no change in CSA were seen in this study it is possible that in postnatal life, when growth hormones rapidly increase muscle mass, a compensatory hypertrophy would have occurred.

As muscles age there is a natural decline in mass and because most myofibres are laid down in prenatal life a decrease in number at birth could exacerbate this later decline. When muscle begins to naturally lose mass those born with a reduced number of myofibres could see a more marked decline (see Figure 3.9) and this may have a detrimental effect upon mobility and the risk of falls.

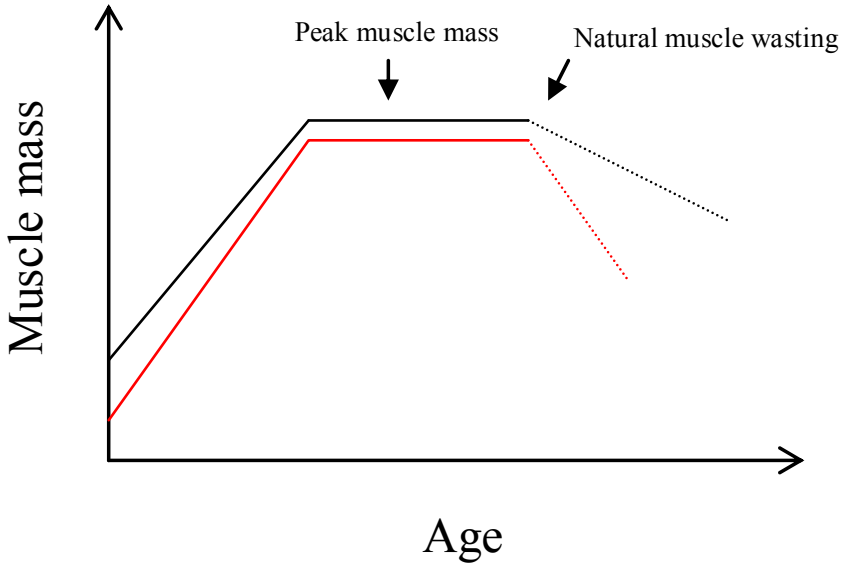


Figure 3-10: The potential effect of lower muscle mass at birth on muscle wasting in later life. Red line represents those born with fewer myofibres. This population may have a reduced peak muscle mass and more pronounced muscle wasting in later life.

3.6.5.2 Metabolism

Undernutrition during the peri-implantation period reduced total myofibre density although it was the late gestation period which had a profound affect upon the slow-oxidative myofibres, coupled to an increase in the message for key mediators of growth and metabolism. This suggests that the L group may have had different postnatal glucose handling. It was beyond the scope of this thesis to determine the impact of the observed changes in muscle composition on postnatal glucose tolerance but muscle is the primary tissue for glucose and fatty acid utilisation (Guo & Zhou, 2004;Krebs & Roden, 2004) and insulin resistance is associated with a shift towards a higher proportion of the relatively insulin-resistant fast-twitch fibres at the expense of the insulin-sensitive slow-twitch fibres (Marin *et al.*, 1994). Thus, it is possible that the reduction in slow-twitch fibres in the L group could have reduced postnatal glucose tolerance. Indeed, a late gestational challenge from 110 dGA to term in sheep resulted in glucose intolerant offspring at 1 year of age (Gardner *et al.*, 2005). Furthermore, from the same study there were changes in the insulin-signalling pathway in peri-renal fat of the late-gestation group, but with no changes in the skeletal muscle. Sampling of the muscle in their study was not bed specific and so samples of varying composition may have been studied and may in part explain the negative results. Similar studies of

peri-implantation nutrient restriction (50 % 1-31 dGA and 0-30 dGA) did not see any differences in postnatal glucose handling (Poore *et al.*, 2007;Gardner *et al.*, 2005). Further evidence for the importance of the late gestation period in determining later glucose metabolism came from studies of the Dutch Winter Famine, with those exposed in late gestation having impaired glucose tolerance (Ravelli *et al.*, 1998). Since this chapter has shown a reduction in slow-twitch myofibre density, following a late-gestation nutrient challenge, altered muscle fibre type composition may have mediated the changes in postnatal glucose tolerance from previous late-gestation challenge studies.

Changes in the microcirculation are a characteristic of diabetes, and insulin resistance has been associated with reduced capillary density alongside changes in muscle fibre composition (Marin *et al.*, 1994). Changes in skeletal muscle capillary basement membrane thickness are also associated with diabetes, although this was not studied in this thesis. Therefore, the reduction in capillary density is not only an important observation in trying to elucidate the mechanisms behind the reduced myofibre density, but is also interesting in terms of later metabolic function and insulin action. In fact many onset complications of diabetes stem from damage to the microcirculation. In clinical studies glucose tolerance has been correlated with muscle morphology measurements, with the strongest correlation between cross-sectional area of the myofibres and the density of the capillaries (mean fibre area per capillary, Lithell *et al.*, 1981). This is thought to be due to changes in the diffusion distance within the muscle.

3.7 Conclusions

These data showed that reduced maternal nutrition in either the peri-implantation or late gestation period reduced muscle fibre density. The late gestation nutrient restriction had particularly profound effects on the slow-twitch (insulin sensitive) fibres. The decrease in myofibres was found in the triceps brachii muscle and not in the soleus suggesting that not all muscle beds are affected in the same way by a nutritional challenge. A decrease in capillary density and capillary : myofibre ratio was seen in the triceps muscle and not the soleus, following both challenges. Expression (mRNA) of key markers of growth and metabolism (InsR, GLUT-4 and IGF-IR) were also increased in the triceps brachii of L fetuses.

As skeletal muscle is the primary tissue for the utilisation of glucose and fatty acids, decreased myofibre number and altered fibre type composition could impair glucose and fatty acid metabolism, predisposing to diabetes and obesity in postnatal life. The observed increase of key mediators in the insulin metabolic pathway following a late gestation challenge may therefore have been a compensatory mechanism aimed at maintaining metabolic function following a loss of myofibres. ***It was unknown whether this adaptation would have been adequate in maintaining normal postnatal glucose tolerance or insulin sensitivity.***

The association between the capillary and myofibre density may suggest a link between local blood flow and muscle growth in times of nutrient deprivation. However, from these data alone it is not known whether it was the reduction in capillary density which drove the reduction in myofibre density or whether the capillary reduction was a reflection of reduced myofibre number. ***A reduction in capillary density may have impaired myofibre development and the reduced angiogenesis have been mediated by reduced upstream blood flow, since angiogenesis can be a flow-mediated process***

Thus, this study raised two important questions. Firstly, what were the mechanisms whereby nutrient intake was able to impair muscle development? This may have been through reduced blood flow to the developing tissue, reducing capillary formation and therefore impairing muscle development and this will be pursued in Chapter 4. Second, does a reduction in myofibre density and altered fibre type composition impair postnatal skeletal muscle function? Functional changes can encompass both muscle strength and metabolism but only the metabolism story will be pursued in Chapter 5.

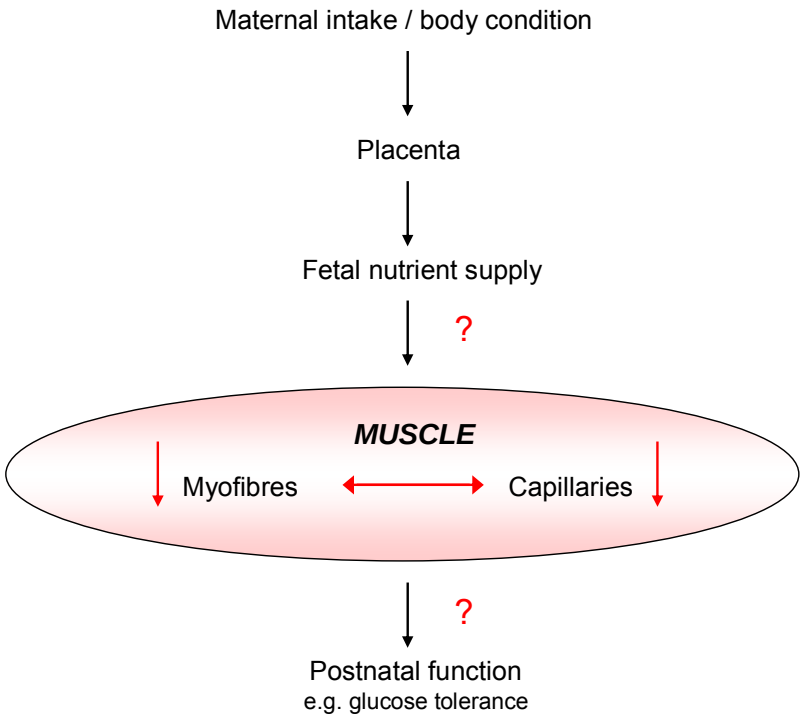


Figure 3-11: Overview of Chapter 3. Peri-implantation and late gestation nutrient restriction reduced myofibre density in the triceps brachii of the late gestation fetus and the association between the capillary and myofibre density may suggest a link between blood flow and skeletal muscle growth in times of nutrient deprivation. The mechanism whereby reduced nutrient intake impaired skeletal muscle development and whether the reduction in myofibre density and altered fibre type composition would affect postnatal glucose tolerance was not known.

4 The effect of two distinct periods of nutrient restriction on the brachial resistance artery function

4.1 Introduction

In Chapter 3 it was shown that both peri-implantation and late gestation maternal undernutrition reduced myofibre density in the fetal triceps brachii but not in the soleus muscle. The reduction was associated with a reduced capillary density and capillary : myofibre ratio which suggested a link between local blood flow and muscle growth in response to periods of undernutrition.

The reduction in capillary number may have been a reflection of reduced myofibre density i.e. fewer fibres to support. However, the capillary : myofibre ratio was also reduced (fewer capillaries supplying each fibre) suggesting that the capillaries were hit harder by the challenge and this may have been due to peripheral redistribution of blood. Since angiogenesis can be a flow-mediated process (Prior *et al.*, 2004) it is possible that a reduction in the upstream blood flow to the developing muscle (in this case the brachial artery) may have attenuated capillary formation. A redistribution of blood flow away from the periphery occurred in the hypoxic challenged fetus (Cohn *et al.*, 1974;Reuss *et al.*, 1982) and (from preliminary data) in nutrient restricted sheep (Burrage *et al.*, 2005). Cardiovascular function in the sheep can be altered by a mild nutrient challenge, with 15 % global nutrient restriction for the first half of gestation decreasing blood pressure and increasing basal femoral artery vascular resistance in the late gestation fetus, followed by elevated blood pressure in postnatal life (Hawkins *et al.*, 2000a;Hawkins *et al.*, 2000b) plus chronically hypoglycemic late gestation sheep fetuses were found to have a significantly lower basal femoral blood flow than controls (Gardner *et al.*, 2002). Therefore, during times of reduced nutrient availability a fetus can redistribute blood flow away from the peripheral tissues through vasoconstriction of the peripheral arteries. Changes in blood flow through a vessel are mediated via altered vascular tone. Vascular tone is maintained by a complex balance of vasoconstrictor and vasodilator factors which act on both endothelium and vascular smooth muscle (see 1.4.3). Alterations in any component part of either the vasoconstriction or vasodilatation pathways can upset this balance and thus the

regulation of blood flow and blood pressure. Endothelium and smooth muscle dysfunction have been found in both rats and sheep following maternal nutrient restriction. In the rat, endothelium-dependent and -independent (factors acting directly on smooth muscle) relaxation was impaired in isolated femoral arteries following a 30 % global restriction from 0 to 18 dGA (Ozaki *et al.*, 2001) and in mesenteric arteries following a 50 % protein restriction throughout gestation (Brawley *et al.*, 2003). In the sheep, endothelium-dependent and -independent relaxation was impaired in isolated femoral arteries following both a 50 % global nutrient restriction from 0 to 70 dGA (Ozaki *et al.*, 2000). and a 30 % protein restriction from -12 to 70 dGA (Nishina *et al.*, 2003). It is important to note that those changes in endothelium and smooth muscle function in the sheep, following maternal undernutrition, were both detected during fetal life. In Chapter 3, both myofibre and capillary density were reduced in the triceps brachii, but not in the soleus muscle, of nutrient restricted fetuses, suggesting a link between local blood flow and myofibre development following maternal nutrient restriction. **Thus, the next logical step of this thesis was to investigate the possibility of altered upstream blood flow, which may have caused the reduction in capillary and myofibre density in the triceps brachii muscle, by characterising vasoactive properties of the fetal brachial artery.**

4.2 Specific hypothesis

Reduced blood flow to the developing skeletal muscle mediates in part the observed reduction in myofibre and capillary density following periods of undernutrition and this will be reflected by changes in the vasoactive properties of the vessel.

4.3 Aims and Objectives

To assess whether the specific periods of peri-implantation and late gestation maternal nutrient restriction have an impact on the vasoactive properties of the isolated fetal femoral artery in late gestation which may indicate a reduction in blood flow through the vessel.

4.4 Methods

4.4.1 Animals and Study design

4.4.1.1 Diet

Ewes were penned individually on straw and fed either 100 % nutrient requirements (C, *n* = 8) or a restricted diet peri-implantation (PI, *n* = 9. 40 %, 1-31 dGA (term ~147)) or in late gestation (L, *n* = 6. 50 %, 104 dGA - postmortem), with 100% at all other times. For full details see section 2.1.1

4.4.1.2 Tissue collection

At post-mortem (see section 2.3.1) third order branches (diameter < 300 μm) of the fetal brachial artery were dissected. Fetal blood was sampled from a pre-implanted femoral artery catheter (see section 2.1.1.1) before PM and blood gas, glucose and lactate levels assessed with a blood gas analyser (ABL735 Blood gas analyser, Radiometer Limited, Crawley, UK).

4.4.2 Small vessel wire myography

Small vessel wire myography is a means of obtaining vascular properties of small resistance arteries. The myograph chamber consists of two jaws, one connected to a transducer, capable of measuring force with a sensitivity of 0.01 mN and the other to a micrometer to allow adjustment of the jaw (Figure 4.1). Two wires are mounted through the lumen of the vessel segment and the ends secured with tension at both ends of the jaw, allowing for measurements of isometric tension across the vessel. Vasoactive substances can be then added to the myograph chamber and the response of the vessel, through changes in lumen diameter, observed.

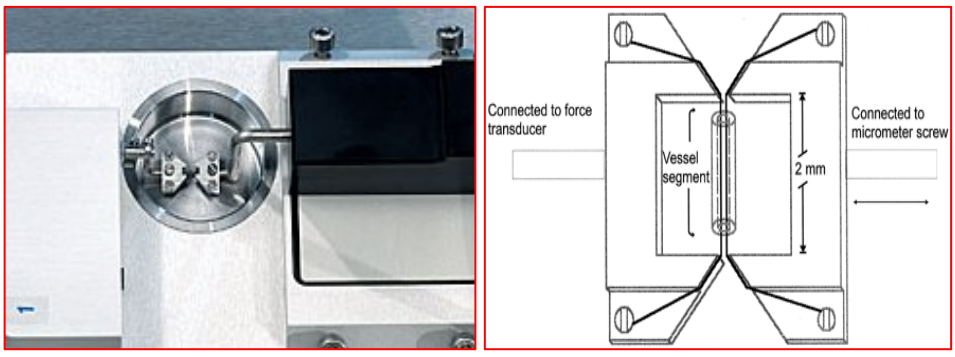


Figure 4-1: A photograph and cartoon representation of the wire myograph chamber (from Torrens, 2004).

4.4.2.1 Vessel normalisation

Before any analysis on vessel function can be performed it is necessary to normalise the vessel, as any measurement of an artery will only have meaning if the conditions are clearly defined and standardised. Vessel size is best described by a fully relaxed vessel under an appropriate transmural pressure (Mulvany & Halpern, 1977) and in the fetal sheep this is ~ 40 mmHg (Nishina *et al.*, 2003). Normalisation thus determines the internal circumference of the mounted vessel under a transmural pressure of 40 mmHg, denoted as $IC_{100.}$ The internal circumference was calculated by adding the distance between and the diameter of the mounting wires, and wall length and tension were used to determine the pressure. Normalisation of the artery was performed by the stepwise distension of the vessel until the effective pressure was in excess of 40 mmHg (5.34 kPa, Figure 4.2). This was plotted in order to calculate the IC_{100}. From this value the internal circumference was then set to 90 % of the IC_{100} value to produce the maximal active force (Mulvany & Halpern, 1977).

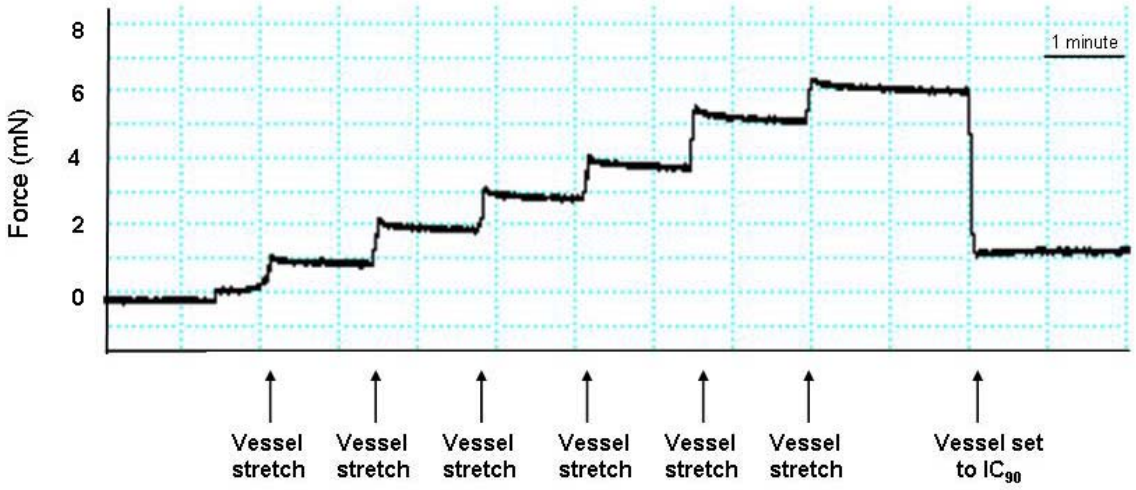


Figure 4-2: Representative trace of the normalisation, by step-wise increases in stretch, of a late gestation sheep brachial artery mounted on a wire myograph.

4.4.3 Assessment of vascular function

The fetal forelimb was removed and placed into cold PSS and third order brachial arteries were dissected out and cleaned for small wire myography (performed in association with Dr Christopher Torrens). Four artery segments (~ 250 µm in diameter) of 2 mm in length from each animal were mounted on to a pair of 40 µm diameter wires, which were then secured under tension to each jaw of the myograph (M610; Multi myography, J.P. Trading, Denmark). Once mounted, the arteries were bathed in physiological saline solution (PSS), heated to 37 ºC to provide the optimum physiological temperature and continually gassed with a composition of 5 % CO_2 and 95 % O_2.

Once normalised, the functional integrity of the vessel was tested by 2 x 2 minute washes with a depolarising KPSS solution (PSS with an equimolar substitution of KCl for NaCl, Figure 4.3) and one with NAK (10 µM NA in KPSS). Vessels failing to achieve an active pressure equal to 5.34 kPa were assumed to have smooth muscle damage and were eliminated from the study. After each incubation the vessels were washed with warmed PSS and allowed 15 minutes of recovery before any further treatments. Chart recordings and data were collected using a Powerlab® data acquisition system (AD Instruments, Chalgrove, UK).

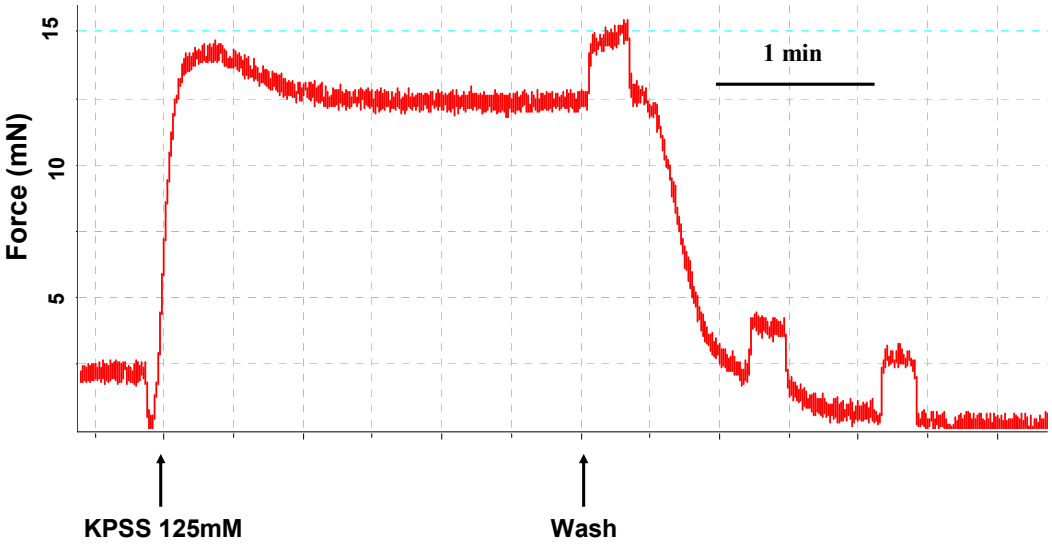


Figure 4-3: Representative trace of the addition of KPSS to a late gestation sheep brachial artery mounted on a wire myograph.

4.4.3.1 Vasoconstriction

A cumulative concentration-response curve (CRC) to the α_1- adrenoceptor agonist noradrenaline (NA 10 nM – 100 μM) was examined under isometric conditions (Figure 4.4).

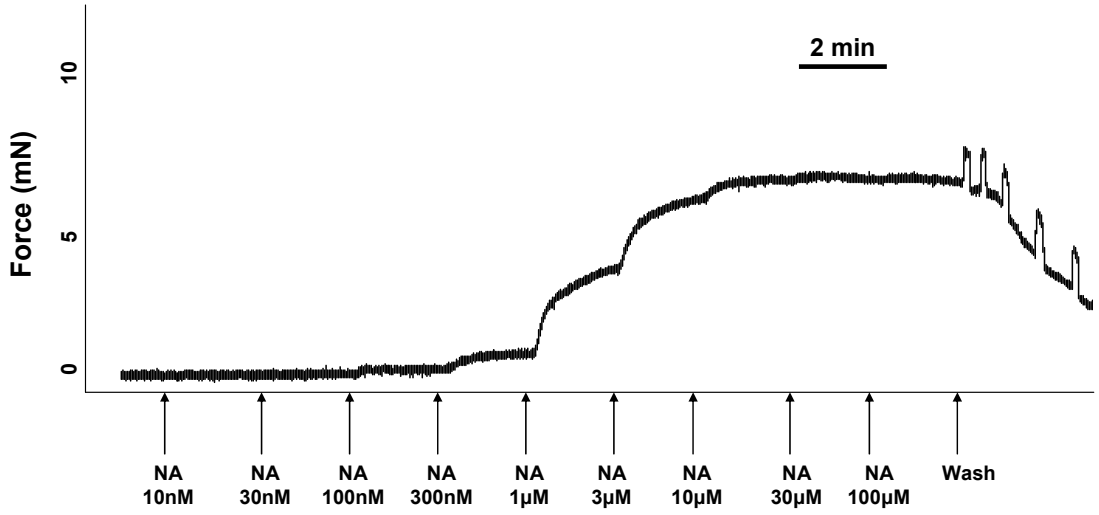


Figure 4-4: Representative trace of a cumulative concentration response curve to NA of a late gestation sheep brachial artery mounted on a wire myograph.

4.4.3.2 Vasodilatation

Arteries were pre-constricted with a submaximal concentration of NA (EC_{80}) and the integrity of the endothelium assessed with cumulative concentrations of the endothelium-dependent vasodilator acetylcholine (ACh; 100 pM – 10 μM, Figure 4.5). The vessel was then washed in warm PSS and allowed 15 minutes for recovery. Arteries were pre-constricted with a submaximal concentration of NA (EC_{80}) and the integrity of the smooth muscle assessed with cumulative concentrations of the endothelium-independent vasodilator sodium nitroprusside (SNP; 100 pM – 10 μM).

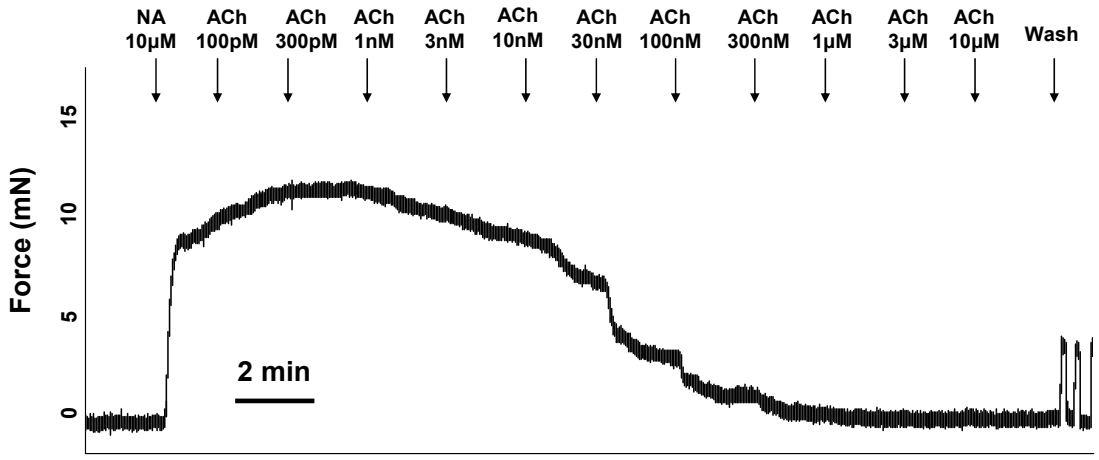


Figure 4-5: Representative trace of a cumulative concentration response curve to ACh of a late gestation sheep brachial artery mounted on a wire myograph.

4.4.4 Analysis and statistics

For a general description of the analysis strategy and statistics packages used, see 2.9.

- Data were tested for normal distribution and are expressed as mean ± SEM.

- Differences in vasoconstriction and dilatation between nutritional groups were analysed by one-way ANOVA with Bonferroni *post-hoc* correction, however, since the animal number was low (C, $n = 2$) these results are interpreted with caution.

- The sensitivity of the individual vessels (pEC_{50}) was correlated with fetal circulating nutrients and metabolites by linear regression. The pEC_{50} is the concentration required to produce a half–maximal response and is therefore a measurement of the vessels sensitivity, whereby a high concentration would indicate a low sensitivity.

- Statistical significance was accepted when $P < 0.05$ and were considered as trends when $0.05 < P < 0.1$.

4.5 Results

4.5.1 Vasoconstriction

Noradrenaline produced a concentration-dependent contraction in isolated brachial arteries to a similar extent between the groups (Figure 4.6).

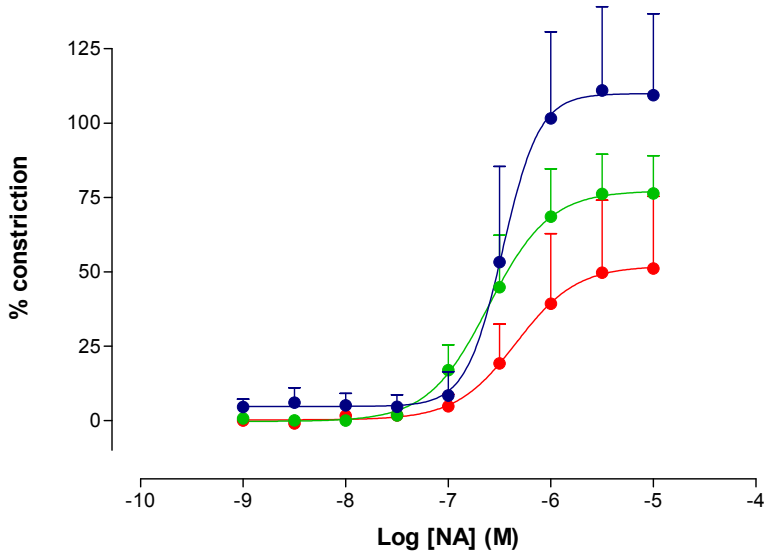


Figure 4-6: Cumulative additions of the vasoconstrictor noradrenaline to isolated brachial arteries of late gestation sheep fetuses. NA was added to vessels from control (● n = 2), peri-implantation nutrient restricted (● n = 5) and late gestation nutrient restricted (● n = 4) fetuses at 127 ± 1 dGA. Data are shown as the mean ± SEM and ANOVA with Bonferroni *post-hoc* test was used.

4.5.2 Endothelial-dependent vasodilatation

The endothelium-dependent vasodilator ACh produced a concentration–dependent vasodilatation of NA-induced tone to a similar extent between the groups (Figure 4.7).

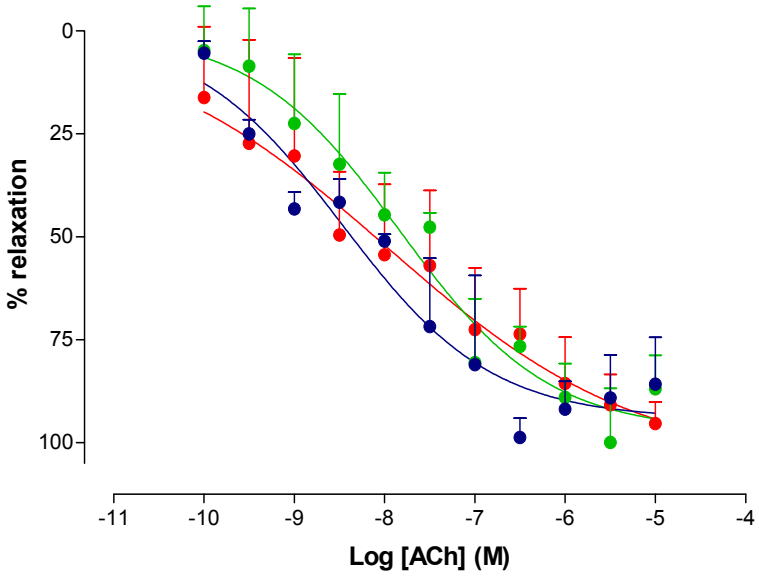


Figure 4-7: Cumulative additions of the endothelium-dependent vasodilator acetylcholine to isolated brachial arteries of late gestation sheep fetuses. ACh was added to vessels from control (● *n* = 2), peri-implantation nutrient restricted (● *n* = 4) and late gestation nutrient restricted (● *n* = 4) fetuses at 127 ± 1 dGA. Data are shown as the mean ± SEM and ANOVA with Bonferroni *post-hoc* test was used.

4.5.3 Endothelial-independent vasodilatation

The exogenous NO donor sodium nitroprusside (SNP) produced a concentration-dependent vasodilatation of NA-induced tone in all brachial vessels. SNP- induced vasodilatation was reduced in PI compared to C ($p < 0.01$) and L ($p < 0.05$) fetuses (Figure 4.8).

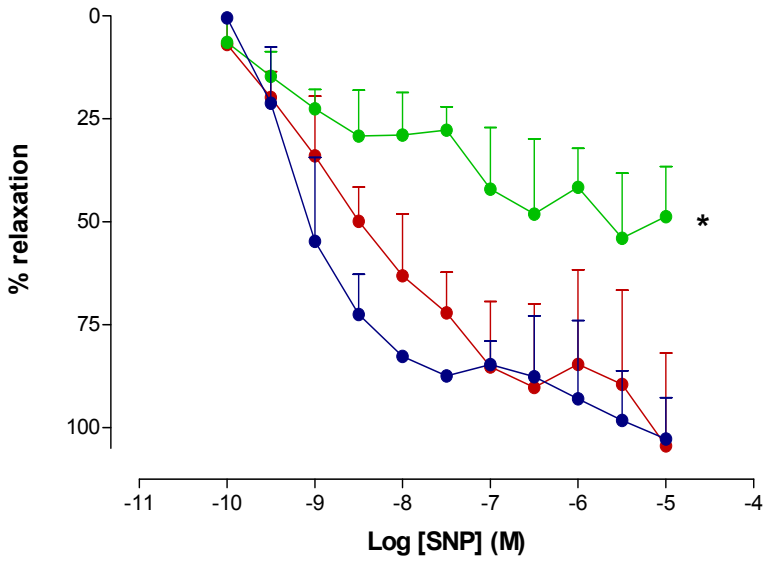


Figure 4-8: Cumulative additions of the nitric oxide donor sodium nitroprusside to isolated brachial arteries of late gestation sheep fetuses. SNP was added to vessels from control (● $n = 2$), peri-implantation nutrient restricted (● $n = 4$) and late gestation nutrient restricted (● $n = 3$) fetuses at 127 ± 1 dGA, * PI vs C, $p < 0.01$; PI vs L, $p < 0.05$. Data are shown as the mean ± SEM and ANOVA with Bonferroni *post-hoc* test was used.

4.5.4 Vascular reactivity and circulating nutrients

For all animals, vascular sensitivity (pEC_{50}) to ACh was positively related ($p < 0.01$, Figure 4.9a) and sensitivity to NA was inversely related ($p < 0.01$, Figure 4.9b) to blood glucose at PM. These were not related to any of the other physiological markers (lactate, pO_2, pCO_2, pH, fetal weight, ewe weight; Table 4.1). SNP sensitivity was not related to glucose (Figure 4.9c), but tended to be negatively related to circulating lactate (Figure 4.9d, $p < 0.1$). SNP was not related to any of the other physiological markers (glucose, pO_2, pCO_2, fetal weight, ewe weight; Table 4.1).

	NA EC_{50}	**ACh EC_{50}**	**SNP EC_{50}**
Arterial blood glucose	- 0.72 **	0.54 **	0.03
Arterial blood lactate	0.03	0.04	-0.43 ~
Arterial $p0_2$	0.05	0.00	0.23
Arterial $pC0_2$	0.01	0.03	0.01
Fetal weight	0.08	0.01	0.12
Ewe weight	0.06	0.28	0.03

Table 4-1: The correlation of brachial artery vascular sensitivity to physiological markers in the late gestation sheep fetus. The vascular sensitivity of the brachial artery to NA, ACh and SNP was correlated against physiological markers taken from the fetus at 127 ± 1 dGA, ** $p < 0.01$, ~ $p < 0.1$. Data are correlation coefficients r^2.

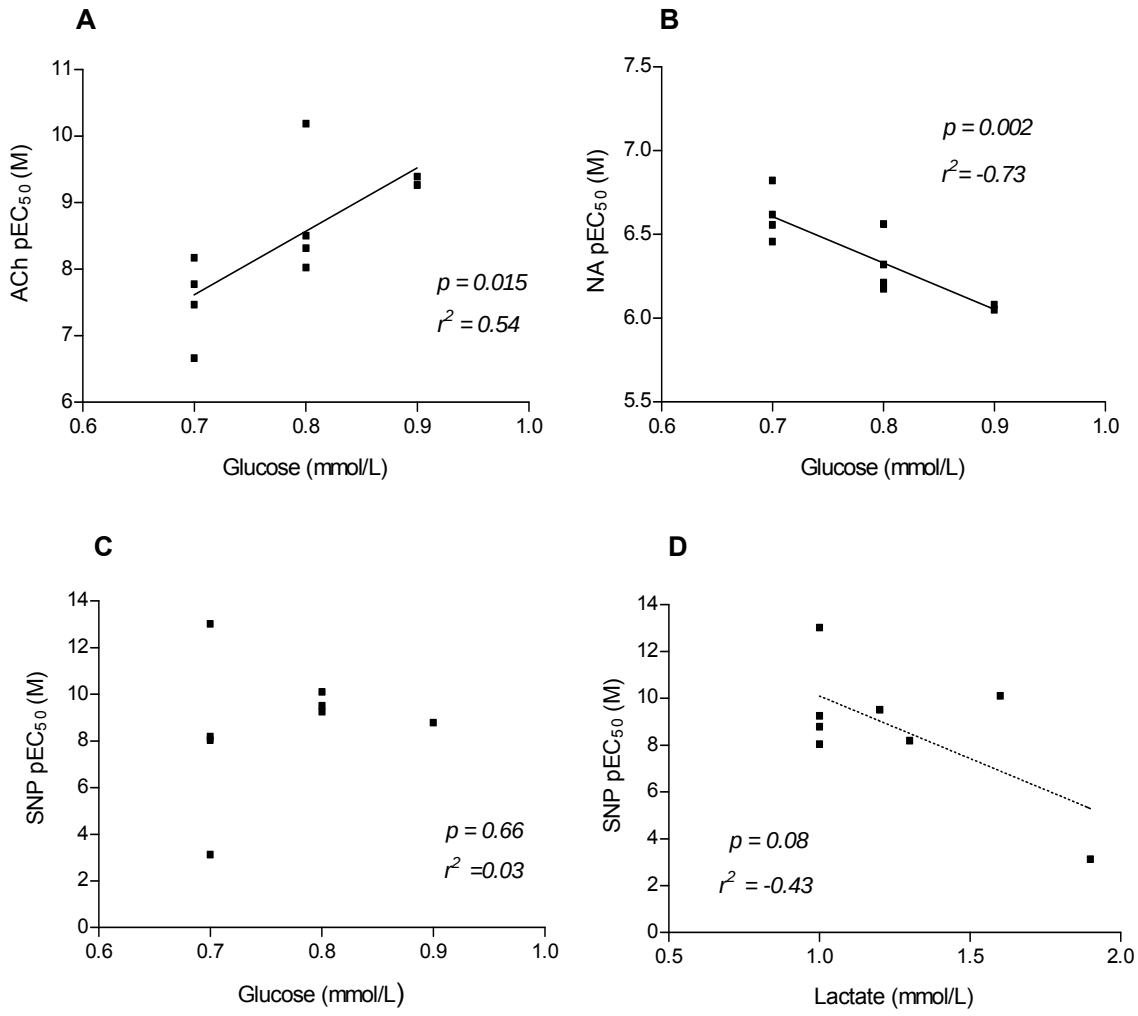


Figure 4-9: The correlation of brachial artery vascular sensitivity to circulating glucose and lactate in the late gestation sheep fetus. The vascular sensitivity (pEC50) of the brachial artery to ACh, NA and SNP from fetuses at 127 ± 1 dGA was correlated against circulating glucose and lactate. Circulating glucose and vessel sensitivity to (a) ACh, (b) NA and c) SNP and (d) circulating lactate and vessel sensitivity to SNP.

4.6 Discussion

This chapter has shown that peri-implantation maternal undernutrition reduced the brachial artery response to SNP in the late gestation fetus, indicative of smooth-muscle dysfunction. There were no changes in the vasoactive properties of the vessel following the late gestation nutrient challenge. For all animals, vascular sensitivity to ACh was positively related, and sensitivity to NA was inversely related to circulating glucose. These changes in vessel reactivity may be a remnant of reduced blood flow and could have mediated in part the decrease in capillary density of the triceps brachii muscle which was seen in Chapter 3.

4.6.1 Maternal undernutrition and isolated brachial artery function in the late gestation fetus

In this study SNP-, but not ACh-, induced vasodilatation was reduced in peri-implantation nutrient restricted fetuses. NA induced vasoconstriction did not differ between the nutritional groups. A decreased sensitivity to ACh and SNP were found in the femoral artery of mid-gestation sheep fetuses following a 30 % protein restriction (12 to 70 dGA; Nishina *et al.*, 2003). In the same study a global nutrient restriction reduced ACh sensitivity but did not affect SNP sensitivity, suggesting that dietary balance was important in determining the cardiovascular outcome. In contrast, a 50 % global restriction (0-70 dGA) femoral artery responses to both ACh and SNP were attenuated in the late-gestation sheep fetus (Ozaki *et al.*, 2000), reflecting the global nature of the dietary restriction or the later study time. The Nishina (2003) and Ozaki (2000) studies all saw attenuation in the ACh response and therefore it is surprising that in this chapter only the SNP- and not the ACh- induced vasodilatation was impaired.

The attenuated response to the NO donor SNP in the PI group suggests a reduced sensitivity to NO in the vascular smooth muscle and may be indicative of an overall reduced vasodilatation in this vessel. The response to ACh was unaltered in the PI group and since one of the ways through which ACh can bring about vasodilatation is NO release this suggests that other pathways in ACh vasodilatation (e.g. EDHF) are compensating for the reduced sensitivity to NO in the vascular smooth muscle. The

blunted SNP response indicates that the impairment is downstream in the NO pathway, perhaps in the enzyme guanyl cyclase (GC) (Nishina *et al.*, 2003). However, the numbers in this study (C, $n = 2$) were small and so the interpretation of the data should be approached with caution.

It has been well established in chronically instrumented sheep that during hypoxia CVO is redistributed in favour of vital organs such as the heart and brain but at the expense of the periphery, as shown by a fall in peripheral blood flow. There is now evidence that a similar redistribution can occur in response to a nutrient challenge (Burrage, 2006;Gardner *et al.*, 2002;Haugen *et al.*, 2005). The decrease in peripheral flow is likely to be caused by an overall vasoconstriction in the vessels, via a change in the dilator/constrictor balance. The effects of the PI challenge in this chapter, with reduced SNP-induced vasodilatation, suggest that an early gestation challenge can have persisting effects on fetal cardiovascular function. As the PI challenge was so early in development it is unlikely that the changes to vascular function were caused by a direct CVO redistribution response since the system was not fully functional at that point (Ozaki *et al.*, 2000) and the early embryo is not constrained by nutrient supply in the same way as the late gestation fetus (Schneider, 1996). Challenges very early in gestation may affect the assignment of embryonic cells to the placental or fetal lineages, and later challenges may affect tissues undergoing rapid growth at that time. Alternatively, it is possible that the effects on the vascular smooth muscle were a predictive adaptive response made by the fetus, in preparation for a poor postnatal environment. Other studies have shown how the fetus can respond with altered vascular properties to challenges that occurred before the control mechanisms of the system were fully developed (Nishina *et al.*, 2003; Ozaki *et al.*, 2000). A relatively mild (15 %) maternal restriction from 0 to 70 dGA showed that basal femoral vascular resistance was higher than in controls (Hawkins *et al.*, 2000a). Although this challenge was mild, relative to the current study, it did have twice the duration of the PI nutrient restriction and so would have impacted on further stages of vascular development. The vascular changes may also have been due to changes in the development and function of the placenta. If placental function is compromised early in development the fetus may experience a further nutrient challenge later in gestation because placental transport is impaired (Jansson & Powell, 2006). If nutrient transport across the placenta were impaired the cardiovascular system may respond with a peripheral

redistribution of blood flow, much in the same way as an acute nutrient challenge.

The preliminary brachial artery data suggests that vascular reactivity was altered in the PI group and so it is possible that this group had reduced blood flow through the brachial artery. A reduced supply to the triceps muscle could have caused the reduction in capillary density. However, since capillary density was reduced in both PI and L fetuses (Figure 3.5) and reduced sensitivity to SNP was only seen in the PI group, the mechanisms underlying the capillary changes may therefore be quite different. The PI challenge may have affected placental development and later nutrient transfer which caused a peripheral vasoconstriction and reduced flow through the brachial artery. This may have reduced flow-mediated angiogenesis and capillary density, which may have had a subsequent effect on myofibre density. There were no differences in vascular reactivity following the L challenge and so a reduction in blood flow may not have occurred. Instead, capillary density may have been reduced due to a change in local angiogenic factors, such as VEGF. Placental VEGF mRNA expression has been found reduced following a nutrient challenge in the sheep (McMullen *et al.*, 2005).

4.6.2 Fetal circulating glucose and isolated brachial artery function

The group numbers from the vascular data were small and in the case of the control group vascular properties from only two animals were successfully studied which made the interpretation of the results difficult. Blood data was available for all animals from ~ 15 minutes prior to the post-mortem and so these were then used as a proxy measurement of nutritional status (e.g. circulating glucose). A summary measurement of vascular sensitivity (pEC_{50}, which is the concentration that produces 50 % of maximal response) was plotted for each animal against the various blood physiological parameters taken on the day of the post-mortem. For all animals, vascular sensitivity to ACh was positively related and sensitivity to NA was inversely related to circulating glucose. So at lower circulating glucose levels it may have taken lower concentrations of NA and higher concentrations of ACh to produce half-maximal response and this is likely to be an indication of greater overall vasoconstriction. Thus, it appears that while there were no differences between groups in circulating glucose, glucose levels are related to individual vascular sensitivity. From the same cohort of animals femoral artery and skin blood flow (as measured by microspheres) fell during maternal insulin-induced acute fetal hypoglycaemia, along with an increase in femoral vascular resistance (as measured by femoral flow probe) (Burrage *et al.*, 2005).

Chronically hypoglycaemic late gestation sheep fetuses were also observed to have significantly lower basal femoral arterial blood flow than controls (Gardner *et al.*, 2002). Together, these findings support the idea that the fetus will redistribute blood flow away from the periphery as a response to hypoglycaemia.

4.7 Conclusions

These preliminary data suggest that a peri-implantation maternal nutrient restriction reduces the sensitivity to NO in the vascular smooth muscle of the brachial artery in the near-term fetal sheep. This is consistent with peripheral blood flow redistribution and a reduced blood supply to the developing muscle which caused a reduction in capillary density. However, since capillary density was reduced in both PI and L fetuses and changes in vasoactive properties of the brachial artery were only seen in the PI group, this suggests that the mechanisms behind the reduction in myofibre density are dependent upon the timing of the challenge and that although the outcome may be similar (reduced myofibre density) the stimulus for this change is different between the groups. Increased sensitivity to NA and decreased sensitivity to ACh, at lower circulating glucose levels, is likely to be associated with greater overall vasoconstriction, suggesting a link between fetal nutritional status and vascular reactivity. Hence, it is possible that the dilator/constrictor balance is altered in the nutrient restricted fetus, which may impair vessel function in postnatal life and predispose to heart disease. This change in the vasoactive properties of the vessel may also have reduced blood flow through the vessel and therefore impaired development of downstream tissues such as the triceps brachii muscle.

5 The effect of maternal diet and body condition on skeletal muscle morphology and glucose tolerance in the adult offspring

5.1 Introduction

In Chapter 3 it was found that 50 % late-gestation nutrient restriction reduced fetal myofibre density, particularly of the slow-twitch myofibres, along with increased markers of growth and glucose metabolism. These changes were associated with a reduction in capillary density and capillary : myofibre ratio. Changes in myofibre composition and capillary density are linked with type-II diabetes (Marin *et al.*, 1994) and obesity (Tanner *et al.*, 2002). The changes in muscle composition and increased molecular markers of glucose uptake could therefore be indicative of long-term changes in offspring metabolism. Since the study only looked at the late-gestation fetus it is not known whether these changes would have persisted into adult life to affect muscle function and glucose tolerance. The effect of maternal nutrition on postnatal offspring glucose tolerance (Cripps *et al.*, 2008;Gardner *et al.*, 2005;Poore et al., 2007) and on skeletal muscle structure (Zhu *et al.*, 2006;Bayol *et al.*, 2004) (see 1.5.10 for details) have been studied separately but few studies have brought these two elements together. One study published after the inception of work in this chapter has shown that maternal obesity in the mouse increases basal glucose and insulin and reduces glucose tolerance in the offspring at 6 months of age, with associated decreased mass of the tibialis anterior muscle and no difference in capillary : myofibre ratio (Samuelsson *et al.*, 2008), but there was no information on fibre type.

Maternal body composition is linked to maternal dietary intake and is important since the mother will provide nutrition for the fetus from both her dietary intake and own body reserves, but there are relatively few studies which have investigated its effects on fetal growth and later health outcomes. In humans the offspring of low body weight or low body mass index mothers have been shown at increased risk of insulin resistance in studies around the world (Fall *et al.*, 1998;Shiell *et al.*, 2000;Mi *et al.*, 2000). Maternal fat free mass in late gestation has been positively linked to birth weight in humans (Mardones-Santander *et al.*, 1998;Larciprete *et al.*, 2003). Tall or heavy women with a higher weight gain during pregnancy gave birth to heavier

offspring (Kirchengast *et al.*, 1998) but the biggest impact was pre-pregnancy weight, with pregnancy weight gain (an indication of environmental conditions of the mother) only having a minor impact on newborn size. In another study, pregnancy weight gain has been found related to child adiposity at 3 years (Oken *et al.*, 2007). Pre-pregnancy BMI is also positively associated with infant birth weight (Frederick *et al.*, 2007). Previously in our group, two groups of Welsh Mountain ewes were established, by manipulation of nutrient intake, at a body condition score of 2 (LBCS) or >3 (HBCS) prior to and during pregnancy (Cripps *et al.*, 2008). At 1.5 years an intravenous glucose tolerance test (GTT) was performed. LBCS were found to have increased fasting plasma glucose concentrations and glucose area under the curve compared to HBCS. Insulin secretion was also reduced during the first five minutes of the GTT in the LBCS group. An increased fasting glycaemia, mild glucose intolerance and impaired initial insulin secretory response are established indicators of increased diabetes risk in humans. Since skeletal muscle is the main site of glucose utilisation the difference in glucose tolerance may be related to muscle morphology. ***This same cohort became available to study at 4 years of age and provided the perfect opportunity to assess the effects of maternal nutrition on skeletal muscle morphology in a much older cohort, and to investigate its impact on glucose tolerance.***

5.2 Specific hypothesis

1. A reduction in maternal body condition, through dietary manipulation, will reduce skeletal muscle myofibre density and alter fibre type composition in the mature adult offspring.

2. These anticipated changes in myofibre density and composition will be associated with a reduced glucose tolerance in mature adulthood.

5.3 Aims

1. To investigate the effects of low and high maternal body condition on skeletal muscle morphology (fibre density and type) in mature adult offspring.

2. To relate these changes directly to isolated skeletal muscle glucose uptake from three different muscle beds (vastus, soleus and gastrocnemius) and whole body glucose tolerance.

5.4 Methods

5.4.1 Animals and study design

5.4.1.1 Diet

Pregnant Welsh Mountain ewes were housed individually on straw and established at a Body Condition Score (BCS) of 2 (n = 30) (LBCS) or equal or greater than 3 (n = 30) (HBCS) by adjusting daily ration of complete pelleted diet. The ewes were allowed to deliver spontaneously and female offspring were removed from the study. Ewes and male offspring were housed in two groups according to the maternal BCS; HBCS (n = 17) or LBCS (n = 12). The lambs were weaned at 12 weeks and housed in a single flock. The flock was studied at 1.5 years (Cripps *et al.*, 2008) and was studied again at 4 years (HBCS, n = 14; LBCS, n = 10) as outlined below

5.4.1.2 Tissue collection

At post-mortem (see section 2.3.2) samples of the vastus, gastrocnemius and soleus muscle were removed and immediately frozen by immersion into freezing isopentane for histological analysis (see section 2.3.3.1) and liquid nitrogen for molecular analysis.

5.4.2 Skeletal muscle immunohistochemistry

Transverse sections (10 μM) were cut on a cryostat and kept frozen at -80 ºC until staining. The presence of fast skeletal myosin was revealed by incubation with anti-skeletal fast myosin (Sigma, USA; see section 2.4.2.1).

Frozen sections (10 μm) were stained with anti-human von Willebrand factor (DakoCytomation, Denmark; see section 2.4.2.2) but this method was unsuccessful when applied to the adult muscle; the staining was weak and it was hard to identify the capillaries. Therefore, the frozen samples were re-embedded in paraffin which allowed thinner sections of 4 μm to be cut. This greatly improved the tissue morphology and the capillaries were then identifiable when using the same antibody.

Thus, the capillary staining method for the adult muscle was as follows:

Muscle cryosections (4 μm) were re-embedded in paraffin and then deparaffinised in Clearene (2 x 5 minutes) and rehydrated through graded alcohols (5 minutes in each) to 70 %. Endogenous peroxidase activity was inhibited by incubation with 0.5 % hydrogen peroxide in methanol for 10 minutes. Slides were covered with working pronase solution (DakoCytomation, Denmark) and incubated at room temperature for 10 minutes. Sections were then washed in tris-buffered saline (TBS; 3 washes x 2 minutes), immersed in avidin solution for 20 minutes, rinsed in TBS then immersed in biotin solution for 20 minutes and rinsed in TBS. Sections were incubated in DMEM containing 20 % calf serum and 1 % BSA culture medium for 20 minutes to block non-specific protein interactions. Following this, anti-human von Willebrand factor antibody (1:300) was applied to the sections and incubated at 4 °C overnight. After rinsing with TBS, sections were incubated with biotinylated anti-rabbit (1:400) antibody for 30 minutes. Sections were washed and treated with streptavidin biotin-peroxidase complex (1 + 1:200) for 30 minutes and then with Diaminobenzidine (DAB) for 5 minutes. Sections were rinsed in TBS and washed in running tap water (5 minutes). Finally, sections were counterstained with Mayers haematoxylin and dehydrated through graded alcohols, cleared in Clearene and mounted in Pertex (Surgipath, Peterborough, UK). A negative control section was processed simultaneously (methodology as above, replacing the primary antibody with TBS buffer). All chemicals were from Sigma, USA unless otherwise stated.

5.4.3 Skeletal muscle image analysis

The image analysis in this chapter was undertaken by Natashsa Bearpark.

Cross-sectional fields from the muscles were captured on a photomicroscope and analysed using the KS-400 image analysing system, as described in section 2.4.3.

5.4.3.1 Myofibre density

5.4.3.1.1 Intra-observer error

The image analysis in this section was performed by a different observer to that of Chapter 3 and so the intra-observer error was repeated accordingly. The intra-observer variability was tested by reproducing the counts from the same field, at different times. The intra-observer variability was less than 2.2 % (Table 5.1).

	Same section counted		
	All fibres per mm^2	*Fast fibres per mm^2*	*Slow fibres per mm^2*
Count 1	329	212	117
Count 2	339	223	117
Count 3	339	223	117
Count 4	339	223	117
Count 5	339	223	117
CV	**1.3**	**2.2**	**0**

Table 5-1: The intra-observer error associated with repeated myofibre counts of the same adult muscle field. Counting of capillaries from the same muscle field was repeated on different days, by the same observer, in the vastus muscle and the error between these counts was then calculated.

5.4.3.1.2 Number of counting fields

From one cross-section of muscle the average myofibre density was calculated from 10 fields of view and this was considered the 'gold standard'. The percentage error of calculating density from random combinations of fields was then compared to this gold standard. The error dropped dramatically at 5 fields and plateaued (Table 5.2) and so it was concluded that 5 fields were sufficient to obtain an accurate myofibre density.

VASTUS	
No. of fields	**Percentage Error**
1	4.5
2	3.4
3	2.7
4	2.2
5	**1.5**
6	1.7
7	1.6
8	1.5
9	1.4

SOLEUS	
No. of fields	**Percentage Error**
1	3.0
2	1.5
3	2.5
4	1.8
5	**0.9**
6	1.6
7	0.8
8	1.4
9	0.1

Table 5-2: Determining the minimum number of counting fields needed to obtain an accurate average myofibre density from adult muscle. These tables show the percentage error that is associated with calculations of myofibre density from varying numbers of fields, as compared to the gold standard of 10, for the vastus and soleus muscle. In both instances 5 frames were found to be sufficient. For both muscles counting from 5 fields of view was sufficient to obtain an accurate myofibre density.

5.4.3.1.3 Myofibre cross-sectional area

The cross-sectional area of the myofibres was defined by manually drawing around them with the cursor. Myofibres were numbered and their size re-calculated on different days, with the intra-observer error between 0.28 - 2.82 % (Table 5.3).

Initially average CSA was calculated by including all fibres obtained from 10 individual frames. However, the number of myofibres available to count varied greatly between the muscle samples (41-131 fast myofibres and 24-98 slow myofibres) and so a ceiling number (based on the sample with the fewest myofibre number) was then applied to all slides.

Fibre No.	First count (μm^2)	Second count (μm^2)	Third count (μm^2)	CV
1	3842.93	3696.36	3815.41	**2.1**
2	2565.48	2669.94	2662.98	**2.2**
3	998.76	995.56	991.15	**0.4**
4	1972.48	1929.68	1969.68	**1.2**
5	1028.42	1034.24	1030.15	**0.3**
6	3942.69	4092.53	3978.96	**2.0**
7	1491.13	1502.65	1501.89	**0.4**
8	3046.69	2994.99	2996.95	**1.0**
9	2324.13	2327.69	2342.84	**0.4**
10	4968.05	4971.89	4969.94	**0.0**
11	2681.75	2835.85	2781.83	**2.8**
12	4207.87	4345.9	4222.09	**1.8**
13	3462.55	3529.18	3640.04	**2.5**
14	3703.05	3807.37	3758.94	**1.4**
15	1248.79	1217	1234.89	**1.3**
16	1905.28	1935.72	1959.68	**1.4**
17	1882.81	1885.45	1901.41	**0.5**
18	932.7	894.91	901.71	**2.2**

Table 5-3: The intra-observer error associated with repeated calculations of myofibre cross-sectional area, in adult muscle. A total of 18 individual myofibres were numbered (so as to allow identification of the same fibre) and the CSA of these were determined on different days, by the same observer, and the error between these areas was then calculated.

5.4.4 Capillary density

The image analysis validation which was performed for myofibre density was then repeated for capillary density.

5.4.4.1 Intra-observer error

Same section counted		
	Capillaries per mm^2	*Capillary: myofibre*
Count 1	297	1.22
Count 2	307	1.16
Count 3	329	1.29
Count 4	318	1.30
Count 5	307	1.16
CV	**3.9**	**5.7**

Table 5-4: The intra-observer error associated with repeated capillary counts of the same adult muscle section. Counting of capillary density from the same muscle field was repeated on different days, by the same observer, in the vastus muscle and the error between counts was then calculated.

5.4.4.2 Number of counting frames

VASTUS	
No. of fields	**Percentage error**
1	6.2
2	4.6
3	3.4
4	2.7
5	3.1
6	**1.9**
7	3.3
8	2.4
9	1.2

Table 5-5: Determining the minimum number of counting frames needed to obtain an accurate average capillary density from adult muscle. This table shows the percentage error that is associated with calculations of capillary density from varying numbers of fields, as compared to the gold standard of 10, for the vastus muscle. It was found that 6 fields of view were sufficient to obtain an accurate capillary density.

5.4.5 Glucose tolerance test

An *in vivo* glucose tolerance test was performed following an overnight fast. Glucose (0.5 g/kg body weight) was administered as an intravenous bolus of 2 minute duration and arterial blood samples were collected for analysis of plasma glucose and insulin concentrations at 15 minute, 7 minute, and immediately (0 minute) before and 5, 10, 15, 20, 30, 45, 60, 90 and 120 minute after the start of the glucose administration (time 0), see section 2.6.

5.4.6 Glucose uptake

Muscle strips were incubated at 37 ºC in Tyrodes solution either without (basal) or with (insulin-stimulated) 16 nM insulin for 20 minutes, then in Tyrodes solution containing 8 mM [^{3}H]methyl glucose (437 μCi/mmol) and 32 mM [^{14}C]mannitol for 10 minutes. After incubation the strips were homogenised and centrifuged and radioactivity in the supernatant was determined by liquid scintillation counting, with channels for simultaneous quantitation of ^{3}H and ^{14}C. Glucose uptake was then corrected for the amount of protein and extracellular space by subtracting the mannitol levels from the glucose, leaving actual glucose uptake, see section 2.7.

5.4.7 Western blotting

The Western blots were performed by Dr Roselle Cripps at the University of Cambridge. Total protein was extracted from vastus muscle and abdominal fat. The cleared protein lysates from each animal were standardised to a final concentration of 1 mg/ml in Laemmli's sample buffer and equal amounts of protein for each animal (20 μg) were loaded onto 10 % SDS polyacramide gels for separation by electrophoresis. The separated proteins were transferred onto polyvinylidene difluoride membrane in singleton and Western blotting was carried out. The antibodies used were to insulin receptor β-subunit (InsR), PI3-kinase p85α regulatory subunit (p85), protein kinase C zeta (PKCζ), insulin-like growth factor-1 receptor β-subunit (IGF-IR), glucose transporter-4 (GLUT-4) and Akt1. Autoradiographs of Western blots were imaged and the optical densities of the immunoreactive protein bands were measured (see section 2.8).

5.4.8 Analysis and statistics

For a general description of the analysis strategy and statistics packages used see section 2.9.

- Data were tested for normal distribution. Parametric data are shown as the mean ± SEM. Nonparametric data were log transformed prior to testing to achieve normality and are shown as the geometric mean (95 % confidence intervals).

- The intra-observer error in determining myofibre/capillary density and fibre cross-sectional area and the variation with muscle sample site was calculated using a coefficient of variation.

- For each GTT experiment, the area under the glucose and insulin response curve (AUC) was calculated [integrated plasma concentrations following glucose administration (5–210 min) above the mean pre-GTT (–15 to 0 min) concentrations].

- Differences in glucose tolerance and myofibre/capillary density between nutritional groups were analysed by an unpaired Student's t-test.

- Glucose uptake into isolated muscle strips was corrected for amount of protein and the extracellular space was corrected for by subtracting the mannitol levels away from the glucose, leaving actual glucose uptake. Differences in uptake between nutritional groups were analysed by an unpaired Student's t-test.

- To test the effect of age and its interaction with nutritional group a repeated measures ANOVA was used. Where a significant interaction was found between age and nutritional group a t-test was used to compare age within group.

- Linear relationships between variables expression were investigated by linear regression analysis.

- Statistical significance was accepted when $P < 0.05$ and were considered as trends when $0.05 < P < 0.1$.

5.5 Results

5.5.1 Body biometry and organ data

There were no differences between nutritional groups at 4 years of age in body biometry or organ weights, apart from an increased muscle depth in LBCS offspring ($p < 0.05$, Table 5.6).

POST MORTEM MEASURMENTS	HBCS		LBCS	
Absolute weight	*n*		*n*	
Body weight (kg)	14	80.5 ± 1.8	10	82.5 ± 1.5
BCS (1-5)	14	2.6 (2.4-2.9)	10	2.6 (2.5-2.8)
Crown-rump length (cm)	14	116 ± 2	10	115 ± 2
Abdominal circumference (cm)	14	121 ± 2	10	122 ± 2
Biparietal diameter (cm)	14	11.9 ± 0.3	10	12.5 ± 0.3
Femur length (cm)	14	32.5 ± 0.5	10	32.8 ± 0.6
Shoulder height (mm)	14	91 ± 7	10	83 ± 1
Muscle depth (mm)	14	29.2 ± 0.9	10	32.3 ± 0.7 *
Fat depth (mm)	14	3.4 ± 0.4	10	3.3 ± 0.3
Left adrenal (g)	14	2.2 ± 0.1	9	2.1 ± 0.3
Right adrenal (g)	14	2.3 ± 0.1	10	2.08 ± 0.25
Left kidney (g)	14	92.9 ± 2.4	10	90.9 ± 2.5
Right kidney (g)	14	91.3 ± 3.2	10	92.1 ± 2.5
Liver (g)	14	890.4 ± 20.1	9	886.0 ± 24.3
Heart (g)	14	352.8 ± 18.0	10	4028 ± 31.3
Lung (g)	11	901.5 ± 82.2	10	969.3 ± 110.0
Soleus (g)	14	3.0 ± 0.2	10	3.2 ± 0.4
Gastrocnemius (g)	14	61.4 ± 3.0	10	66.7 ± 2.9
As % of body weight				
Left kidney	14	0.116 ± 0.003	10	0.110 ± 0.003
Right kidney	14	0.113 ± 0.003	10	0.112 ± 0.002
Liver	14	1.11 ± 0.03	9	1.08 ± 0.03
Heart	14	0.44 ± 0.02	10	0.49 ± 0.03
Lung	11	1.10 ± 0.09	10	1.17 ± 0.12
Circumference				
Soleus (cm)	12	2.73 ± 0.13	10	2.58 ± 0.18
Gastrocnemius (cm)	12	13.13 ± 0.20	10	12.68 ± 0.24

Table 5-6: Body biometry and organ data from mature adult sheep. Body biometry and organ weights were taken in 4 year old offspring from high body condition score (HBCS, $n = 14$) and low body condition score (LBCS, $n = 10$) ewes, * $p < 0.05$. Data are shown as the mean ± SEM, except for body condition score (BCS) which is shown as the geometric mean (95 % CI), and an independent samples t-test was used.

5.5.2 Body biometry with age

There was a significant increase with age in body weight ($p < 0.001$, Figure 5.1a), BCS ($p < 0.001$, Figure 5.1b) and back fat depth ($p < 0.001$, Figure 5.1c) that was not affected by nutritional group. For back muscle depth there was a significant interaction between age and group ($p < 0.05$). Subsequent analysis showed that muscle depth increased with age in LBCS offspring only ($p < 0.05$, Figure 5.1d).

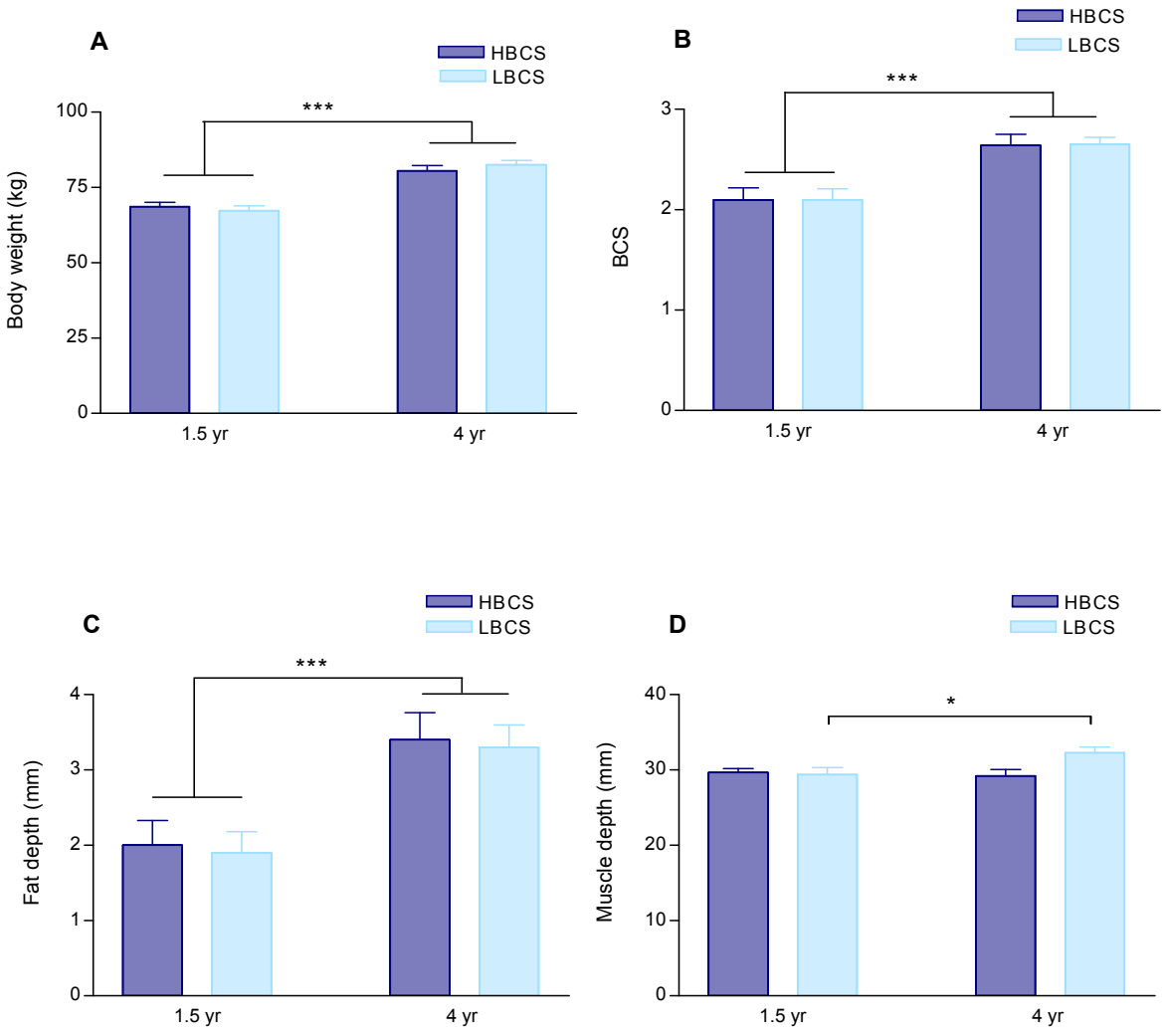


Figure 5-1: Changes with age in body weight, body condition score and back fat and muscle depth in adult sheep. (a) body weight (b) BCS (c) back fat depth and (d) back muscle depth in 1.5 and 4 year old offspring from high body condition score (HBCS, $n = 14$) and low body condition score (LBCS, $n = 10$) ewes, * $p < 0.05$, *** $p < 0.001$. Data are shown as the mean ± SEM and repeated measures ANOVA was used, with an independent samples t-test for figure d.

5.5.3 Glucose tolerance test

5.5.3.1 Basal levels

There were no differences in basal plasma cortisol (Figure 5.2a) glucose (Figure 5.2b) or insulin (Figure 5.2c) between the nutritional groups. Data sets for lactate, triglycerides and free fatty acids are incomplete (levels fell below assay standards) and are to be re-analysed as part of future work.

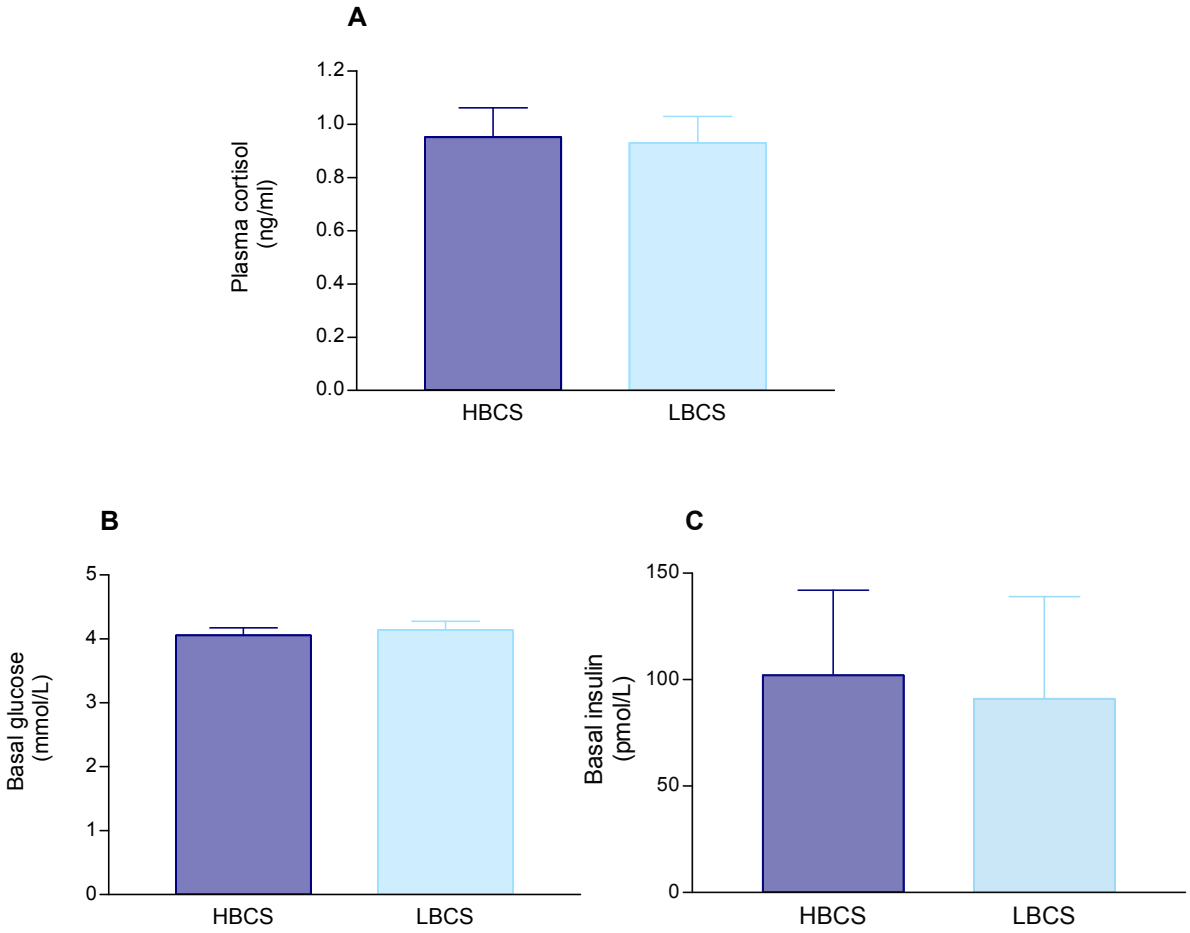


Figure 5-2: Fasting plasma levels of glucose, insulin and cortisol in mature adult sheep. Fasting levels of (a) cortisol, (b) glucose and (c) insulin in 4 year old offspring from high body condition score (HBCS, *n* = 14) and low body condition score (LBCS, *n* = 10) ewes. Data are shown as the mean ± SEM or the geometric mean (95 % CI; basal insulin) and an independent samples t-test was used.

5.5.3.2 Glucose response

Plasma glucose levels increased after the addition of the glucose bolus (at 0 minute) and returned to basal levels by 210 minute (Figure 5.3a). There was no difference in glucose AUC (Figure 5.3b) or peak glucose (Figure 5.3c) between the nutritional groups.

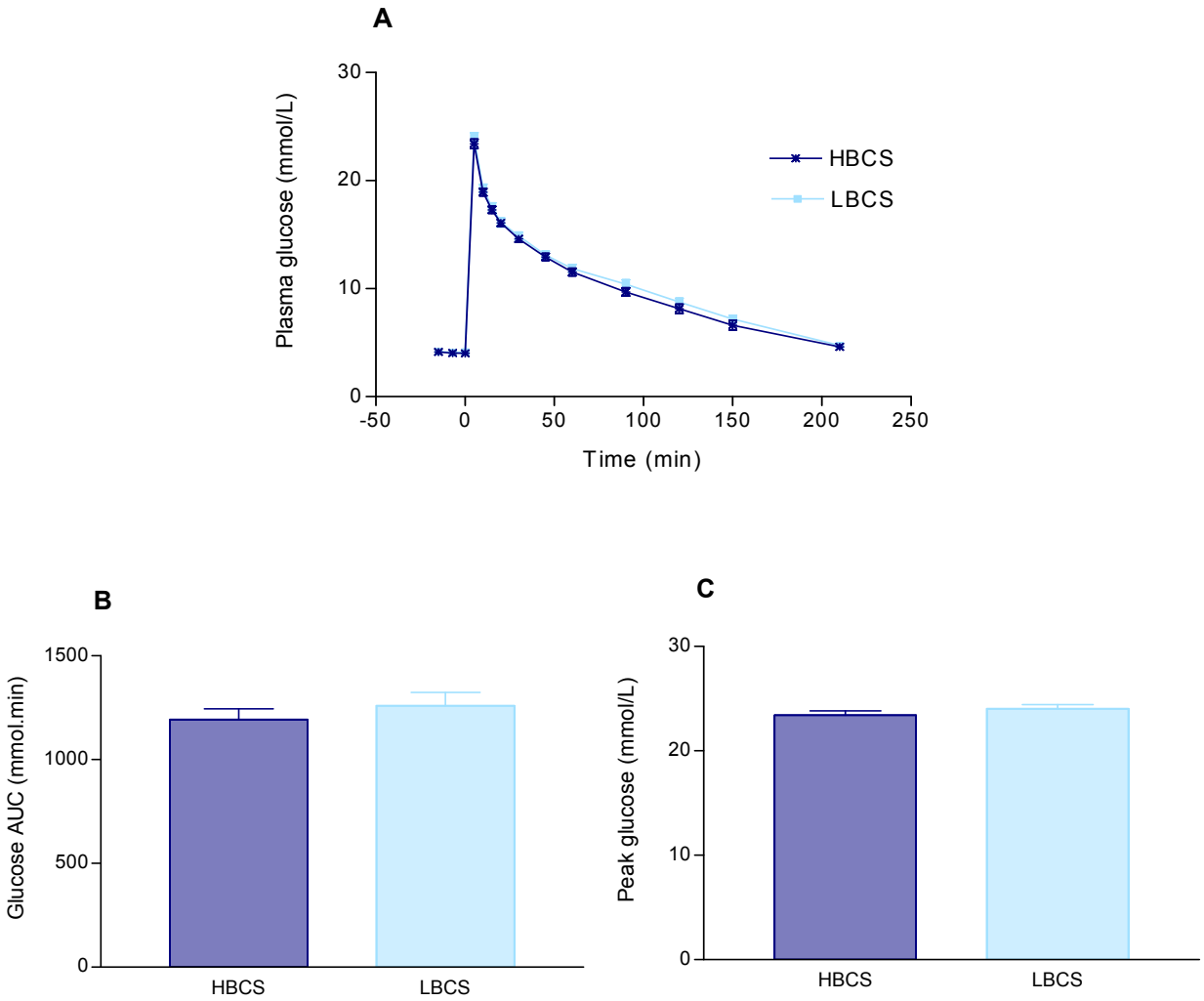


Figure 5-3: Plasma glucose levels during a glucose tolerance test in mature adult sheep. a) plasma glucose levels over time (b) glucose AUC and (c) peak glucose in 4 year old offspring from high body condition score (HBCS, $n = 14$) and low body condition score (LBCS, $n = 10$) ewes. Data are shown as the mean ± SEM and an independent samples t-test was used.

5.5.3.3 Insulin response

Plasma insulin levels increased after the addition of the glucose bolus (at 0 minute) and returned to basal levels by 210 minute (Figure 5.4a). There was no difference in insulin AUC (Figure 5.4b), peak insulin (Figure 5.4c), or time to peak (HBCS, 71 ± 15; LBCS, 62 ± 12 minute) between the nutritional groups.

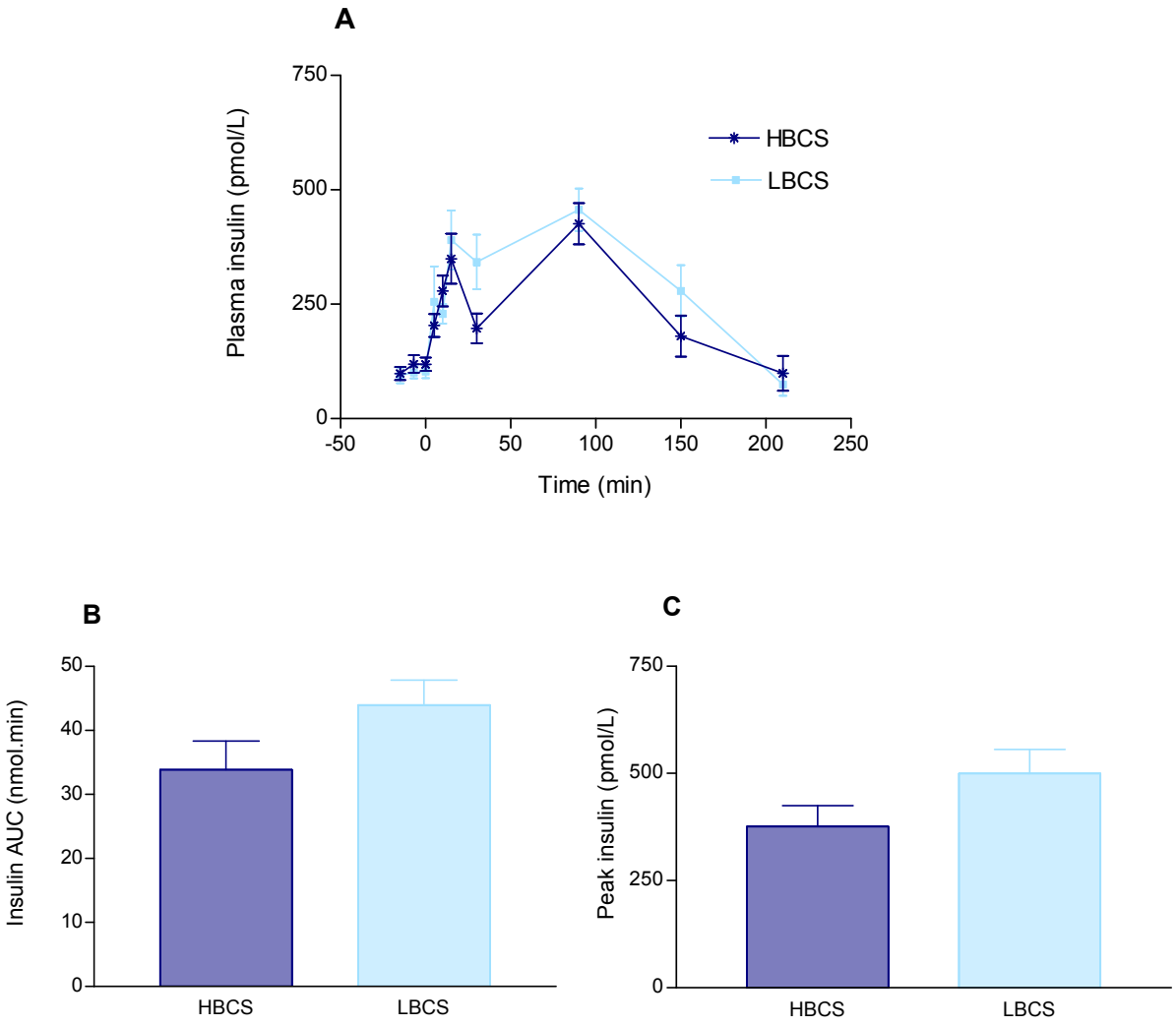


Figure 5-4: Plasma insulin levels during a glucose tolerance test in mature adult sheep. (a) plasma insulin levels over time (b) insulin AUC and (c) peak insulin in 4 year old offspring from high body condition score (HBCS, *n* = 14) and low body condition score (LBCS, *n* = 10) ewes. Data are shown as the mean ± SEM and an independent samples t-test was used.

There was no difference in initial insulin release at 0 to 5 minute (Figure 5.5a) or 0 to 10 minute (Figure 5.5b) between the nutritional groups. There was no difference in first phase insulin AUC (0 to 30 minute) between the nutritional groups (Figure 5.5c). Second phase insulin AUC (30 to 210 minute) tended to be greater in the LBCS as compared to HBCS offspring ($p < 0.1$, Figure 5.5d).

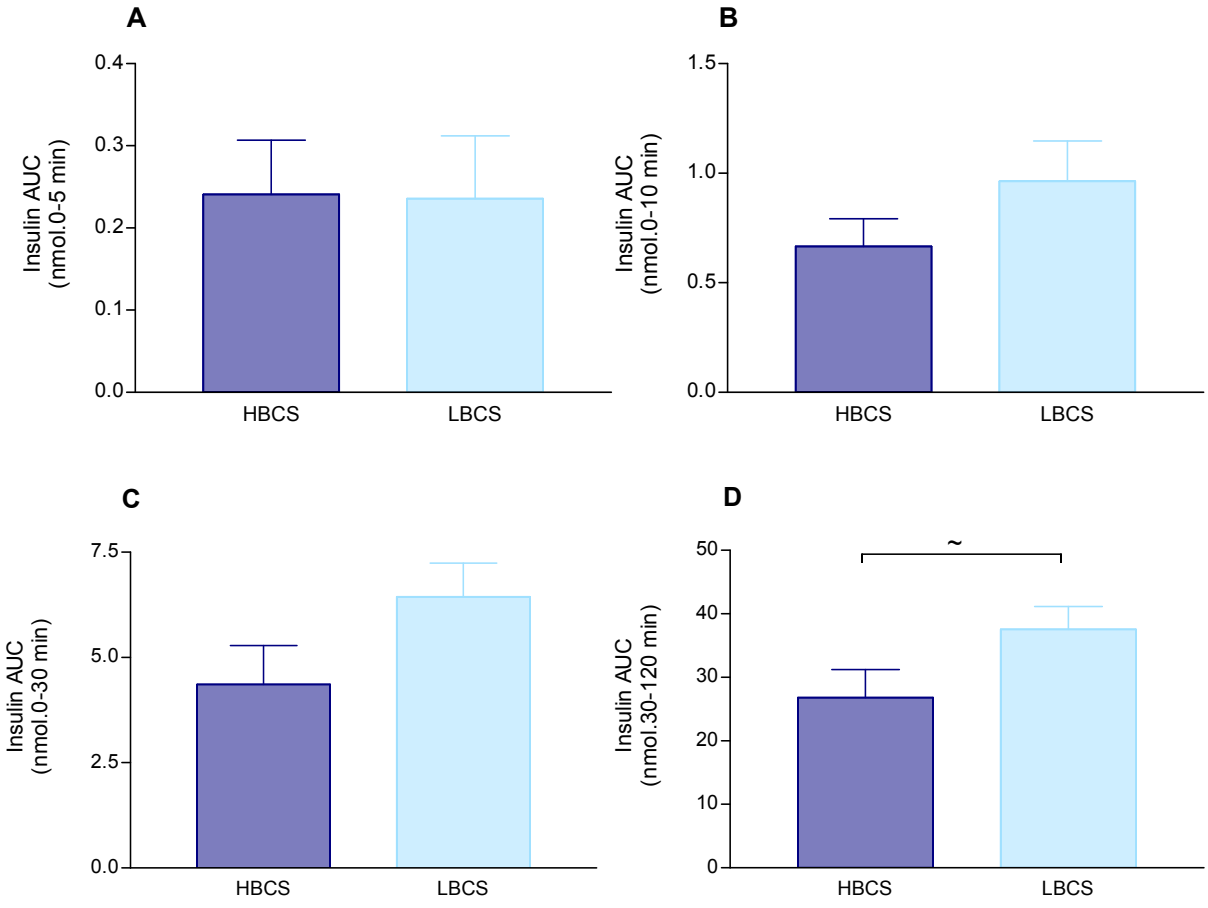


Figure 5-5: Phases of insulin release during glucose tolerance test in mature adult sheep. Plasma insulin AUC for (a) 0-5 min (b) 0-10 min (c) 0-30 min and, (d) 30-210 min of GTT in 4 year old offspring from high body condition score (HBCS, $n = 14$) and low body condition score (LBCS, $n = 10$) ewes, ~ $p < 0.1$. Data are shown as the mean ± SEM and an independent samples t-test was used.

5.5.4 Glucose tolerance and age

5.5.4.1 Basal level comparisons

There was a significant increase with age in basal glucose ($p < 0.01$, Figure 5.6a), insulin ($p < 0.001$, Figure 5.6b) and basal insulin : glucose ratio ($p < 0.001$, Figure 5.6c) that was not affected by nutritional group.

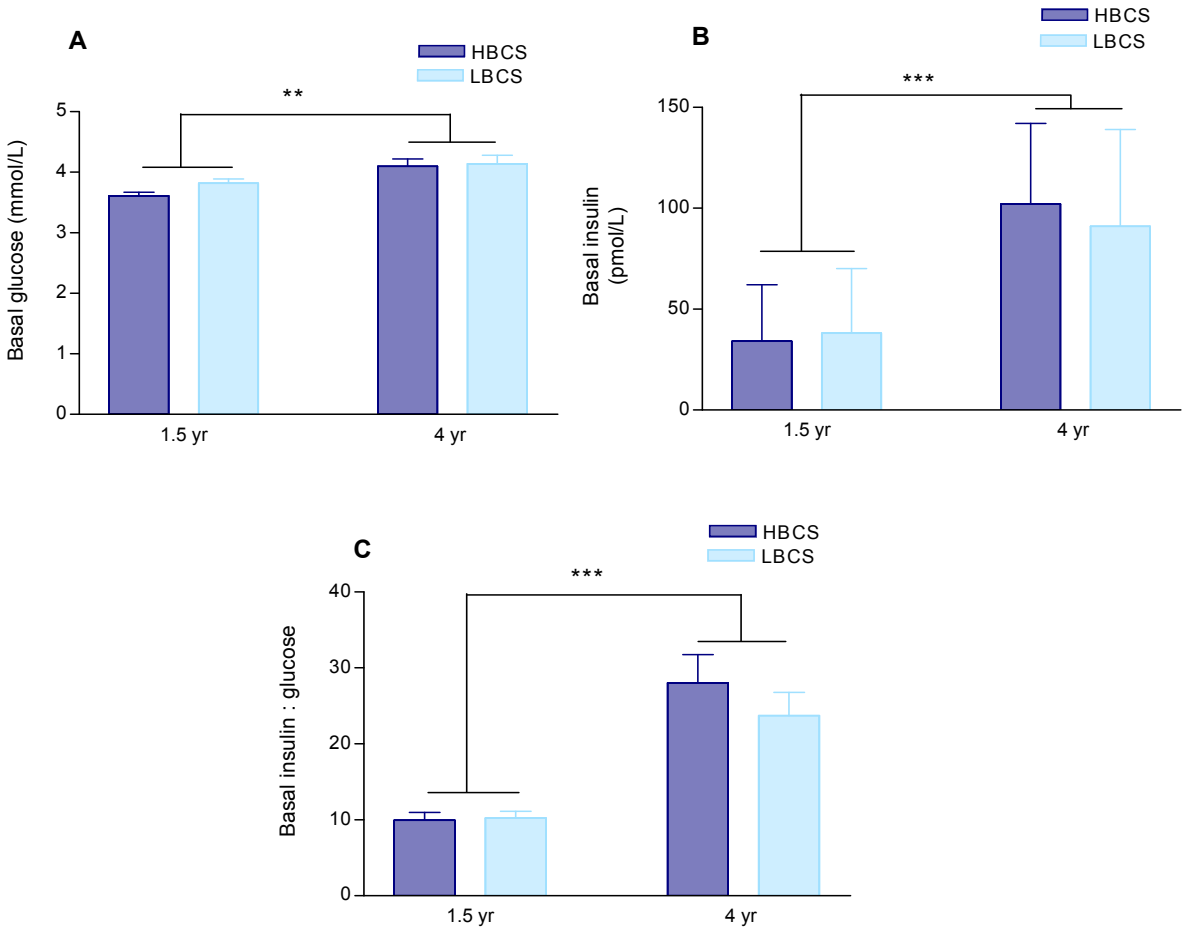


Figure 5-6: Changes with age in basal plasma glucose and insulin in adult sheep. (a) basal glucose, (b) basal insulin, and (c) basal insulin : basal glucose in 4 year old offspring from high body condition score (HBCS, *n* = 14) and low body condition score (LBCS, *n* = 10) ewes, ** $p < 0.01$, *** $p < 0.001$. Data are shown as the mean ± SEM or the geometric mean (95 % CI, basal insulin) and a repeated measures ANOVA was used.

5.5.4.2 Glucose AUC comparisons

The previous study at 1.5 years included baseline levels when calculating AUC, but the results in section 5.5.3.2 are minus baseline. For the purposes of comparison, two sets of analysis between the years have therefore been performed – those with baseline and those without.

There was a significant decrease with age in glucose AUC ($p < 0.01$, Figure 5.7a) that was not affected by nutritional group. There was no effect of age on glucose AUC with baseline (Figure 5.7b).

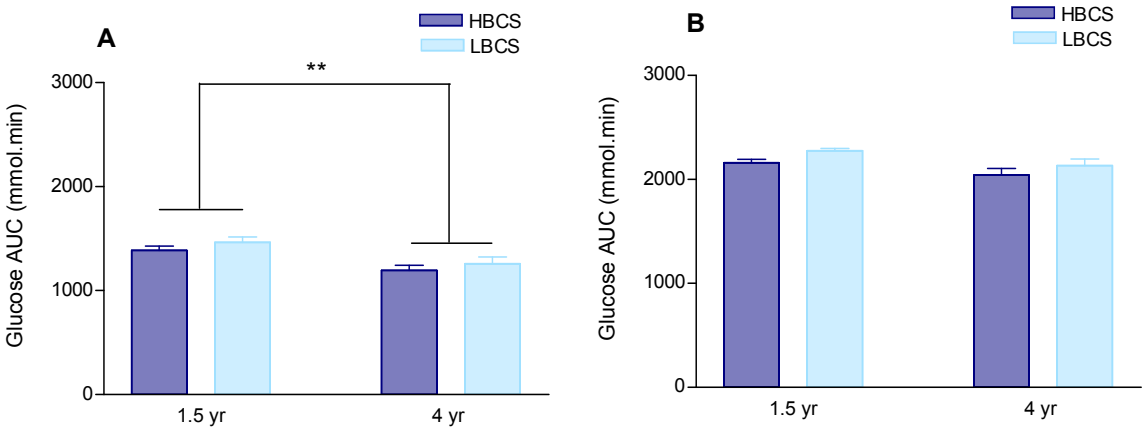


Figure 5-7: Changes with age in glucose AUC during a glucose tolerance test in adult sheep. (a) glucose AUC without baseline and (b) glucose AUC with baseline glucose in 4 year old offspring from high body condition score (HBCS, *n* = 14) and low body condition score (LBCS, *n* = 10) ewes, ** $p < 0.01$. Data are shown as the mean ± SEM and a repeated measures ANOVA was used.

5.5.4.3 Insulin AUC comparisons

Total insulin AUC cannot be compared due to sampling differences between the years – at 1.5 years the final sample was taken at 120 minutes and at 4 years the final sample was taken at 210 minutes, however the 120 minute sample at 4 years was not analysed for insulin and so the 4 year data cannot simply be truncated at this time point. Instead, insulin : glucose AUC and initial insulin response were compared between the two ages. At 1.5 years of age initial insulin AUC were calculated with the inclusion of basal insulin levels. For the purposes of comparison between the different ages two sets of analysis have therefore been performed - those with baseline and those without.

5.5.4.3.1 Insulin : Glucose AUC

There was a trend for an increase with age in insulin : glucose AUC ($p < 0.1$, Figure 5.8) that was not affected by nutritional group.

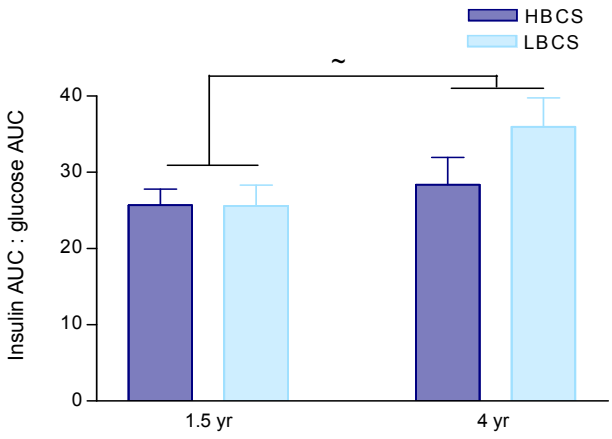


Figure 5-8: Changes with age in insulin : glucose AUC during a glucose tolerance test in adult sheep. Insulin : glucose AUC in 4 year old offspring from high body condition score (HBCS, *n* = 14) and low body condition score (LBCS, *n* = 10) ewes ~ $p < 0.1$. Data are shown as the mean ± SEM and a repeated measures ANOVA was used.

5.5.4.4 Initial insulin response

There was a significant decrease with age in initial insulin response ($p < 0.01$, Figure 5.9a) that was not affected by nutritional group. There was no effect of age on initial insulin response with baseline (Figure 5.9b).

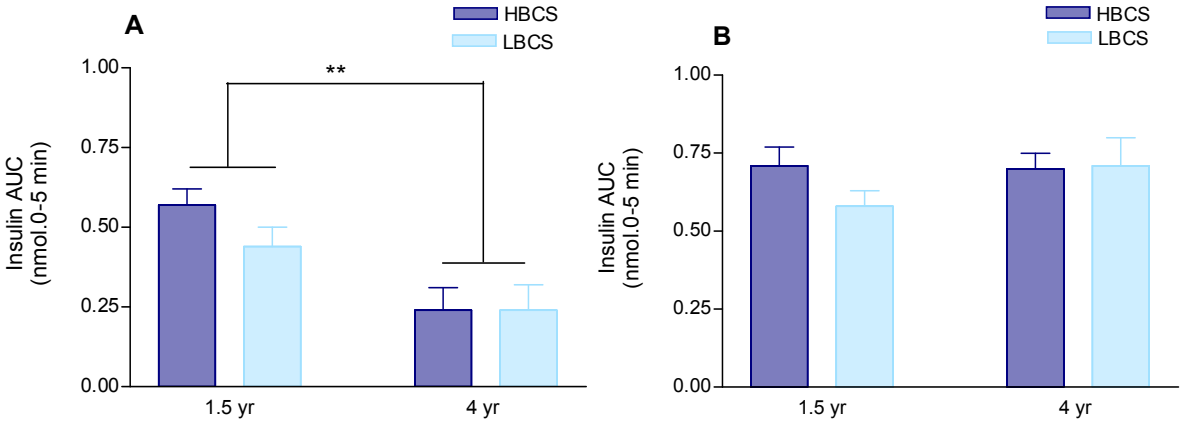


Figure 5-9: Changes with age in initial insulin response to a glucose tolerance test in adult sheep. (a) insulin response minus baseline and (b) insulin response with baseline in 4 year old offspring from high body condition score (HBCS, *n* = 14) and low body condition score (LBCS, *n* = 10) ewes, ** p <0.01. Data are shown as the mean ± SEM and a repeated measures ANOVA was used

5.5.5 Pancreatic insulin

There were no differences between groups in pancreatic insulin (Figure 5.10) and pancreatic insulin was not correlated to any of the GTT outcomes (Table 5.7).

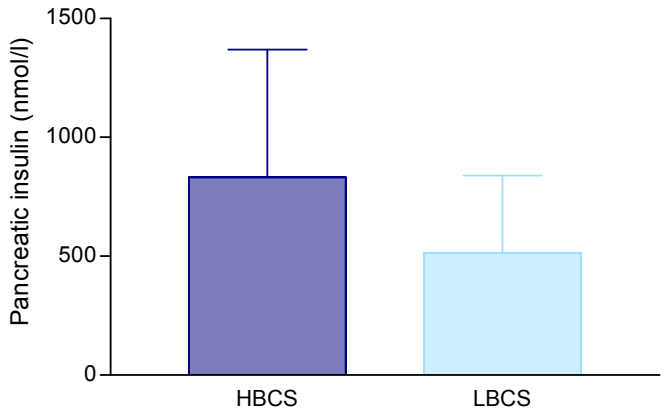


Figure 5-10: Pancreatic insulin levels in mature adult sheep. Amount of pancreatic insulin in 4 year old offspring from high body condition score (HBCS, *n* = 14) and low body condition score (LBCS, *n* = 14) ewes. Data are shown as the geometric mean (95 % CI) and an independent samples t-test was used.

	Pancreatic insulin
Glucose AUC	0.021
Insulin AUC	0.008
Basal glucose	0.001
Basal insulin	0.010
Peak glucose	0.004
Peak insulin	0.076

Table 5-7: The correlation of glucose tolerance and pancreatic insulin levels in mature adult sheep. The GTT outcomes were correlated against pancreatic insulin levels in 4 year old rams. Data are correlation coefficients r^2.

5.5.6 Glucose uptake into isolated muscle strips

5.5.6.1 Insulin concentration

Muscle strips from the three muscle beds (soleus, vastus and gastrocnemius) from two animals were incubated with either 16 nM or 32 nM insulin and in all three muscle beds insulin-stimulated glucose uptake was similar with either concentration (Figure 5.11). Thus, subsequent glucose uptake incubations were with 16 nM insulin only as this was sufficient to produce a maximal response.

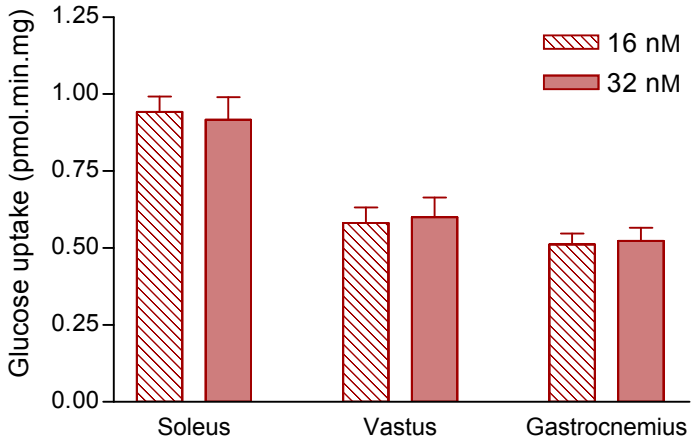


Figure 5-11: Effect of different insulin concentrations on [^{3}H] methylglucose uptake into isolated muscle strips of mature adult sheep. Insulin-stimulated glucose uptake following incubation with 16 nM or 32 nM insulin in soleus (n = 2), vastus (n = 2) and gastrocnemius (n = 2) muscle strips of 4 year old rams. Data are shown as the mean ± SEM and an independent samples t-test was used.

5.5.6.2 Time-course incubation

Muscle strips from two animals were incubated for 5, 10 or 15 minutes in the presence of 16 nM insulin. Glucose uptake increased over time in all three muscle beds (Figure 5.12). Thus, all subsequent glucose uptake incubations were for 10 minutes only as it was established that glucose uptake was still increasing at this time point and a plateu in uptake would not be reached.

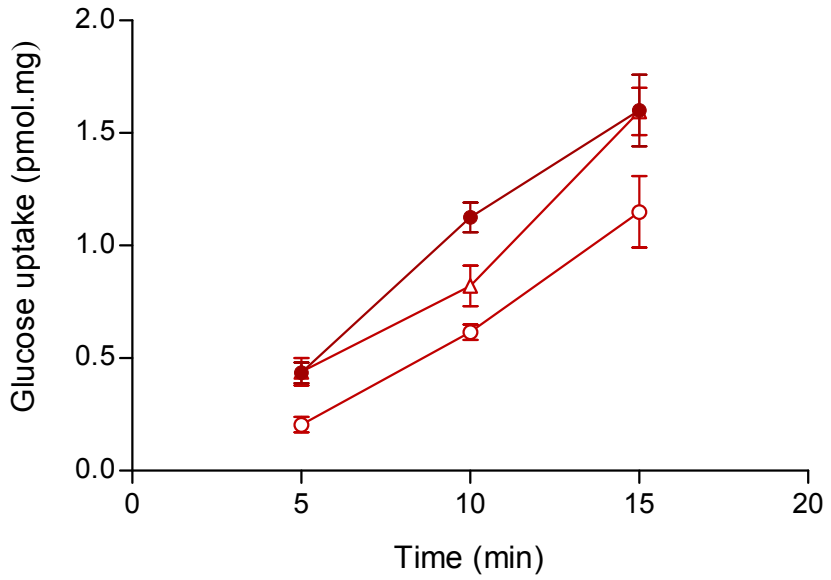


Figure 5-12: Uptake of [^{3}H] methylglucose over time into isolated muscle strips of mature adult sheep. Insulin-stimulated glucose uptake into soleus (● n = 2), vastus (○ n = 2) and gastrocnemius (Δ n = 2) muscle strips of 4 year old rams over a period of 15 minutes. Data are shown as the mean ± SEM.

5.5.6.3 Basal glucose uptake

In all three muscle beds there was no difference in basal glucose uptake between the nutritional groups (Figure 5.13). However, basal glucose uptake was found to be greater in the soleus compared to the gastrocnemius and vastus muscles ($p < 0.001$, Figure 5.14).

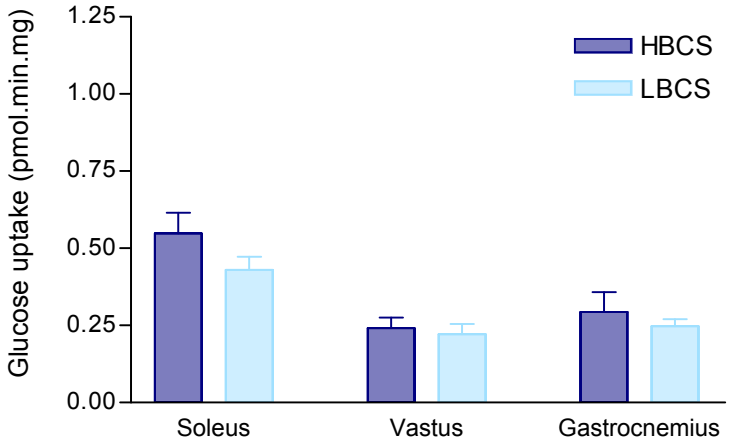


Figure 5-13: Basal uptake of [^{3}H] methylglucose into isolated muscle strips of mature adult sheep, by muscle bed and nutritional group. Basal and insulin stimulated glucose uptake into soleus, vastus and gastrocnemius muscle strips of 4 year old offspring from high body condition score (HBCS, $n = 10$) and low body condition score (LBCS, $n = 7$) ewes. Data are shown as the mean ± SEM and an independent samples t-test was used.

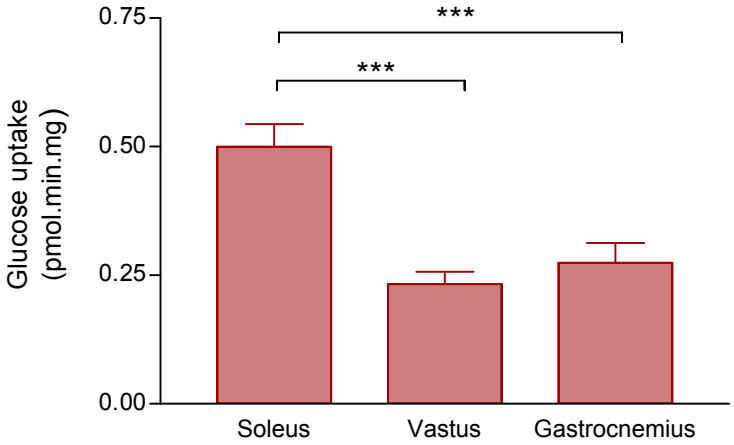


Figure 5-14: Basal uptake of [^{3}H] methylglucose into isolated muscle strips of mature adult sheep, by muscle bed. Basal and insulin stimulated glucose uptake into soleus ($n = 17$), vastus ($n = 17$), and gastrocnemius ($n = 17$) muscle strips of 4 year old rams, *** $p < 0.001$. Data are shown as the mean ± SEM and an independent samples t-test was used.

5.5.6.4 Insulin stimulated glucose uptake

Insulin increased glucose uptake in all three muscles beds ($p < 0.001$, Figure 5.15). In the soleus muscle insulin-stimulated glucose uptake tended to be lower in the LBCS as compared to HBCS offspring ($p < 0.1$), but there were no differences between the nutritional groups in the vastus and gastrocnemius muscles (Figure 5.16).

Insulin-stimulated uptake was greater in the soleus muscle than in the vastus and gastrocnemius ($p < 0.01$, Figure 5.17).

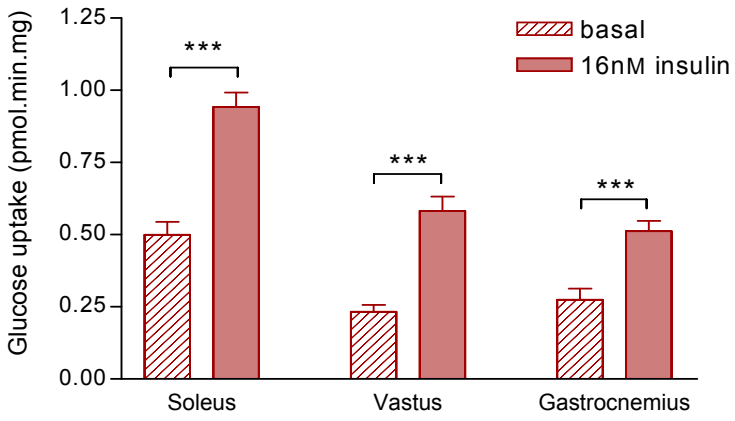


Figure 5-15: Basal and insulin stimulated [^{3}H] methylglucose uptake into isolated muscle strips of mature adult sheep, by muscle bed. Basal and insulin stimulated glucose uptake into the soleus (n = 17), vastus (n = 17) and gastrocnemius (n = 17) muscle strips of 4 year old rams, *** $p < 0.001$. Data are shown as the mean ± SEM and an independent samples t-test was used.

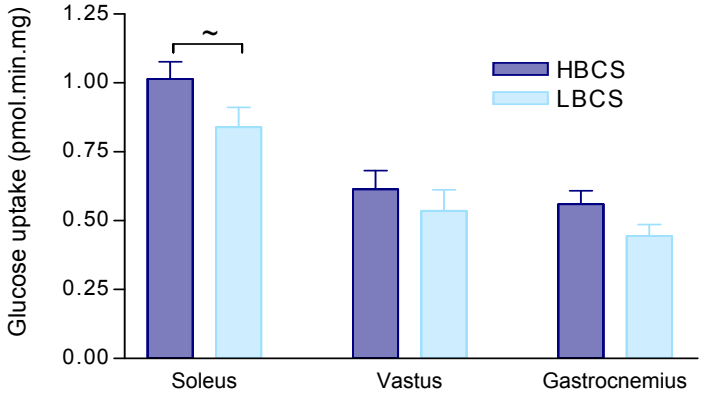


Figure 5-16: Insulin-stimulated [^{3}H] methylglucose uptake into isolated muscle strips of mature adult sheep, by muscle bed and nutritional group. Insulin-stimulated glucose uptake into soleus, vastus and gastrocnemius muscle strips of 4 year old offspring from high body condition score (HBCS, n = 10) and low body condition score (LBCS, n = 7) ewes, ~ $p < 0.1$. Data are shown as the mean ± SEM and an independent samples t-test was used.

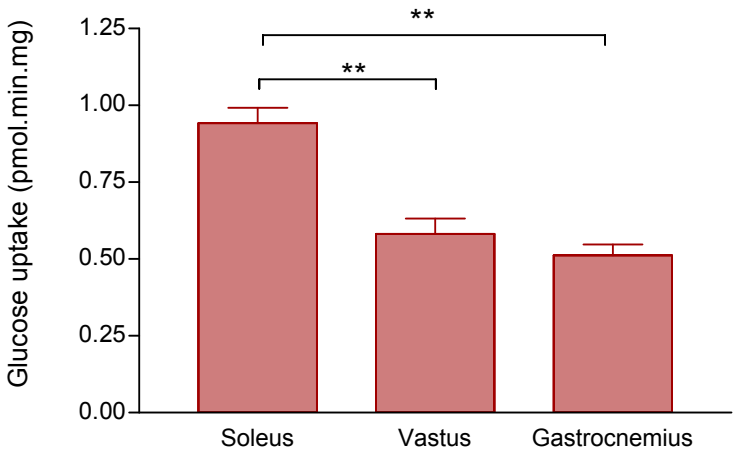


Figure 5-17: Insulin-stimulated [^{3}H] methylglucose uptake into isolated muscle strips of mature adult sheep, by muscle bed. Insulin-stimulated glucose uptake into soleus (n = 17), vastus (n = 17) and gastrocnemius (n = 17) muscle strips of 4 year old rams, ** $p < 0.01$. Data are shown as the mean ± SEM and an independent samples t-test was used.

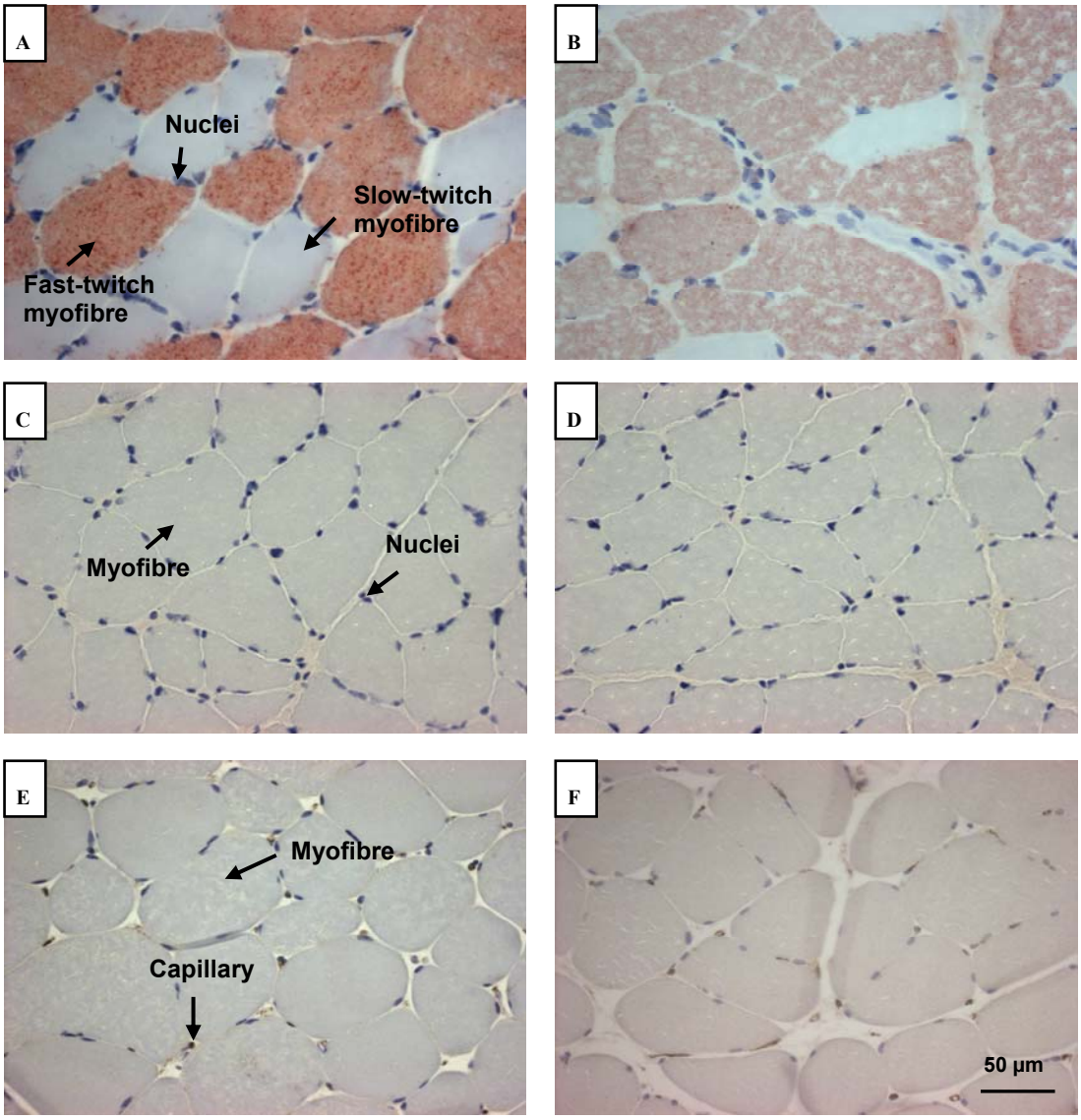


Figure 5-18: Representative counting fields of the vastus and soleus muscle in mature adult sheep from each nutritional group. Slides were immunostained to visualise myofibres and capillaries in the soleus and vastus muscles of 4 year old offspring from high body condition score (HBCS) and low body condition score (LBCS) ewes, (a) myofibre staining in vastus of HBCS offspring (b) myofibre staining in vastus of LBCS offspring (c) myofibre staining in soleus of HBCS offspring (d) myofibre staining in soleus of LBCS offspring (e) capillary staining in vastus of HBCS offspring and, (f) capillary staining in vastus of LBCS offspring at x 40 objective.

5.5.7 Myofibre density

5.5.7.1 Vastus

There was a reduction in total myofibre density in the LBCS compared to HBCS offspring ($p < 0.05$, Figure 5.19a). Fast fibre density was reduced in LBCS compared to HBCS offspring ($p < 0.05$, Figure 5.19b). Slow fibre density tended to be lower in LBCS compared to HBCS offspring ($p < 0.1$, Figure 5.19c).

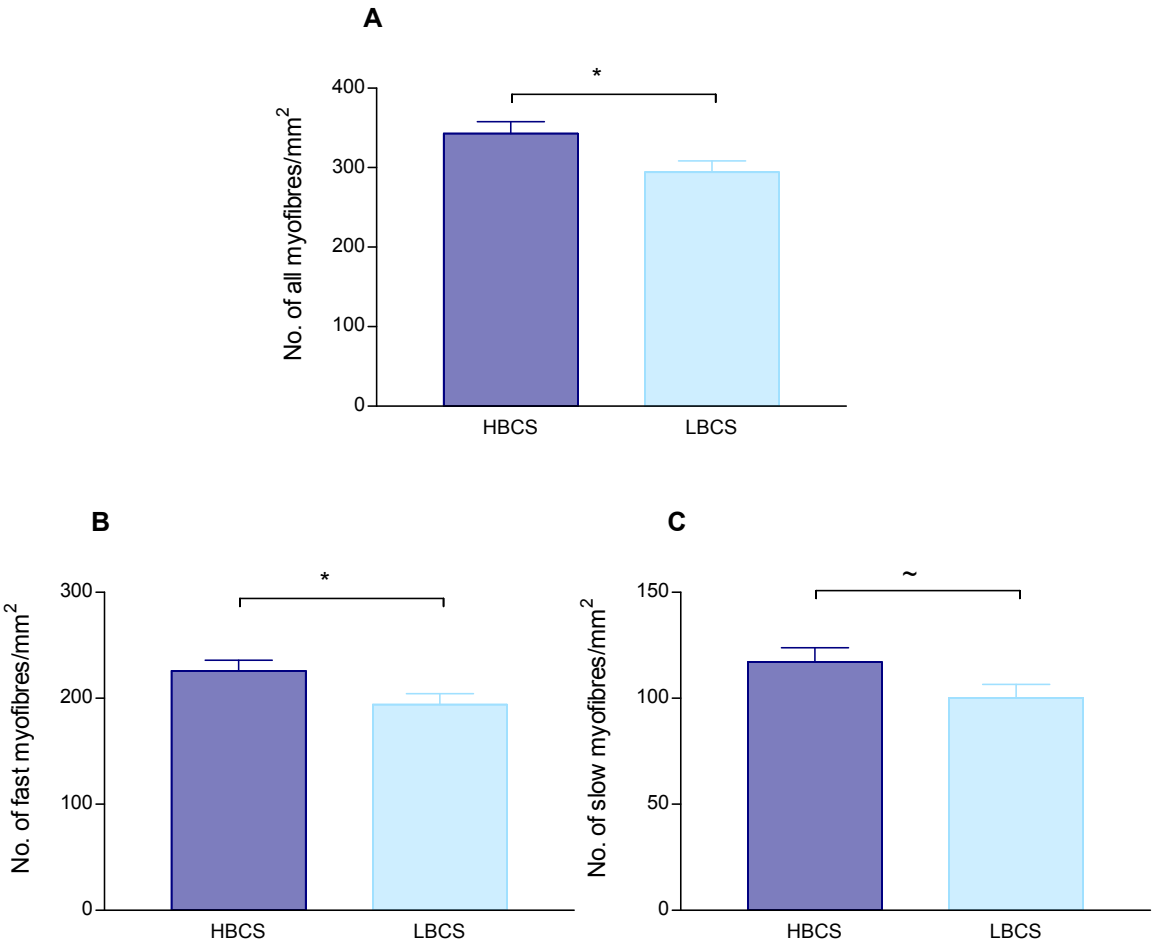


Figure 5-19: Myofibre density in the vastus muscle of mature adult sheep. Density of (a) all myofibres, (b) fast-twitch fibres and (c) slow-twitch fibres in 4 year old offspring from high body condition score (HBCS, $n = 14$) and low body condition score (LBCS, $n = 14$) ewes, *$p < 0.05$; ~$p < 0.1$. Data are shown as the mean ± SEM and an independent samples t-test was used.

When averaging from as many fibres as possible in the 10 captured fields there was no difference between groups in fast-fibre CSA (Figure 5.20a) but there was an increase in slow-fibre CSA in LBCS as compared to HBCS offspring ($p < 0.05$, Figure 5.20b).

However, using this approach there was a big range in the number of fibres analysed (41-131 fast myofibres and 24-98 slow myofibres). When a cut-off number of 41 fibres for fast-twitch and 24 for slow-twitch (lowest number obtained from one muscle sample) was used to obtain the average CSA there were no differences between the groups for fast-fibre (Figure 5.20c) or slow-fibre (Figure 5.20d) CSA.

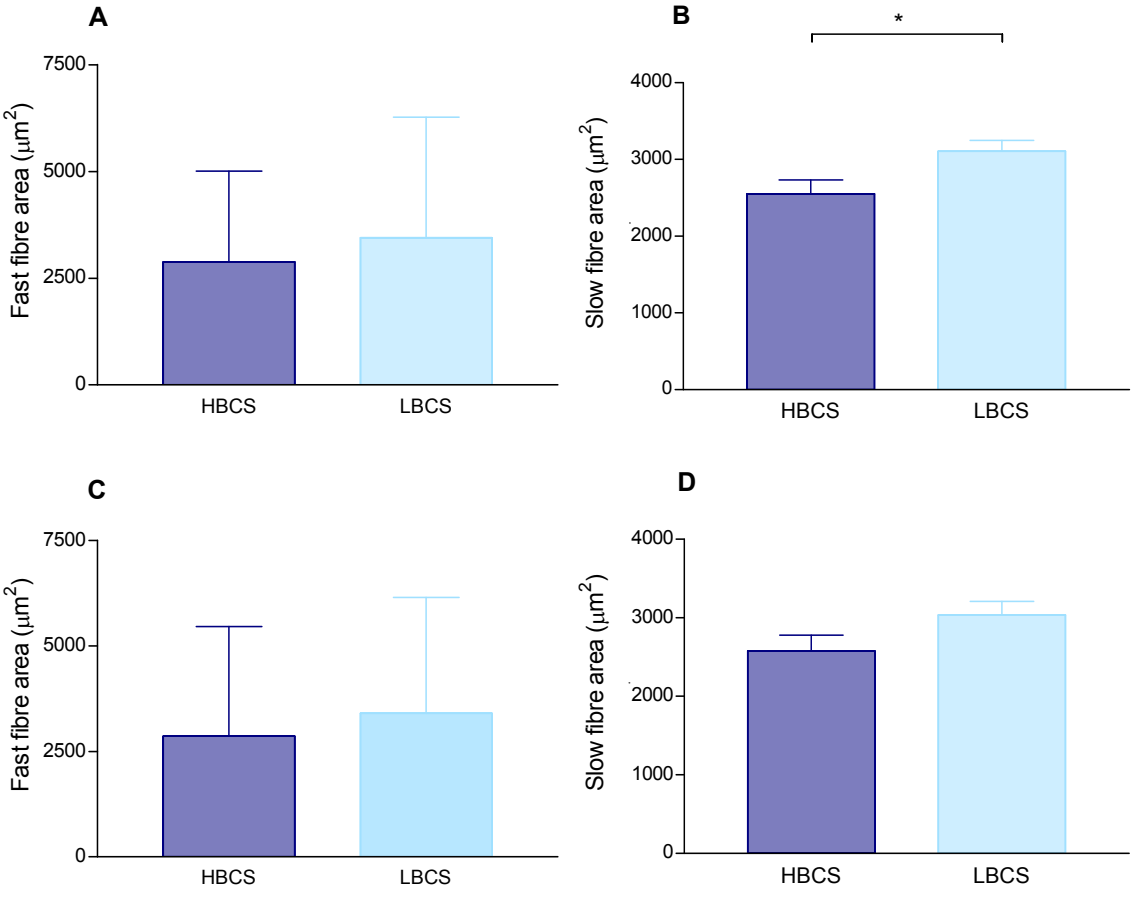


Figure 5-20: Cross-sectional area of myofibres in the vastus muscle of mature adult sheep. Average CSA of (a) fast-fibre CSA from 10 fields, (b) slow-fibre CSA from 10 fields, (c) fast-fibre CSA from 41 fibres and (d) slow-fibre CSA from 24 fibres in 4 year old offspring from high body condition score (HBCS, $n = 14$) and low body condition score (LBCS, $n = 14$) ewes, *$p < 0.05$. Data are shown as the mean ± SEM or geometric mean (95 % CI; Figures a & c) and an independent samples t-test was used.

5.5.7.2 Soleus

There was no difference between the groups in myofibre density (Figure 5.21a) or myofibre CSA (5.21b). Soleus myofibre density was not related to weight (Figure 5.21c) or circumference (Figure 5.21d).

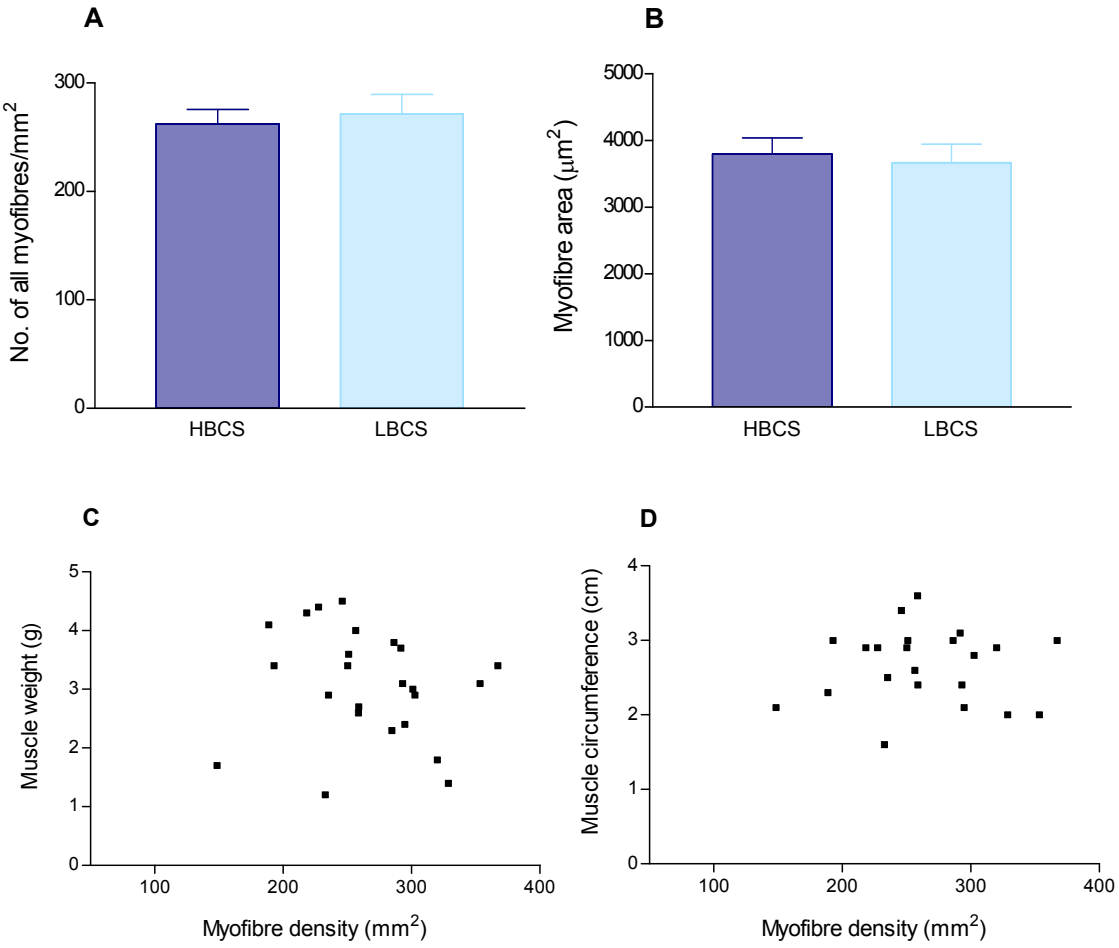


Figure 5-21: Myofibre density, cross-sectional area of myofibres and biometry of the soleus muscle in mature adult sheep. (a) myofibre density and (b) myofibre CSA in 4 year old offspring from high body condition score (HBCS, *n* = 14) and low body condition score (LBCS, *n* = 14) ewes (c) correlation of muscle weight and myofibre density and, (d) correlation of muscle circumference and myofibre density. Data are shown as the mean ± SEM and an independent samples t-test or linear regression was used.

5.5.8 Capillary density

5.5.8.1 Vastus

Capillary density tended to be reduced in the LBCS compared to HBCS offspring ($p < 0.1$, Figure 5.22a). The capillary : myofibre ratio was significantly reduced in LBCS compared to HBCS offspring ($p < 0.05$, Figure 5.22b).

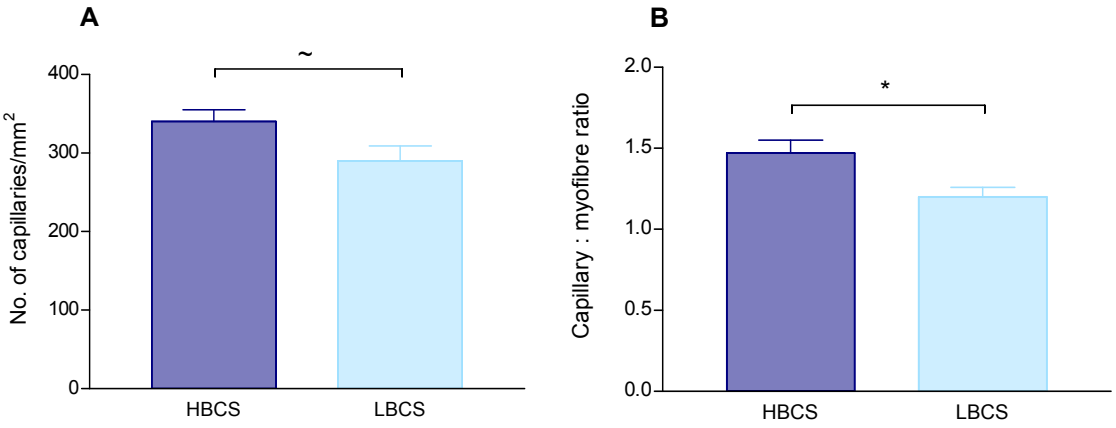


Figure 5-22: Capillary density and capillary : myofibre ratio in the vastus muscle of mature adult sheep. (a) capillary density and (b) capillary : myofibre ratio in 4 year old offspring from high body condition score (HBCS, *n* = 14) and low body condition score (LBCS, *n* = 10) ewes, *$p < 0.05$; ~$p < 0.1$. Data are shown as the mean ± SEM and an independent samples t-test was used.

5.5.9 Muscle composition and glucose tolerance

Muscle composition was not related to any of the GTT outcomes at 4 yr, except for insulin AUC which tended to be positively related to soleus myofibre density ($p < 0.1$). There were no correlations between muscle composition and glucose uptake into isolated muscle strips (Table 5.8). Vastus myofibre density was negatively related ($p < 0.05$) and vastus fast fibre density tended to be negatively related to basal glucose at 4 yr ($p < 0.1$). Vastus slow fibre density tended to be positively related to initial insulin release at 1.5 yr ($p < 0.1$).

	Basal glucose	Basal insulin	Glucose AUC	Insulin AUC	Peak glucose	Peak insulin	Initial insulin release	1st phase insulin release	2nd phase insulin release	Basal glucose uptake (strip)	Insulin-stimulated glucose uptake (strip)
1.5 yr											
Vastus total myofibre density	-0.175 *	0.005	0.007	0.010	0.086	0.030	0.074	/	/	/	/
Vastus fast fibre density	-0.160 ~	0.006	0.008	0.007	0.098	0.013	0.030	/	/	/	/
Vastus slow fibre density	0.112	0.002	0.001	0.008	0.033	0..050	0.126 ~	/	/	/	/
Soleus total myofibre density	0.032	0.007	0.147	0.001	0.005	0.016	0.019	/	/	/	/
4 yr											
Vastus total myofibre density	0.046	0.056	0.001	0.003	0.003	0.000	0.109	0.072	0.002	0.045	0.013
Vastus fast fibre density	0.093	0.068	0.002	0.000	0.012	0.000	0.087	0.082	0.064	0.029	0.002
Vastus slow fibre density	0.001	0.020	0.020	0.029	0.087	0.002	0.088	0.028	0.117	0.054	0.037
Soleus total myofibre density	0.020	0.031	0.002	0.123 ~	0.007	0.031	0.010	0.008	0.077	0.000	0.000

Table 5-8: The correlation of myofibre density of the soleus and triceps brachii muscle to outcomes of the glucose tolerance test and glucose uptake into isolated muscle strips from adult sheep. The myofibre density of the soleus and triceps muscle were correlated against outcomes of the glucose tolerance test from 1.5 and 4 year old rams and glucose uptake into isolated muscle strips from 4 year old rams only, ~ $p < 0.1$, * $p < 0.05$. Data are correlation coefficients.

5.5.10 Insulin signalling pathway

Protein levels of Akt1 were lower ($p < 0.05$) and IGF-IR levels tended to be lower ($p < 0.1$) in the vastus muscle of LBCS offspring. GLUT-4 protein levels were increased in the vastus muscle of LBCS offspring ($p < 0.001$, Figure 5.23). Protein levels of Akt1 tended to be reduced in the abdominal fat of LBCS offspring ($p < 0.1$, Figure 5.24).

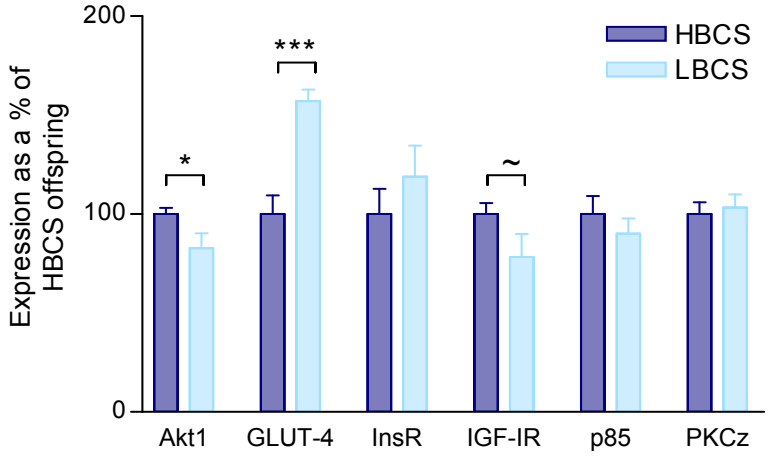


Figure 5-23: Expression of proteins from the insulin signalling pathway in the vastus muscle of mature adult sheep. Expression of Akt1, GLUT-4, InsR, IGF-IR, p85 and PKCζ in 4 year old offspring from high body condition score (HBCS, *n* = 14) and low body condition score (LBCS, *n* = 10) ewes, ~p < 0.1, *p < 0.05, *** p < 0.001. Data are shown as the mean ± SEM and an independent samples t-test was used.

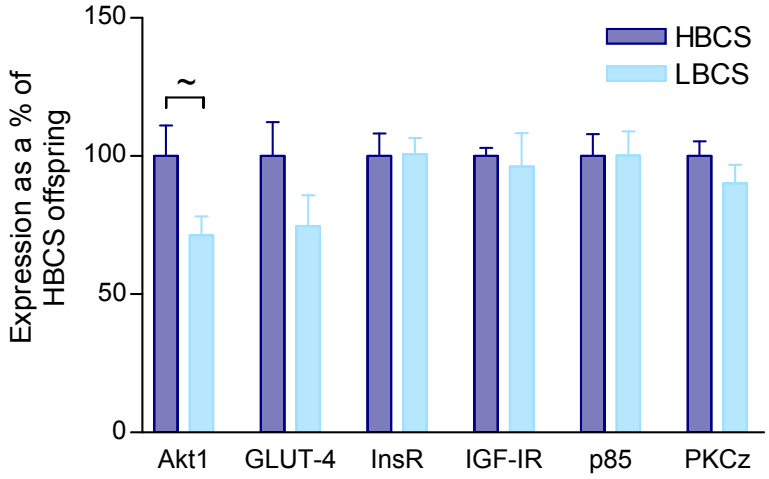


Figure 5-24: Expression of proteins from the insulin signalling pathway in the abdominal fat of mature adult sheep. Expression of Akt1, GLUT-4, InsR, IGF-IR, p85 and PKCζ in 4 year old offspring from high body condition score (HBCS, *n* = 14) and low body condition score (LBCS, *n* = 10) ewes, ~p < 0.1. Data are shown as the mean ± SEM and an independent samples t-test was used.

5.5.11 Vastus muscle insulin signalling proteins, muscle morphology and glucose tolerance

Levels of proteins (arbitrary values) in the insulin signalling pathway from the vastus muscle were correlated against GTT outcomes, muscle morphology and glucose into isolated vastus strips (Table 5.9). Akt1 was positively related to vastus slow fibre density ($p < 0.05$) and Akt1 was negatively related to second phase insulin release ($p < 0.05$) and tended to be negatively related to insulin AUC ($p < 0.1$) and peak glucose ($p < 0.1$). GLUT-4 was negatively related to total myofibre density ($p < 0.05$) and fast fibre density ($p < 0.01$). The p85 subunit of PI 3-kinase was positively related to glucose AUC ($p < 0.05$).

	Basal glucose	Basal insulin	Glucose AUC	Insulin AUC	Peak glucose	Peak insulin	Initial insulin release	1st phase insulin release	2nd phase insulin release	Basal glucose uptake (muscle)	Stimulated glucose uptake (muscle)	Myofibre density	Fast fibre density	Slow fibre density
Akt1	0.063	0.060	0.002	- 0.153 ~	- 0.119 ~	0.040	0.004	0.019	- 0.201 *	0.046	0.018	0.042	0.000	0.230 *
GLUT-4	0.012	0.008	0.031	0.019	0.020	0.000	0.049	0.035	0.015	0.007	0.013	- 0.227 *	- 0.277 *	0.072
InsR	0.014	0.015	0.018	0.000	0.000	0.017	0.013	0.054	0.006	0.017	0.024	0.002	0.000	0.018
IGF-IR	0.005	0.010	0.000	0.096	0.007	0.010	0.005	0.001	0.097	0.005	0.024	0.001	0.000	0.012
p85	0.049	0.000	0.204 *	0.014	0.044	0.000	0.017	0.004	0.010	0.004	0.025	0.000	0.005	0.007
PKCζ	0.000	0.003	0.053	0.094	0.070	0.017	0.033	0.017	0.091	0.069	0.042	0.006	0.016	0.000

Table 5-9: The correlation of myofibre density of the triceps brachii muscle, outcomes of the glucose tolerance test and glucose uptake into isolated muscle strips to expression of components of the insulin signalling pathway from adult sheep. The myofibre density of the triceps muscle and outcomes of the glucose tolerance test and glucose uptake into the isolated strips was correlated against protein levels of of Akt1, GLUT-4, InsR, IGF-IR, p85 and PKCζ in the triceps muscle of 4 year old rams, $p < 0.1$, * $p < 0.05$, ** $p < 0.01$. Data are correlation coefficients r^2.

5.5.12 Ewe gestational energy intake

Average daily energy intake (allowing for food refusals) was calculated per ewe for each week of gestation. The average daily intake was split into three periods; early intake (0-28 dGA HBCS, 11.0 ± 0.4; LBCS, 3.7 ± 0.3 MJ/day), late intake (105-140 dGA. HBCS, 17.6 ± 1.1; LBCS, 9.1 ± 0.4 MJ/day) and overall intake (0 to 140 dGA. HBCS, 15.5 ± 0.5; LBCS, 7.3 ± 0.3 MJ/day) and these were correlated against the 4 yr GTT data and muscle morphology (Table 5.10).

Early and late energy intakes were not related to any of the GTT outcomes. Total energy intake was negatively related to first ($p < 0.05$) and second phase ($p < 0.05$) insulin release, and tended to be negatively related to insulin AUC ($p < 0.1$) and peak insulin ($p < 0.1$). Early energy intake was positively related to vastus total fibre and fast fibre density ($p < 0.05$). Late energy intake tended to be positively related with vastus fast fibre density ($p < 0.1$). Total energy intake tended to be positively related to vastus total fibre and fast fibre density ($p < 0.1$).

	Early intake	Late intake	Overall intake
Glucose AUC	0.015	0.018	0.061
Insulin AUC	0.032	0.050	- 0.161 ~
Basal glucose	0.000	0.005	0.000
Basal insulin	0.036	0.000	0.025
Peak glucose	0.000	0.012	0.068
Peak insulin	0.017	0.092	- 0.136 ~
Initial insulin release	0.030	0.079	0.069
First phase insulin release	0.065	0.090	- 0.169 *
Second phase insulin release	0.050	0.044	- 0.182 *
Pancreatic insulin	0.020	0.002	0.002
Vastus total myofibre density	0.188 *	0.092	0.144~
Vastus fast density	0.225*	0.127 ~	0.141~
Vastus slow density	0.063	0.019	0.080
Soleus myofibre density	0.030	0.001	0.045

Table 5-10: The correlation of ewe gestational energy intake to skeletal muscle morphology and outcomes of the glucose tolerance test in mature adult sheep offspring. The average ewe gestational energy intake was split into periods of early gestation (0-28 dGA), late gestation (105 – 140 dGA) and overall gestation and these periods were correlated against myofibre density of the soleus and triceps brachii muscles and outcomes of the GTT in 4 year old offspring, $p < 0.1$, * $p < 0.05$. Data are correlation coefficients r^2.

5.5.13 Ewe gestational weight gain

Ewe weight was recorded prior to pregnancy and for each week of gestation. Ewe weight gain was split into three periods; early weight gain (0-28 dGA. HBCS, 3.5 ± 0.4; LBCS, 1.3 ± 0.4 kg), late weight gain (105-140 dGA. HBCS, 5.2 ± 0.8; LBCS 1.7 ± 0.8 kg) and total weight gain (0-140 dGA. HBCS, 19.7 ± 1.0; LBCS 8.4 ± 0.7 kg) and these were correlated against the 4 yr GTT data and muscle morphology (Table 5.11).

Early weight gain was not related to any of the GTT outcomes. Late weight gain tended to be negatively related to second phase insulin release ($p < 0.1$) and total weight gain tended to be negatively related to insulin AUC ($p < 0.1$) and second phase insulin release ($p < 0.1$).

Early weight gain tended to be positively related vastus slow-fibre density ($p < 0.1$). Late weight gain tended to be positively related to vastus slow-fibre density ($p < 0.1$). Total weight gain tended to be related to vastus total myofibre and slow-fibre density ($p < 0.1$).

	Early weight gain	Late weight gain	Overall weight gain
Glucose AUC	0.000	0.000	0.027
Insulin AUC	0.043	0.104	- 0.128 ~
Basal glucose	0.109	0.104	0.067
Basal insulin	0.009	0.003	0.009
Peak glucose	0.052	0.002	0.008
Peak insulin	0.005	0.001	0.014
Initial insulin release	0.012	0.069	0.000
First phase insulin release	0.012	0.000	0.023
Second phase insulin release	0.083	- 0.136 ~	- 0.151 ~
Pancreatic insulin	0.099	0.084	0.061
Vastus total myofibre density	0.075	0.074	0.145 ~
Vastus fast density	0.030	0.025	0.091
Vastus slow density	0.130 ~	0.140 ~	0.161 ~
Soleus myofibre density	0.000	0.040	0.018

Table 5-11: The correlation of ewe gestational weight gain to skeletal muscle morphology and outcomes of the glucose tolerance test in mature adult sheep offspring. The average ewe gestational weight gain was split into periods of early gestation (0-28 dGA), late gestation (105 – 140 dGA) and overall gestation and these periods were correlated against myofibre density of the soleus and triceps brachii muscles and outcomes of the GTT in 4 year old offspring, ~ $p < 0.1$. Data are correlation coefficients r^2.

5.5.14 Postnatal growth and GTT

Postnatal growth rate was split into three periods; suckling (0-12 weeks. HBCS, 29.6 ± 0.8; LBCS, 29.3 ± 0.7 kg), weaning to young adulthood (12 weeks – 1.5 years. HBCS, 35.0 ± 1.3; LBCS, 34.0 ± 1.7 kg) and young adulthood to mature adulthood (1.5 years – 2.5 years. HBCS, 14.2 ± 1.6; LBCS, 16.9 ± 1.7 kg) and at 2.5 years they were considered fully grown. These were correlated against the 4 year GTT and muscle morphology data (Table 5.12).

Weight gain during suckling was negatively related to basal glucose ($p < 0.05$). Weight gain from 12 weeks to 1.5 yr was positively related to peak glucose ($p < 0.05$) and tended to be positively related to peak insulin ($p < 0.1$).

	0- 12 weeks	**12 weeks - 1.5 yr**	**1.5 – 2.5 yr**
Glucose AUC	0.014	0.002	0.001
Insulin AUC	0.104	0.057	0.006
Basal glucose	- 0.197 *	0.003	0.008
Basal insulin	0.023	0.004	0.003
Peak glucose	0.022	0.175 *	0.030
Peak insulin	0.051	0.119 ~	0.077
Initial insulin release	0.006	0.027	0.007
First phase insulin release	0.000	0.000	0.092
Second phase insulin release	0.110	0.062	0.012
Pancreatic insulin	0.035	0.001	0.044
Vastus total myofibre density	0.036	0.112	0.007
Vastus fast density	0.040	0.014	0.017
Vastus slow density	0.015	0.032	0.000
Soleus myofibre density	0.001	0.013	0.013

Table 5-12: The correlation of postnatal growth to skeletal muscle morphology and outcomes of the glucose tolerance test in mature adult sheep offspring. The postnatal growth rates of the rams were split into periods of suckling (0 – 12 weeks), weaning to young adulthood (12 weeks – 1.5 yrs) and young adulthood to mature adulthood (1.5 yrs – 2.5 yrs) and these periods were correlated against myofibre density of the soleus and triceps brachii muscles and outcomes of the GTT from the animals at 4 years of age, ~ $p < 0.1$. Data are correlation coefficients r^2.

5.6 Discussion

This chapter has shown that there is no effect of reduced maternal diet and body condition on the glucose tolerance of mature adult offspring. However, the glucose tolerance of the offspring changed with age with an increased basal glucose, basal insulin and worsened initial insulin response, along with decreased glucose AUC between 1.5 and 4 yrs and this was associated with an increase in body weight, BCS and back fat depth. A reduced maternal diet and body condition decreased myofibre and capillary density in the vastus muscle of the adult offspring and this was associated with a decrease in Akt1 and IGF-IR expression.. However, glucose tolerance and uptake into isolated muscle strips was not affected by muscle composition and thus the impact of reduced myofibre density may have been offset in part by increased GLUT-4.

5.6.1 Glucose tolerance

5.6.1.1 Effect of maternal body condition and diet

Maternal body condition did not have any effect on basal glucose or insulin levels at 4 years of age. This is consistent with data from the Dutch winter famine where the exposed population had similar basal glucose and insulin levels, as compared to the unexposed population at age 50 (Ravelli *et al.*, 1998) and 58 (de Rooij *et al.*, 2006). In other sheep studies there were no differences in basal levels in postnatal offspring following an early (Poore *et al.*, 2007;Gardner *et al.*, 2005) mid- (Ford *et al.*, 2007) or late gestation (Gardner *et al.*, 2005) nutrient restriction. However, in rats an increased basal glucose (Ozanne *et al.*, 2003) and insulin (Fernandez-Twinn *et al.*, 2005) have been observed following a low protein diet throughout pregnancy and lactation. Higher basal glucose was also observed in young Danish low-birth-weight men (Jensen *et al.*, 2002), and a low body mass index in early and late pregnancy was associated with elevated plasma glucose and insulin in adult offspring (Mi *et al.*, 2000). In humans, a high fasting level of glucose is considered indicative of diabetes (Alberti & Zimmet, 1998). Hyperinsulinemia in the basal state has been attributed to both hypersecretion (Ferrannini *et al.*, 1997) and decreased hepatic insulin clearance (Rossell *et al.*, 1983).

Plasma glucose and insulin levels increased after the addition of the glucose bolus but there were no differences in AUC or peak values between the nutritional groups. Other

studies have found changes in glucose tolerance following gestational nutrient restriction. Glucose tolerance (increased levels 2 h after a standard glucose bolus) was reduced among men and women exposed to undernutrition during late gestation and this was more pronounced in those exposed to famine who then became obese as adults (Ravelli *et al.*, 1998). In sheep offspring, exposure to a late gestation undernutrition challenge increased insulin and glucose AUC at 1 yr (Gardner *et al.*, 2005), and exposure to a 50 % reduction from 28-78 dGA increased glucose AUC in 63 and 250 day old offspring (Ford *et al.*, 2007). An early gestation undernutrition did not alter glucose tolerance but did increase insulin AUC in adult male offspring (Poore *et al.*, 2007) and a 60 % maternal undernutrition from -60 dGA to 30 dGA increased insulin AUC in the late gestation sheep fetus (Oliver *et al.*, 2001). In rats, 15 month old male offspring of low protein fed rat dams were shown to have a greater glucose AUC and insulin AUC tended to be higher (Ozanne *et al.*, 2003). In contrast, there were no differences in female low protein offspring of the same age (Hales *et al.*, 1996). A greater glucose AUC is described as an impaired glucose tolerance because the subject takes longer to clear the same amount of glucose. Hyperinsulinaemia is thought to be a compensatory response to the development of insulin resistance, but there are also data which suggest that hyperinsulinaemia may be the cause of or may exacerbate insulin resistance (Shanik *et al.*, 2008). The differences between sexes may reflect different strategies adopted by males and females following a nutrient challenge. The study in this chapter was males only since changes in glucose tolerance and insulin signalling pathways were previously found in male rats (Ozanne *et al.*, 2003;Hales *et al.*, 1996).

Plasma insulin levels rapidly increased following administration of the glucose bolus and then fell at around 15 minutes, probably due to the depletion of pre-formed insulin (first-phase insulin response). Levels then rose again at around 30 minutes, and this was probably due to the synthesis of more insulin (second-phase insulin response). Initial insulin release was measured in the first 5 minutes, to allow comparison to that at 1.5 yrs, and also as the first 10 minutes, since other studies have defined this as the acute phase. Initial insulin secretion for both periods was not different between the groups. A decreased initial insulin response was found in the sheep following mid-gestation maternal undernutrition (Ford *et al.*, 2007). Reduced first-phase insulin release is the first detectable sign in patients who go on to develop diabetes (Leahy, 2005) and this is thought to reflect a reduction of pre-formed insulin (Ferrannini,

1998). Second phase insulin AUC tended to be greater in the LBCS offspring as compared to HBCS. The greater secondary response suggests that the LBCS group were more insulin resistant since it took more insulin to clear the bolus of glucose (glucose AUC was not different and so more insulin did not speed up clearance time) and although the LBCS animals are clearing the glucose bolus it is taking higher amounts of insulin to do so. This is similar to the first stages of type II diabetes, where the pancreas is able to compensate for the insulin resistance by releasing more insulin, but eventually it may not be able to maintain this level and the beta cells start to fail (Kahn, 2000). However, pancreatic insulin levels were not different between groups and were not related to any of the GTT outcomes. Previous studies have indicated that undernutrition during gestation affects the development of the pancreas, leading to impaired function of the beta cells and consequently insulin deficiency (Cherif *et al.*, 1998;Snoeck *et al.*, 1990). In rats, maternal nutrient restriction during pregnancy and lactation result in glucose intolerance and type II diabetes with ageing and this is associated with reduced β-cell mass and function (Garofano *et al.*, 1998;Bertin *et al.*, 1999).

This study used a broad challenge throughout gestation and so the critical windows cannot be dissected as they were in Chapter 3. However, using early and late gestation maternal energy intake and weight gain it is possible to try to decipher the effects of impaired nutrition at specific periods of gestation. The GTT outcomes from the mature adult offspring were plotted against maternal energy intake and weight gain for different periods in gestation. Early and late gestation energy intakes were not related to glucose tolerance. However, total energy intake was negatively related to insulin AUC and peak insulin; a higher energy intake improves insulin sensitivity since it takes less insulin to clear the same bolus of glucose. Similarly, late and total gestational weight gains were negatively related to insulin AUC. This shows that although there are no differences in glucose tolerance when the cohort are analysed by BCS groups, maternal energy intake and subsequent weight gain are important in determining the insulin sensitivity of the adult offspring. In humans, maternal weight gain has been found to be related to offspring birth weight (Kirchengast *et al.*, 1998) and child adiposity at 3 years of age (Oken *et al.*, 2007). Similarly, the offspring of low body weight mothers have been shown at increased risk of insulin resistance as adults (Fall *et al.*, 1998).

There are several studies which have proposed a connection between intrauterine growth restriction, postnatal catch-up growth and the incidence of obesity and type 2 diabetes mellitus (for review see Hales & Ozanne, 2003) and insulin resistance is positively related to catch-up growth in small for gestational age infants at 1 year of age (Soto *et al.*, 2003). There was no difference in weight or BCS between LBCS and HBCS groups at birth. The deposition of adipose tissue on the back of the animals did not differ between the 2 groups and at 8 weeks only muscle depth was reduced in the LBCS group (Cripps *et al.*, 2008). This was during the pre-weaning period when the ewes were still being individually penned according to their BCS group, and so this may have been caused by impaired milk production which has been observed in other undernutrition studies (Tygesen *et al.*, 2008). The postnatal growth of the offspring was similar between the two groups and at the time of study at 1.5 yrs they were indistinguishable in terms of body weight and size. At 4 yrs there were no significant differences in BCS, back fat or weight between the two groups. The only difference observed was in back muscle depth which was increased in the LBCS group. Muscle depth was previously found lower in LBCS at 8 weeks of age, but there were no differences at any other postnatal time points and so the increase in muscle depth occurred in mature adult life. There were no differences between dietary groups in body biometry or organ weight at post-mortem, both in absolute and as % of body weight values. However, weight gain in the cohort overall during the period of suckling was negatively related to basal glucose, suggesting that poor growth during this period reduced glucose tolerance. Indeed, the suckling period of growth is significant because final maturation of the ovine pancreas occurs ~ 2 months of postnatal age (Titlbach *et al.*, 1985). However, growth following weaning and up to young adulthood was positively related to peak glucose and insulin (trend) during the GTT. This suggests that increased growth during this period reduced glucose tolerance and was related to insulin resistance. This is similar to another study whereby adult sheep glucose tolerance was greater (lower glucose AUC during GTT) and basal insulin was reduced in females, and insulin AUC and ratio of insulin AUC to glucose AUC was reduced in males exposed to postnatal undernutrition (reduction of body weight to 85 % of target 12-25 weeks of age) (Poore *et al.*, 2007). Poor early postnatal growth was associated with an increased fat in females and this may explain the difference in glucose tolerance between the sexes in response to the postnatal challenge.

5.6.1.2 Effects of age

At 1.5 years of age basal glucose levels were higher in the LBCS group (Cripps *et al.*, 2008). This was not seen at 4 yrs but basal glucose, insulin and the insulin : glucose ratio were all increased at 4 yrs compared to at 1.5 yrs, independent of group. The increased basal insulin : glucose ratio may suggest that the cohort were more insulin resistant at 4 yrs and since glucose levels also rose this implies that the increased insulin release was not enough to overcome the developing insulin resistance and maintain basal glucose levels. An increase in both basal glucose and insulin have long been known to increase with age in humans (Jackson *et al.*, 1982).

Glucose AUC was previously found to be greater in the LBCS group at 1.5 yrs (Cripps *et al.*, 2008). However, an important difference between the two GTT experiments was in their analysis. At 1.5 yrs the AUC was calculated using basal plasma levels but since basal levels are reported as a separate observation a true test of glucose tolerance is to measure the area above baseline and therefore compare all animals from an even starting point. For the benefit of comparison the 1.5 year data were re-analysed without baseline, and similarly the 4 year data were analysed with baseline. The increased glucose AUC seen at 1.5 years did not hold once the baseline was excluded. By including this in the analysis of glucose AUC it biased the result and gave the appearance of reduced glucose tolerance at 1.5 years. When analysing the 4 year data inclusive of baseline there were still no differences seen between the groups since there were no differences in basal glucose levels at this age.

Glucose AUC was lower at 4 yr of age than at 1.5 yrs, independent of group, suggesting that glucose tolerance had improved with age. However, as discussed above basal glucose levels were seen to rise with age. Blood glucose levels have been shown to rise (1 h after an oral glucose challenge) with age and independent of basal glucose, even among people who have normal glucose tolerance (Rhee et al., 2006). Thus, there is a precedent for dissociation between basal glucose and response to a glucose bolus. Insulin AUC could not be compared between the ages due to sampling differences but insulin AUC : glucose AUC tended to be increased at 4 yr of age than at 1.5 yrs, independent of group. This suggests that the improved glucose tolerance was due to increased insulin concentrations or either that insulin resistance increased with age. However, if peripheral insulin resistance had increased this makes it difficult to

explain the improvement in glucose AUC with age but a possible explanation may be changes in post InsR pathway. Initial insulin response decreased from 1.5 to 4 yr and this is consistent with several human studies that have reported a significant age-dependent decrease in glucose-stimulated insulin secretion (Chen *et al.*, 1985; Gumbiner *et al.*, 1989; Muller *et al.*, 1996). The prevalence of type II diabetes increases with age in both men and women (Wingard *et al.*, 1990) and glucose tolerance worse ns with age (Jackson, 1990). At age 50 those exposed to the Dutch famine had decreased glucose tolerance (higher 120 minute glucose concentrations) and this was associated with insulin resistance (increased basal and 120 minute insulin concentrations; Ravelli *et al*., 1998). Glucose tolerance deteriorated between the age of 50 and 58 in these men and women (de Rooij *et al.*, 2006) and a large part of this decline could be attributed to an increase in BMI. There were no differences in the increase of glucose and insulin levels (with age) between the famine-exposed group and the unexposed so although undernutrition was linked to decreased glucose tolerance this effect did not become more pronounced with age. In the sheep, insulin sensitivity was shown to decrease from pre-weaning to young adulthood (Gatford *et al.*, 2004). The decrease in glucose tolerance with age is not thought to be an affect of age *per se* but may be a combination of associated characteristics such as obesity and inactivity (Shimokata *et al.*, 1991). Indeed, in this study the animals were found to be heavier and had a higher back fat thickness at 4 years compared to 1.5 years which may have contributed to increased basal glucose, although glucose tolerance was found to be improved with age.

Basal glucose is a reflection of the general requirements of brain and muscle and that synthesised and released by the liver. In the fed state the liver will absorb and store glucose as glycogen (glycogen synthesis). In fasting conditions the liver can then convert this glycogen back into glucose (glycogenolysis) or can synthesise glucose from other precursors such as certain amino acids or lactate (gluconeogenesis), and this is released back into the blood. Gluconeogenesis is stimulated by glucagon (released by the pancreas) and inhibited by insulin. An increase with age indicates impairment in this control of basal glucose, whereas the response to the glucose bolus is an indication of peripheral sensitivity to insulin. Since insulin is involved in the maintenance of both basal glucose and glucose tolerance, the difference between the two at 4 years (basal glucose increased but glucose tolerance improved) implies there

is a problem further downstream in the control of basal glucose. This may be in the enzyme phosphoenolpyruvate carboxykinase (PEPCK) which is a rate-limiting enzyme in gluconeogenesis and plays an important role in regulating glucose production. Indeed, PEPCK expression is elevated in type II diabetes (Consoli *et al.*, 1989;DeFronzo *et al.*, 1989).

5.6.2 Glucose uptake into isolated muscle strips

The range of glucose uptake into the muscle beds (0.25 to 0.8 pmol.min.mg) was far less than that seen in rat studies (2 to 25 nmol.min.mg; Ozanne *et al.*, 1996) but was comparable to a study in sheep that used Fick's principle (the uptake of a marker substance by an organ can be calculated if blood flow to the organ is known, together with arterial and venous concentrations of the marker substance) to calculate glucose uptake into hindlimb muscle (Anderson *et al.*, 2005). When transforming the values from this study they were found to be more comparable to the results of this chapter (33 – 45 pmol.min.mg). Basal glucose uptake was significantly higher in the soleus compared to the vastus and gastrocnemius muscles. This is comparable to a study which found basal glucose uptake to be greater in the soleus as compared to extensor digitorum longus in the rat (Shoji, 1986).

Increased glucose uptake in the presence of insulin was observed in all three of the muscle beds. Insulin stimulated uptake was greater in the soleus muscle as compared to the vastus or gastrocnemius. This was as expected since muscle fibres are thought to follow an order of type I > type IIa > type IIb in their insulin sensitivity (James *et al.*, 1985;Kern *et al.*, 1990) and the soleus muscle consists entirely of type I fibres. Insulin sensitivity in skeletal muscle is thought to be largely determined by the levels of GLUT-4 (Kern *et al.*, 1990) and GLUT-4 is found in larger amounts in type I and IIa muscle fibres than type IIb (Daugaard & Richter, 2001). Insulin-stimulated uptake in the soleus tended to be reduced in the LBCS as compared to HBCS offspring. Initially this would suggest reduced insulin sensitivity in the LBCS offspring, however there were no differences in the response to the *in vivo* glucose tolerance test between the groups. The soleus is a small postural muscle with a potentially minor contribution to glucose metabolism compared to much larger muscles such as the vastus (for which uptake was not changed). Therefore, the trend for a reduction in insulin stimulated uptake in the soleus would probably have little or no effect on whole body glucose

tolerance, and it was unknown whether this would have occurred in other postural muscles. In another study, basal glucose uptake was unchanged but insulin-stimulated uptake was impaired in the soleus muscle of low protein rat offspring (Ozanne *et al.*, 2003).

5.6.3 Skeletal muscle morphology

5.6.3.1 Myofibre density

The offspring of LBCS ewes had reduced total myofibre and fast-twitch myofibre density and slow-twitch density tended to be reduced in the vastus muscle. Changes in fast fibre number have been seen following a 50 % nutrient reduction 30-70 dGA (Fahey *et al.*, 2005b;Daniel *et al.*, 2007). The decrease in total myofibre density is consistent with the findings in Chapter 3 where fetal muscle density was decreased by two different periods of nutrient restriction. However, in that study it was the slow-twitch fibres which were significantly reduced following a 50 % restriction in late gestation. This study instead encompassed the whole of gestation and weaning and so would therefore have impacted on all periods of muscle development. A reduction in fast fibres is more likely to have a greater effect on muscle strength than a reduction in slow fibres due to their faster speed of contraction and reduced fatigue resistance. A decrease in strength with ageing has been associated with a reduction in type II fibres (Hortobagyi *et al.*, 1995) and an increase in strength as a result of high resistance training is through hypertrophy of the type II fibres (Campos *et al.*, 2002). Low birth weight is correlated with a reduction in muscle strength in adult life (Sayer *et al.*, 2004;Kuh *et al.*, 2002). The decrease in myofibre number as a result of impaired fetal development was therefore still apparent in mature adult life and as expected shows that muscle is unable to reverse a reduction in number. The myofibre density of the soleus was not different between the groups and this again highlights the resistance of the soleus muscle to a nutrient challenge, probably due to its high composition of primary myofibres (Ward & Stickland, 1991).

A change in myofibre density can reflect a change in the size of the myofibres. With analysis of equal numbers of fibres per group in the vastus muscle there was no change in CSA and so this would suggest that a change in CSA is unlikely to account for the decrease in density. The draw back with this approach were the small numbers of fibres (fast-twitch, 41; slow-twitch, 24) and so further analysis was undertaken of all

available fibres within the 10 fields. With all fibres analysed the slow-twitch fibres were increased in the LBCS as compared to the HBCS offspring. Therefore, it cannot be ruled out that a change in CSA did not contribute to the trend in decreased slow-twitch myofibres.

The amount of Akt1 protein was decreased in the vastus muscle and tended to be lower in abdominal fat of LBCS offspring. Muscle-specific Akt1 knockout mice display impairment of growth but have normal glucose tolerance, whereas muscle-specific Akt2 knockout mice develop diabetes (Cho *et al.*, 2001c;Cho *et al.*, 2001b). Akt1 is therefore thought to be involved in the growth aspects of insulin signalling and Akt2 the metabolic. IGF-IR protein tended to be reduced in the vastus muscle of LBCS offspring and along with the reduction in Akt1 is consistent with the reduction in fibre density since these are both muscle growth promoters.

Maternal gestational energy intake and weight gain were plotted against vastus and soleus muscle morphology of the mature adult offspring. Early gestation energy intake was positively related to myofibre density; so less energy intake during this period may have reduced the myofibre density, possibly through a reduction of the myoblast population. However, late gestation and total energy intake also tended to be positively related to myofibre density and so this analytical approach does not aid differentiation between the effects of the different periods of undernutrition since dietary manipulation to reduce BCS was throughout gestation. Similarly, ewe weight gain for all three periods tended to be positively related to slow-twitch myofibre density. There was no relationship between offspring postnatal growth and myofibre density, which confirms that myofibre number are set during the prenatal period.

5.6.3.2 Capillary density

The capillary density in the vastus muscle tended to be reduced and so capillary density is likely to have been reduced along with myofibres. Capillary: myofibre ratio was also reduced in the LBCS offspring and indicates that the capillary reduction is more than just a simple reflection of fewer myofibres because if they had been reduced in parallel this ratio would not have changed. Rather, the reduction in capillary: myofibre ratio indicates that each fibre was being supplied by fewer blood vessels. Although blood flow or artery function were not measured in this study it is possible

that a reduced blood flow reduced capillary density due to the evidence that a fetus may respond to a nutrient challenge by redistributing blood flow away from the peripheral tissues (Burrage *et al.*, 2005;Gardner *et al.*, 2002).

5.6.4 Glucose tolerance and muscle composition

5.6.4.1 Isolated muscle strips

Insulin-stimulated uptake of glucose into isolated muscle strips tended to be reduced in the soleus muscle of LBCS offspring, despite there being no changes in myofibre density for this muscle. Myofibre density was reduced in the vastus muscle of LBCS offspring but glucose uptake was not altered for this muscle. These results are interesting because they show that changes in glucose uptake can occur without changes in muscle morphology and that muscle morphology changes may not necessarily affect glucose uptake. This suggests that the LBCS offspring may have adapted to the morphological changes in the vastus muscle, thereby maintaining normal glucose uptake, and this may be through alterations in the insulin signalling pathway (see below). Changes in this pathway may also have impaired glucose uptake in the soleus muscle.

5.6.4.2 Glucose tolerance test

Skeletal muscle morphology was not linked to whole body glucose handling at 4 years except for a higher soleus myofibre density which tended to be positively related to insulin AUC. This was unexpected as the soleus is made up of insulin-sensitive slow fibres and so a higher myofibre density should be related to better and not worse insulin sensitivity. A peri-implantation challenge (1-31 dGA) has been found to have no effect on later body glucose function, although muscle morphology was not studied (Poore *et al.*, 2007). This was the same challenge used in Chapter 3 which did find differences in myofibre density and therefore it is probable that these changes in muscle morphology would have occurred in the Poore study since it was the same challenge and species of sheep. Taken together these findings suggest that there is a precedent for changes in skeletal muscle morphology to occur without affecting glucose tolerance. The reduction in fibre density observed in this chapter may have contributed to the reduced glucose tolerance of the young adult offspring (1.5 year; Cripps *et al.*, 2008). Indeed, vastus myofibre density (and trend for fast fibre density) was negatively related to basal glucose at 1.5 years. A high proportion of fast-twitch

myofibres has previously been associated with increased basal insulin levels in middle-aged subjects (Venojarvi *et al.*, 2005).

5.6.4.3 Compensatory changes in the insulin-signalling pathway

Skeletal myofibre and capillary density was reduced in the offspring of low maternal BCS ewes but these changes in muscle morphology were not related to glucose tolerance at 4 years and this may be due to compensatory changes in the insulin-signalling pathway. Insulin-stimulated glucose transport is achieved by transportation of GLUT-4 (the major insulin-responsive glucose transporter in skeletal muscle) from intracellular vesicular storage to the plasma membrane. GLUT-4 was increased in the vastus muscle of LBCS offspring. In other studies a 50 % nutrient restriction in ewes in early to mid-gestation did not alter GLUT-4 in fetal skeletal muscle (Zhu *et al*. 2006) but a 50 % restriction in late gestation reduced GLUT-4 in the adipose tissue of the offspring (Gardner *et al.* 2005). The expression of GLUT-4 is not altered in patients with insulin resistant or type-II diabetes (Krook *et al.*, 2000;Pedersen *et al.*, 1990;Garvey *et al.*, 1992) and so it is thought that instead there may be a defect in GLUT-4 translocation (Krook *et al.*, 2000). GLUT-4 protein content was unchanged but translocation was increased in the muscle of offspring from protein restricted dams and this was associated with normal glucose tolerance (Gavete *et al.*, 2005). GLUT-4 has been found to be increased after swimming training in the rat and this was associated with an increased insulin-stimulated glucose uptake into isolated muscle strips (Kawanaka *et al.*, 1997). In the current study there were no differences in glucose tolerance between the HBCS and LBCS groups and so the increase in GLUT-4 protein may have offset the impact of the reduction in myofibres on glucose handling in the LBCS offspring. Levels of GLUT-4 were not measured at the time of the first GTT and so it was unknown whether they were altered at this time.

There were no differences in InsR, PI3-kinase p85 subunit and PKCζ protein in the vastus muscle between HBCS and LBCS groups. This was similar to findings at 1.5 years in vastus muscle (Cripps *et al.*, 2008), but since the Western blot only gives an arbitrary value it is impossible to compare between the years. Western blots were not performed on the soleus muscles and so there was no information on the insulin signalling pathway and how this may have caused the trend for reduced insulin-stimulated glucose uptake in the soleus muscle strip. In other studies, late-gestation maternal undernutrition increased InsR and p110β but decreased GLUT-4 levels in the

peri-renal fat of sheep offspring at 1 year of age (Gardner *et al.*, 2005). In the same study there were no differences in the skeletal muscle following a late restriction but an early restriction increased InsR expression. However, the site of the muscle sampling was not specified and thus it is possible that the sampling site may not have been consistent. This may have increased variability in the western results and hence led to the negative results in the skeletal muscle. Young Danish low-birth weight men had reduced expression of PKCζ, the regulatory p85α and catalytic p110β subunits of PI3-kinase and GLUT-4 in the vastus muscle (Ozanne *et al.*, 2005). Protein restriction throughout gestation and lactation did not change InsR or GLUT-4 levels but did reduce expression of PKC ζ at 15 months of age in the soleus muscle (Ozanne *et al.*, 2003). The PKC family play a major role in pathways that mediate insulin action (Newton, 1995) with PKC ζ a downstream target of PI 3-kinase in insulin-stimulated glucose uptake (Standaert *et al.*, 1997). Over expression of PKC ζ in rat skeletal muscle improves both basal and insulin-stimulated glucose uptake (Etgen *et al.*, 1999). Thus it appears that PKC ζ expression may be important in determining glucose tolerance and levels were not different between LBCS and HBCS offspring at 4 years.

5.7 Conclusions

This chapter has shown that reduced maternal diet/body composition during gestation reduces skeletal myofibre and capillary density of the offspring into mature adult life. However, at 4 years these changes in muscle morphology are not related to total body glucose handling or glucose uptake into isolated muscle strips. Therefore, changes in the muscle can occur without overall effects on glucose tolerance and this may be due to a compensatory up-regulation of GLUT-4 which helps to maintain glucose homeostasis despite a reduction in myofibre number.

6 General Discussion

6.1 Summary of results

This thesis has shown that decreased maternal nutrient intake or body condition can reduce skeletal myofibre density in the offspring and that this may be mediated by a reduction in muscle capillary density. Depending on the timing of this nutritional insult (late-gestation) this also increases markers of glucose tolerance during fetal life. These changes in muscle morphology do not affect glucose tolerance in mature adulthood and this may be due to compensatory increases in glucose uptake mechanisms.

In Chapter 3, it was found that 60 % maternal nutrient restriction for the first 31 days of gestation led to a decrease in triceps muscle myofibre density. A 50 % maternal nutrient restriction for 3 weeks in late gestation led to a decrease in myofibre density and particularly that of the slow-twitch myofibres. The late restriction was associated with an increase in insulin receptor (InsR), insulin-like growth factor I receptor (IGF-IR) and glucose transporter-4 (GLUT-4) mRNA levels. Both challenges affected only the mixed fibre triceps muscle and not the slow-twitch soleus muscle. Capillary density and capillary : myofibre ratio were reduced in the triceps muscle following both challenges, but there were no differences in the soleus which suggested a link between blood flow and muscle development following nutrient restriction.

In Chapter 4, it was found that 60 % maternal nutrient restriction 1-31 dGA reduced the brachial artery response to sodium nitroprusside in late gestation, which may indicate smooth muscle dysfunction. A late gestation nutrient restriction did not alter the vasoactive properties of the artery following the undernutrition challenge. Vascular sensitivity to ACh was positively related, and sensitivity to NA was inversely related, to circulating glucose. This suggested that in times of low glucose the vessel was prone to vasoconstriction. These changes in the reactivity of the vessel may indicate a reduced peripheral blood flow, a fetal response to maternal undernutrition which has been observed in previous studies, and this could have contributed to the decrease in capillary density and hence myofibre number seen in Chapter 3.

In Chapter 5, it was found that a lower maternal body condition score maintained throughout pregnancy and lactation reduced myofibre density in the vastus muscle. This effect was predominantly due to a reduction in fast-twitch myofibre density, although slow-twitch myofibre density tended to be reduced in low body condition score (LBCS) offspring. The soleus muscle myofibre density was not affected. Capillary density tended to be reduced and capillary : myofibre ratio was reduced in the vastus muscle (measurements pending in the soleus). This reinforced the link between capillary and myofibre density following maternal nutrient restriction, as suggested in Chapter 3. There were no differences in basal insulin and glucose levels or in glucose tolerance between the groups. However, basal levels of both glucose and insulin were found to increase, and initial insulin response worsened, with age. Despite these changes, glucose tolerance improved with age (decreased glucose AUC). Basal and insulin-stimulated glucose uptake was greater in the soleus muscle compared to gastrocnemius and vastus muscles. There was no effect of maternal BCS on glucose uptake in gastrocnemius and vastus muscles, but insulin-stimulated uptake tended to be reduced in the soleus muscle of LBCS offspring. Protein levels of Akt1 were lower, and IGF-IR tended to be lower, in the vastus muscle of LBCS muscle and in light of the decrease in myofibre density this is consistent with their roles in mediating growth. GLUT-4 levels were increased in the vastus muscle of LBCS offspring and may indicate a compensatory increase in skeletal muscle glucose uptake mechanisms to maintain glucose tolerance in the face of reduced myofibre density.

6.2 The suitability of the sheep model and nutrient challenges

The general benefits of the sheep model, which was used in this thesis, are that several of their organs are thought to develop at a similar rate to humans, including the heart and kidneys (Lumbers, 1995). In terms of muscle development sheep and humans have three waves of myofibre development (Draeger *et al.*, 1987;Maier *et al.*, 1992), whereas smaller animals such as rats have only two waves. Myofibre number is set at birth in most mammals (Brameld *et al.*, 1998) but in the rat numbers continue to rise and can be altered in early postnatal life (Desai *et al.*, 1996) making sheep the better model for studying muscle development. Sheep have a gestation length that allows

specific windows of nutrient restriction to be targeted and have a high rate of singleton births, making them comparable to humans and a good model for fetal research. They are also considered docile and are of an appropriate size to allow surgical instrumentation.

A disadvantage of the sheep model is that they are ruminants. These are animals which digest their food in two stages through the regurgitation and chewing of partially digested food. Ruminants derive little glucose from their diet and meet their requirements through gluconeogenesis (Leng, 1970). The rate of gluconeogenesis in ruminants is highest after feeding, whereas in non-ruminant animals it is lowest after feeding and highest during an energy deficit (Nafikov & Beitz, 2007). However, the aim of an experiment such as a GTT is to test the limitations of a system rather than to recreate a precise physiological situation. Due to rumen fermentation, ruminant animals derive far more energy from fibre and volatile fatty acids and more of their amino acids from non-protein nitrogen and ammonia (microbial protein synthesis) than non-ruminant animals (Baker, 2008). Therefore, studies looking at the reduction of specific nutrients e.g. low protein may be harder to interpret in the sheep model. The nutrient restrictions used in this thesis were all a reduction in total nutrient intake. A further consideration is that while the waves of myofibre formation are similar to humans it is important to note that sheep are in some respects more precocial than humans since they are born with fully differentiated skeletal muscles and are able to stand and walk soon after birth. In contrast, humans are born with immature muscles, in respect to postural and locomotor control, and full development proceeds slowly in the postnatal period (Walker & Luff, 1995). Another disadvantage of the sheep model is that they are expensive and are unsuitable for transgenerational studies due to the length of gestation and the time taken to reach puberty (30-50 weeks, Foster & Karsch, 1976).

Statistical power is an important issue in animal studies due to the need to keep numbers to a minimum, in line with the reduction recommended by the 3 Rs of animal research (replace, reduce, refine; Research Defence Society, 2008). These are the guiding principles underpinning the humane use of animals in scientific research. Reduction states that the number of animals should be kept to a minimum in order to obtain information from fewer animals or more information from the same number of animals. In order to plan the appropriate number of animals to include in the study a

power calculation was used to estimate the numbers needed to give the necessary statistical power. For the myofibre density studies presented in Chapters 3 and 5, the power calculation indicated 6 animals per group (see 2.9.1). The final numbers of the three treatment groups in Chapter 3 were 6, 9 and 6 and in Chapter 5 they were 10 and 14 and studies were adequately powered to detect any differences between the groups. However, in Chapter 4 the final numbers for which a successful myography experiment was performed were 2, 5 and 4 and so these studies are underpowered and the data has been interpreted with caution accordingly.

Two types of nutrient challenge were used in this thesis, maternal dietary restriction and reduction of maternal body condition (maintained through maternal dietary manipulation). The first study (Chapters 3 and 4) used two distinct periods of undernutrition (peri-implantation and late gestation) since it had been shown previously that the period of gestation in which the challenge occurs will determine the postnatal outcome (Roseboom *et al.*, 2006), the assumption being that the adaptations depend on which organ or tissue is developing at the time of the challenge. The aim of the second study (Chapter 5) was to observe the effects of modest changes in maternal body composition within the normal range. Maternal body composition can affect birth outcome since the mother will provide for the fetus from both her dietary intake and own body reserves. This study is more comparable to that of the human situation, where women are advised to avoid dieting or weight loss during pregnancy (Food Standards Agency, 2008) and normal pregnancy weight gain is calculated using body mass index (BMI) before pregnancy (Cedergren, 2007). It is therefore unlikely that a pregnant women would suddenly alter her body composition for a short period during pregnancy, unless due to illness or famine. The LBCS ewes had a reduction in muscle depth over gestation that was proportional to their change in weight while fat depth was reduced considerably more, confirming a change in body composition (Cripps *et al.*, 2008). However, the range of BCS was not simply a natural variance within the cohort and maintenance of high or low BCS was maintained through dietary manipulation. The study could have been improved if the range of BCS within the flock was natural i.e. low and high body condition score ewes without the need for nutritional intervention, but this would have required far larger numbers of animals to achieve.

The ewes were of the same parity within each study (first parity in Chapters 3 & 4 and second parity for Chapter 5) to eliminate the interactions of the differences in parity. Size at birth varies between firstborn and subsequent offspring (Ong *et al.*, 2002). Maternal parity not only influences birth weight but also postnatal growth and morbidity (Bai *et al.*, 2002) and first-born infants are more likely to be growth restricted *in utero* and exhibit compensatory growth after birth (Ong *et al.*, 2002). Increasing maternal parity can also predict neonatal adiposity (Joshi *et al.*, 2005).

Welsh Mountain sheep were chosen because of their tendency to produce singleton offspring rather than twins and all pregnancies in this thesis were singletons. This is important because there are distinct fetal growth patterns in singleton and twin pregnancies (Blickstein, 2005) and the constraint of fetal growth and development following dietary manipulation is likely to be greater in twin than singleton pregnancies. Indeed, the strategy adopted by the fetus to a nutrient challenge in order to enhance immediate survival i.e. growth rate or gestation length, has been shown to be dependent on both offspring number and sex (Cleal *et al.*, 2007) and a restricted periconceptual undernutrition increased arterial blood pressure in twin but not singleton fetuses (Edwards & McMillen, 2002).

In Chapter 5, male offspring only were studied in postnatal life since previous studies in rats had shown the predominant changes in glucose tolerance to be in males and not females (Hales *et al.*, 1996;Ozanne *et al.*, 2003). Sex differences should be considered because males may be more vulnerable to prenatal undernutrition as they grow more rapidly *in utero* than females (Clarke & Mittwoch, 1995). There were approximately equal numbers of males and females in Chapter 3 (C, female (f) = 4, male (m) = 4; PI, f = 4, m = 5 and L, f = 2, m = 4) but these were analysed together as nutritional groups because when combined the study was sufficiently powered, but if split into sexes the study would have been under-powered.

6.3 Maternal nutrient restriction alters skeletal muscle morphology in the offspring

A key aim of this thesis was to investigate the effects of maternal nutrient restriction on skeletal muscle morphology. The peri-implantation and late gestation period were chosen because it had previously been shown that the timing of nutrient restriction will determine the postnatal health outcomes (Roseboom *et al.*, 2006) and muscle development in the sheep occurs in three distinct stages (Maier *et al.*, 1992;Wilson *et al.*, 1992). A 60 % reduction in total nutrient intake was imposed for the first 31 days of gestation in the peri-implantation (PI) challenged group and a 50 % reduction from 104 – 127 dGA in the late gestation (L) challenged group. Both periods of nutrient restriction reduced myofibre density in the triceps brachii of the late gestation fetus. The late gestation nutrient restriction had particularly profound effects on the slow-twitch insulin sensitive fibres. A lower maternal BCS, maintained throughout pregnancy and lactation reduced myofibre density in the vastus muscle of 4 year old male offspring. In the LBCS offspring this appeared also to be due to a significant reduction in fast-twitch myofibre density. Thus, maternal nutrition can not only reduce myofibre density but, depending on intensity and duration of the challenge, have differing effects on the myofibre types. While the BCS challenge (Chapter 5) was throughout the whole of gestation and encompassed all stages of muscle development, the energy intake of the ewe was recorded each week and therefore it was possible to correlate energy intake in critical periods of gestation with offspring muscle morphology. Interestingly, it was early gestation energy intake (0-28 dGA) that was positively related to vastus total and fast myofibre density (Chapter 5) and it was a maternal nutrient restriction during the same period (1-31 dGA) which reduced triceps total myofibre and fast-fibre density (trend) in Chapter 3. However, late gestation total energy intake also tended to be positively related to myofibre density and so this analytical approach does not aid differentiation between the effects of the different periods of undernutrition. Few muscle studies have focused on the effects of maternal undernutrition in late gestation. Dwyer *et al*. (1995) identified a critical period in the guinea pig, with maternal feed restriction during the first half of gestation inducing the same reduction in myofibre number as a restriction throughout gestation, whereas maternal undernutrition during late gestation had no effect, but in rats a reduced maternal protein intake in late gestation did reduce myofibre density (Mallinson *et al.*,

2007). Other studies have targeted the early period of gestation including the temporary exposure of ovine embryos to an advanced uterine environment, which increased myofibre number in the late gestation fetus (Maxfield *et al.*, 1998), and a 50 % nutrient restriction from -18 to 6 dGA decreased total myofibre number in the mid-gestation fetus (Quigley *et al.*, 2005). This early period of development is important in determining the number of myogenic cells since it is the period of their proliferation, and a decrease in their number is likely to affect the number of mature myofibres. Muscle satellite cells will also be derived from this pool of myoblasts. The number of muscle nuclei has been correlated with postnatal growth and this is likely to be a reflection of a large pool of satellite cells (Greenwood *et al.*, 1999). Thus, maternal undernutrition may not only reduce myofibre number at birth but may reduce the number of satellite cells and the ability of the muscle to grow and repair in future life.

The early period of undernutrition may also have effects on placental function since placental growth is maximal during the first half of gestation (Schneider, 1996). In Chapter 3 there were no differences in total placentome weight or types between the nutritional groups, but this was not studied in Chapter 5. Similarly, a 50 % nutrient challenge from -18 to 6 dGA decreased myofibre number but no changes were found in placental weight (Quigley *et al.*, 2005). However, weight and type is not a conclusive indication of placental function and changes in nutrient transfer may still have occurred. Treatment with somatotropin from 10 to 127 dGA has been shown to improve placental function (Rehfeldt *et al.*, 2001b) and the same treatment increased total myofibre number in the neonatal pig (Rehfeldt *et al.*, 2001a) and so the effect of the treatment on myofibre number is likely to have been mediated by the improvement in placental function.

The nutrient restriction from both studies may also have had an effect on skeletal muscle through interactions with growth factors. In the fetus the insulin-like growth factors are the dominant mediators of growth. IGF-I also has an important role in muscle, regulating both myofibre formation and postnatal growth (Coleman *et al.*, 1995;Mitchell *et al.*, 2002). IGF-IR was increased in the triceps muscle of L fetuses and while this appears to be inconsistent with fewer myofibres it may reflect a compensatory increase in the expression of this gene in response to decreased growth. However, IGF-IR was decreased in the vastus muscle of LBCS offspring at 4 years

and this is consistent with the reduction in myofibre density. The discrepancy between the two studies is likely to reflect the timing of the measurements i.e. fetus vs. adulthood since there are major changes in the IGF axis at term (Fowden, 2003).

Birth weight was similar between groups in both studies which show that changes in skeletal muscle development can occur without a change in birth weight. Although birth weight has been correlated with later disease such as type II diabetes (Hales *et al.*, 1991) and reduced hand grip strength (indicative of muscle weakness) (Kuh *et al.*, 2002), other studies have not found a relationship between birth weight and later disease (Huxley *et al.*, 2008). Therefore, it is important to note that phenotypes can be induced in offspring without necessarily being accompanied by low birth weight and it is clear that reduction of fetal growth *per se* does not cause later disease. Rather, low birth weight is a marker of the effects of a restricted environment on the fetus and one aspect of the fetal response to that environment. It is also important to remember that birth weight is an inadequate measurement of fetal growth since fetuses can be of the same weight and yet vastly differ in their body measurements or composition. Similarly, a reduction in myofibre density can occur without changes in muscle weight (Quigley *et al.*, 2005;Maxfield *et al.*, 1998). It is thought that any space created by the decrease in myofibre density, in the absence of myofibre hypotrophy, can be filled by connective tissues such as the endomysium. This has been shown in the bovine fetus following *in vitro* fertilisation (Crosier *et al.*, 2002). Muscle weights were not collected in Chapter 3, and in Chapter 5 only gastrocnemius weight, for which myofibre density was not studied, and soleus weight, for which there were no reduction in myofibre density, were taken. Thus, it is unknown whether a reduction in myofibre density (as observed in the triceps and vastus muscles) would have decreased muscle weight.

6.4 Reduced blood flow and capillary density may mediate the reduction in myofibre density

Capillary density was found to be reduced alongside myofibre density in both Chapters 3 and 5 (trend). The decrease in capillary density may have been due to overall decreased myofibre density i.e. fewer fibres need fewer capillaries. However, the capillary : myofibre ratio was also reduced which indicates that the remaining myofibres were being supplied by *fewer* capillaries. There were no changes in

myofibre or capillary density in the soleus muscle. Together these findings suggest that local blood flow is linked to muscle development during a nutrient challenge. Analysis of soleus capillary density in Chapter 5 has not been performed at the time of writing but this is planned for the future since the absence of changes in capillary density would further strengthen the finding that capillary density and myofibre density are related following maternal nutrient restriction. It was thought (Chapter 4) that a decrease in blood flow to the developing muscle may have reduced capillary formation and therefore reduced myofibre number. The preliminary myography data from Chapter 4 suggested that the peri-implantation nutrient restriction altered smooth muscle function of the brachial artery, with a reduced sensitivity to the vasodilator SNP. The changes in the reactivity of the vessel may indicate a reduced peripheral blood flow, a fetal response which has been well characterised in the hypoxic model (Cohn *et al.*, 1974). Although peripheral blood flow was not directly measured to the muscle bed, an acute hypoglycaemia in the same cohort was shown to reduce femoral artery and skin blood flow (Burrage, 2006). Thus, a reduced blood flow may have down-regulated flow-mediated angiogenesis and reduced capillary density. However, there is some discrepancy between the data since capillary density was reduced in both PI and L fetuses and the vascular changes were only seen in the PI group. This suggests that the mechanism behind the reduction in muscle capillary density may be different between the two nutritional groups and this is likely to be dependent upon the timing of the challenge, in relation to circulation development. Indeed, the response to hypoxia has been found to differ between the mid- and late gestation sheep fetus (Iwamoto *et al.*, 1989). Increased sensitivity to NA and decreased sensitivity to ACh, at lower circulating glucose levels, was also seen in Chapter 3 and this is likely to be associated with greater overall vasoconstriction, suggesting a link between fetal nutritional status and vascular reactivity.

In Chapter 5, Akt1 (protein kinase b) was reduced in the vastus muscle of LBCS offspring. Akt is a protein kinase in the insulin signalling pathway and the family consists of Akt1, Akt2 and Akt3 (Barnett *et al.*, 2005). Akt1 is thought to be involved in the growth aspects of insulin signalling and Akt2 the metabolic (Cho *et al.*, 2001c;Cho *et al.*, 2001b). Akt1 has also been implicated in angiogenesis (Somanath *et al.*, 2006) and is the predominant isoform of Akt in endothelial cells (Chen *et al.*, 2005). Hypertrophy of myotubes, in response to IGF-I or insulin, increases secretion of vascular endothelial growth factor (VEGF; Takahashi *et al.*, 2002). Myofibre

hypertrophy and hypertrophy-associated VEGF expression is inhibited by a dominant-negative mutant of Akt1 and conversely, transduction of a constitutively active form of Akt1 increases myofibre size and VEGF production (Takahashi *et al.*, 2002). Thus, since Akt1 is a mechanism through which blood vessel recruitment and muscle growth may be coupled it is tempting to speculate that it may play a role in linking the reduced myofibre and capillary density in Chapters 3 and 5. Of interest, the insulin-stimulated activity of all three isoforms of Akt have been found to be reduced in diabetic patients (Cozzone *et al.*, 2008).

6.5 Altered muscle composition may not be detrimental to later glucose handling

It was hypothesised that changes in muscle fibre composition i.e. percentages of fibre types would reduce glucose tolerance since the fibre types have differing metabolic and insulin sensitivities (Kern *et al.*, 1990;James *et al.*, 1985). Previous studies have shown that fibres types are possible determinants of insulin resistance (Lillioja *et al.*, 1987;Mårin *et al.*, 1994). However, despite the changes in the vastus muscle in the LBCS group (Chapter 5) there were no changes seen in glucose uptake in the isolated muscle strips. No difference was observed between the groups in soleus myofibre density yet insulin-stimulated glucose uptake tended to be reduced in the soleus muscle of LBCS offspring. There was no difference to *in vivo* glucose tolerance between the nutritional groups, despite a decrease in myofibre density in the vastus muscle of LBCS offspring. Furthermore, skeletal muscle morphology was not correlated to whole body glucose handling except for a higher soleus myofibre density, which tended to be positively related to insulin AUC. These results are interesting because they show that a tendency for reduced glucose uptake can occur without a reduction in myofibre density and that reduced myofibre density may not necessarily affect glucose tolerance. A peri-implantation challenge (1-31 dGA) was found to have no effect on later body glucose function, although muscle morphology was not studied (Poore *et al.*, 2007). Since this was the same challenge (and species of sheep) used in Chapter 3, which did find a reduction in myofibre density, it is therefore probable that skeletal myofibre number would also have been decreased in the Poore study.

Protein levels of GLUT-4 were increased in the LBCS muscle and this may have offset the reduction in myofibres in order to maintain glucose homeostasis. The decrease in myofibre density and altered myofibre composition in late gestation fetal muscle following a period of undernutrition was coupled to an increase in GLUT-4 and InsR and this may have been a compensatory mechanism aimed at maintaining glucose homeostasis. An increase in GLUT-4 can improve glucose handling, with over-expression improving peripheral glucose utilisation in the mouse (Treadway *et al.*, 1994) and an increase following training increasing insulin-stimulated glucose uptake into muscle strips in the rat (Kawanaka *et al.*, 1997). The study in Chapter 3 assessed GLUT-4 mRNA levels whereas Chapter 5 looked at protein levels. Both were found to be increased and so I speculate that the increased mRNA levels seen in the fetal muscle would have translated to an increase in protein levels. However, even the protein content of GLUT-4 is not a clear indication of function, since the transporter needs to be present in the cell membrane for glucose uptake and the translocation of the protein was not studied. Data have indicated that insulin resistance in skeletal muscle is the result of impaired translocation of intracellular GLUT-4 to the sarcolemma (Garvey *et al.*, 1998;Zierath *et al.*, 1996). Indeed, in rat pups of nutrient restricted dams total GLUT-4 content was not affected by undernutrition but the protein was more efficiently translocated to the cell membrane (Gavete *et al.*, 2005). The increase in GLUT-4 protein suggests that the LBCS offspring may have adapted to the reduction in myofibre density in the vastus muscle through alterations in glucose uptake mechanisms. Changes in this pathway may have also affected the insulin sensitivity of the soleus muscle and so an investigation of this is planned as future work. Such compensatory mechanisms (i.e. increased GLUT-4) may have an upper limit and it remains to be determined whether if the animals were further challenged by an over abundant nutritional environment then the reduction in myofibre density may be detrimental to function. Indeed, recent concepts state that where a mismatch occurs between the predicted and actual postnatal environment, the adaptive response which the fetus has made *in utero* may lead to an increased risk of disease (Gluckman *et al.*, 2007).

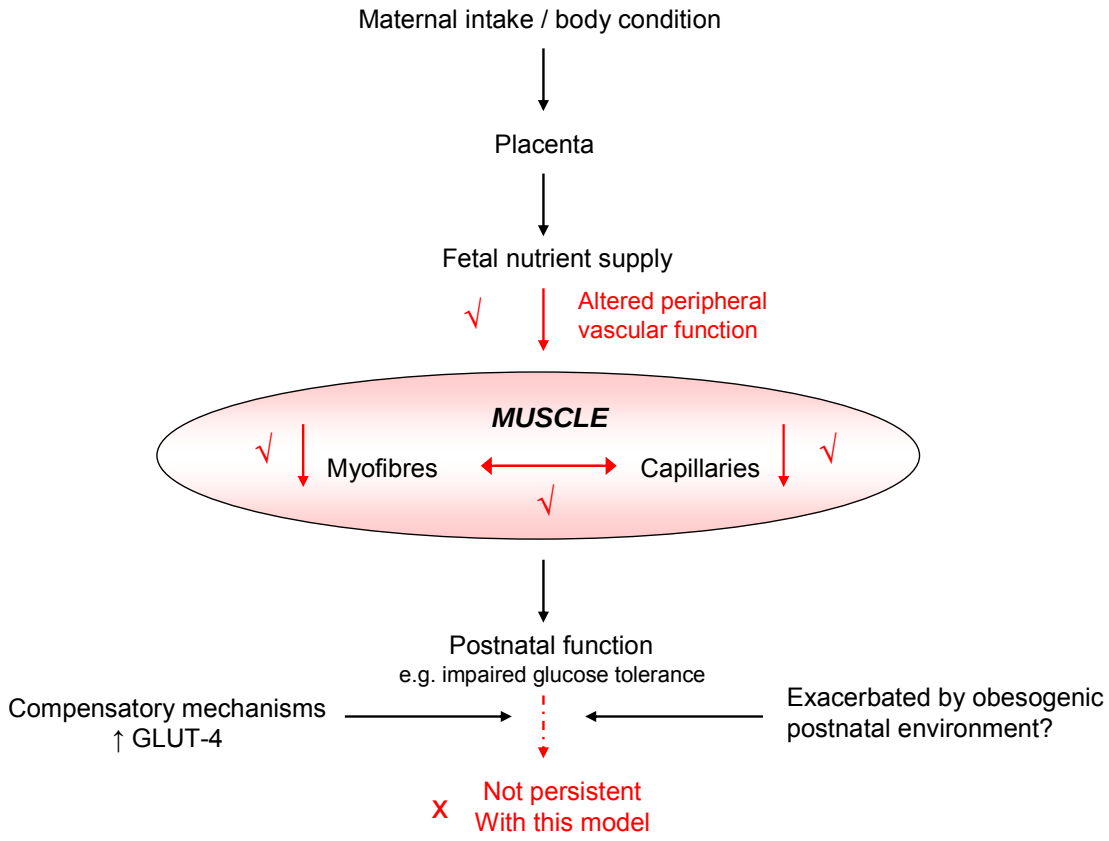


Figure 6-1: Thesis overview. This thesis has shown that maternal undernutrition can reduce both skeletal myofibre and capillary density in the offspring and alter fibre type composition and these changes can be detected in both fetal and mature adult life. The resultant changes in muscle morphology are dependent upon the timing of the nutrient restriction and the muscle bed studied and may be related to local blood flow and capillary formation. The changes in muscle morphology were not detrimental to glucose tolerance in mature adult life and this may in part be due to compensatory mechanisms such as increased GLUT-4. However, these mechanisms may have limitations and it is possible that in an over-abundant postnatal environment the change in muscle morphology would be detrimental to glucose tolerance.

6.6 Future work

There are several areas which would be interesting to investigate in light of the results presented here but that were beyond the scope of this thesis.

6.6.1 Immediate questions arising from thesis

The capillary density in soleus muscle from Chapter 5 needs to be determined and these results may strengthen the link between capillary density and myofibre density following a maternal nutrient restriction.

The insulin-signalling pathway in the soleus muscle from Chapter 5 should also be studied since there was a trend for reduced insulin-stimulated glucose uptake in the isolated muscle strip. This was without any reduction in myofibre density and so it will be interesting to determine what might be mediating this trend in reduced insulin sensitivity.

The gluconeogenic pathway in the liver is extremely important in glucose homeostasis and so components of this (PEPCK and glucose-6-phosphatase) are currently being studied in the same model.

Myofibres in Chapters 3 and 5 were split into just the fast and slow fibres types and so any changes in the proportion of fast-twitch isoforms i.e. types IIa and IIb were not determined. A change in the fast fibre population is likely to have a bigger impact upon postnatal function since they make up a larger proportion of total fibres. Other studies have found a change in the proportion of the fast fibre types following maternal undernutrition (Daniel *et al.*, 2007; Zhu *et al.*, 2006). The fast-fibre isoforms could be separated through either protein electrophoresis (Bamman *et al.*, 1999) or the use of more specific antibodies.

The amount of intramuscular fat can reduce glucose tolerance since fat metabolism interferes with glucose uptake into cells (Boden & Chen, 1995). Since the animals in Chapter 5 were shown to be fatter with age it would be interesting to see if there were any differences in intramuscular fat between the nutritional groups and if this was related to glucose tolerance. Also, adipokine production from fat such as adiponectin has been shown to have both vascular effects (Fésüs *et al.*, 2007) as well as being a

mediator of insulin sensitivity (Yamauchi *et al.*, 2001). Unlike other adipokines, adiponectin production is reduced with obesity (Weyer *et al.*, 2001) and increased after weight reduction (Yang *et al.*, 2001).

6.6.2 Longer term research goals

The effect of an overabundant postnatal environment should be tested to see whether any adaptations, which aim to maintain glucose homeostasis in the face of reduced myofibre density, are appropriate in an unexpectedly abundant postnatal environment.

This thesis has looked at the metabolic outcomes of changes in the muscle morphology but another important factor to consider is the effect on peak muscle mass, strength and ageing. A reduction in myofibre number at birth may exacerbate muscle wasting in later life (Sayer *et al.*, 2004) and so this approach should be considered for future studies, for which a small animal model is likely to be more suitable.

It will be important to determine interventions that can reduce disease risk (caused by changes in skeletal muscle) and one of these may be exercise in postnatal life since this can increase both muscle hypertrophy (McDonagh & Davies, 1984) and alter myofibre composition (Abernethy *et al.*, 1990). This could be studied by a gestational nutrient restriction, which drives changes in muscle composition, followed by a dietary or exercise intervention in postnatal life.

6.7 Final conclusion

This thesis has demonstrated that maternal undernutrition can reduce both skeletal myofibre and capillary density and alter fibre type composition in the offspring and that the outcomes are dependent upon the timing of the nutrient restriction and the muscle bed studied. Resultant changes in muscle morphology can be detected in both fetal and mature adult life and appear to be related to local blood flow and capillary formation. While a reduction in myofibre density may not be detrimental to later glucose tolerance this may be due to compensatory mechanisms e.g. increased GLUT-4. However, these adaptations may have limitations in an over-abundant postnatal environment, where there would be increased propensity for weight gain and obesity. In the long term, this work contributes to the understanding of the mechanisms underlying the early origins of strength/degenerative (e.g. sarcopenia) or metabolic disorders, and to the debate over dietary advise given to pregnant women aimed at reducing such disease risk.

Reference List

Aberle ED (1984). Myofiber Differentiation in Skeletal Muscles of Newborn Runt and Normal Weight Pigs. *J Anim Sci* **59**, 1651-1656.

Abernethy P, Thayer R, & Taylor PD (1990). Acute and chronic responses of skeletal muscle to endurance and sprint exercise. A review. *Sports medicine* **10**, 365-89.

AFRC (1993). Energy and Protein Requirements of Ruminants. In *An advisory manual prepared by the AFRC technical committee on responses to nutrients* Wallingford, UK: CAB International.

Agote M, Goya L, Ramos S, Alvarez C, Gavete ML, Pascual-Leone AM, & Escriva F (2001). Glucose uptake and glucose transporter proteins in skeletal muscle from undernourished rats. *Am J Physiol Endocrinol Metab* **281**, E1101-E1109.

Aiello L (2005). Angiogenic pathways in diabetic retinopathy. *N Engl J Med* **353**, 839-41.

Alberti K & Zimmet P (1998). Definition, diagnosis and classification of diabetes mellitus and its complications. Part 1: diagnosis and classification of diabetes mellitus provisional report of a WHO consultation. *Diabet Med* **15**, 539-53.

Albo D, Wang X, & Tuszynski G (2004). Antiangiogenic therapy. *Curr Pharm Des* **10**, 27-37.

Alexander D, Britton H, Cohen N, & Nixon D (1972). The permeability of the sheep placenta to insulin: studies with the perfused placental preparation. *Biol Neonate* **21**, 361-368.

Andersen P (1975). Capillary density in skeletal muscle of man. *Acta Physiol Scand* **95**, 203-205.

Andersen P & Henriksson J (1977). Capillary supply of the quadriceps femoris muscle of man: adaptive response to exercise. *J Physiol* **270**, 677-690.

Anderson MS, Thamotharan M, Kao D, Devaskar SU, Qiao L, Friedman JE, & Hay WW, Jr. (2005). Effects of acute hyperinsulinemia on insulin signal transduction and glucose transporters in ovine fetal skeletal muscle. *Am J Physiol Regul Integr Comp Physiol* **288**, R473-R481.

Anderson RH, Webb S, Brown NA, Lamers W, & Moorman A (2003). Development of the heart: (2) Septation of the atriums and ventricles. *Heart* **89**, 949-958.

Au Y (2004). The muscle ultrastructure: a structural perspective of the sarcomere. *Cell Mol Life Sci* **61**, 3016-33.

Baar K & Esser K (1999). Phosphorylation of p70S6k correlates with increased skeletal muscle mass following resistance exercise. *Am J Physiol Cell Physiol* **276**, C120-C127.

Backer J, Myers M, Shoelson S, Chin D, Sun X, Miralpeix M, Hu P, Margolis B, Skolnik E, & Schlessinger J (1992). Phosphatidylinositol 3'-kinase is activated by association with IRS-1 during insulin stimulation. *The EMBO journal* **11**, 3469-3479.

Bai J, Wong F, Bauman A, & Mohsin M (2002). Parity and pregnancy outcomes. *Am J Obstet Gynecol* **186**, 274-8.

Baker DH (2008). Animal Models in Nutrition Research. *J Nutr* **138**, 391-396.

Balagopal P, Schimke JC, Ades P, Adey D, & Nair KS (2001). Age effect on transcript levels and synthesis rate of muscle MHC and response to resistance exercise. *Am J Physiol Endocrinol Metab* **280**, E203-E208.

Bamman MM, Shipp JR, Jiang J, Gower BA, Hunter GR, Goodman A, McLafferty CL, Jr., & Urban RJ (2001). Mechanical load increases muscle IGF-I and androgen receptor mRNA concentrations in humans. *Am J Physiol Endocrinol Metab* **280**, E383-E390.

Bamman M, Clarke M, Talmadge R, & Feeback D (1999). Enhanced protein electrophoresis technique for separating human skeletal muscle myosin heavy chain isoforms. *Electrophoresis* **20**, 468.

Bar A & Pette D (1988). Three fast myosin heavy chains in adult rat skeletal muscle. *FEBS Letters* **235**, 153-155.

Barker DJ, Osmond C, Golding J, Kuh D, & Wadsworth ME (1989a). Growth in utero, blood pressure in childhood and adult life, and mortality from cardiovascular disease. *BMJ* **298**, 564-567.

Barker DJ, Winter PD, Osmond C, Margetts B, & Simmonds SJ (1989b). Weight in infancy and death from ischaemic heart disease. *Lancet* 577-580.

Barker DJP (1998). In utero programming of chronic disease. *Clinical Science* **95**, 115-128.

Barker DJP, Osmond C, Forsen TJ, Kajantie E, & Eriksson JG (2005). Trajectories of Growth among Children Who Have Coronary Events as Adults. *N Engl J Med* **353**, 1802-1809.

Barnett S, Bilodeau M, & Lindsley C (2005). The Akt/PKB Family of Protein Kinases: A Review of Small Molecule Inhibitors and Progress Towards Target Validation. *Curr Top Med Chem* **2005**, 109-25.

Barton-Davis E, Shoturma D, & Sweeney H (1999). Contribution of satellite cells to IGF-I induced hypertrophy of skeletal muscle. *Acta Physiol Scand* **167**, 301-5.

Battaglia FC & Regnault TRH (2001). Placental Transport and Metabolism of Amino Acids. *Placenta* **22**, 145-161.

Bauman D, Eisemann J, & Currie W (1982). Hormonal effects on partitioning of nutrients for tissue growth: role of growth hormone and prolactin. *Federation proceedings* **41**, 2538-44.

Bayol S, Jones D, Goldspink G, & Stickland N (2004). The influence of undernutrition during gestation on skeletal muscle cellularity and on the expression of genes that control muscle growth. *British Journal of Nutrition* **91**, 331-339.

Bayol SA, Simbi BH, & Stickland NC (2005). A maternal cafeteria diet during gestation and lactation promotes adiposity and impairs skeletal muscle development and metabolism in rat offspring at weaning. *J Physiol (Lond)* **567**, 951-961.

Bedi K, Birzgalis A, Mahon M, Smart J, & Wareham A (1982). Early life undernutrition in rats. 1. Quantitative histology of skeletal muscles from underfed young and refed adult animals. *Br J Nutr* **47**, 417-31.

Beermann DH, Hood LF, & Liboff M (1983). Satellite Cell and Myonuclei Populations in Rat Soleus and Extensor Digitorum Longus Muscles after Maternal Nutritional Deprivation and Realimentation. *J Anim Sci* **57**, 1618-1625.

Benjamin M, Kaiser E, & Milz S (2008). Structure-function relationships in tendons: a review. *Journal of Anatomy* **212**, 211-228.

Benthem L, Mundinger TO, & Taborsky GJ, Jr. (2000). Meal-induced insulin secretion in dogs is mediated by both branches of the autonomic nervous system. *Am J Physiol Endocrinol Metab* **278**, E603-E610.

Bertin E, Gangnerau MN, Bailbe D, & Portha B (1999). Glucose metabolism and beta -cell mass in adult offspring of rats protein and/or energy restricted during the last week of pregnancy. *Am J Physiol Endocrinol Metab* **277**, E11-E17.

Blickstein I (2005). Growth aberration in multiple pregnancy. *Obstet Gynecol Clin North Am* **32**, 39-54.

Bloomfield S (1997). Changes in musculoskeletal structure and function with prolonged bed rest. *Med Sci Sports Exerc* **29**, 197-206.

Boddy K, Dawes G, Fisher R, Pinter S, & Robinson J (1974). Fetal respiratory movements, electrocortical and cardiovascular responses to hypoxaemia and hypercapnia in sheep. *Journal of Physiology* **243**, 599-618.

Boden G & Chen X (1995). Effects of fat on glucose uptake and utilization in patients with non-insulin-dependent diabetes. *J Clin Invest* **96**, 1261-8.

Borg TK & Caulfield JB (1980). Morphology of connective tissue in skeletal muscle. *Tissue and Cell* **12**, 197-207.

Borrell A (2004). The ductus venosus in early pregnancy and congenital anomalies. *Prenatal Diagnosis* **9**, 688-692.

Bottinelli R, Betto R, Schiaffino S, & Reggiani C (1994). Unloaded shortening velocity and myosin heavy chain and alkali light chain isoform composition in rat skeletal muscle fibres. *J Physiol (Lond)* **478**, 341-349.

Brahm H, Piehl-Aulin K, Saltin B, & Ljunghall S (1997). Net Fluxes Over Working Thigh of Hormones, Growth Factors and Biomarkers of Bone Metabolism During Short Lasting Dynamic Exercise. *Calcified Tissue International* **60**, 175-180.

Brameld JM, Buttery PJ, Dawson JM, & Harper JMM (1998). Nutritional and hormonal control of skeletal-mucle cell growth and differentiation. *Proceedings of the Nutrition Society* **57**, 207-217.

Brameld JM, Mostyn A, Dandrea J, Stephenson TJ, Dawson JM, Buttery PJ, & Symonds ME (2000). Maternal nutrition alters the expression of insulin-like growth factors in fetal sheep liver and skeletal muscle. *J Endocrinol* **167**, 429-437.

Brand-Saberi B & Christ B (1999). Genetic and epigenetic control of muscle development in vertebrates. *Cell and Tissue Research* **296**, 199-212.

Brawley L, Itoh S, Torrens C, Barker A, Bartram C, Poston L, & Hansons M (2003). Dietary Protein Restriction in Pregnancy Induces Hypertension and Vascular Defects in Rat Male Offspring. *Pediatr Res* **54**, 83-90.

Breen EC, Johnson EC, Wagner H, Tseng HM, Sung LA, & Wagner PD (1996). Angiogenic growth factor mRNA responses in muscle to a single bout of exercise. *J Appl Physiol* **81**, 355-361.

Brennan TJ, Edmondson DG, Li L, & Olson EN (1991). Transforming Growth Factor {beta} Represses the Actions of Myogenin Through a Mechanism Independent of DNA Binding. *PNAS* **88**, 3822-3826.

Brooks AA, Johnson MR, Steer PJ, Pawson ME, & Abdalla HI (1995). Birth weight: nature or nurture? *Early Human Development* **42**, 29-35.

Bryson-Richardson RJ & Currie PD (2008). The genetics of vertebrate myogenesis. *Nat Rev Genet* **9**, 632-646.

Buller AJ, Eccles JC, & Eccles RM (1960). Interactions between motoneurones and muscles in respect of the characteristic speeds of their responses. *J Physiol (Lond)* **150**, 417-439.

Burrage D. The impact of reduced nutrition on growth and cardiovascular control in the fetus. 2006. University of Southampton.
Ref Type: Thesis/Dissertation

Burrage D, Braddick L, Noakes D, Hanson M, & Green L (2005). Fetal cardiovascular responses to acute hypoglycaemia are modified by early gestation nutrient restriction in sheep. *Journal of the Society for Gynecologic Investigation* **12**, 113A.

Burrage D, Green L, Moss T, Sloboda D, Nitsos I, Newnham J, & Hanson M (2008). The carotid bodies influence growth responses to moderate maternal undernutrition in late-gestation fetal sheep. *BJOG* **115**, 261-8.

Byzova TV, Goldman CK, Jankau J, Chen J, Cabrera G, Achen MG, Stacker SA, Carnevale KA, Siemionow M, Deitcher SR, & DiCorleto PE (2002). Adenovirus encoding vascular endothelial growth factor-D induces tissue-specific vascular patterns in vivo. *Blood* **99**, 4434-4442.

Bzoskie I, Blount I, Kashiwai K, Humme J, & Padbury J (1997). Placental norepinephrine transporter development in the ovine fetus. *Placenta* **18**, 65-70.

Campos G, Luecke T, Wendeln H, Toma K, Hagerman F, Murray T, Ragg K, Ratamess N, Kraemer W, & Staron R (2002). Muscular adaptations in response to three different resistance-training regimens: specificity of repetition maximum training zones. *Eur J Appl Physiol* **88**, 50-60.

Carrow R, Brown R, & Van Huss WD (1967). Fiber sizes and capillary to fiber ratios in skeletal muscle of exercised rats. *Anat Rec* **159**, 33-9.

Cartee GD, Douen AG, Ramlal T, Klip A, & Holloszy JO (1991). Stimulation of glucose transport in skeletal muscle by hypoxia. *J Appl Physiol* **70**, 1593-1600.

Carter BS, Moores RR, Jr., Battaglia FC, & Meschia G (1993). Ovine fetal placental lactate exchange and decarboxylation at midgestation. *Am J Physiol Endocrinol Metab* **264**, E221-E225.

Cedergren MI (2007). Optimal Gestational Weight Gain for Body Mass Index Categories. *Obstet Gynecol* **110**, 759-764.

Cetin I (2003). Placental transport of amino acids in normal and growth-restricted pregnancies. *European Journal of Obstetrics & Gynecology and Reproductive Biology* **110**, S50-S54.

Chandler K, Leury B, Bird A, & Bell A (1985). Effects of undernutrition and exercise during late pregnancy on uterine, fetal and uteroplacental metabolism in the ewe. *Br J Nutr* **53**, 625-35.

Charlton V & Johengen M (1987). Fetal intravenous nutritional supplementation ameliorates the development of embolization-induced growth retardation in sheep. *Pediatr Res* 55-61.

Chen J, Somanath P, Razorenova O, Chen W, Hay N, Bornstein P, & Byzova T (2005). Akt1 regulates pathological angiogenesis, vascular maturation and permeability in vivo. *Nature Medicine* **11**, 1188-96.

Chen M, Bergman RN, Pacini G, & Porte D, Jr. (1985). Pathogenesis of age-related glucose intolerance in man: insulin resistance and decreased beta-cell function. *J Clin Endocrinol Metab* **60**, 13-20.

Cherif H, Reusens B, Ahn MT, Hoet JJ, & Remacle C (1998). Effects of taurine on the insulin secretion of rat fetal islets from dams fed a low-protein diet. *J Endocrinol* **159**, 341-348.

Cho H, Mu J, Kim JK, Thorvaldsen JL, Chu Q, Crenshaw EB, III, Kaestner KH, Bartolomei MS, Shulman GI, & Birnbaum MJ (2001b). Insulin Resistance and a Diabetes Mellitus-Like Syndrome in Mice Lacking the Protein Kinase Akt2 (PKBbeta). *Science* **292**, 1728-1731.

Cho H, Mu J, Kim JK, Thorvaldsen JL, Chu Q, Crenshaw EB, III, Kaestner KH, Bartolomei MS, Shulman GI, & Birnbaum MJ (2001a). Insulin Resistance and a Diabetes Mellitus-Like Syndrome in Mice Lacking the Protein Kinase Akt2 (PKBbeta). *Science* **292**, 1728-1731.

Cho H, Thorvaldsen JL, Chu Q, Feng F, & Birnbaum MJ (2001c). Akt1/PKBalpha Is Required for Normal Growth but Dispensable for Maintenance of Glucose Homeostasis in Mice. *J Biol Chem* **276**, 38349-38352.

Clamann HP (1993). Motor unit recruitment and the gradation of muscle force. *PHYS THER* **73**, 830-843.

Clarke C & Mittwoch U (1995). Changes in the male to female ratio at different stages of life. *Br J Obstet Gynaecol* **102**, 677-9.

Cleal J, Poore K, Newman J, Noakes D, Hanson M, & Green L (2007). The effect of maternal undernutrition in early gestation on gestation length and fetal and postnatal growth in sheep. *Pediatr Res* **62**, 422-7.

Cohn H, Sacks E, Heymann M, & Rudolph A (1974). Cardiovascular responses to hypoxemia and acidemia in fetal lambs. *American journal of obstetrics and gynecology* **15**, 817-824.

Coleman ME, DeMayo F, Yin KC, Lee HM, Geske R, Montgomery C, & Schwartz RJ (1995). Myogenic Vector Expression of Insulin-like Growth Factor I Stimulates Muscle Cell Differentiation and Myofiber Hypertrophy in Transgenic Mice. *J Biol Chem* **270**, 12109-12116.

Colorado State University Hypertextbook. Pathophysiology of the Reproductive System. http://www.vivo.colostate.edu/hbooks/pathphys/reprod/placenta/ruminants.html . 2000.
Ref Type: Electronic Citation

Conejo R & Lorenzo M (2001). Insulin signaling leading to proliferation, survival, and membrane ruffling in C2C12 myoblasts. *J Cell Physiol* **187**, 96-108.

Consoli A, Nurjhan N, Capani F, & Gerich J (1989). Predominant role of gluconeogenesis in increased hepatic glucose production in NIDDM. *Diabetes* **38**, 550-7.

Constancia M, Hemberger M, Hughes J, Dean W, Ferguson-Smith A, Fundele R, Stewart F, Kelsey G, Fowden A, Sibley C, & Reik W (2002). Placental-specific IGF-II is a major modulator of placental and fetal growth. *Nature* **417**, 945-948.

Cooke R (1989). Structure of the myosin head. *Cell Motil Cytoskeleton* **14**, 183-186.

Costa A, Zoppetti G, Benedetto C, Bertino E, Marozio L, Fabris C, Arisio R, Giraudi G, Testori O, & Ariano M (1993). Immunolike growth hormone substance in tissues from human embryos/fetuses and adults. *J Endocrinol Invest* **16**, 625-33.

Costill D, Daniels J, Evans W, Fink W, Krahenbuhl G, & Saltin B (1976). Skeletal muscle enzymes and fiber composition in male and female track athletes. *J Appl Physiol* **40**, 149-54.

Cozzone D, Fröjdö S, Disse E, Debard C, Laville M, Pirola L, & Vidal H (2008). Isoform-specific defects of insulin stimulation of Akt/protein kinase B (PKB) in skeletal muscle cells from type 2 diabetic patients. *Diabetologia* **51**, 512-21.

Craig R & Lehman W (2001). Crossbridge and tropomyosin positions observed in native, interacting thick and thin filaments. *Journal of Molecular Biology* **311**, 1027-1036.

Cripps R, Green L, Thompson J, Martin-Gronert M, Monk M, Sheldon I, Hanson M, Hales CD, & Ozanne S (2008). The Effect of Maternal Body Condition Score Before and During Pregnancy on the Glucose Tolerance of Adult Sheep Offspring. *Reproductive Sciences* **15**, 448-456.

Crosier AE, Farin CE, Rodriguez KF, Blondin P, Alexander JE, & Farin PW (2002). Development of Skeletal Muscle and Expression of Candidate Genes in Bovine Fetuses from Embryos Produced In Vivo or In Vitro. *Biol Reprod* **67**, 401-408.

Cross DAE, Alessi DR, Cohen P, Andjelkovich M, & Hemmings BA (1995). Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. *Nature* **378**, 785-789.

Crowther N, Cameron N, Trusler J, & Gray I (1998). Association between poor glucose tolerance and rapid post natal weight gain in seven-year-old children. *Diabetologia* **41**, 1163-7.

Curry D, Bennett L, & Grodsky GM (1968). Dynamics of insulin secretion by the perfused rat pancreas. *Endocrinology* **83**, 572-84.

Daniel Z, Brameld JM, Craigon J, Scollan ND, & Buttery PJ (2007). Effect of maternal dietary restriction during pregnancy on lamb carcass characteristics and muscle fiber composition. *J Anim Sci* **85**, 1565-1576.

Das UG, He J, Ehrhardt RA, Hay WW, Jr., & Devaskar SU (2000). Time-dependent physiological regulation of ovine placental GLUT-3 glucose transporter protein. *Am J Physiol Regul Integr Comp Physiol* **279**, R2252-R2261.

Das UG, Sadiq HF, Soares MJ, Hay WW, Jr., & Devaskar SU (1998). Time-dependent physiological regulation of rodent and ovine placental glucose transporter (GLUT-1) protein. *Am J Physiol Regul Integr Comp Physiol* **274**, R339-R347.

Daugaard JR & Richter EA (2001). Relationship between muscle fibre composition, glucose transporter protein 4 and exercise training: possible consequences in non-insulin-dependent diabetes mellitus. *Acta Physiologica Scandinavica* **171**, 267-276.

Dauncey MJ & Gilmour RS (1996). Regulatory factors in the control of muscle development. *Proceedings of the Nutrition Society* **55**, 543-559.

Davidson HW, Rhodes CJ, & Hutton JC (1988). Intraorganellar calcium and pH control proinsulin cleavage in the pancreatic [beta] cell via two distinct site-specific endopeptidases. *Nature* **333**, 93-96.

Davies P, Olesen S, Clapham D, Morrel E, & Schoen F (1988). Endothelial communication. State of the art lecture. *Hypertension* 563-572.

de Rooij S, Painter R, Roseboom T, Phillips D, Osmond C, Barker D, Tanck M, Michels R, Bossuyt P, & Bleker O (2006). Glucose tolerance at age 58 and the decline of glucose tolerance in comparison with age 50 in people prenatally exposed to the Dutch famine. *Diabetologia* **49**, 637-643.

De Vriese AS, Verbeuren TJ, Van de Voorde J, Lameire NH, & Vanhoutte PM (2000). Endothelial dysfunction in diabetes. *Br J Pharmacol* **130**, 963-974.

DeFronzo R, Ferrannini E, & Simonson D (1989). Fasting hyperglycemia in non-insulin-dependent diabetes mellitus: contributions of excessive hepatic glucose production and impaired tissue glucose uptake. *Metabolism* **38**, 387-95.

Desai M, Crowther N, Lucas A, & Hales C (1996). Organ-selective growth in the offspring of protein-restricted mothers. *Br J Nutr* **76**, 591-603.

DeVol DL, Rotwein P, Sadow JL, Novakofski J, & Bechtel PJ (1990). Activation of insulin-like growth factor gene expression during work-induced skeletal muscle growth. *Am J Physiol Endocrinol Metab* **259**, E89-E95.

Dickson MC, Saunders JC, & Gilmour RS (1991). The ovine insulin-like growth factor-I gene: characterization, expression and identification of a putative promoter. *Journal of Molecular Endocrinology* **6**, 17-31.

Djonov V, Baum O, & Burri P (2003). Vascular remodeling by intussusceptive angiogenesis. *Cell Tissue Res* **314**, 107-117.

Draeger A, Weeds AG, & Fitzsimons RB (1987). Primary, secondary and tertiary myotubes in developing skeletal muscle: a new approach to the analysis of human myogenesis. *Journal of the Neurological Sciences* **81**, 19-43.

Duckworth WC (1998). Insulin degradation: mechanisms, products, and significance. *Endocr Rev* **9**, 319-45.

Duclos MJ, Wilkie RS, & Goddard C (1991). Stimulation of DNA synthesis in chicken muscle satellite cells by insulin and insulin-like growth factors: evidence for exclusive mediation by a type-I insulin-like growth factor receptor. *J Endocrinol* **128**, 35-42.

Dupouy J, Chatelain A, & Allaume P (1980). Absence of transplacental passage of ACTH in the rat: direct experimental proof. *Biol Neonate* **37**, 96-102.

Duyne CMV, Parker HR, Havel RJ, & Holm LW (1960). Free fatty acid metabolism in fetal and newborn sheep. *Am J Physiol* **199**, 987-990.

Dwyer CM, Fletcher JM, & Stickland NC (1993). Muscle cellularity and postnatal growth in the pig. *J Anim Sci* **71**, 3339-3343.

Dwyer CM, Stickland NC, & Fletcher JM (1994). The influence of maternal nutrition on muscle fiber number development in the porcine fetus and on subsequent postnatal growth. *J Anim Sci* **72**, 911-917.

Dwyer C, Madgwick A, Crook A, & Stickland N (1992). The effect of maternal undernutrition on the growth and development of the guinea pig placenta. *Journal of developmental physiology* **18**, 295-302.

Dwyer C, Madgwick A, Ward S, & Stickland N (1995). Effect of maternal undernutrition in early gestation on the development of fetal myofibres in the guinea-pig. *Reprod Fertil Dev* **7**, 1285-92.

Dwyer C & Stickland N (1992). Does the anatomical location of a muscle affect the influence of undernutrition on muscle fibre number? *J Anat* **181**, 373-376.

Dwyer CM & Stickland N (1991). Sources of variation in myofiber number within and between litters of pigs. *Animal Production* **52**, 527-533.

Dyce KM, Sack WO & Wensing CJG (1987) *Textbook of Veterinary Anatomy*. WB Saunders Company.

Easton RM, Cho H, Roovers K, Shineman DW, Mizrahi M, Forman MS, Lee VMY, Szabolcs M, de Jong R, Oltersdorf T, Ludwig T, Efstratiadis A, & Birnbaum MJ (2005). Role for Akt3/Protein Kinase B{gamma} in Attainment of Normal Brain Size. *Mol Cell Biol* **25**, 1869-1878.

Edwards LJ & McMillen IC (2001). Maternal undernutrition increases arterial blood pressure in the sheep fetus during late gestation. *The Journal of Physiology* **533**, 561-570.

Edwards LJ & McMillen IC (2002). Periconceptional nutrition programs development of the cardiovascular system in the fetal sheep. *Am J Physiol Regul Integr Comp Physiol* **283**, R669-R679.

Edwards LJ, Symonds ME, Warnes KE, Owens JA, Butler TG, Jurisevic A, & McMillen IC (2001). Responses of the Fetal Pituitary-Adrenal Axis to Acute and Chronic Hypoglycemia during Late Gestation in the Sheep. *Endocrinology* **142**, 1778-1785.

Egginton S, Zhou A, Brown M, & Hudlicka O (2001). Unorthodox angiogenesis in skeletal muscle. *Cardiovasc Res* **49**, 634-646.

el-Saleh S, Warber K, & Potter J (1986). The role of tropomyosin-troponin in the regulation of skeletal muscle contraction. *J Muscle Res Cell Motil* **7**, 387-404.

Eriksson JG, Forsen T, Tuomilehto J, Osmond C, & Barker DJP (2001). Early growth and coronary heart disease in later life: longitudinal study. *BMJ* **322**, 949-953.

Eriksson J, Forsén T, Tuomilehto J, Jaddoe V, Osmond C, & Barker D (2002). Effects of size at birth and childhood growth on the insulin resistance syndrome in elderly individuals. *Diabetologia* **45**, 342-8.

Eskridge EM & Shields D (1983). Cell-free processing and segregation of insulin precursors. *J Biol Chem* **258**, 11487-11491.

Etgen GJ, Valasek KM, Broderick CL, & Miller AR (1999). In Vivo Adenoviral Delivery of Recombinant Human Protein Kinase C-zeta Stimulates Glucose Transport Activity in Rat Skeletal Muscle. *J Biol Chem* **274**, 22139-22142.

Fahey AJ, Brameld JM, Parr T, & Buttery PJ (2005a). Ontogeny of factors associated with proliferation and differentiation of muscle in the ovine fetus. *J Anim Sci* **83**, 2330-2338.

Fahey AJ, Brameld JM, Parr T, & Buttery PJ (2005b). The effect of maternal undernutrition before muscle differentiation on the muscle fiber development of the newborn lamb. *J Anim Sci* **83**, 2564-2571.

Fall C, Stein C, Kumaran K C, V, Osmond C, Barker D, & Hales CN (1998). Size at birth, maternal weight, and type 2 diabetes in South India. *Diabetic medicine* **15**, 220-227.

Fernandez AM, Kim JK, Yakar S, Dupont J, Hernandez-Sanchez C, Castle AL, Filmore J, Shulman GI, & Le Roith D (2001). Functional inactivation of the IGF-I and insulin receptors in skeletal muscle causes type 2 diabetes. *Genes Dev* **15**, 1926-1934.

Fernandez-Twinn DS, Wayman A, Ekizoglou S, Martin MS, Hales CN, & Ozanne SE (2005). Maternal protein restriction leads to hyperinsulinemia and reduced insulin-signaling protein expression in 21-mo-old female rat offspring. *Am J Physiol Regul Integr Comp Physiol* **288**, R368-R373.

Ferrannini E, Natali A, Bell P, Cavallo-Perin P, Lalic N, & Mingrone G (1997). Insulin resistance and hypersecretion in obesity. *J Clin Invest* **100**, 1166-73.

Ferrannini E (1998). Insulin Resistance versus Insulin Deficiency in Non-Insulin-Dependent Diabetes Mellitus: Problems and Prospects. *Endocr Rev* **19**, 477-490.

Ferrara N & vis-Smyth T (1997). The Biology of Vascular Endothelial Growth Factor. *Endocr Rev* **18**, 4-25.

Fésüs G, Dubrovska G, Gorzelniak K, Kluge R, Huang Y, Luft F, & Gollasch M (2007). Adiponectin is a novel humoral vasodilator. *Cardiovasc Res* **75**, 719-27.

Finnell RH, Waes JG, Eudy JD, & Rosenquist TH (2002). Molecular basis of environmentally induced birth defects. *Annual Review of Pharmacology and Toxicology* **42**, 181-208.

Florini JR, Ewton DZ, & Coolican SA (1996). Growth hormone and the insulin-like growth factor system in myogenesis. *Endocr Rev* **17**, 481-517.

Floyd JJ, Fajans S, Conn J, Knopf R, & Rull J (1966). Stimulation of insulin secretion by amino acids. *J Clin Invest* **45**, 1487-502.

Fluck M (2006). Functional, structural and molecular plasticity of mammalian skeletal muscle in response to exercise stimuli. *J Exp Biol* **209**, 2239-2248.

Food Standards Agency. Eat Well: When You're Pregnant. http://www.eatwell.gov.uk/agesandstages/pregnancy/whenyrpregnant/ . 2008.
Ref Type: Electronic Citation

Forbes GB (1985). The effect of anabolic steroids on lean body mass: The dose response curve. *Metabolism* **34**, 571-573.

Ford SP, Hess BW, Schwope MM, Nijland MJ, Gilbert JS, Vonnahme KA, Means WJ, Han H, & Nathanielsz PW (2007). Maternal undernutrition during early to mid-gestation in the ewe results in altered growth, adiposity, and glucose tolerance in male offspring. *J Anim Sci* **85**, 1285-1294.

Foster D & Karsch F (1976). Development of the mechanism regulating the preovulatory surge of luteinizing hormone in sheep. *Endocrinology* **97**, 1205-9.

Fowden AL (1993). Insulin deficiency: effects on fetal growth and development. *J Paediatr Child Health* **29**, 6-11.

Fowden AL & Forhead AJ (2004). Endocrine mechanisms of intrauterine programming. *Reproduction* **127**, 515-526.

Fowden AL (2003). The Insulin-like Growth Factors and feto-placental Growth. *Placenta* **24**, 803-812.

Fowden AL, Giussani DA, & Forhead AJ (2005). Endocrine and metabolic programming during intrauterine development. *Early Human Development* **81**, 723-734.

Fowden A (1995). Endocrine regulation of fetal growth. *Reprod Fertil Dev* **7**, 351-363.

Fowden A, Sibley C, Reik W, & Constancia M (2006). Imprinted genes, placental development and fetal growth. *Horm Res* **65**, 50-58.

Franco MdC, Fortes ZB, Akamine EH, Kawamoto EM, Scavone C, de Britto LRG, Muscara MN, Teixeira SA, Tostes RCA, Carvalho MH, & Nigro D (2004). Tetrahydrobiopterin improves endothelial dysfunction and vascular oxidative stress in microvessels of intrauterine undernourished rats. *J Physiol (Lond)* **558**, 239-248.

Franzini-Armstrong C (1999). The sarcoplasmic reticulum and the control of muscle contraction. *FASEB J* **13**, 266S-270.

Frederick I, Williams M, Sales A, Martin D, & Killien M (2007). Pre-pregnancy Body Mass Index, Gestational Weight Gain, and Other Maternal Characteristics in Relation to Infant Birth Weight. *Matern Child Health J* **[Epub ahead of print]**.

Gallaher BW, Breier BH, Keven CL, Harding JE, & Gluckman PD (1998). Fetal programming of insulin-like growth factor (IGF)-I and IGF-binding protein-3: evidence for an altered response to undernutrition in late gestation following exposure to periconceptual undernutrition in the sheep. *J Endocrinol* **159**, 501-508.

Gardner DS, Fletcher AJW, Bloomfield MR, Fowden AL, & Giussani DA (2002). Effects of prevailing hypoxaemia, acidaemia or hypoglycaemia upon the cardiovascular, endocrine and metabolic responses to acute hypoxaemia in the ovine fetus. *J Physiol (Lond)* **540**, 351-366.

Gardner DS, Tingey K, Van Bon BWM, Ozanne SE, Wilson V, Dandrea J, Keisler DH, Stephenson T, & Symonds ME (2005). Programming of glucose-insulin metabolism in adult sheep after maternal undernutrition. *Am J Physiol Regul Integr Comp Physiol* **289**, R947-R954.

Garofano A, Czernichow P, & Bréant B (1998). Beta-cell mass and proliferation following late fetal and early postnatal malnutrition in the rat. *Diabetologia* **41**, 1114-20.

Garvey WT, Maianu L, Hancock JA, Golichowski AM, & Baron A (1992). Gene expression of GLUT4 in skeletal muscle from insulin-resistant patients with obesity, IGT, GDM, and NIDDM. *Diabetes* **41**, 465-475.

Garvey W, Maianu L, Zhu J, Brechtel-Hook G, Wallace P, & Baron A (1998). Evidence for defects in the trafficking and translocation of GLUT4 glucose transporters in skeletal muscle as a cause of human insulin resistance. *J Clin Invest* **101**, 2377-86.

Gatford KL, De Blasio MJ, Thavaneswaran P, Robinson JS, McMillen IC, & Owens JA (2004). Postnatal ontogeny of glucose homeostasis and insulin action in sheep. *Am J Physiol Endocrinol Metab* **286**, E1050-E1059.

Gavete ML, Martin MA, Alvarez C, & Escriva F (2005). Maternal Food Restriction Enhances Insulin-Induced GLUT-4 Translocation and Insulin Signaling Pathway in Skeletal Muscle from Suckling Rats. *Endocrinology* **146**, 3368-3378.

Gerich JE (2002). Is Reduced First-Phase Insulin Release the Earliest Detectable Abnormality in Individuals Destined to Develop Type 2 Diabetes? *Diabetes* **51**, S117-S121.

Gerrard DE, Okamura CS, Ranalletta MA, & Grant AL (1998). Developmental expression and location of IGF-I and IGF-II mRNA and protein in skeletal muscle. *J Anim Sci* **76**, 1004-1011.

Gilbert S (2006). The early development of vertebrates: Fish, birds, and mammals. In *Developmental Biology*, ed. Gilbert S, pp. 345-388. Sinauer Associates Incorporated.

Giussani D, Spencer J, Moore P, Bennet L, & Hanson M (1993). Afferent and efferent components of the cardiovascular reflex responses to acute hypoxia in term fetal sheep. *J Physiol* **461**, 431-449.

Giussani DA, Forhead AJ, Gardner DS, Fletcher AJW, Allen WR, & Fowden AL (2003). Postnatal cardiovascular function after manipulation of fetal growth by embryo transfer in the horse. *J Physiol (Lond)* **547**, 67-76.

Gluckman PD & Hanson MA (2004a). Living with the Past: Evolution, Development, and Patterns of Disease. *Science* **305**, 1733-1736.

Gluckman PD & Hanson MA (2004b). The developmental origins of the metabolic syndrome. *Trends in Endocrinology and Metabolism* **15**, 183-187.

Gluckman P & Butler J (1983). Parturition-related changes in insulin-like growth factors-I and -II in the perinatal lamb. *J Endocrinol* **99**, 223-32.

Gluckman P, Hanson M, & Beedle A (2007). Early life events and their consequences for later disease: a life history and evolutionary perspective. *Am J Hum Biol* **19**, 1-19.

Godfrey KM & Barker DJ (2000). Fetal nutrition and adult disease. *Am J Clin Nutr* **71**, 1344S-1352.

Godfrey K, Lillycrop K, Burdge G, Gluckman P, & Hanson M (2007). Epigenetic mechanisms and the mismatch concept of the developmental origins of health and disease. *Pediatr Res* **61**, 5R-10R.

Goldspink DF, Cox VM, Smith SK, Eaves LA, Osbaldeston NJ, Lee DM, & Mantle D (1995). Muscle growth in response to mechanical stimuli. *Am J Physiol Endocrinol Metab* **268**, 288-297.

Gollnick P, Armstrong R, Saubert C, Piehl K, & Saltin B (1972). Enzyme activity and fiber composition in skeletal muscle of untrained and trained men. *Journal of applied physiology* **33**, 312-9.

Goodpaster BH, Park SW, Harris TB, Kritchevsky SB, Nevitt M, Schwartz AV, Simonsick EM, Tylavsky FA, Visser M, Newman AB, & for the Health ABC Study (2006). The Loss of Skeletal Muscle Strength, Mass, and Quality in Older Adults: The Health, Aging and Body Composition Study. *J Gerontol A Biol Sci Med Sci* **61**, 1059-1064.

Gould G & Holman G (1993). The glucose transporter family: structure, function and tissue-specific expression. *Biochem J* **295**, 329-41.

Graziotti GH, Rios CM, & Rivero JL (2001). Evidence for Three Fast Myosin Heavy Chain Isoforms in Type II Skeletal Muscle Fibers in the Adult Llama (Lama glama). *J Histochem Cytochem* **49**, 1033-1044.

Green H, Daub B, Houston M, Thomson J, Fraser I, & Ranney D (1981). Human vastus lateralis and gastrocnemius muscles. A comparative histochemical and biochemical analysis. *Journal of the Neurological Sciences* **52**, 201-10.

Greenlund LJS & Nair KS (2003). Sarcopenia--consequences, mechanisms, and potential therapies. *Mechanisms of Ageing and Development* **124**, 287-299.

Greenwood PL, Slepetis RM, Hermanson JW, & Bell AW (1999). Intrauterine growth retardation is associated with reduced cell cycle activity, but not myofibre number, in ovine fetal muscle. *Reprod Fertil Dev* **11**, 281-291.

Gregory P, Low R, & Stirewalt W (1986). Changes in skeletal-muscle myosin isoenzymes with hypertrophy and exercise. *Biochem J* **15**, 55-63.

Grimby G & Saltin B (1983). The ageing muscle. *Clin Physiol* **3**, 209-218.

Grottel K & Celichowski J (1999). The influence of changes in the stimulation pattern on force and fusion in motor units of the rat medial gastrocnemius muscle. *Exp Brain Res* **127**, 298-306.

Gruetter CA, Gruetter DY, Lyon JE, Kadowitz PJ, & Ignarro LJ (1981). Relationship between cyclic guanosine 3':5'-monophosphate formation and relaxation of coronary arterial smooth muscle by glyceryl trinitrate, nitroprusside, nitrite and nitric oxide: effects of methylene blue and methemoglobin. *J Pharmacol Exp Ther* **219**, 181-186.

Guillomot M (1995). Cellular interactions during implantation in domestic ruminants. *J Reprod Fertil Suppl* **49**, 39-51.

Guillomot M, Turbe A, Hue I, & Renard JP (2004). Staging of ovine embryos and expression of the T-box genes Brachyury and Eomesodermin around gastrulation. *Reproduction* **127**, 491-501.

Gumbiner B, Polonsky KS, Beltz WF, Wallace P, Brechtel G, & Fink RI (1989). Effects of aging on insulin secretion. *Diabetes* **38**, 1549-1556.

Guo Z & Zhou L (2004). Evidence for increased and insulin-resistant lipolysis in skeletal muscle of high-fat-fed rats. *Metabolism* **53**, 794-798.

Hafner E, Schuchter K, Metzenbauer M, & Philipp K (2000). Uterine artery Doppler perfusion in the first and second pregnancies. *Ultrasound Obstet Gynecol* **16**, 625-9.

Haggarty P (2002). Placental regulation of fatty acid delivery and its effect on fetal growth--a review. *Placenta* **23**, S28-38.

Hales CN, Barker DJ, Clark PM, Cox LJ, Fall C, Osmond C, & Winter PD (1991). Fetal and infant growth and impaired glucose tolerance at age 64. *BMJ* **303**, 1019-1022.

Hales CN & Ozanne SE (2003). The dangerous road of catch-up growth. *J Physiol (Lond)* **547**, 5-10.

Hales C, Desai M, Ozanne S, & Crowther N (1996). Fishing in the stream of diabetes: from measuring insulin to the control of fetal organogenesis. *Biochem Soc Trans* **24**, 341-350.

Hameed M, Orrell RW, Cobbold M, Goldspink G, & Harridge SDR (2003). Expression of IGF-I splice variants in young and old human skeletal muscle after high resistance exercise. *J Physiol (Lond)* **547**, 247-254.

Hanahan D & Folkman J (1996). Patterns and Emerging Mechanisms of the Angiogenic Switch during Tumorigenesis. *Cell* **86**, 353-364.

Handwerger S & Freemark M (2000). The roles of placental growth hormone and placental lactogen in the regulation of human fetal growth and development. *J Pediatr Endocrinol Metab* **4**, 343-356.

Hanson J & Huxley H (1954). Changes in the cross-striations of muscle during contraction and stretch and their structural interpretation. *Nature* **173**, 973-976.

Hardt SE & Sadoshima J (2002). Glycogen Synthase Kinase-3{beta}: A Novel Regulator of Cardiac Hypertrophy and Development. *Circ Res* **90**, 1055-1063.

Harvey M, Leco K, Arcellana-Panlilio M, Zhang X, Edwards D, & Schultz G (1995). Endocrinology and paracrinology: Roles of growth factors during peri-implantation development. *Hum Reprod* **10**, 712-718.

Hasty P, Bradley A, Morris JH, Edmondson DG, Venuti JM, Olson EN, & Klein WH (1993). Muscle deficiency and neonatal death in mice with a targeted mutation in the myogenin gene. *Nature* **364**, 501-506.

Haugen G, Hanson M, Kiserud T, Crozier S, Inskip H, & Godfrey KM (2005). Fetal Liver-Sparing Cardiovascular Adaptations Linked to Mother's Slimness and Diet. *Circ Res* **96**, 12-14.

Hawke TJ & Garry DJ (2001). Myogenic satellite cells: physiology to molecular biology. *J Appl Physiol* **91**, 534-551.

Hawkins P, Steyn C, McGarrigle HH, Calder NA, Saito T, Stratford LL, Noakes DE, & Hansona MA (2000a). Cardiovascular and hypothalamic-pituitary-adrenal axis development in late gestation fetal sheep and young lambs following modest maternal nutrient restriction in early gestation. *Reprod Fertil Dev* **12**, 443-456.

Hawkins P, Steyn C, Ozaki T, Saito T, Noakes DE, & HANSON MA (2000b). Effect of maternal undernutrition in early gestation on ovine fetal blood pressure and cardiovascular reflexes. *Am J Physiol Regul Integr Comp Physiol* **279**, R340-R348.

Haworth SG & Hislop AA (2003). Lung development--the effects of chronic hypoxia. *Seminars in Neonatology* **8**, 1-8.

Hay WW, Jr., Sparks JW, Wilkening RB, Battaglia FC, & Meschia G (1983). Partition of maternal glucose production between conceptus and maternal tissues in sheep. *Am J Physiol Endocrinol Metab* **245**, E347-E350.

Hay WW, Jr., Sparks JW, Wilkening RB, Battaglia FC, & Meschia G (1984). Fetal glucose uptake and utilization as functions of maternal glucose concentration. *Am J Physiol Endocrinol Metab* **246**, E237-E242.

Hay WJr (1994). Placental transport of nutrients to the fetus. *Hormone Research* **42**, 215-22.

Hay WJr (1991). The role of placental-fetal interaction in fetal nutrition. *Semin Perinatol* **15**, 424-433.

Hediger ML, Overpeck MD, Kuczmarski RJ, McGlynn A, Maurer KR, & Davis WW (1998). Muscularity and Fatness of Infants and Young Children Born Small- or Large-for-Gestational-Age. *Pediatrics* **102**, e60.

Helmreich E (2001). *The Biochemistry of Cell Signalling* Oxford University Press.

Hennessy BT, Smith DL, Ram PT, Lu Y, & Mills GB (2005). Exploiting the PI3K/AKT Pathway for Cancer Drug Discovery. *Nat Rev Drug Discov* **4**, 988-1004.

Henriksen EJ, Bourey RE, Rodnick KJ, Koranyi L, Permutt MA, & Holloszy JO (1990). Glucose transporter protein content and glucose transport capacity in rat skeletal muscles. *Am J Physiol Endocrinol Metab* **259**, E593-E598.

Hiles I, Volinia S, Otsu M, Fry M, Gout I, Dhand R, Panayotou G, Ruiz-Larrea F, Thompson A, & Totty N (1992). Phosphatidylinositol 3-kinase: structure and expression of the 110 kd catalytic subunit. *Cell* **70**, 419-429.

Hinchliffe S, Lynch M, Sargent P, Howard C, & Van Velzen D (1992). The effect of intrauterine growth retardation on the development of renal nephrons. *Br J Obstet Gynaecol* **99**, 296-301.

Hoh JFY (1991). Myogenic Regulation of Mammalian Skeletal Muscle Fibres. *News Physiol Sci* **6**, 1-6.

Holt R (2004). The Metabolic Effects of Growth Hormone. *CME Bulletin Endocrinology & Diabete* **5**, 11-17.

Hortobagyi T, Zheng D, Weidner M, Lambert NJ, Westbrook S, & Houmard JA (1995). The influence of aging on muscle strength and muscle fiber characteristics with special reference to eccentric strength. *J Gerontol A Biol Sci Med Sci* **50**, B399-B406.

Huxley R, Neil A, & Collins R (2008). Unravelling the fetal origins hypothesis: is there really an inverse association between birthweight and subsequent blood pressure? *The Lancet* **360**, 659-65.

Icardo J (1996). Developmental biology of the vertebrate heart. *The Journal of experimental zoology* **275**, 144-161.

Ignarro LJ, Adams JB, Horwitz PM, & Wood KS (1986). Activation of soluble guanylate cyclase by NO-hemoproteins involves NO- heme exchange. Comparison of heme-containing and heme-deficient enzyme forms. *J Biol Chem* **261**, 4997-5002.

Ijkema-Paassen J & Gramsbergen A (2005). Development of postural muscles and their innervation. *Neural Plast* **12**, 141-51.

International Diabetes Federation. www.idf.org . 2008.
Ref Type: Electronic Citation

Iwamoto H, Kaufman T, Keil L, & Rudolph A (1989). Responses to acute hypoxemia in fetal sheep at 0.6-0.7 gestation. *Am J Physiol* **256**, 613-20.

Jackson C, Dickinson A, & Ringel S (1990). Skeletal muscle fiber area alterations in two opposing modes of resistance-exercise training in the same individual. *Eur J Appl Physiol Occup Physiol* **61**, 37-41.

Jackson R (1990). Mechanisms of age-related glucose intolerance. *Diabetes Care* **13**, 9-19.

Jackson R, Blix P, Matthews J, Hamling J, Din B, Brown D, Belin J, Rubenstein A, & Nabarro J (1982). Influence of ageing on glucose homeostasis. *J Clin Endocrinol Metab* **55**, 840-8.

James DE, Jenkins AB, & Kraegen EW (1985). Heterogeneity of insulin action in individual muscles in vivo: euglycemic clamp studies in rats. *Am J Physiol Endocrinol Metab* **248**, E567-E574.

James DE, Zorzano A, Boni-Schnetzler M, Nemenoff RA, Powers A, Pilch PF, & Ruderman NB (1986). Intrinsic differences of insulin receptor kinase activity in red and white muscle. *J Biol Chem* **261**, 14939-14944.

Jansson T & Powell TL (2006). Human Placental Transport in Altered Fetal Growth: Does the Placenta Function as a Nutrient Sensor? - A Review. *Placenta* **27**, 91-97.

Jensen CB, Storgaard H, Dela F, Holst JJ, Madsbad S, & Vaag AA (2002). Early Differential Defects of Insulin Secretion and Action in 19-Year-Old Caucasian Men Who Had Low Birth Weight. *Diabetes* **51**, 1271-1280.

Jones D, DeHaan A, & Round J (2004). *Skeletal Muscle: From Molecules to Movement* Churchill Livingstone.

Jones HN, Powell TL, & Jansson T (2007). Regulation of Placental Nutrient Transport - A Review. *Placenta* **28**, 763-774.

Joshi N, Kulkarni S, Yajnik C, Joglekar C, Rao S, Coyaji K, Lubree H, Rege S, & Fall C (2005). Increasing maternal parity predicts neonatal adiposity: Pune Maternal Nutrition Study. *Am J Obstet Gynecol* **193**, 783-9.

Kaarsholm N & Ludvigsen S (1995). The high resolution solution structure of the insulin monomer determined by NMR. *Receptor* **5**, 1-8.

Kabsch W, Mannherz HG, Suck D, Pai EF, & Holmes KC (1990). Atomic structure of the actin: DNase I complex. *Nature* **347**, 37-44.

Kad NM, Kim S, Warshaw DM, VanBuren P, & Baker JE (2005). Single-myosin crossbridge interactions with actin filaments regulated by troponin-tropomyosin. *PNAS* **102**, 16990-16995.

Kadi F, Eriksson A, Holmner S, & Thornell L (1999a). Effects of anabolic steroids on the muscle cells of strength-trained athletes. *Med Sci Sports Exerc* **11**, 1528-34.

Kadi F, Eriksson A, Holmner S, Butler-Browne GS, & Thornell LE (1999b). Cellular adaptation of the trapezius muscle in strength-trained athletes. *Histochemistry and Cell Biology* **111**, 189-195.

Kadi F, Schjerling P, Andersen LL, Charifi N, Madsen JL, Christensen LR, & Andersen JL (2004). The effects of heavy resistance training and detraining on satellite cells in human skeletal muscles. *J Physiol (Lond)* **558**, 1005-1012.

Kahn S (2000). The importance of the beta-cell in the pathogenesis of type 2 diabetes mellitus. *Am J Med* **108**, 2-8.

Karabulut A, Layfield R, & Pratten M (2001). Growth promoting effects of human placental lactogen during early organogenesis: a link to insulin-like growth factors. *Journal of Anatomy* **198**, 651-62.

Kasuga M, Karlsson F, & Kahn C (1982). Insulin stimulates the phosphorylation of the 95,000-dalton subunit of its own receptor. *Science* **215**, 185-187.

Kawanaka K, Tabata I, Katsuta S, & Higuchi M (1997). Changes in insulin-stimulated glucose transport and GLUT-4 protein in rat skeletal muscle after training. *J Appl Physiol* **83**, 2043-2047.

Kern M, Wells J, Stephens J, Elton C, Friedman J, Tapscott E, Pekala P, & Dohm G (1990). Insulin responsiveness in skeletal muscle is determined by glucose transporter (Glut4) protein level. *Biochem J* **270**, 397-400.

Kim Js, Cross JM, & Bamman MM (2005). Impact of resistance loading on myostatin expression and cell cycle regulation in young and older men and women. *Am J Physiol Endocrinol Metab* **288**, E1110-E1119.

Kinoshita T & Miyajima A (2002). Cytokine regulation of liver development. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research* **1592**, 303-312.

Kirchengast S & Hartmann B (2003). Impact of maternal age and maternal somatic characteristics on newborn size. *American Journal of Human Biology* **15**, 220-228.

Kirchengast S, Hartmann B, Schweppe K, & Husslein P (1998). Impact of maternal body build characteristics on newborn size in two different European populations. *Hum Biol* **70**, 761-774.

Kleemann DO, Walker SK, Hartwich KM, Fong L, Seamark RF, Robinson JS, & Owens JA (2001). Fetoplacental Growth in Sheep Administered Progesterone during the First Three Days of Pregnancy. *Placenta* **22**, 14-23.

Kleemann D, Walker S, & Seamark R (1994). Enhanced fetal growth in sheep administered progesterone during the first three days of pregnancy. *Journal of reproduction and fertility* **102**, 411-417.

Knudsen KA (1985). The calcium-dependent myoblast adhesion that precedes cell fusion is mediated by glycoproteins. *J Cell Biol* **101**, 891-897.

Kohn AD, Summers SA, Birnbaum MJ, & Roth RA (1996). Expression of a Constitutively Active Akt Ser/Thr Kinase in 3T3-L1 Adipocytes Stimulates Glucose Uptake and Glucose Transporter 4Translocation. *J Biol Chem* **271**, 31372-31378.

Koistinen H & Zierath J (2002). Regulation of glucose transport in human skeletal muscle. *Annals of Medicine* **6**, 410-418.

Komi P, Viitasalo J, Havu M, Thorstensson A, Sjödin B, & Karlsson J (1977). Skeletal muscle fibres and muscle enzyme activities in monozygous and dizygous twins of both sexes. *Acta Physiol Scand* **100**, 385-392.

Koster JC, Permutt MA, & Nichols CG (2005). Diabetes and Insulin Secretion. *Diabetes* **54**, 3065-3072.

Kraemer WJ, Marchitelli L, Gordon SE, Harman E, Dziados JE, Mello R, Frykman P, McCurry D, & Fleck SJ (1990). Hormonal and growth factor responses to heavy resistance exercise protocols. *J Appl Physiol* **69**, 1442-1450.

Krebs M & Roden M (2004). Nutrient-induced insulin resistance in human skeletal muscle. *Current medicinal chemistry* **2004**, 901-8.

Krook A, Bjornholm M, Galuska D, Jiang XJ, Fahlman R, Myers MG, Wallberg-Henriksson H, & Zierath JR (2000). Characterization of signal transduction and glucose transport in skeletal muscle from type 2 diabetic patients. *Diabetes* **49**, 284-292.

Kugelberg E & Lindegren B (1979). Transmission and contraction fatigue of rat motor units in relation to succinate dehydrogenase activity of motor unit fibres. *J Physiol (Lond)* **288**, 285-300.

Kuh D, Bassey J, Hardy R, Aihie Sayer A, Wadsworth M, & Cooper C (2002). Birth Weight, Childhood Size, and Muscle Strength in Adult Life: Evidence from a Birth Cohort Study. *Am J Epidemiol* **156**, 627-633.

Kwong WY, Wild AE, Roberts P, Willis AC, & Fleming TP (2000). Maternal undernutrition during the preimplantation period of rat development causes blastocyst abnormalities and programming of postnatal hypertension. *Development* **127**, 4195-4202.

Lamontagne D, Pohl U, & Busse R (1992). Mechanical deformation of vessel wall and shear stress determine the basal release of endothelium-derived relaxing factor in the intact rabbit coronary vascular bed. *Circ Res* **70**, 123-130.

Lane R, Kelley D, Ritov V, Tsirka A, & Gruetzmacher E (2001). Altered expression and function of mitochondrial beta-oxidation enzymes in juvenile intrauterine-growth-retarded rat skeletal muscle. *Pediatr Res* **50**, 83-90.

Lang D, Matthews D, Peto J, & Turner R (1979). Cyclic oscillations of basal plasma glucose and insulin concentrations in human beings. *N Engl J Med* **301**, 1023-7.

Langley S & Jackson A (2005). Increased systolic blood pressure in adult rats induced by fetal exposure to maternal low protein diets. *Clin Sci (Lond)* **86**, 217-222.

Langley-Evans SC, Welham SJ, & Jackson AA (1999). Fetal exposure to a maternal low protein diet impairs nephrogenesis and promotes hypertension in the rat. *Life Sciences* **64**, 965-974.

Larciprete G, Valensise H, Vasapollo B, Di Pierro G, Menghini S, Magnani F, De Lorenzo A, & Arduini D (2003). Maternal body composition at term gestation and birth weight: is there a link? *Acta diabetologica* **40**, S222-4.

Larsen WJ (2001). *Human embryology*, 3rd ed. Churchill Livingstone.

Lassar A, Skapek S, & Novitch B (1994). Regulatory mechanisms that coordinate skeletal muscle differentiation and cell cycle withdrawal. *Curr Opin Cell Biol* **6**, 788-794.

Lea RG & Sandra O (2007). Immunoendocrine aspects of endometrial function and implantation. *Reproduction* **134**, 389-404.

Leahy JL (2005). Pathogenesis of Type 2 Diabetes Mellitus. *Archives of Medical Research* **36**, 197-209.

Leary SC, Lyons CN, Rosenberger AG, Ballantyne JS, Stillman J, & Moyes CD (2003). Fiber-type differences in muscle mitochondrial profiles. *Am J Physiol Regul Integr Comp Physiol* **285**, R817-R826.

Lefaucheur L, Hoffman RK, Gerrard DE, Okamura CS, Rubinstein N, & Kelly A (1998). Evidence for three adult fast myosin heavy chain isoforms in type II skeletal muscle fibers in pigs. *J Anim Sci* **76**, 1584-1593.

Lehman W, Rosol M, Tobacman LS, & Craig R (2001). Troponin organization on relaxed and activated thin filaments revealed by electron microscopy and three-dimensional reconstruction. *Journal of Molecular Biology* **307**, 739-744.

Leng R (1970). Glucose synthesis in ruminants. *Adv Vet Sci Comp Med* **14**, 209-260.

Leon DA, Lithell HO, Vagero D, Koupilova I, Mohsen R, Berglund L, Lithell UB, & McKeigue PM (1998). Reduced fetal growth rate and increased risk of death from ischaemic heart disease: cohort study of 15á000 Swedish men and women born 1915-29. *BMJ* **317**, 241-245.

Levine RJ, Maynard SE, Qian C, Lim KH, England LJ, Yu KF, Schisterman EF, Thadhani R, Sachs BP, Epstein FH, Sibai BM, Sukhatme VP, & Karumanchi SA (2004). Circulating Angiogenic Factors and the Risk of Preeclampsia. *N Engl J Med* **350**, 672-683.

Li J, Forhead AJ, Dauncey MJ, Gilmour RS, & Fowden AL (2002). Control of growth hormone receptor and insulin-like growth factor-I expression by cortisol in ovine fetal skeletal muscle. *J Physiol (Lond)* **541**, 581-589.

Lillioja S, Young A, Culter C, Ivy J, Abbott W, Zawadzki J, Yki-Järvinen H, Christin L, Secomb T, & Bogardus C (1987). Skeletal muscle capillary density and fiber type are possible determinants of in vivo insulin resistance in man. *The Journal of Clinical Investigation* **80**, 415-24.

Lillycrop K, Phillips E, Jackson A, Hanson M, & Burdge G (2005). Dietary protein restriction of pregnant rats induces and folic acid supplementation prevents epigenetic modification of hepatic gene expression in the offspring. *J Nutr* **135**, 1382-6.

Lincoln TM (1989). Cyclic GMP and mechanisms of vasodilation. *Pharmacology & Therapeutics* **41**, 479-502.

Lithell H, F L, Hellsing K, Lundqvist G, Nygaard E, Vessby B, & Saltin B (1981). Body weight, skeletal muscle morphology, and enzyme activities in relation to fasting serum insulin concentration and glucose tolerance in 48-year-old men. *Diabetes* **1**, 19-25.

Liu X, Yang C, Gupta N, Zuo J, Chang Y, & Fang F. Protein kinase C-zeta regulation of GLUT4 translocation through actin remodeling in CHO cells. J Mol Med. 85, 851-61. 2007.
Ref Type: Abstract

Louvi A, Accili D, & Efstratiadis A (1997). Growth-Promoting Interaction of IGF-II with the Insulin Receptor during Mouse Embryonic Development. *Developmental Biology* **189**, 33-48.

Lowell BB & Shulman GI (2005). Mitochondrial Dysfunction and Type 2 Diabetes. *Science* **307**, 384-387.

Ludwig T, Eggenschwiler J, Fisher P, D'Ercole AJ, Davenport ML, & Efstratiadis A (1996). Mouse Mutants Lacking the Type 2 IGF Receptor (IGF2R) Are Rescued from Perinatal Lethality inIgf2andIgf1rNull Backgrounds. *Developmental Biology* **177**, 517-535.

Lumbers E (1995). Development of renal function in the fetus: a review. *Reprod Fertil Dev* **7**, 415-26.

Lund S, Holman G, Schmitz O, & Pedersen O (1995). Contraction Stimulates Translocation of Glucose Transporter GLUT4 in Skeletal Muscle Through a Mechanism Distinct from that of Insulin. *PNAS* **92**, 5817-5821.

Lüscher T & Tanner F (1993). Endothelial regulation of vascular tone and growth. *Am J Hypertens* **6**, 283S-293S.

Maier A, McEwan JC, Dodds KG, Fischman DA, Fitzsimons RB, & Harris AJ (1992). Myosin heavy chain composition of single fibres and their origins and distribution in developing fascicles of sheep tibialis cranialis muscles. *Journal of Muscle Research and Cell Motility* **13**, 551-572.

Mallinson J, Sculley D, Craigon J, Plant R, Langley-Evans S, & Brameld J (2007). Fetal exposure to a maternal low-protein diet during mid-gestation results in muscle-specific effects on fibre type composition in young rats. *Br J Nutr* **98**, 292-9.

Mardones-Santander F, Salazar G, Rosso P, & Villarroel L (1998). Maternal body composition near term and birth weight. *Obstet Gynecol* **91**, 873-877.

Marin P, Andersson B, Krotkiewski M, & Bjorntorp P (1994). Muscle fiber composition and capillary density in women and men with NIDDM. *Diabetes Care* **17**, 382-386.

Mårin P, Andersson B, Krotkiewski M, & Björntorp P (1994). Muscle fiber composition and capillary density in women and men with NIDDM. *Diabetes Care* **17**, 382-386.

Marsál K, Persson P, Larsen T, Lilja H, Selbing A, & Sultan B (1996). Intrauterine growth curves based on ultrasonically estimated foetal weights. *Acta Paediatr* **85**, 843-8.

Marsden PA, Schappert KT, Chen HS, Flowers M, Sundell CL, Wilcox JN, Lamas S, & Michel T (1992). Molecular cloning and characterization of human endothelial nitric oxide synthase. *FEBS Letters* **307**, 287-293.

Mascarello F, Stecchini ML, Rowlerson A, & Ballocchi E (1992). Tertiary myotubes in postnatal growing pig muscle detected by their myosin isoform composition. *J Anim Sci* **70**, 1806-1813.

Maxfield EK, Sinclair KD, Dunne LD, Broadbent PJ, Robinson JJ, Stewart E, Kyle DG, & Maltin CA (1998). Temporary Exposure of Ovine Embryos to an Advanced Uterine Environment Does Not Affect Fetal Weight but Alters Fetal Muscle Development. *Biol Reprod* **59**, 321-325.

Maxwell L, Faulkner J, & Murphy R (1982). Relationship among fibre type, myosin ATPase activity and contractile properties. *Histochem J* **14**, 981-97.

McComas A (1995). Motor unit estimation: anxieties and achievements. *Muscle and Nerve* **18**, 369-379.

McDonagh M & Davies C (1984). Adaptive response of mammalian skeletal muscle to exercise with high loads. *Eur J Appl Physiol Occup Physiol* **52**, 139-55.

McEvoy TG, Robinson JJ, Aitken RP, Findlay PA, & Robertson IS (1995). Relationship between pre-ovulatory feed intake, progesterone priming and the subsequent in vitro development of OVA collected from superovulated ewes. *Theriogenology* **43**, 276.

McGuire MA, Vicini JL, Bauman DE, & Veenhuizen JJ (1992). Insulin-like growth factors and binding proteins in ruminants and their nutritional regulation. *J Anim Sci* **70**, 2901-2910.

McMullen S, Osgerby J, Milne J, Wallace J, & Wathes D (2005). The effects of acute nutrient restriction in the mid-gestational ewe on maternal and fetal nutrient status, the expression of placental growth factors and fetal growth. *Placenta* **26**, 25-33.

McPherron AC, Lawler AM, & Lee SJ (1997). Regulation of skeletal muscle mass in mice by a new TGF-beta superfamily member. *Nature* **387**, 83-90.

McPherron AC & Lee SJ (1997). Double muscling in cattle due to mutations in the myostatin gene. *PNAS* **94**, 12457-12461.

Mellor D & Murray L (1981). Effects of placental weight and maternal nutrition on the growth rates of individual fetuses in single and twin bearing ewes during late pregnancy. *Res Vet Sci* **30**, 198-204.

Mi J, Law C, Zhang K, Osmond C, Stein C, & Barker D (2000). Effects of Infant Birthweight and Maternal Body Mass Index in Pregnancy on Components of the Insulin Resistance Syndrome in China. *Ann Intern Med* **132**, 253-260.

Miller JB & Stockdale FE (1986). Developmental Origins of Skeletal Muscle Fibers: Clonal Analysis of Myogenic Cell Lineages Based on Expression of Fast and Slow Myosin Heavy Chains. *PNAS* **83**, 3860-3864.

Miller S, Green L, Peebles D, Hanson M, & Blanco C (2002). Effects of chronic hypoxia and protein malnutrition on growth in the developing chick. *Am J Obstet Gynecol* **186**, 261-267.

Mitchell E, Louey S, Cock M, Harding R, & Black M (2004). Nephron Endowment and Filtration Surface Area in the Kidney after Growth Restriction of Fetal Sheep. *Pediatr Res* **55**, 769-773.

Mitchell P, Johnson S, & Hannon K (2002). Insulin-like growth factor I stimulates myoblast expansion and myofiber development in the limb. *Developmental dynamics* **223**, 12-23.

Montarras D, Morgan J, Collins C, Relaix F, Zaffran S, Cumano A, Partridge T, & Buckingham M (2005). Direct isolation of satellite cells for skeletal muscle regeneration. *Science* **309**, 2064-7.

Moorman A, Webb S, Brown NA, Lamers W, & Anderson RH (2003). Development of the heart: (1) Formation of the cardiac chambers and arterial trunks. *Heart* **89**, 806-814.

Morgane P, Austin-LaFrance R, Bronzino J, Tonkiss J, Díaz-Cintra S, Cintra L, Kemper T, & Galler J (1993). Prenatal malnutrition and development of the brain. *Neurosci Biobehav Rev* 91-128.

Muller D, Elahi D, Tobin J, & Andres R (1996). Insulin response during the oral glucose tolerance test: the role of age, sex, body fat and the pattern of fat distribution. *Aging* **8**, 13-21.

Mulvany MJ & Halpern W (1977). Contractile properties of small arterial resistance vessels in spontaneously hypertensive and normotensive rats. *Circ Res* **41**, 19-26.

Nafikov RA & Beitz DC (2007). Carbohydrate and Lipid Metabolism in Farm Animals. *J Nutr* **137**, 702-705.

Nakae J, Kido Y, & Accili D (2001). Distinct and Overlapping Functions of Insulin and IGF-I Receptors. *Endocr Rev* **22**, 818-835.

Napier J, Thomas MF, Sharma M, Hodgkinson SC, & Bass JJ (1999). Insulin-like growth factor-I protects myoblasts from apoptosis but requires other factors to stimulate proliferation. *J Endocrinol* **163**, 63-68.

Neil D (1964). *The Anatomy of the Sheep* University of Queensland Press.

Newton A (1995). Protein kinase C: structure, function, and regulation. *J Biol Chem* **270**, 28495-8.

Nishina H, Green LR, McGarrigle HHG, Noakes DE, Poston L, & HANSON MA (2003). Effect of nutritional restriction in early pregnancy on isolated femoral artery function in mid-gestation fetal sheep. *The Journal of Physiology* **553**, 637-647.

Nolte L, Abdel-Halim S, Martin I, Guenifi A, Zierath J, Ostenson C, & Wallberg-Henriksson H (1995). Development of decreased insulin-induced glucose transport in skeletal muscle of glucose-intolerant hybrids of diabetic GK rats. *Clin Sci (Lond)* **88**, 301-306.

O'Connor PMJ, Bush JA, Suryawan A, Nguyen HV, & Davis TA (2003). Insulin and amino acids independently stimulate skeletal muscle protein synthesis in neonatal pigs. *Am J Physiol Endocrinol Metab* **284**, E110-E119.

Oken E, Taveras E, Kleinman K, Rich-Edwards J, & Gillman M (2007). Gestational weight gain and child adiposity at age 3 years. *Am J Obstet Gynecol* **196**.

Oliver MH, Hawkins P, Breier BH, Van Zijl PL, Sargison SA, & Harding JE (2001). Maternal Undernutrition during the Periconceptual Period Increases Plasma Taurine Levels and Insulin Response to Glucose But Not Arginine in the Late Gestational Fetal Sheep. *Endocrinology* **142**, 4576-4579.

Ong K, Kratzsch J, Kiess W, Costello M, Scott C, & Dunger D (2000). Size at Birth and Cord Blood Levels of Insulin, Insulin-Like Growth Factor I (IGF-I), IGF-II, IGF-Binding Protein-1 (IGFBP-1), IGFBP-3, and the Soluble IGF-II/Mannose-6-Phosphate Receptor in Term Human Infants. *J Clin Endocrinol Metab* **85**, 4266-4269.

Ong K, Preece M, Emmett P, Ahmed M, Dunger D, & ALSPAC Study Team (2002). Size at birth and early childhood growth in relation to maternal smoking, parity and infant breast-feeding: longitudinal birth cohort study and analysis. *Pediatr Res* **52**, 863-867.

Ortiz L, Quan A, Weinberg A, & Baum M (2001). Effect of prenatal dexamethasone on rat renal development. *Kidney International* **59**, 1663-1669.

Osmond C, Barker DJ, Winter PD, Fall CH, & Simmonds SJ (1993). Early growth and death from cardiovascular disease in women. *BMJ* **307**, 1519-1524.

Ozaki T, Nishina H, Hanson MA, & Poston L (2001). Dietary restriction in pregnant rats causes gender-related hypertension and vascular dysfunction in offspring. *The Journal of Physiology* **530**, 141-152.

Ozaki T, Hawkins P, Nishina H, Steyn C, Poston L, & Hanson MA (2000). Effects of undernutrition in early pregnancy on systemic small artery function in late-gestation fetal sheep. *American Journal of Obstetrics and Gynecology* **183**, 1301-1307.

Ozanne SE, Olsen GS, Hansen LL, Tingey KJ, Nave BT, Wang CL, Hartil K, Petry CJ, Buckley AJ, & Mosthaf-Seedorf L (2003). Early growth restriction leads to down regulation of protein kinase C zeta and insulin resistance in skeletal muscle. *J Endocrinol* **177**, 235-241.

Ozanne SE, Wang CL, Coleman N, & Smith GD (1996). Altered muscle insulin sensitivity in the male offspring of protein-malnourished rats. *Am J Physiol Endocrinol Metab* **271**, E1128-E1134.

Ozanne S, Jensen C, Tingey K, Storgaard H, Madsbad S, & Vaag A (2005). Low birthweight is associated with specific changes in muscle insulin-signalling protein expression. *Diabetologia* **48**, 547-542.

Painter RC, Roseboom TJ, & Bleker OP (2005a). Prenatal exposure to the Dutch famine and disease in later life: An overview. *Reproductive Toxicology* **20**, 345-352.

Painter RC, Roseboom TJ, van Montfrans GA, Bossuyt PMM, Krediet RT, Osmond C, Barker DJP, & Bleker OP (2005b). Microalbuminuria in Adults after Prenatal Exposure to the Dutch Famine. *J Am Soc Nephrol* **16**, 189-194.

Paleolog E (2002). Angiogenesis in rheumatoid arthritis. *Arthritis Res* **4**, S81-90.

Palmer RMJ, Rees DD, Ashton DS, & Moncada S (1988). L-arginine is the physiological precursor for the formation of nitric oxide in endothelium-dependent relaxation. *Biochemical and Biophysical Research Communications* **153**, 1251-1256.

Parr R (1992). Nutrition-progesterone interactions during early pregnancy in sheep. *Reproduction, fertility, and development* **4**, 297-300.

Patti M & Kahn C (1998). The insulin receptor--a critical link in glucose homeostasis and insulin action. *J Basic Clin Physiol Pharmacol* **9**, 89-109.

Pedersen O, Bak JF, Andersen PH, Lund S, Moller DE, Flier JS, & Kahn BB (1990). Evidence against altered expression of GLUT1 or GLUT4 in skeletal muscle of patients with obesity or NIDDM. *Diabetes* **39**, 865-870.

Pessin JE, Thurmond DC, Elmendorf JS, Coker KJ, & Okada S (1999). Molecular Basis of Insulin-stimulated GLUT4 Vesicle Trafficking. LOCATION! LOCATION! LOCATION! *J Biol Chem* **274**, 2593-2596.

Petrella JK, Kim Js, Cross JM, Kosek DJ, & Bamman MM (2006). Efficacy of myonuclear addition may explain differential myofiber growth among resistance-trained young and older men and women. *Am J Physiol Endocrinol Metab* **291**, E937-E946.

Pette D & Staron R (1990). Cellular and molecular diversities of mammalian skeletal muscle fibers. *Rev Physiol Biochem Pharmacol* **116**, 1-76.

Pette D & Staron R (1997). Mammalian skeletal muscle fiber type transitions. *Int Rev Cytol* **170**, 143-223.

Pettitt D, Forman M, Hanson R, Knowler W, & Bennett PH (1997). Breastfeeding and incidence of non-insulin-dependent diabetes mellitus in Pima Indians. *Lancet* **350**, 166-8.

Phillips DI, Barker DJ, Hales CN, Hirst S, & Osmond C (1994). Thinness at birth and insulin resistance in adult life. *Diabetologia* **37**, 150-4.

Picard B, Lefaucheur L, Berri C, & Duclos MJ (2002). Muscle fibre ontogenesis in farm animal species. *Reprod Nutr Dev* **42**, 415-431.

Pin C, Hrycyshyn A, Rogers K, Rushlow W, & Merrifield P (2002). Embryonic and fetal rat myoblasts form different muscle fiber types in an ectopic in vivo environment. *Dev Dyn* **224**, 253-66.

Ploug T, van Deurs B, Ai H, Cushman SW, & Ralston E (1998). Analysis of GLUT4 Distribution in Whole Skeletal Muscle Fibers: Identification of Distinct Storage Compartments That Are Recruited by Insulin and Muscle Contractions. *J Cell Biol* **142**, 1429-1446.

Pohl U, De Wit C, & Gloe T (2000). Large arterioles in the control of blood flow: role of endothelium-dependent dilation. *Acta Physiologica Scandinavica* **168**, 505-510.

Poore KR, Cleal JK, Newman JP, Boullin JP, Noakes DE, HANSON MA, & Green LR (2007). Nutritional challenges during development induce sex-specific changes in glucose homeostasis in the adult sheep. *Am J Physiol Endocrinol Metab* **292**, E32-E39.

Pourquie O, Coltey M, Breant C, & Douarin NML (1995). Control of Somite Patterning by Signals from the Lateral Plate. *PNAS* **92**, 3219-3223.

Powell-Braxton L, Hollingshead P, Warburton C, Dowd M, Pitts-Meek S, Dalton D, Gillett N, & Stewart T (1993). IGF-I is required for normal embryonic growth in mice. *Genes & development* **7**, 2609-2617.

Prior BM, Yang HT, & Terjung RL (2004). What makes vessels grow with exercise training? *J Appl Physiol* **97**, 1119-1128.

Punkt K (2002). Fibre types in skeletal muscles. *Adv Anat Embryol Cell Biol* **162**, 1-109.

Purves W, Sadava D, Orians G, & Heller C (2003). *Life: The Science of Biology*, 7th ed. Sinauer Associates and W. H. Freeman.

Quigley SP, Kleemann DO, Kakar MA, Owens JA, Nattrass GS, Maddocks S, & Walker SK (2005). Myogenesis in sheep is altered by maternal feed intake during the peri-conception period. *Animal Reproduction Science* **87**, 241-251.

Rantanen T, Harris T, Leveille SG, Visser M, Foley D, Masaki K, & Guralnik JM (2000). Muscle Strength and Body Mass Index as Long-Term Predictors of Mortality in Initially Healthy Men. *J Gerontol A Biol Sci Med Sci* **55**, M168-M173.

Ravelli ACJ, van der Meulen JHP, Michels RPJ, Osmond C, Barker DJP, Hales CN, & Bleker OP (1998). Glucose tolerance in adults after prenatal exposure to famine. *The Lancet* **351**, 173-177.

Ravelli AC, van der Meulen JH, Osmond C, Barker DJ, & Bleker OP (1999). Obesity at the age of 50 y in men and women exposed to famine prenatally. *Am J Clin Nutr* **70**, 811-816.

Rawls A, Morris J, Rudnicki M, Braun T, Arnold H-H, Klein W, & Olson E. Myogenin's Functions Do Not Overlap with Those of MyoD or Myf-5 during Mouse Embryogenesis. Developmental Biology 172, 37-50. 1995.
Ref Type: Abstract

Rayment I, Rypniewski W, Schmidt-Bäse K, Smith R, Tomchick D, Benning M, Winkelmann D, Wesenberg G, & Holden H (1993). Three-dimensional structure of myosin subfragment-1: a molecular motor. *Science* **261**, 35-36.

Rayment I (1996). The Structural Basis of the Myosin ATPase Activity. *J Biol Chem* **271**, 15850-15853.

Razin A (1998). CpG methylation, chromatin structure and gene silencing—a three-way connection. *EMBO J* **17**, 4905-8.

Rehfeldt C, Kuhn G, Vanselow J, Fürbass R, Fiedler I, Nürnberg G, Clelland A, Stickland N, & Ender K (2001a). Maternal treatment with somatotropin during early gestation affects basic events of myogenesis in pigs. *Cell and Tissue Research* **306**, 429-440.

Rehfeldt C, Kuhn G, Nurnberg G, Kanitz E, Schneider F, Beyer M, Nurnberg K, & Ender K (2001b). Effects of exogenous somatotropin during early gestation on maternal performance, fetal growth, and compositional traits in pigs. *J Anim Sci* **79**, 1789-1799.

Rennie MJ, Wackerhage H, Spangenburg EE, & Booth FW (2004). Control of the Size of the Human Muscle Mass. *Annual Review of Physiology* **66**, 799-828.

Research Defence Society. The 3Rs. http://www.rds-online.org.uk/ . 2008.
Ref Type: Electronic Citation

Reuss M, Parer J, Harris J, & Krueger T (1982). Hemodynamic effects of alpha-adrenergic blockade during hypoxia in fetal sheep. *American journal of obstetrics and gynecology* **15**, 410-415.

Rhee M, Ziemer D, Kolm P, & Phillips L (2006). Postchallenge glucose rises with increasing age even when glucose tolerance is normal. *Diabet Med* **23**, 1174-9.

Rice J & Allis C (2001). Histone methylation versus histone acetylation: new insights into epigenetic regulation. *Curr Opin Cell Biol* **13**, 263-73.

Rich-Edwards JW, Stampfer MJ, Manson JE, Rosner B, Hankinson SE, Colditz GA, Hennekens CH, & Willet WC (1997). Birth weight and risk of cardiovascular disease in a cohort of women followed up since 1976. *British Medical Journal* **315**, 396-400.

Risau W & Flamme I (1995). Vasculogenesis. *Annual Review of Cell and Developmental Biology* **11**, 73-91.

Rooyackers O, Adey D, Ades P, & Nair Ká (1996). Effect of age on in vivo rates of mitochondrial protein synthesis in humanáskeletalámuscle. *PNAS* **93**, 15364-15369.

Roseboom TJ, de Rooij S, & Painter R (2006). The Dutch famine and its long-term consequences for adult health. *Early Human Development* **82**, 485-91.

Roseboom TJ, van der Meulen JHP, Osmond C, Barker DJP, Ravelli ACJ, Schroeder-Tanka JM, van Montfrans GA, Michels RPJ, & Bleker OP (2000a). Coronary heart disease after prenatal exposure to the Dutch famine, 1944-45. *Heart* **84**, 595-598.

Roseboom TJ, van der Meulen JH, Osmond C, Barker DJ, Ravelli AC, & Bleker OP (2000b). Plasma lipid profiles in adults after prenatal exposure to the Dutch famine. *Am J Clin Nutr* **72**, 1101-1106.

Rosenthal SM, Hsiao D, & Silverman LA (1994). An insulin-like growth factor-II (IGF-II) analog with highly selective affinity for IGF-II receptors stimulates differentiation, but not IGF-I receptor down-regulation in muscle cells. *Endocrinology* **135**, 38-44.

Ross JJ, Duxson MJ, & Harris AJ (1987). Neural determination of muscle fibre numbers in embryonic rat lumbrical muscles. *Development* **100**, 395-409.

Rossell R, Gomis R, Casamitjana R, Segura R, Vilardell E, & Rivera F (1983). Reduced hepatic insulin extraction in obesity: relationship with plasma insulin levels. *J Clin Endocrinol Metab* **56**, 608-11.

Rosso P & Kava R (1980). Effects of Food Restriction on Cardiac Output and Blood Flow to the Uterus and Placenta in the Pregnant Rat. *J Nutr* **110**, 2350-2354.

Roth SM, Martel GF, Ferrell RE, Metter EJ, Hurley BF, & Rogers MA (2003). Myostatin Gene Expression Is Reduced in Humans with Heavy-Resistance Strength Training: A Brief Communication. *Experimental Biology and Medicine* **228**, 706-709.

Rother K & Accili D (2000). Role of insulin receptors and IGF receptors in growth and development. *Pediatr Nephrol* **14**, 558-61.

Rudnicki MA, Schnegelsberg PNJ, Stead RH, Braun T, Arnold HH, & Jaenisch R (1993). MyoD or Myf-5 is required for the formation of skeletal muscle. *Cell* **75**, 1351-1359.

Rudolph AM (1979). Fetal and Neonatal Pulmonary Circulation. *Annual Review of Physiology* **41**, 383-395.

Russell A (1991). *Body condition scoring of sheep*, pp. 3-10. Bailliere Tindall, London.

Sacks D (2004). Determinants of fetal growth. *Curr Diab Rep* 281-7.

Salathe EP & Chen C (1993). The role of myoglobin in retarding oxygen depletion in skeletal muscle. *Mathematical Biosciences* **116**, 1-20.

Saltiel AR & Kahn CR (2001). Insulin signalling and the regulation of glucose and lipid metabolism. *Nature* **414**, 799-806.

Samuelsson AM, Matthews PA, Argenton M, Christie MR, McConnell JM, Jansen EHJ, Piersma AH, Ozanne SE, Twinn DF, Remacle C, Rowlerson A, Poston L, & Taylor PD (2008). Diet-Induced Obesity in Female Mice Leads to Offspring Hyperphagia, Adiposity, Hypertension, and Insulin Resistance: A Novel Murine Model of Developmental Programming. *Hypertension* **51**, 383-392.

Sanes JR (2003). The Basement Membrane/Basal Lamina of Skeletal Muscle. *J Biol Chem* **278**, 12601-12604.

Sartore S, Gorza L, & Schiaffino S (1982). Fetal myosin heavy chains in regenerating muscle. *Nature* **298**, 294-296.

Sayer AA, Syddall HE, Gilbody HJ, Dennison EM, & Cooper C (2004). Does Sarcopenia Originate in Early Life? Findings From the Hertfordshire Cohort Study. *J Gerontol A Biol Sci Med Sci* **59**, M930-M934.

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

Schilling WP, Rajan L, & Strobl-Jager E (1989). Characterization of the bradykinin-stimulated calcium influx pathway of cultured vascular endothelial cells. Saturability, selectivity, and kinetics. *J Biol Chem* **264**, 12838-12848.

Schmidt HH, Gagne GD, Nakane M, Pollock JS, Miller MF, & Murad F (1992). Mapping of neural nitric oxide synthase in the rat suggests frequent co- localization with NADPH diaphorase but not with soluble guanylyl cyclase, and novel paraneural functions for nitrinergic signal transduction. *J Histochem Cytochem* **40**, 1439-1456.

Schneider H (1996). Ontogenic changes in the nutritive function of the placenta. *Placenta* **17**, 15-26.

Scott PH, Brunn GJ, Kohn AD, Roth RA, & Lawrence JC, Jr. (1998). Evidence of insulin-stimulated phosphorylation and activation of the mammalian target of rapamycin mediated by a protein kinase B signaling pathway. *PNAS* **95**, 7772-7777.

Seale P & Rudnicki MA (2000). A New Look at the Origin, Function, and "Stem-Cell" Status of Muscle Satellite Cells. *Developmental Biology* **218**, 115-124.

Selak MA, Storey BT, Peterside I, & Simmons RA (2003). Impaired oxidative phosphorylation in skeletal muscle of intrauterine growth-retarded rats. *Am J Physiol Endocrinol Metab* **285**, E130-E137.

Sengupta T, Datta C, Dasgupta J, & Sengupta D (1986). Age related changes of gluconeogenic activity in human placenta during foetal development. *Indian J Med Res* **84**, 385-9.

Shanik M, Xu Y, Skrha J, Dankner R, Zick Y, & Roth J (2008). Insulin Resistance and Hyperinsulinemia - is hyperinsulinemia the cart or the horse? *Diabetes Care* **31**, S262-8.

Sheldon R, Peeters L, Jones MJ, Makowski E, & Meschia G (1979). Redistribution of cardiac output and oxygen delivery in the hypoxemic fetal lamb. *Am J Obstet Gynecol* **15**, 1071-8.

Shepherd PR & Kahn BB (1999). Glucose Transporters and Insulin Action -- Implications for Insulin Resistance and Diabetes Mellitus. *N Engl J Med* **341**, 248-257.

Shiell AW, Campbell DM, Hall MH, & Barker DJP (2000). Diet in late pregnancy and glucose-insulin metabolism of the offspring 40 years later. *BJOG: An International Journal of Obstetrics and Gynaecology* **107**, 890-895.

Shimokata H, Muller DC, Fleg JL, Sorkin J, Ziemba AW, & Andres R (1991). Age as independent determinant of glucose tolerance. *Diabetes* **40**, 44-51.

Shoji S (1986). Effect of denervation on glucose uptake in rat soleus and extensor digitorum longus muscles. *Muscle and Nerve* **9**, 69-72.

Shulman GI (2000). Cellular mechanisms of insulin resistance. *J Clin Invest* **106**, 171-176.

Simoneau JA & Bouchard C (1995). Genetic determinism of fiber type proportion in human skeletal muscle. *FASEB J* **9**, 1091-1095.

Sinha-Hikim I, Roth SM, Lee MI, & Bhasin S (2003). Testosterone-induced muscle hypertrophy is associated with an increase in satellite cell number in healthy, young men. *Am J Physiol Endocrinol Metab* **285**, E197-E205.

Sjogren K, Liu JL, Blad K, Skrtic S, Vidal O, Wallenius V, LeRoith D, Tornell J, Isaksson OG, Jansson JO, & Ohlsson C (1999b). Liver-derived insulin-like growth factor I (IGF-I) is the principal source of IGF-I in blood but is not required for postnatal body growth in mice. *PNAS* **96**, 7088-7092.

Sjogren K, Liu JL, Blad K, Skrtic S, Vidal O, Wallenius V, LeRoith D, Tornell J, Isaksson OG, Jansson JO, & Ohlsson C (1999a). Liver-derived insulin-like growth factor I (IGF-I) is the principal source of IGF-I in blood but is not required for postnatal body growth in mice. *PNAS* **96**, 7088-7092.

Skolnik E, Margolis B, Mohammadi M, Lowenstein E, Fischer R, Drepps A, Ullrich A, & Schlessinger J (1991). Cloning of PI3 kinase-associated p85 utilizing a novel method for expression/cloning of target proteins for receptor tyrosine kinases. *Cell* **65**, 83-90.

Smerdu V, Karsch-Mizrachi I, Campione M, Leinwand L, & Schiaffino S (1994). Type IIx myosin heavy chain transcripts are expressed in type IIb fibers of human skeletal muscle. *Am J Physiol Cell Physiol* **267**, C1723-C1728.

Snell CR & Smyth DG (1975). Proinsulin: a proposed three-dimensional structure. *J Biol Chem* **250**, 6291-6295.

Snoeck A, Remacle C, Reusens B, & Hoet J (1990). Effect of a low protein diet during pregnancy on the fetal rat endocrine pancreas. *Biol Neonate* **57**, 107-18.

Somanath P, Razorenova O, Chen J, & Byzova T (2006). Akt1 in endothelial cell and angiogenesis. *Cell Cycle* **5**, 512-8.

Soto N, Bazaes RA, Pena V, Salazar T, Avila A, Iniguez G, Ong KK, Dunger DB, & Mericq MV (2003). Insulin Sensitivity and Secretion Are Related to Catch-Up Growth in Small-for-Gestational-Age Infants at Age 1 Year: Results from a Prospective Cohort. *J Clin Endocrinol Metab* **88**, 3645-3650.

Sparks J, Hay WJ, Bonds D, Meschia G, & Battaglia F (1982). Simultaneous measurements of lactate turnover rate and umbilical lactate uptake in the fetal lamb. *J Clin Invest* **70**, 179-92.

Spencer T, Johnson G, Bazer F, & Burghardt R (2004). Implantation mechanisms: insights from the sheep. *Reproduction* **128**, 657-668.

Spencer T, Johnson G, Bazer F, & Burghardt R (2007). Fetal-maternal interactions during the establishment of pregnancy in ruminants. *Soc Reprod Fertil Suppl* **64**, 379-96.

Standaert ML, Galloway L, Karnam P, Bandyopadhyay G, Moscat J, & Farese RV (1997). Protein Kinase C-zeta as a Downstream Effector of Phosphatidylinositol 3-Kinase during Insulin Stimulation in Rat Adipocytes. POTENTIAL ROLE IN GLUCOSE TRANSPORT. *J Biol Chem* **272**, 30075-30082.

Stein CE, Fall CHD, Kumaran K, Osmond C, Cox V, & Barker DJP (1996). Fetal growth and coronary heart disease in South India. *The Lancet* **348**, 1269-1273.

Steppel J & Horton E (2004). Beta-cell failure in the pathogenesis of type 2 diabetes mellitus. *Curr Diab Rep* **4**, 169-75.

Stewart CE & Rotwein P (1996). Growth, differentiation, and survival: multiple physiological functions for insulin-like growth factors. *Physiol Rev* **76**, 1005-1026.

Stockdale FE (1992). Myogenic cell lineages. *Developmental Biology* **154**, 284-298.

Stuehr DJ, Cho HJ, Kwon NS, Weise MF, & Nathan CF (1991). Purification and Characterization of the Cytokine-Induced Macrophage Nitric Oxide Synthase: An FAD- and FMN-Containing Flavoprotein. *PNAS* **88**, 7773-7777.

Sun XJ, Rothenberg P, Kahn CR, Backer JM, Araki E, Wilden PA, Cahill DA, Goldstein BJ, & White MF (1991). Structure of the insulin receptor substrate IRS-1 defines a unique signal transduction protein. *Nature* **352**, 73-77.

Taddei S, Virdis A, Ghiadoni L, Sudano I, & Salvetti A (2001). Endothelial dysfunction in hypertension. *J Cardiovasc Pharmacol* **38**, S11-14.

Takahashi A, Kureishi Y, Yang J, Luo Z, Guo K, Mukhopadhyay D, Ivashchenko Y, Branellec D, & Walsh K (2002). Myogenic Akt Signaling Regulates Blood Vessel Recruitment during Myofiber Growth. *Mol Cell Biol* **22**, 4803-4814.

Tanabe R, Muroya S, & Chikuni K (1998). Sequencing of the 2a, 2x, and slow isoforms of the bovine myosin heavy chain and the different expression among muscles. *Mamm Genome* **9**, 1056-8.

Tanner C, Barakat H, Dohm G, Pories W, MacDonald K, Cunningham P, Swanson M, & Houmard J (2002). Muscle fiber type is associated with obesity and weight loss. *Am J Physiol Endocrinol Metab* **282**, E1191-E1196.

Tesch PA, Thorsson A, & Fujitsuka N (1989). Creatine phosphate in fiber types of skeletal muscle before and after exhaustive exercise. *J Appl Physiol* **66**, 1756-1759.

Thibodeau G & Patton K (2003). *Anatomy and Physiology*, 5th ed. Mosby.

Thomas M, Langley B, Berry C, Sharma M, Kirk S, Bass J, & Kambadur R (2000). Myostatin, a Negative Regulator of Muscle Growth, Functions by Inhibiting Myoblast Proliferation. *J Biol Chem* **275**, 40235-40243.

Thorens B (1992). Molecular and cellular physiology of GLUT-2, a high-Km facilitated diffusion glucose transporter. *Int Rev Cytol* **137**, 209-38.

Titlbach M, Fält K, & Falkmer S (1985). Postnatal maturation of the islets of Langerhans in sheep. Light microscopic, immunohistochemical, morphometric, and ultrastructural investigations with particular reference to the transient appearance of argyrophil insulin immunoreactive cells. *Diabetes Res* **2**, 5-15.

Tonnesen M, Feng X, & Clark R (2000). Angiogenesis in wound healing. *J Investig Dermatol Symp Proc* **5**, 40-46.

Torrens C. Transgenerational programming of the cardiovascular system by protein restriction in pregnancy. 2004. University of Southampton.
Ref Type: Thesis/Dissertation

Tortora G & Grabowski S (1996). Principles of Anatomy and Physiology. Harper Collins.

Toschi E, Camastra S, Sironi AM, Masoni A, Gastaldelli A, Mari A, Ferrannini E, & Natali A (2002). Effect of Acute Hyperglycemia on Insulin Secretion in Humans. *Diabetes* **51**, S130-S133.

Treadway JL, Hargrove DM, Nardone NA, McPherson RK, Russo JF, Milici AJ, Stukenbrok HA, Gibbs EM, Stevenson RW, & Pessin JE (1994). Enhanced peripheral glucose utilization in transgenic mice expressing the human GLUT4 gene. *J Biol Chem* **269**, 29956-29961.

Tsika RW, Herrick RE, & Baldwin KM (1987). Interaction of compensatory overload and hindlimb suspension on myosin isoform expression. *J Appl Physiol* **62**, 2180-2186.

Tygesen M, Nielsen M, Nørgaard P, Ranvig H, Harrison A, & Tauson AH (2008). Late gestational nutrient restriction: effects on ewes' metabolic and homeorhetic adaptation, consequences for lamb birth weight and lactation performance. *Arch Anim Nutr* **62**, 44-59.

UAB Health System. www.health.uab.edu . 2008.
Ref Type: Electronic Citation

Underwood L & D'Ercole A (1984). Insulin and insulin-like growth factors/somatomedins in fetal and neonatal development. *Clin Endocrinol Metab* **13**, 69-89.

Urban RJ, Bodenburg YH, Gilkison C, Foxworth J, Coggan AR, Wolfe RR, & Ferrando A (1995). Testosterone administration to elderly men increases skeletal muscle strength and protein synthesis. *Am J Physiol Endocrinol Metab* **269**, E820-E826.

Vaidya TB, Rhodes SJ, Taparowsky EJ, & Konieczny SF (1989). Fibroblast growth factor and transforming growth factor beta repress transcription of the myogenic regulatory gene MyoD1. *Mol Cell Biol* **9**, 3576-3579.

Vallance P, Collier J, & Moncada S (1989). Effects of endothelium-derived nitric oxide on peripheral arteriolar in man. *The Lancet* **334**, 997-1000.

Vartanian SM & Sarkar R (2007). Therapeutic Angiogenesis. *Vascular and Endovascular Surgery* **41**, 173-185.

Vatnick I, Schoknecht P, Darrigrand R, & Bell A (1991). Growth and metabolism of the placenta after unilateral fetectomy in twin pregnant ewes. *Journal of developmental physiology* **15**, 351-356.

Velázquez A AN, Rosado A, Bernal A, & Noriega L (1976). Amino acid pools in the feto-maternal system. *Biol Neonate* **29**, 28-40.

Venojarvi M, Puhke R, Hamalainen H, Marniemi J, Rastas M, Rusko H, Nuutila P, Hanninen O, & Aunola S (2005). Role of skeletal muscle-fibre type in regulation of glucose metabolism in middle-aged subjects with impaired glucose tolerance during a long-term exercise and dietary intervention. *Diabetes, Obesity and Metabolism* **7**, 745-754.

von Kries R, Koletzko B, Sauerwald T, von Mutius E, Barnert D, Grunert V, & von Voss H (1999). Breast feeding and obesity: cross sectional study. *BMJ* **319**, 147-50.

Vyas DR, Spangenburg EE, Abraha TW, Childs TE, & Booth FW (2002). GSK-3beta negatively regulates skeletal myotube hypertrophy. *Am J Physiol Cell Physiol* **283**, C545-C551.

Wackerhage H & Rennie MJ (2006). How nutrition and exercise maintain the human musculoskeletal mass. *Journal of Anatomy* **208**, 451-458.

Walker D & Luff A. Functional development of fetal limb muscles: a review of the roles of activity, nerves and hormones. Reprod.Fertil.Dev 7, 391-398. 1995.
Ref Type: Generic

Wallace J, Bourke D, Da Silva P, & Aitken R (2001). Nutrient partitioning during adolescent pregnancy. *Reproduction* **122**, 347-357.

Walton A & Hammond J (1938). The maternal effects on growth and conformation in Shire horse-Shetland crosses. *Proc R Soc Lond B* **125**, 311-335.

Wang X, Chu W, & van Breemen C (1996). Potentiation of acetylcholine-induced responses in freshly isolated rabbit aortic endothelial cells. *J Vasc Res* **33**, 414-424.

Ward S & Stickland N (1991). Why are slow and fast muscles differentially affected during prenatal undernutrition? *Muscle and Nerve* **14**, 259-67.

Ware J & Simons M (1997). Angiogenesis in ischemic heart disease. *Nature Medicine* **3**, 158-164.

Warnotte C, Gilon P, Nenquin M, & Henquin JC (1994). Mechanisms of the stimulation of insulin release by saturated fatty acids. A study of palmitate effects in mouse beta-cells. *Diabetes* **43**, 703-711.

Waugh A & Grant A (2006). *Anatomy and Physiology*, 10th ed. Churchill Livingstone.

Weyer C, Funahashi T, Tanaka S, Hotta K, Matsuzawa Y, Pratley R, & Tataranni P (2001). Hypoadiponectinemia in obesity and type 2 diabetes: close association with insulin resistance and hyperinsulinemia. *J Clin Endocrinol Metab* **86**, 1930-5.

White MF, Shoelson SE, Keutmann H, & Kahn CR (1988). A cascade of tyrosine autophosphorylation in the beta-subunit activates the phosphotransferase of the insulin receptor. *J Biol Chem* **263**, 2969-2980.

Wigmore P & Stickland N (1983). Muscle development in large and small pig fetuses. *Journal of Anatomy* **137**, 235-245.

Wilson MT & Reeder BJ (2008). Oxygen-binding haem proteins. *Exp Physiol* **93**, 128-132.

Wilson SJ, McEwan JC, Sheard PW, & Harris AJ (1992). Early stages of myogenesis in a large mammal: formation of successive generations of myotubes in sheel tibialis cranialis muscle. *Journal of Muscle Research and Cell Motility* **13**, 534-550.

Wingard D, Sinsheimer P, Barrett-Connor E, & McPhillips J (1990). Community based study of prevalence of NIDDM in older adults. *Diabetes Care* **13**, 3-8.

Woodall SM, Johnston BM, Breier BH, & Gluckman PD (1996). Chronic maternal undernutrition in the rat leads to delayed postnatal growth and elevated blood pressure of offspring. *Pediatr Res* **40**, 438-443.

Wooding FB (2006). Analysis of the Structure of the Ruminant Placenta. *Methods Mol Med* **121**, 315-22.

Wozniak A, Kong J, Bock E, Pilipowicz O, & Anderson J (2004). Signaling satellite-cell activation in skeletal muscle: Markers, models, stretch, and potential alternate pathways. *Muscle and Nerve* **31**, 283-300.

Yakar S, Liu JL, Stannard B, Butler A, Accili D, Sauer B, & LeRoith D (1999). Normal growth and development in the absence of hepatic insulin-like growth factor I. *PNAS* **96**, 7324-7329.

Yamauchi T, Kamon J, Waki H, Terauchi Y, Kubota N, Hara K, Mori Y, Ide T, Murakami K, Tsuboyama-Kasaoka N, Ezaki O, Akanuma Y, Gavrilova O, Vinson C, Reitman M, Kagechika H, Shudo K, Yoda M, Nakano Y, Tobe K, Nagai R, Kimura S, Tomita M, Froguel P, & Kadowaki T (2001). The fat-derived hormone adiponectin reverses insulin resistance associated with both lipoatrophy and obesity. *Nature Medicine* **7**, 941-6.

Yancopoulos GD, Davis S, Gale NW, Rudge JS, Wiegand SJ, & Holash J (2000). Vascular-specific growth factors and blood vessel formation. *Nature* **407**, 242-248.

Yang H, Alnaqeeb M, Simpson H, & Goldspink G (1997). Changes in muscle fibre type, muscle mass and IGF-I gene expression in rabbit skeletal muscle subjected to stretch. *The American Journal of Anatomy* **190**, 613-622.

Yang W, Lee W, Funahashi T, Tanaka S, Matsuzawa Y, Chao C, Chen C, Tai T, & Chuang L (2001). Weight Reduction Increases Plasma Levels of an Adipose-Derived Anti-Inflammatory Protein, Adiponectin. *J Clin Endocrinol Metab* **86**, 3815-9.

Yang Z & Ming XF (2006). Recent Advances in Understanding Endothelial Dysfunction in Atherosclerosis. *CLINICAL MEDICINE & RESEARCH* **4**, 53-65.

Yarasheski KE, Welle S, Nair KS, Volpi E, Rasmussen BB, Sheffield-Moore M, & Wolfe RR (2002). Muscle Protein Synthesis in Younger and Older Men. *JAMA* **287**, 317-318.

Zammit PS, Partridge TA, & Yablonka-Reuveni Z (2006). The Skeletal Muscle Satellite Cell: The Stem Cell That Came in From the Cold. *J Histochem Cytochem* **54**, 1177-1191.

Zheng X, Kwan C, & Daniel E (1994). Role of intracellular Ca2+ in EDRF release in rat aorta. *J Vasc Res* **31**, 18-24.

Zhou H, Li XM, Meinkoth J, & Pittman RN (2000). Akt Regulates Cell Survival and Apoptosis at a Postmitochondrial Level. *J Cell Biol* **151**, 483-494.

Zhu MJ, Ford SP, Means WJ, Hess BW, Nathanielsz PW, & Du M (2006). Maternal nutrient restriction affects properties of skeletal muscle in offspring. *J Physiol (Lond)* **575**, 241-250.

Zhu MJ, Ford SP, Nathanielsz PW, & Du M (2004). Effect of Maternal Nutrient Restriction in Sheep on the Development of Fetal Skeletal Muscle. *Biol Reprod* **71**, 1968-1973.

Zierath J, He L, Gumà A, Odegoard Wahlström E, Klip A, & Wallberg-Henriksson H (1996). Insulin action on glucose transport and plasma membrane GLUT4 content in skeletal muscle from patients with NIDDM. *Diabetologia* **10**, 1180-9.

Appendices

Appendix 1 – Ewe diet composition

Nutrient	Feed content	Nutrient	Feed content
Dry matter (DM)	89.205 %	Sodium	0.692 %
Metabolisable Energy	10.725 MJ/kg DM	Chloride	0.086 %
Protein	14.750 %	Salt	0.691 %
Oil	2.493 %	Copper	1.500 mg/kg
Starch	15.200 %	Iodine	4.086 mg/kg
Fibre	16.653 %	Cobalt	2.033 mg/kg
Ash	8.175 %	Selenium	200.037 mg/kg
Lysine	0.766 %	Zinc	65.610 mg/kg
Methionine	0.224 %	Manganese	65.050 mg/kg
Threonine	0.615 %	Iron	39.500 mg/kg
Rumen degradable protein	1.375 %	Molybdenum	0.053 mg/kg
Calcium	0.688 %	Vitamin A	10000 iu/kg
Phosphorus	0.313 %	Vitamin D3	2000 iu/kg
Magnesium	0.150 %	Vitamin E	10.000 mg/kg

Table A1: Nutrient composition of complete pelleted diet fed to ewes in Chapters 3 and 5. Dry matter content was 89.205 % and as fed provided 9.57 MJ/kg.

Appendix 2 – Ewe high energy diet composition

Nutrient	Feed content	Nutrient	Feed content
Dry matter (DM)	89.205 %	Sodium	0.599 %
Metabolisable Energy	12.263 MJ/kg DM	Chloride	0.123 %
Protein	14.560 %	Salt	0.752 %
Oil	6.633 %	Copper	1.800 mg/kg
Starch	15.163 %	Iodine	4.124 mg/kg
Fibre	14.066 %	Cobalt	2.048 mg/kg
Ash	8.300 %	Selenium	200.042 mg/kg
Lysine	0.759 %	Zinc	66.610 mg/kg
Methionine	0.221 %	Manganese	65.425 mg/kg
Threonine	0.597 %	Iron	44.500 mg/kg
Rumen degradable protein	1.375 %	Molybdenum	0.058 mg/kg
Calcium	1.077 %	Vitamin A	10000 iu/kg
Phosphorus	0.311 %	Vitamin D3	2000 iu/kg
Magnesium	0.151 %	Vitamin E	10.000 mg/kg

Table A2: Nutrient composition of high energy complete pelleted diet fed to ewes in Chapter 5. Dry matter content was 89.205 % and as fed provided 10.94 MJ/kg.

Appendix 3 – Lamb creep pellets composition

Nutrient	Feed content	Nutrient	Feed content
Dry matter (DM)	86.7 %	Fibre	11.24 %
Metabolisable Energy	12.22 MJ/kg DM	Ash	9.50 %
Protein	18.00 %	Vitamin A	8000 iu/kg
Oil	4.19 %	Vitamin D3	2500 iu/kg
Starch	17.76 %	Vitamin E	30 iu/kg

Table A3: Nutrient composition of lamb creep pellets fed to weaning offspring in Chapter 5. Dry matter content was 86.7 % and as fed provided 10.59 MJ/kg.

Appendix 4 – Ewbol 18 diet composition

Nutrient	Feed content	Nutrient	Feed content
Metabolisable Energy, as fed	10.38 MJ/kg	Ash	9.50 %
Protein	18 %	Vitamin A	8000 iu/kg
Oil	6 %	Vitamin D3	2500 iu/kg
Starch	16.93 %	Vitamin E	150 iu/kg
Fibre	11.24 %		

Table A4: Nutrient composition of Ewbol 18 complete pelleted diet fed to offspring in Chapter 5

Appendix 5 - Fetal post-mortem

Organ	Part Sampled		Treatment			
		Weigh	*Fast Freeze*	*Slow Freeze*	*Fix*	*other*
Cotyledons	*A*	+	+			
	B	+	+			
	C	+	+			
	D	+	+			
Pancreas			+		+	**10ml HCL on ice**
Peri-renal fat			+			
Adrenal	*Left*	+		1/2	1/2	
Kidney	*Left*	+			+	**perfusion fix**
Adrenal	*Right*	+				
Kidney		+				
	Right cortex		**Bottom 1/2**			
	Right medulla		**Bottom 1/2**			
Liver		+	+			
	Left		+			
	Right		+			
Heart		+				
	Left ventricle		+			
	Right ventricle		+			
Thoracic Aorta			+			
Lung		+	+			
	Left		+			
	Right		+			
Skeletal Muscle	*Soleus*		**Rt 1/2**	**Rt 1/2**		
	FDL		**Rt 1/2**	**Rt 1/2**		
	Triceps		**Rt**	**Lt**		
Brain		+				
	brain stem			+		
	front cortex		+			
Hypothalamus	*block*			+		
Pituitary	*stalk up*			+		
Hippocampus		+	+			

Table A5: Treatment of tissue samples taken from fetal sheep at post-mortem. Fixed tissue was fixed in formalin overnight, frozen tissue was stored at -80 ^{o}C.

Appendix 6 - Fetal post mortem results

POST MORTEM MEASUREMENTS	C		PI		L	
Absolute	***n***		***n***		***n***	
Body weight (Kg)	8	2.91 ± 0.08	9	2.92 ± 0.12	6	2.80 ± 0.16
Crown-rump length (cm)	8	44.4 ± 0.7	9	43.5 ± 1.0	6	43.7 ± 0.8
Abdominal circumference cm)	8	31.0 ± 1.4	9	32.9 ± 0.6	6	32.0 ± 1.0
Biparietal diameter (cm)	8	5.7 ± 0.1	9	5.6 ± 0.1	6	5.7 ± 0.1
Femur length (cm)	6	8.2 ± 0.1	7	8.1 ± 0.2	5	8.0 ± 0.2
Shoulder height (cm)	8	33.7 ± 0.8	9	33.7 ± 0.7	6	33.5 ± 1.0
Type A cotyledons (g)	8	99.00 ± 11.19	9	95.00 ± 27.60	6	155.08 ± 45.76
Type B cotyledons (g)	8	149.45 ± 16.43	9	109.46 ± 19.68	6	143.05 ± 28.25
Type C cotyledons (g)	8	78.28 ± 27.34	9	68.11 ± 23.94	5	30.08 ± 11.87
Type D cotyledons (g)	8	18.25 ± 7.94	9	35.17 ± 15.50	5	17.28 ± 15.84
Total placental weight (g)	8	344.97 ± 20.79	9	307.73 ± 14.50	6	337.59 ± 28.51
Left adrenal (g)	7	0.22 ± 0.04	8	0.21 ± 0.01	6	0.23 ± 0.02
Right adrenal (g)	7	0.16 ± 0.02	9	0.17 ± 0.02	6	0.19 ± 0.02
Right kidney (g)	7	9.66 ± 0.61	8	9.80 ± 0.40	6	9.66 ± 0.42
Liver (g)	7	101.06 ± 6.21	9	101.14 ± 3.89	6	102.59 ± 11.05
Heart (g)	8	18.99 ± 0.63	9	19.40 ± 0.56	6	17.44 ± 1.10
Lung (g)	8	79.30 ± 3.38	9	76.82 ± 6.11	6	71.33 ± 4.27
Brain (g)	7	36.40 ± 0.99	9	33.90 ± 0.81	6	35.93 ± 1.01
Pituitary (g)	7	0.10 ± 0.01	8	0.10 ± 0.01	5	0.09 ± 0.01
As % of body weight						
Total placental weight	8	11.85 ± 0.64	9	10.57 ± 0.43	6	12.03 ± 0.71
Left adrenal	7	0.008 ± 0.001	8	0.007 ± 0.001	6	0.008 ± 0.001
Right adrenal	7	0.006 ± 0.001	9	0.006 ± 0.001	6	0.007 ± 0.001
Right kidney	7	0.34 ± 0.02	8	0.34 ± 0.02	6	0.35 ± 0.02
Liver	7	3.43 ± 0.18	9	3.48 ± 0.12	6	3.63 ± 0.18
Heart	8	0.65 ± 0.02	9	0.67 ± 0.03	6	0.62 ± 0.03
Lung	8	2.72 ± 0.09	9	2.62 ± 0.15	6	2.56 ± 0.13
Brain	7	1.24 ± 0.04	9	1.17 ± 0.04	6	1.30 ± 0.06
Pituitary	7	0.004 ± 0.000	8	0.004 ± 0.000	5	0.003 ± 0.001

Table A6: Fetal biometery (mean ± SEM) obtained at post-mortem. There was no significant difference between the dietary groups.

Appendix 7 - Adult post mortem

Organ	Part Sampled		Treatment		
		Weigh	*Fast Freeze*	*Slow Freeze*	*other*
Pancreas			+		10ml HCL on ice
Abdominal fat			+		
Peri-renal fat			+		
Back fat			+		
Adrenal	*Left*	+	+		
	Right	+		+	
Kidney	*Left*	+			
	Right	+			
	Right cortex		+		
	Right medulla		+		
Liver		+			
	Left lobe		+		
	Right lobe		+		
	Quadrate lobs		+		
	Caudate lobe		+		
Heart		+			
	Left ventricle		+		
	Right ventricle		+		
Lung		+	+		
	Left		+		
	Right		+		
Skeletal Muscle	*Soleus*	+	**Rt**	**Rt**	**Circumference**
	Vastus		**Rt**	**Rt**	
	Gastrocnemius	+	**Rt**	**Rt**	**Circumference**

Table A7: Treatment of tissue samples taken from adult sheep at post-mortem. Frozen tissue was stored at -80 ^{o}C

Abstract 8 - Abstract 1

The Physiological Society 2006 Main Meeting, University College London
Proc Physiol Soc 3 C116

Fetal skeletal muscle fibre number and type are altered by early and late gestation maternal undernutrition in sheep

Costello PM[1], Rowlerson A[2], Braddick L[1], Burrage D[1], Cooper C[3], Hanson MA[1], Aihie Sayer A[2], Green LR[1]

[1.] Institute of Developmental Sciences, University of Southampton, UK.
[2.] Centre for Applied Biomedical Research, King's College London, UK
[3.] MRC Epidemiology Resource Centre, Southampton General Hospital, UK.

Epidemiological studies have shown that small size at birth is associated with reduced muscle strength (Sayer *et al*. 2004) and type 2 diabetes (Hales *et al*. 1991) in adulthood. In sheep, reduced early gestation maternal nutrient intake reduces fetal skeletal muscle fibre number (Zhu *et al*. 2004) and alters myofibre composition in postnatal life (Fahey *et al*. 2005). Insulin resistance is associated with a shift towards a higher proportion of the relatively insulin-resistant type 2 fast-twitch fibres at the expense of the insulin-sensitive type 1 slow-twitch fibres (Marin *et al*.1994). In this study we investigated the effect of early or late gestation nutrient restriction on muscle fibre number and type in late gestation fetal sheep.

Pregnant Welsh Mountain ewes of uniform body weight were housed individually, and received either, 100 % of total nutrient requirements throughout gestation (C n = 6: f = 3, m = 3), 40% for first 30 days gestation (dGA) (ER n = 9: f = 4, m = 5) or 50 % from 104 dGA until post mortem (LR n = 6: f = 2, m = 4), with 100 % requirements at all other times. All fetuses were singletons. At ± 127 dGA (term ~147 dGA) ewes were killed by an overdose of barbiturate (i.v., 145 mg/kg) and the fetal triceps brachii removed and immersed in freezing isopentane. A 10 μm section was cut, stained with anti-fast skeletal myosin and five random fields (magnification x40) were captured. Fibre density, type and cross-sectional area (CSA) were measured and analysed by ANOVA with Bonferroni post-hoc tests. Data are expressed as mean ± SEM.

The total density of myofibres was reduced in both the ER ($p < 0.01$) and LR ($p < 0.05$) compared to C fetuses (C, 3869 ± 130; ER, 3013 ± 160; LR, 3075 ± 218 fibres/mm^2). Slow fibre density was significantly lower in LR ($p < 0.05$), but not ER fetuses (C, 603 ± 80; ER 494 ± 51; LR 370 ± 76 fibres/mm^2). Fast fibre density tended

to be lower in ER ($p = 0.07$) but not LR fetuses (C, 3286 ± 164; ER 2680 ± 136; LR 2855 ± 243 fibres/mm^2). Fast and slow fibre CSA was similar in all nutritional groups.

These data show that reduced maternal nutrition in either the peri-implantation period or late gestation affects myofibre density, but that late gestation nutrient restriction has particularly profound effects on slow-twitch fibres. These observations may have important implications for understanding the determinants of muscle strength and glucose tolerance in later life.

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Appendix 9 - Abstract 2

The 4th World Congress on DOHaD (2006), University of Utrecht, The Netherlands
Early Human Development 2006 82(8), 518-519 (C-07)

Circulating glucose affects vascular reactivity in the late gestation sheep

Costello PM, Torrens C, Braddick L, Burrage D, Hanson MA, Green LR

Institute of Developmental Sciences, University of Southampton, UK.

Background: Cardiovascular control in late gestation fetuses is altered by undernutrition (Hawkins, 2000 *Am.J.Physiol*. 279, R340-48) and prevailing hypoglycaemia (Gardner, 2002 *J.Physiol*. 540, 351-66). Our aim was to relate circulating glucose to isolated vascular function in the late gestation fetal sheep, after early and late gestation undernutrition.

Methods: Welsh Mountain ewes were fed 100% total nutrient requirements (C, n = 2), 40 % for first 31 days gestation (dGA) (ER, n = 5) or 50 % from 104 dGA until post-mortem (LR, n = 4), with 100 % requirements at all other times. At 127 ± 1dGA (term ~147 dGA) fetal blood glucose was measured, brachial artery dissected and vascular reactivity assessed using noradrenaline (NA; 10nM–100μM), acetylcholine (ACh; 100pM –10μM) and sodium nitroprusside (SNP; 100pM–10μM). Analysis by ANOVA/Bonferroni, and linear regression.

Results: SNP-, but not ACh-, induced vasodilatation was reduced in ER compared to C ($p < 0.01$) and LR ($p < 0.05$) fetuses. NA-induced vasoconstriction was unaltered. For all animals, vascular sensitivity to ACh was positively related ($p < 0.01$) and sensitivity to NA was inversely related ($p < 0.01$) to circulating glucose.

Conclusion: These data suggest that brachial artery vascular responses are altered in nutrient restricted fetuses. Increased sensitivity to NA and decreased sensitivity to ACh, at lower circulating glucose levels, is likely to be associated with greater overall vasoconstriction, suggesting a link between fetal nutritional status and vascular reactivity.

Supported by BBSRC and Gerald Kerkut Trust.

Appendix 10 - Abstract 3

The Physiological Society Focused Meeting 2007 (Perinatal physiology: from uterus to brain), University of Edinburgh
Proc Physiol Soc 6, PC2

Fetal muscle capillary density is reduced by both early and late gestation maternal undernutrition in sheep

Costello PM[1], Rowlerson A[2], Braddick L[1], Burrage D[1], Cooper C[3], Aihie Sayer A[2], Hanson MA[1], Green LR[1]

[1.] Institute of Developmental Sciences, University of Southampton, UK.
[2.] Centre for Applied Biomedical Research, King's College London, UK
[3.] MRC Epidemiology Resource Centre, Southampton General Hospital, UK.

In humans, insulin resistance is associated with a shift towards a higher proportion of the relatively insulin-resistant type 2 fast-twitch fibres at the expense of the insulin-sensitive type 1 slow-twitch fibres, along with a reduction in capillary density (Marin *et al*. 1994). Previously we have shown in sheep that an early or late gestation maternal nutrition restriction reduces myofibre number and alters fibre type composition in the fetal triceps brachii (Costello *et al*. 2006) but not in the slow-twitch soleus muscle (unpublished observations). The aim of the study was therefore to investigate the effect of an early or late gestation nutrient restriction on muscle capillary density in the late gestation fetal sheep.

Pregnant Welsh Mountain ewes of uniform body weight were housed individually, and received either 100 % of total nutrient requirements throughout gestation (C, n = 8), 40 % from 1-31 days gestation (dGA) (ER, n = 9) or 50 % from 104 dGA until post mortem (LR, n = 6), with 100 % requirements at all other times. All fetuses were singletons and groups contained equal numbers of males and females. At 127 ±1 dGA (term ~147 dGA) ewes were killed by an overdose of barbiturate (i.v., 145 mg/kg), and the fetal triceps brachii and soleus muscles were removed and immersed in freezing isopentane. 10 μm sections of muscle were cut, stained with anti-human von Willebrand factor and five random fields (magnification x40) were captured. From these fields the capillary density and capillary:muscle fibre ratio were measured, averaged and analysed by ANOVA with Bonferroni post-hoc tests. Data are expressed as mean ± SEM.

The density of capillaries in the triceps brachii was reduced in both the ER ($p < 0.01$) and LR ($p < 0.05$) fetuses as compared to C (C, 1874 ± 57; ER, 1480 ± 65; LR, 1389 ± 98 capillaries/mm^2). Capillary:muscle fibre ratios were reduced in both the ER ($p < 0.01$) and LR ($p < 0.01$) as compared to C fetuses (C, 1.80 ± 0.09; ER, 1.48 ± 0.06; LR, 1.44 ± 0.12 capillaries:muscle fibre). No differences were seen in the soleus muscle.

Our findings of decreased capillary density following reduced maternal nutrition in either the peri-implantation period or in late gestation is muscle bed dependent and parallels the effect on myofibre density previously observed (Costello *et al*. 2006). Moreover, the reduction in capillary:muscle fibre ratio in the triceps brachii indicates that the reduction in capillary density was greater than that in the myofibres, i.e. that each fibre would be supplied by fewer vessels. These data suggest a link between blood flow and skeletal muscle growth, and the reductions in both capillary and myofibre density may have long-term implications for skeletal muscle function.

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Appendix 11 - Abstract 4

The 5th International Congress on DOHaD (2007), University of Western Australia
Early Human Development 2007 83(1), S135 (P2-23)

Peri-implantation and late gestation maternal undernutrition differentially alter pathways of insulin and IGF-I action in fetal sheep skeletal muscle

Costello PM [1], Astaman NA [1], Anthony FW [1], Aihie Sayer A[2], Cooper C [2], Hanson, MA [1], Green LR [1]

[1.] Institute of Developmental Sciences, University of Southampton, UK.
[2.] MRC Epidemiology Resource Centre, Southampton General Hospital, UK.

Aims: To investigate the effect of peri-implantation (PI) or late gestation (LR) undernutrition on mRNA levels of insulin receptor (InsR), GLUT-4 and insulin-like growth factor receptor 1 (IGF-IR) in the fetal triceps brachii muscle. Both periods of undernutrition have been previously shown to decrease myofibre density, and LR predominantly decreased slow-oxidative (insulin-sensitive) myofibres (Costello *et al*. 2006 *Proc. Physiol. Soc* 3 C116).
Subjects: Welsh Mountain ewes were fed 100% total nutrient requirements (C, n = 8), 40 % for first 31 days gestation (dGA, term=147) (PI n = 9) or 50 % from 104 dGA until post-mortem (LR n = 6), with 100 % requirements at all other times.
Study design: At 127 ± 1dGA triceps brachii muscle was dissected and fast frozen. RNA was extracted and mRNA levels measured by reverse transcription and quantitative real-time PCR (normalised to GAPDH). Data were analysed by Kruskal-Wallis and appropriate post-hoc tests.
Outcome measures: mRNA for a) InsR b) GLUT4 and c) IGF-IR.
Results: InsR mRNA levels were greater in LR than CC ($p < 0.01$) and PI ($p < 0.01$) fetuses. GLUT-4 mRNA levels tended to be higher in LR than CC (P=0.087), and was significantly higher than PI fetuses ($p < 0.05$). IGF-IR mRNA levels were higher in LR than CC ($p < 0.01$) and PI ($p < 0.001$) fetuses.
Conclusions: Increased mRNA for insulin and IGF action in the LR group may constitute a compensatory response by the fetus to conserve metabolic function in the face of altered myofibre density and composition (Costello *et al*. 2006) and may be important in determining later metabolic function.

Appendix 12 - Abstract 5

The Physiological Society 2008 Main Meeting, University of Cambridge
Proc Physiol Soc 11, C32

Effect of Maternal Diet and Body Condition on Glucose Metabolism and Skeletal Muscle Structure in Mature Adult Sheep Offspring.

Costello PM[1], Cripps RL[2], Bearpark N, Rowlerson A[3], Hollis L[1], Patel H[4], Aihie Sayer A[4], Hanson MA[1], Ozanne SE[2], Green LR[1]

[1.] Institute of Developmental Sciences, University of Southampton, UK
[2.] Institute of Metabolic Science, University of Cambridge, UK
[3.] Centre for Applied Biomedical Research, King's College London, UK
[4.] MRC Epidemiology Resource Centre, Southampton General Hospital, UK

Early life nutrition is implicated in the risk of metabolic diseases (e.g. type 2 diabetes) in adulthood. Low birth-weight was associated with defects in the skeletal muscle insulin-signalling pathway of young adult men (1), and insulin resistance was associated with changes in myofibre composition (2). In sheep, maternal undernutrition reduced fetal skeletal muscle myofibre density and composition (3). Recently we reported that lower body condition score (BCS) led to increased fasting glycaemia, mild glucose intolerance and impaired initial insulin secretory response in adult offspring (4). We hypothesised that this would worsen with age, and that altered skeletal muscle structure and insulin signalling pathways are involved.

Ewes were established, by dietary manipulation, at a BCS of 2 (lower (L) n = 10) or >3 (higher (H) n = 14) before and during pregnancy (4). In male offspring at 4.04±0.02 years plasma glucose and insulin concentrations were measured during a glucose tolerance test (0.5 g/kg body weight *i.v.*) and rams were killed by an overdose of barbiturate (*i.v.* 145 mg/kg). We analysed a) insulin-signalling proteins by Western blotting in abdominal fat and vastus muscle (*m.*); b) glucose uptake in isolated strips of vastus and soleus *m.*(5); c) myofibre density and cross-sectional area (CSA) by immunostaining with anti-fast skeletal myosin (3). Data are mean±SE and were analysed by Student's t test.

Glucose tolerance was similar between groups. Basal glucose uptake was similar in L and H group soleus and vastus *m.* isolated strips. However insulin-stimulated uptake tended to be reduced in the soleus *m.* only of L rams (H 1.01±0.06; L 0.84±0.07 pmol.min.mg, $p < 0.1$). In vastus, but not soleus, *m.* total myofibre density (H 343±15;

L 294 ± 14 fibres/mm^2, $p < 0.05$) and fast myofibre density (H, 226 ± 10; L, 194 ± 10 fibres/mm^2, $p < 0.05$) were lower in L rams. Slow myofibre density tended to be lower in L rams (H 117 ± 7; L 100 ± 6 fibres/mm^2, $p < 0.1$). Myofibre CSA was unaltered. Protein levels of (i) Akt1 were lower in the vastus *m.* (L = 83 ± 7 % of H, $p < 0.05$), and tended to be lower in abdominal fat (L = 71 ± 7 % of H, $p<0.1$), of L rams; (ii) GLUT-4 were increased (L = 157 ± 6 % of H, $p < 0.001$), and (iii) IGF-IR tended to be reduced (L = 78 ± 12 % of H, $p < 0.1$), in the vastus *m.* of L rams.

Reduced signalling through Akt1 may therefore mediate the decreased vastus *m.* myofibre density in L rams resulting in reduced glucose tolerance of the young adult offspring (4). However in mature adulthood, glucose tolerance and glucose uptake into vastus *m*. was not altered by maternal BCS, and thus the impact of reduced myofibre density may be offset in part by increased GLUT-4. Such adaptations may lead to complications in metabolic control in an overabundant postnatal nutrient environment.

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1. Ozanne S.E. *et al.* (2006). *Diabetologia* **48**, 547-542
2. Marin P. *et al.* (1994). *Diabetes Care* **17**, 382-386
3. Costello P.M. *et al.* (2008). *Journal of Physiology* [Epub]
4. Cripps R.L. *et al.* (2008). *Reproductive Sciences* [Epub]
5. Ozanne S.E. *et al.* (2003). *Journal of Endocrinology* **177**, 235-241