The effects of uterine environment upon embryonic, fetal, neonatal and post-natal development and glucose metabolism in sheep

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Abstract

Studies of humans and domestic animals have shown that there is a linkage between the neonatal and post-natal health of an individual and its uterine environment during gestation. However, very little information exists for sheep and there have been no studies that have directly examined the stage of gestation at which such effects could be introduced to the conceptus.

In the present study, pure-breed embryos were transferred within and reciprocally between large (Suffolk: S) and small (Cheviot: C) breeds of sheep to establish different uterine environments; SinS (large control), SinC (restricted environment), CinS (luxurious environment) and CinC (small control) and their effects upon embryonic, fetal, neonatal and post-natal development and glucose metabolism of lambs were examined.

By Day 19 of gestation, conceptuses (embryo and trophoblast) developing in a restricted uterine environment (SinC) were smaller ($P<0.05$) than in control (SinS). The head length of SinC fetuses was smaller ($P<0.05$) than in SinS fetuses on Day 55 of gestation and SinC lambs were lighter and smaller ($P<0.05$) than SinS lambs at birth. During subsequent post-natal life, there was no difference ($P>0.05$) in the growth rate of SinC and SinS lambs. The liveweight and body dimensions of SinC lambs were lower ($P<0.05$) than SinS lambs until 9 weeks and 12 weeks of age, respectively. Day 19 peri-implantation embryos and trophoblasts that developed in a luxurious environment were bigger than in control (CinC). However, CinS fetal size did not differ ($P>0.05$) from CinC fetuses by Day 55 of gestation. There was no difference ($P>0.05$) in the birthweight and body dimensions of lambs born from these two groups. Dimension of the placentas of SinC and SinS or CinS and CinC

did not differ ($P<0.05$) during gestation or at lambing. Concentrations of ovine placental lactogen (oPL), progesterone, insulin-like growth factor-1 (IGF-1), glucose and free fatty acid (FFA) differed between uterine environments. During glucose challenge tests, there were no differences in the concentrations of glucose and insulin, between SinC and SinS female lambs, however, glucose concentrations declined more rapidly ($P<0.05$) in CinS than CinC female lambs at one year of age.

It was concluded that restricted uterine environment affects embryonic, fetal and neonatal development of lambs, and that these effects perpetuates until at least one year of age; but there was no effect upon glucose metabolism. Conversely, a luxurious uterine environment enhances the early development of embryos but had no effects upon subsequent fetal, neonatal and post-natal development; however glucose metabolism of post-natal female lambs was improved. It appears that these effects of uterine environment were mediated through the trophoblast during the early embryonic period and via the placenta during subsequent gestation. oPL, progesterone, IGF-1, glucose and FFA were implicated in feto-maternal dialogue. These results suggest that uterine environment significantly influences the biology of young sheep with possible economic consequences.

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List of Abbreviations

AUC	area under curve
BNC	binucleate cells
CIDR	controlled internal drug release
cm	centimetre
cpm	counts per minute
DNA	deoxyribo nucleic acid
FFA	free fatty acid
g	gram(s)
GLUT1	glucose transporter 1
GLUT3	glucose transporter 3
GLUT4	glucose transporter 4
h	hour(s)
IGFs	Insulin-like growth factors
IGF-1	Insulin-like growth factor-1
IGF-1R	Insulin-like growth factor receptor-1
IGF-2	Insulin-like growth factor-2
IGF-2R	Insulin-like growth factor receptor-2
IGFBP	Insulin-like growth factor binding protein
IU	international unit(s)
kDa	kilo Dalton
kg	kilogram(s)
L	litre
LA	long acting
µg/dL	micrograms per decilitre
µIU/L	micro international unit(s) per litre
µIU/mL	micro international units per millilitre
µL	microlitre
µm	micrometre
mg	milligram(s)
min	minute(s)
min µIU/L	minute micro international unit(s) per litre
min mmol/L	minute milli mol per litre
mL	millilitre
mm	millimetre(s)
mmol/L	milli mol per litre
mRNA	messenger ribonucleic acid
n	number
ng/dL	nanograms per decilitre
NIH	National Institute of Health
oPL	ovine placental lactogen
PAS	Periodic Acid Schiff's

RIA	radio-immuno assay
rpm	revolutions per minute
SD	standard deviation
SE	standard error
VHS	video home system
vs	versus
wt	weight
Π	pi
%	percent
^{125}I	^{125} Iodine
^{o}C	degree(s) celsius

Chapter one - Introduction

Optimal health and growth are essential both for human and animals to achieve normal life expectancy and productivity. Various factors such as genotype, nutrition, surrounding conditions and maternal uterine environment have been attributed as regulators of embryonic development, fetal growth, birthweight and consequently post-natal health (Eisen and Durrant, 1980; Dziuk, 1992; Brooks *et al.*, 1995; Cogswell and Yip, 1995; Bell, 2006; Gardner *et al.*, 2007). Indeed there is considerable evidence that post-natal health status is linked with development *in utero* and birthweight (Godfrey and Barker, 2000; Harding, 2003; Gluckman and Hanson, 2004b). Human epidemiological studies have shown that the uterine environment in which a fetus develops is linked not only with its birthweight but also with its lifetime health status (Barker *et al.*, 1993). Based upon such studies, Barker (1995, pp 171-174) proposed his 'fetal origin hypothesis', in which he postulated that "fetal under-nutrition in mid- to late-gestation leads to disproportionate fetal growth and programmes later coronary heart disease and permanently changes the structure and function of the body". Subsequent human epidemiological studies have indicated a linkage between low birthweight and onset of adulthood diseases such as non-insulin-dependent diabetes, coronary heart disease, hypertension and obesity (Barker and Clark, 1997; Barker, 1998; Godfrey and Barker, 2000; Harding, 2003; Gluckman and Hanson, 2004b; Symonds *et al.*, 2007). Similarly, studies of production animals have also shown that the traits such as milk, wool production and carcass characteristics are directly related to both birthweight and post-natal growth (Greenwood *et al.*, 1998; Bell, 2006; Blair *et al.*, 2010). Low birthweight is associated with increased neonatal mortality (Nash *et al.*, 1996; Morel *et al.*, 2008),

poor subsequent productivity (Greenwood *et al.*, 1998) and long-term morbidity (Garite *et al.*, 2004; Redmer *et al.*, 2004).

The National Research Centre for Growth and Development New Zealand (2007) estimated that achieving the optimum birthweight reduces the incidence of cardiovascular disorders and adult diabetes in humans by 44% and 66%, respectively. Moreover, applying similar principles to farm animal health, perinatal lamb mortality could be curtailed to a greater extent if birthweights were optimised. This would greatly benefit sheep industries, since perinatal lamb mortality causes about 1 to 2 million lamb deaths annually in New Zealand. These various studies indicate that the increased knowledge of factors controlling fetal growth has the potential to improve not only long-term human health but also the productivity of farm animals.

Earlier studies, however, have also suggested that an impaired intrauterine development is associated with onset of various post-natal pathologies with or without an apparent alteration in the birthweight (Gatford *et al.,* 2000; Vehaskari *et al.*, 2001). A mild maternal undernutrition during early gestation has been shown to reduce the cardiac output of the fetuses during later in the gestation without affecting fetal growth (Hawkins *et al.,* 2000). Similarly, maternal undernutrition around conception results in an altered fetal growth and endocrine responses throughout the gestation without affecting the birth size (Oliver *et al.,* 2005). These studies thus suggest that birth size is not necessarily always a pathway for the onset of post-natal metabolic disturbanmces.

Studies in animals (Walton and Hammond, 1938; Dickinson *et al.*, 1962; Cowley *et al.*, 1989; Giussani *et al.*, 2002; Allen *et al.*, 2004; Gardner *et al.*, 2007; Gootwine *et*

al., 2007) have reported the effects of maternal environment, and particularly maternal size, on fetal development, birthweight and post-natal growth. In general, such studies have shown that the fetal development and birthweight is reduced in a restricted maternal uterine environment; whereas it is enhanced in a luxurious environment. It has also been suggested that once a developmental impairment has been set-up in the fetal stage, it persists during the post-natal life (Gatford *et al.*, 2000). Furthermore, even if the post-natal liveweight of individuals that experienced *in utero* growth restriction equalises with that of individuals which developed normally *in utero*, differences in the visceral and vital organs remain in the former that compromises their longevity (Vehaskari *et al.*, 2001; Ozanne and Hales, 2005). In other words, such data indicate that, once established, a pattern of low birthweight, mediocre production performance and poor disease resistance might perpetuate across generations.

The placenta is the site for exchange of nutrients between mother and fetus. It is also a metabolically active organ which secretes various hormones, growth factors and cytokines that regulate development of the feto-placental unit (Gootwine, 2004). There is evidence that placental characteristics are modified in an impaired uterine environment to better support adequate fetal growth (Giussani *et al.*, 2002; Wilsher and Allen, 2002; Vonnahme *et al.*, 2006). It is therefore important to understand the effects of maternal environment and size on placental characteristics and its consequent involvement in the feto-maternal dialogue.

The interaction between conceptus and the dam that can potentially result in impaired fetal development starts at a very early stage of pregnancy (Gaviria and Hernandez, 1994; Goff, 2002; Spencer *et al.*, 2004a). The early embryonic period is critical for development because this corresponds to the period of rapid cell division,

implantation, maternal recognition of pregnancy and organogenesis (Dziuk, 1992; Bazer *et al.*, 1997; Fligny *et al.*, 2009; Van Mourik *et al.*, 2009). Hence, it is conceivable that an environmental insult to the conceptus at such a fragile stage can potentially introduce permanent defects in its subsequent development. The sensitivity of peri-implantation embryos to the surrounding environmental conditions has been indicated by various *in vitro* or *in vivo* studies using bovine, equine or ovine models (Bertolini *et al.*, 2002; Tremoleda *et al.*, 2003; Nyborg *et al.*, 2005). These effects of the maternal uterine environment upon embryonic development are believed to be mediated through various maternal epigenetic pathways, which result in the development of abnormal or impaired patterns of gene expression in the phenotypes (Wolff *et al.*, 1998; Cooney *et al.*, 2001; Jaenisch and Bird, 2003). Recent research has suggested that such effects could be permanent, but non-genomic (Jaenisch and Bird, 2003; Symonds *et al.*, 2007). In other words, the DNA sequence itself is not altered, but the ability of the cell to express the genes, largely through the modification in the methylation of the dinucleotides is altered. At such an early stage, the trophoblast is a key component of the interface between the conceptus and its maternal environment. Trophoblast binucleate cells, which first appear in the ovine trophoblast by Day 16 of pregnancy (Boshier, 1969), play a critical role in implantation (Wooding, 1984) and the number of binucleate cells or the degree of trophoblast epithelial development have been reported as reliable indicators of embryonic viability (Rodriguez *et al.*, 2000).

Amongst the studies mentioned above, only a limited number have directly manipulated the uterine environment to observe the effects upon birthweight and subsequent post-natal growth. None of these studies have examined the effects of

uterine environment upon the morphogenesis of peri-implantation embryos and their surrounding trophoblasts.

Many endocrinological and metabolic factors, such as ovine placental lactogen (oPL), progesterone, insulin-like growth factor-1 (IGF-1), glucose and free fatty acids (FFA), have also been implicated in the feto-maternal interaction. oPL is secreted by the trophoblast binucleate cells and during early conceptus-maternal interaction, it regulates the secretion of histotroph which is a nutrient factor for early embryo growth (Stewart *et al.*, 2000). Subsequently with advancing gestation, it modulates fetal metabolism to utilise substrates by acting as a partitioning agent for nutrient supply between mother and the fetus (Anthony *et al.*, 1995b). In general, the maternal plasma oPL concentration of later stage gestation is correlated with placental mass, number of fetuses (Kappes *et al.*, 1992; Gootwine, 2004) and birthweight (Jenkinson *et al.*, 2007). Progesterone is required for embryonic development and providing trophic support to both placenta and fetus (Alexander and Williams, 1966; Bindon, 1971; Ogle *et al.*, 1990). Some studies have found that the concentration of maternal plasma progesterone (particularly around 8 weeks of pregnancy) is positively correlated with placental growth and lamb birthweight (Manalu and Sumaryadi, 1998; Manalu and Sumaryadi, 1999). IGF-1 is considered to be vital for embryogenesis and subsequent placental growth and fetal development (Blair *et al.*, 1989; Gluckman *et al.*, 1992; Gicquel and Le Bouc, 2006; Watson *et al.*, 2006; Blum *et al.*, 2007). Gene knockout experiments have shown that an absence of IGF-1 production is associated with fetal growth retardation, reduction in the body size, severe muscular dystrophy and neonatal mortality (Powell-Braxton *et al.*, 1993)

Glucose is the main source of fuel for metabolism and energy in cells in most animals (Herdt, 1997) and for fetuses and the placenta (Hay and Sparks, 1985;

Boden, 1996; Scholl *et al.*, 2001). The developing fetus produces very little glucose and therefore fetal demands for glucose are met by supply from the mother. Glucose is transferred across the placenta by facilitated diffusion and by the involvement of transporters on the fetal and maternal trophoblasts (Boden, 1996; Marconi *et al.*, 1996). Maternal glucose concentration is directly related to fetal glucose levels. Fetal glucose concentration regulates the secretion of IGF-1, as such an increased fetal IGF-1 concentration enhances the uptake of glucose in the fetal cells and, hence, results in enhanced fetal growth (Oliver *et al.*, 1993; Gluckman and Harding, 1997). With advancing gestation, in order to meet the increased demand of glucose for fetal growth, the mother starts utilising her body fat reserves for her own energy requirements. Consequently, the concentration of FFA in maternal plasma increases (Sivan *et al.*, 1999; Herrera, 2002). Evaluating the maternal concentrations of oPL, progesterone, IGF-1, glucose and FFA at different stages of pregnancy in different maternal environments might therefore be of help to understand their involvement in the feto-maternal dialogue, and perhaps to suggest means to affect fetal growth, birthweight and subsequent health.

An impaired maternal environment has also been reported to have adverse affects on the development of vital physiological and metabolic systems in the fetus, such as the cardiovascular system, the hypothalamic-pituitary-adrenal axis and glucose metabolism (Barker *et al.*, 1993; Giussani *et al.*, 2002; Bloomfield *et al.*, 2004; Bo *et al.*, 2004). Glucose metabolism is believed to be a key metabolic process by which, such individuals are rendered more susceptible for adulthood diseases. Hence, it would be beneficial to understand how differences in the feto-maternal interaction in different maternal environments affect key metabolic pathways (such as glucose

metabolism) of the fetus and dam. To date limited studies in this area have been undertaken in livestock species.

The present study was undertaken to investigate the effects of genetically and physiologically restricted or luxurious maternal uterine environments upon embryonic development, fetal growth, birthweight and subsequent post-natal growth and glucose metabolism of lambs. The sheep contributes to a large proportion of the livestock to meet the ever increasing nutritional demand of human populations. Besides being an economically important livestock species, sheep are also a good model for human studies since they exhibit similar organogenesis for all major systems, have a comparable birthweight and relatively long gestational length (Harding, 2001;Wallace *et al.*, 2005; Symonds *et al.*, 2007).

The objectives of this study are to:

1. Examine the effect of maternal uterine environment upon development of Day 19 embryos.
2. Investigate the role of the Day 19 trophoblast in the embryo-maternal interaction.
3. Study the effects of maternal uterine environment upon fetal development on Day 55 of gestation.
4. To understand the effects of maternal uterine environment upon birthweight and body dimensions of newborn lambs.
5. Understand the role of the placenta in feto-maternal interaction in different maternal environments.
6. Understand the involvement of humoral factors such as oPL, progesterone and IGF-1 and metabolic factors such as FFA and glucose, as modulators of

the effects of maternal uterine environment on fetal development and, consequently, on birthweight

7. Study the effects of antenatal maternal environment on glucose metabolism of post-natal lambs.
8. Study the effects of antenatal maternal environment on post-natal growth of lambs.

Chapter two - Literature review

Preamble

Various factors such as genotype, nutrition, surrounding environment or the conditions of antenatal development regulate the health status of an individual (Polani, 1974; Eisen and Durrant, 1980; Symonds *et al.*, 2004; Symonds *et al.*, 2007). In general, genotype and uterine environment are the two major factors that control antenatal development. In mammals, besides nuclear DNA, the female parent also contributes mitochondrial DNA, oooplasm and the environment in which the conceptus develops (Falconer, 1960; Robison, 1981; Cowley *et al.*, 1989). Thus, the female appears to exert a more crucial role to the progeny than its male counterpart. Therefore, it might be expected that maternal factors can regulate fetal development with consequent effects upon subsequent post-natal life. For example, early research suggested that whereas fetal genotype accounts for about 15% of the variation in the birthweight and more than 30% of the variation can be attributed to the maternal environment (Polani, 1974).

A large body of epidemiological evidence from human populations suggests a linkage between post-natal health status and the antenatal environment. Barker and his co-workers studied the health records of infants born in early 20th century in Hertfordshire, England, and found that the infants born with low birthweight were predisposed to cardiovascular diseases. Based on this, Barker (1995, pp 171-174) proposed 'fetal origin hypothesis', postulating that "fetal under-nutrition in middle to late gestation leads to disproportionate fetal growth, programmes later coronary heart disease and permanently changes the structure and function of the body". Subsequent epidemiological findings suggested a linkage of low birthweight with neonatal

mortality and onset of adulthood diseases such as non-insulin-dependent diabetes, coronary heart disease, hypertension and obesity (Barker and Clark, 1997; Godfrey and Barker, 2000; Harding, 2003; Garite *et al.*, 2004; Gluckman and Hanson, 2004b; Symonds *et al.*, 2007). Thus, it is now widely accepted that the antenatal environment which an individual experiences *in utero* affects its fetal development, birthweight and subsequent health status. Such studies also indicate that, among all the factors that affect development health and growth, uterine environment is perhaps the most important.

Optimum health and growth are also important for animals, since the economical efficiency of the livestock sector depends on the survivability and productive performance of animals. Production traits such as milk and wool production, survival and carcass characteristics are directly related to birthweight and post-natal growth (Greenwood *et al.*, 1998; Bell, 2006). Therefore, advancement in the knowledge of the effects of the maternal uterine environment upon fetal development and post-natal growth, would not only be of help in improving human health, but will equally be valuable for augmenting productivity in livestock.

Birthweight and size

Birthweight is a widely-agreed index of survivability and subsequent growth in both humans and animals. A high birthweight, on the extreme upper end of the scale, is associated with dystocia and maternal death and does not always have better birth outcomes (Alexander, 1974; Cogswell and Yip, 1995). By contrast, low birthweight is associated with increased neonatal mortality (Morel *et al.*, 2008), poor subsequent productivity (Greenwood *et al.*, 1998) and long-term morbidity (Harding, 2003; Redmer *et al.*, 2004). Nash *et al.* (1996) reported that the risk of neonatal mortality in

low birthweight lambs is twice that of heavy born lambs. Recent human studies have shown that infants born with a low birthweight are at a greater risk of developing diabetes and obesity (Harding, 2003).

Birthweight *per se,* however, is not always a reliable parameter for predicting the subsequent growth and health. Barker (1998) proposed that birthweight is relatively a crude measure of the well-being of a newborn, which should be evaluated along with other body dimensions such as body length or head circumference. For example evaluation of head dimensions plus birthweight allows identification of the antenatal pattern of development that a fetus has experienced. Earlier studies have shown that during fetal development, placental somatomammotropin, growth hormone and prolactin are involved in modulating the supply of nutrients towards fetal brain and heart tissue compared to less vital organs (Bauman *et al.*, 1982), which indicates that the development of fetal brain is privileged over other tissues such as skeletal muscles in nutrient deficient conditions. Likewise, Zhu *et al.* (2006) reported a reduction in the number of skeletal muscle fibres in lambs born from a nutrient restricted group compared to controls. Further, De Blasio *et al.* (2007) performed carunclectomy in ewes to induce placental restriction and intrauterine fetal growth retardation. These authors concluded that there was reduced lean tissue growth and impaired muscle development and increased adipose tissue deposition in post-natal lambs born from carunclectomised ewes compared to the lambs born from controls. These studies thus suggest that intrauterine fetal growth retardation might be associated with onset of various morphometric signs. The characteristics morphometric signs such as decreased birthweight to head circumference ratio and stunted growth have also been reported in human epidemiological studies (Barker, 1998). A major pathway for prioritising the growth of vital organs such as brain

could be the redistribution of blood flow, as earlier studies have shown such evidence in fetal lamb (Campbell *et al.,* 1967). Recent epidemiological studies in the human have also indicated a linkage between the stunted development or thinness at birth and the health status during subsequent life (Martyn *et al.*, 1996; Forsen *et al.*, 1997).

Prenatal uterine environment, however, also influences the development of vital organs. In an experiment, Vehaskari *et al*. (2001) found that rat pups born from nutrient restricted dams were not only had low birthweight but, by eight weeks of age, these pups had around 28 to 29% reduction in the number of glomeruli in their kidneys compared to pups born from controls. Gatford *et al*. (2000) experimentally induced hypertension in fetal sheep at Day 27 of gestation by dexamethasone treatment and reported that such lambs remained hypertensive during their post-natal life, although the birthweight of these lambs was similar to that of controls.

Thus, the above studies indicate that birthweight and various body dimensions and characteristics in an individual are good indicators of both *in utero* development and subsequent post-natal growth. Nonetheless, birthweight and birth size do not necessarily always reflect the underlying impairment in the vital organs and, consequently, disturbances in metabolic pathways during the post-natal life.

Maternal effects on fetal development

Various parameters have been used to investigate the effects of different factors of maternal origin upon fetal development, birthweight, body size and post-natal growth in both humans and animals. Such models have investigated the effects of maternal bodyweight at conception, maternal weight gain during pregnancy, maternal pre-conception body mass index, maternal age, height, litter effect (limited maternal

uterine space per fetus due to sharing of space by all littermates), maternal nutrition and maternal size upon fetal, neonatal and post-natal development. In the human population, maternal height, age and pre-conception weight and maternal weight gain during pregnancy have been suggested to be directly linked with infant birthweight, body length and head circumference (Brooks *et al.*, 1995; Cogswell and Yip, 1995; Kirchengast and Hartmann, 1998). In a study on Ethopian Menz sheep, Mukasamugerwa *et al.* (1994) reported a significant positive relationship between the prepartum weight or overall gestational live-weight gain of dam and the birthweight of lambs. Similarly, a positive relationship has also been reported between the litter weight or lamb weight at birth and the post-partum weight of ewe (Michels *et al.*, 2000). In general, lambs born from light ewes have lower birthweight and body size compared to lambs born from heavy ewes (Clarke *et al.*, 1997). Similarly, impaired nutrient supply to the fetus due to poor maternal nutrition, competition between littermates or placental restriction have been suggested as major factors that affect birthweight and have been extensively reviewed (Wallace *et al.*, 2005; Symonds *et al.*, 2007; Kenyon, 2008).

Gardner *et al.* (2007) studied the effects of maternal nutrition, bodyweight and uterine space on birthweight of lambs and concluded that all the above maternal parameters significantly affected the birthweigh. These studies suggest that the fetal genotype is maternally constrained and that the maternal uterine capacity in terms of size and its ability to provide nutrition to the developing fetus has a marked influence on fetal development and consequently that upon the birthweight. Similarly, limited uterine space, due to competition between littermates, has been identified as a constraint upon birthweight of multigravid ewes (Kenyon *et al.*, 2007). McCoard *et al.* (1997) compared muscle development in single- and twin-born fetuses and found

that most of the major muscles in twin fetuses were lighter than in single fetuses, and attributed the limited maternal uterine space of twin fetuses as the cause of their retarded muscle development. Furthermore, an inverse relationship between the litter size and lamb birthweight has been reported (Gootwine, 2005). Thus, maternal uterine capacity, in terms of size and its ability to provide nutrition to the developing fetus, has a marked influence upon fetal development and consequently that on birthweight.

Dam-size models

The role of maternal size as a regulator of birthweight was first investigated in the classical experiment by Walton and Hammond (1938). By crossing large-sized Shire horses with small-sized Shetland ponies, these authors reported that the Shire foals born to Shetland dams were smaller than Shire foals born from Shire dams. Conversely, Shetland foals were larger when born to Shire dams compared to Shetland foals born from Shetland dams. In other words, the restricted uterine environment of small Shetland pony dams resulted in lowered birthweight of Shire foals, whereas the luxurious uterine environment of the Shire dams enhanced the birthweight of Shetland foals. Effects of maternal uterine environment upon birthweight and post-natal growth have subsequently been reported in sheep (Dickinson *et al.*, 1962; Gardner *et al.*, 2007; Gootwine *et al.*, 2007), mice (Cowley *et al.*, 1989) and horses (Allen *et al.*, 2002b; Giussani *et al.*, 2002; Wilsher and Allen, 2002; Allen *et al.*, 2004).

Using embryo transfer between large Thoroughbred and small pony horses, Allen *et al.* (2002b) reported that the Thoroughbred foals born to pony dams (restricted uterine environment) were significantly smaller and lighter compared to

Thoroughbred foals born to Thoroughbred dams (control) (birthweight 33.0 ± 2.4 kg vs 53.1 ± 2.6 kg, respectively). Besides lower birthweight, Thoroughbred-in-pony foals also displayed characteristics signs of prematurity or dysmaturity and had variable degrees of muscle under-development around the body and upper limbs. In contrast, the pony foals born to Thoroughbred dams (luxurious uterine environment) were bigger and heavier compared to pony foals born to pony dams (control) (birthweight 37.9 ± 2.1 kg vs 24.0 ± 1.3 kg, respectively). Unlike Thoroughbred-in-pony foals, pony-in-Thoroughbred foals did not show any signs of prematurity at birth. These authors thus suggested that the restricted uterine enviornment of the samller pony dams impaired the development of larger genotype Thoroughbred foals; whereas, the luxurious uterine environment of the larger Thoroughbred dams enhanced the development of the smaller genotype pony foals. Similarly, Giussani *et al.* (2002), studied the effect of dam-size upon birthweight in foals by transferring embryos reciprocally between Thoroughbred and pony dams. These authors also reported that Thoroughbred-in-pony foals had lower birthweight than Thoroughbred-in-Thoroughbred foals whereas, pony-in-Thoroughbred foals were heavier than pony-in-pony foals.

Cowley *et al.* (1989) studied birthweight and tail development in mice, by reciprocally transferring embryos between dam strains of large or small body size. Birthweights were higher and tails were longer in pups that were born to the large size dam-strain compared to those born to the small body-size dam-strain.

In a crossbreeding experiment using small-sized Cheviot and large-sized Suffolk ewes, Jenkinson *et al.* (2007) reported that lambs born to Cheviot dams were lighter and smaller compared to those born from Suffolk dams. However no reciprocal embryo transfer experiments have as yet been undertaken in sheep. In order to

understand fully the effects of uterine environment upon embyonic development in the sheep, such an experiment needs to be undertaken, so that paternal effects upon development can be ruled out.

The above investigations support the notion that birthweight is constrained by the size of dam such that a restricted uterine environment results in development of smaller and lighter individuals. They also show that a luxurious uterine environment increases the birthweight and size at birth, thus suggesting that the maternal size has a marked influence upon the growth and development in animals.

Post-natal growth

Allen *et al.* (2004) studied the post-natal growth of foals born from restricted (Thoroughbred-in-pony), luxurious (pony-in-Thoroughbred) or control (Thoroughbred-in-Throughbred or pony-in-pony) uterine environments by transferring embryos between large sized Thoroughbred and small sized pony horses. Weight and body dimensions such as crown-rump length, height at withers, chest circumference, poll-to-nose length, elbow-to-knee length, knee-to-fetlock length and circumference of cannon bone of foals were measured at fortnightly intervals from bith to six months of age, and at montly intervals from six months to three years of age. In all the foals, irespective of the treatment groups, there was a steep rise in growth during first 20 weeks of life, followed by a slower growth curve until 100 to 120 weeks and then a sustainable growth until 160 weeks of age. From six months onwards, the growth of Thoroughbred-in-pony foals was comparable to that of Thoroughbred-in-Thoroughbred foals. However, weight and body dimensions of Thoroughbred-in-pony foals generally remained lower than that of Thoroughbred-in-Thoroughbred foals. Conversely, the weight and body dimensions of pony-in-

Thoroughbred foals remained on a higher scale than that of pony-in-pony foals until three years of age.

In mice, Cowley *et al.* (1989) studied post-natal bodyweight and tail development by reciprocally transferring embryos between dam strains of large or small body size. The bodyweight and tail length of the pups were recorded between birth and 70 days of age. The tails were longer in pups born to the large dam strain and these pups generally also remained heavier during the whole study period compared to the pups born to the small sized dam strain. Thus, the uterine genotype in which pups developed largely influenced their post-natal development.

Greenwood *et al.* (2000) reported that during post-natal life the low birthweight and normal birthweight lambs growth at similar rates. However, the low birthweight lambs generally remained fatter than normal birthweight lambs and had limited capacity for bone and muscle growth.

In the pig, providing an increased maternal nutrition from Days 25 to 70 of gestation had no effect upon the muscle fibre number, area of muscle fibre, fibre type or overall meat quality of post-natal piglets (Nissen *et al.*, 2003).

The above studies indicate that individuals born with low birthweight grow at a comparable rate to that of high birthweight individuals. However, to date no studies have reported the effects of dam-size (genetically and physiologically restricted or luxurious uterine environments) upon post-natal development of lambs.

Glucose metabolism

In addition to its effects upon birthweight, birth-size and post-natal growth, an impaired uterine environment has also been shown to have adverse effects upon the

development of key metabolic axes (Hales and Barker, 2001; Giussani *et al.*, 2002; Bloomfield *et al.*, 2004), which appears to render such individuals more susceptible to adulthood diseases. In their human epidemiological studies, Barker *et al.* (1993) reported a positive correlation between the birthweight and body-size at birth, and the onset of Type 2 diabetes during adult life. Subsequent human studies have also reported an association between fetal growth restriction, low birthweight and impaired glucose tolerance in later life (Hattersley *et al.*, 1998; Hales and Barker, 2001; Ozanne and Hales, 2002; Harding, 2003). Epidemiological studies on the Dutch famine in the mid 20th Century have also shown that exposure of mothers to famine during the mid- to late-gestation is associated with decreased glucose tolerance during the adult life of their offspring (Ravelli *et al.*, 1998). It also appears that individuals born with a low birthweight have a marked decrease in the muscle mass (Eriksson *et al.*, 2004). Since the skeletal muscles are the main sites for utilisation of fatty acids and glucose (Petersen and Shulman, 2002), impairment of their development can render low birthweight individuals susceptible to metabolic syndrome and disturbances in the glucose metabolism (Ozanne *et al.*, 2005).

Disruption of post-natal glucose metabolism due to an adverse antenatal environment has also been reported in a few animal studies. Maternal nutrient restriction of dams is associated with development of glucose intolerance, hyper-insulinaemia and insulin resistance in post-natal rats (Fernandez-Twinn *et al.*, 2003; Ozanne *et al.*, 2003). In sheep, Gardner *et al.* (2005) reported a decreased glucose tolerance in one year old lambs that were born from nutrient restricted dams, compared to those born from control. However, Clarke *et al.* (2000) found no difference in the glucose metabolism between high and low birthweight lambs (single and twins) after 6 months of age.

An adverse uterine environment during later stages of gestation may be of particular importance in predisposing to impaired post-natal glucose metabolism (Ravelli *et al.*, 1998; Gardner *et al.*, 2005). Gardner *et al.* (2005) reported a direct association between maternal nutrient restriction during the later stages of gestation and reduction in the glucose tolerance in lambs by one year of age. Similarly, Limesand and Hay (2003) reported that maternal hypoglycemia during later stage of pregnancy decreases the response of fetal insulin to glucose. On the other hand, an early gestational nutrient restriction either has no effect on the action or secretion of insulin during post-natal life (Portha *et al.*, 1995), or even results in an enhanced response of fetal insulin to glucose (Langley *et al.*, 1993; Oliver *et al.*, 2001). Thus, it appears that the stage of gestation at which disruption to fetal development occurs largely decides the outcome of its post-natal glucose metabolism.

It has also been reported that the impairment in glucose homeostasis in itself is an age-dependent phenomenon and might arise when there is a mismatch between the antenatal and post-natal environment (Ozanne and Hales, 2002). Earlier studies with rats have reported that by 4 weeks of age, offspring born from nutrient restricted dams had better glucose metabolism than controls (Holness, 1996). By one year of age, the glucose metabolism of offspring born from nutrient restricted dams was significantly worse than controls; however, this disturbance in the glucose metabolism was observed after the pups born from nutrient restricted dams were fed with highly palatable cafeteria-style diet (Petry *et al.,* 1997).

Cortisol is also implicated with mechanism of action of insulin and, hence, might affect glucose metabolism. The presence of continuously high concentrations of cortisol has detrimental effects on the health of an individual, and it also interferes with the secretion (Lambillotte *et al.*, 1997) and the physiological actions of insulin

(Holmang and Bjorntorp, 1992). Conversely, adequate cortisol levels are also required for the necessary maturation of vital fetal organs which is pivotal for survival (Fowden, 1995).

Conclusion

A substantial amount of evidence exists to show that maternal uterine environment has pronounced effects upon fetal growth, birthweight and birth-size; and that offspring born from restricted uterine environments grow at a comparable rate to those born from control. However, the bodyweight and body dimensions offspring from the restricted uterine environment remain lower than those born from control. It is also suggested that antenatal uterine environment affects key metabolic pathways such as glucose metabolism. However, to date, most of this knowledge is derived from human epidemiological studies and only a few investigations have described such effects in animals. Moreover, the studies involving dam-size models (within and reciprocal transfer of pure-breed embryos between large and small size dams) to understand the effects of uterine environment upon growth and glucose metabolism are very limited and so far none of them have been reported in sheep.

Stage of gestation

Although various studies of humans and animals have indicated that maternal uterine environment is implicated with the life-time health status of their offspring, the exact stage of gestation at which the uterine environment introduces its effects upon the development of the conceptus is not yet clearly known.

Fetal growth is a complex process which depends upon genetic, cellular, nutritional and various other growth and hormonal factors of fetal and maternal origin (Eisen and Durrant, 1980; Dziuk, 1992; Cogswell and Yip, 1995; Bell, 2006; Gardner *et al.*,

2007). Following fertilisation, the zygote undergoes cleavage, which, in sheep, is controlled by maternal gamete mRNA until the 16-cell stage. Subsequent development is regulated by the embryonic genome (McGeady *et al.*, 2006). Cleavage and subsequent compaction of embryonic cells result in the formation of the morula. Soon afterwards, the secretions from the inner cell mass cells form fluid filled spaces which consequently merge to form a single cavity called as blastocoel and the embryo is then referred as blastocyst, which has a superficial layer of cells called trophectoderm and an inner cell mass from which the embryo develops. The formation of these two distinct cell lines viz. trophoblast and inner cell mass are amongst the earliest development stages of the conceptus. Subsequently, the trophoblast cells restrict their differentiation only to trophoblast lineage which thereafter forms the placenta. On the other hand, the inner cell mass cells maintain their pleuripotency to form the different cell types of embryo (Dyce *et al.*, 1987; Gardner, 1996; Johnson and McConnell, 2004). The apposition of trophoblast tissue to maternal uterine epithelial lining starts in the vicinity of the embryo on Day 14 of gestation. Until Day 20 of pregnancy, trophoblast attachment is largely focused around the embryo, and this area of trophoblast attachment increases as pregnancy progresses (Gaviria and Hernandez, 1994).

Recent studies have shown that, after formation of blastocyst, subsequent development of the cells of inner cell mass largely depends on the surrounding environment (McGeady *et al.*, 2006). After hatching and during apposition to the uterus, the developing embryo is exposed directly to the uterine environment and, hence, becomes more sensitive to its surrounding conditions. The sensitivity of peri-implantation embryos to the surrounding environmental conditions has also been

indicated by a number of *in vitro* or *in vivo* studies using bovine, equine or ovine models (Bertolini *et al.*, 2002; Tremoleda *et al.*, 2003; Nyborg *et al.*, 2005).

The early embryonic period is a crucial developmental stage because it corresponds to the initiation of conceptus-maternal interactions (Gaviria and Hernandez, 1994; Spencer *et al.*, 1999; Spencer *et al.*, 2004a), implantation (Goff, 2002; Spencer *et al.*, 2004b) and organogenesis (Dziuk, 1992). In other words, the peri-implantation embryos are not only developmentally highly active but are also fragile and vulnerable to surrounding conditions. Consequently, it is possible that an impairment or alteration in the uterine environment at this stage would result in defects in embryonic development which might have long-term health implications.

Epigenetic effects upon the early embryo

One of the pathways by which the effects of maternal environment might be translated into permanent or semi-permanent effects upon the embryo is by epigenetic modifications, such as DNA methylation and/or imprinting of genes. DNA methylation is largely responsible for maintenance of stable gene expression (Jaenisch and Bird, 2003). For example, there is evidence that alterations in the metabolism of the developing conceptus that are consequent to the surrounding maternal environment are mediated by epigenetic modification of the genome. This would allow the transmission of gene activity from one generation of cells to another, thereby contributing to the development of abnormal or impaired phenotypes (Wolff *et al.*, 1998; Bergh *et al.*, 1999; Cooney *et al.*, 2001). Developmental effects of DNA methylation on gene expression involve long-term silencing of gene expression (Futscher *et al.*, 2002), suggesting that, once set-up, such effects might persist permanently during subsequent life. The less differentiated

cells (such as embryonic stem cells) are more susceptible to epigenetic nuclear reprogramming than are terminally differentiated cells (Eggan *et al.*, 2001; Wakayama and Yanagimachi, 2001). However, it has also been reported that mammals can tolerate a substantial degree of epigenetic abnormality, since cloned phenotypes with widespred gene dysregulations can survive post-natal life despite such abnormalties (Jaenisch and Wilmut, 2001). This probably indicates that the development, but not the immidiate survivability, of such individuals is compromised by the epigentic effects induced by the maternal environment. An alteration or absence of imprinting of genes has also been suggested as a mechanism that can potentially introduce changes in the conceptus or placental development in mice (Frank *et al.*, 2002).

Factors implicated in feto-maternal interaction

Immediately after a successful fertilisation, and until completion of the gestation, a dialogue has to be established between the dam and the developing fetus. This dialogue is implicated in maintenance of successful pregnancy and ensures that there is an appropriate demand of nutrients from the fetus and a sufficient supply of them from the dam. Some of such key factors and their role in feto-maternal dialogue are summarised below:

Trophoblast

The ruminant trophoblast consists of two cell types: mononucleate and binucleate cells. During trophoblast outgrowth and conceptus elongation, some of the mononuclear trophoblast cells undergo acytokinetic mitosis to differentiate into giant binucleate cells (Wooding, 1992; Hoffman and Wooding, 1993). In sheep, these cells appear as early as Day 16 of pregnancy (Boshier, 1969) and contribute to about 15 to

20% of the total trophectodermal cells at implantation and this number increases as the gestation progresses (Boshier, 1969; Wooding and Wathes, 1980). Binucleate cells are randomly scattered throughout the trophoblast, exhibit a dark-stained cytoplasm due to the presence of numerous cytoplasmic ribosomes and well-developed large Golgi bodies (Wango *et al.*, 1990). These Golgi bodies synthesise large membrane bound granules that occupy almost half of the cell volume and are Periodic Acid Schiff (PAS) positive. The binucleate cells can be observed in tissue sections using PAS stain (Rodriguez *et al.*, 2000). Wooding (1984) demonstrated the presence of these granules in the maternal trinucleate cells and in syncytial plaques, suggesting that the process of migration and fusion of binucleate cells with maternal epithelium ensures the delivery of binucleate cell granules to the maternal compartment. Likewise, Rodriguez *et al.* (2000) demonstrated the distribution of trophoblast binucleate cell number in the uterine lining on Days 14, 20 or 24 of gestation and reported an increase in the number of binucleate cells with advancing gestation. These findings not only supported the earlier notion that the trophoblast binucleate cells fuse with the uterine epithelium with advancing gestation but also that the number and development of conceptuses is positively related with the number of binucleate cells.

Binucleate cell granules contain placental lactogen (somatomammotrophin) which is a hormone that affects both fetal growth and maternal metabolism (Anthony *et al.*, 1995a). By Day 16 of pregnancy in sheep, placental lactogen is implicated in stimulating production of uterine histotroph, which provides nutrition to the early embryo during critical period of implantation and early development (Stewart *et al.*, 2000).

Earlier studies have suggested that both the number of binucleate cells and the epithelial development of trophoblast are reliable indicators of the embryonic viability (Rodriguez *et al.*, 2000). During early development of the conceptus, the presence of two layers of cuboidal or columnar cells in the trophoblast is indicative of good development, whereas presence of a single cuboidal layer suggests impairment in development (Rodriguez *et al.*, 2000). Deficient trophoblast invasion of the endometrium has been reported as a cause of gestational complications, intrauterine growth restriction and placental abruption (Younis and Samueloff, 2003).

Therefore, it appears that trophoblast tissue acts as the interface between the embryo and the maternal environment. Whilst, an adequate binucleate cell population should ensure production of sufficient placental lactogen for early embryonic growth; a deficient number of binucleate cells potentially can disrupt this and hence can lead to an altered feto-maternal dialogue.

Placenta

In sheep the process of formation of the placenta starts after around Day 12 of pregnancy with elongation of trophoblast, and establishment of allantois and its consequent fusion with the chorion to form the chorionallantois (Wooding, 1992; Schlafer *et al.*, 2000; Noakes, 2001). The chorioallantois is a highly vascular structure which has trophectodermal epithelium on its external surface, which with subsequent advancement in gestation, develops into placenta. The migration and fusion of mature binucleate cells with themselves and with the columnar cells of uterine epithelium result in formation of syncytial plaques and thus making the ruminant placenta synepitheliochorial (Wooding, 1992).

The ovine placenta is cotyledonary, so it has placentomal and interplacentomal regions. The interplacentomal region has superficial apposition between fetal and maternal surfaces and is involved in histotrophic transfer. The placentomal region is implicated in transfer of nutrients and metabolites between maternal and fetal circulations. Placentomes are formed by interaction of chorioallantois and endometrium (Boshier, 1969; Schlafer *et al.*, 2000) and thus have fetal and maternal parts called cotyledons and caruncles, respectively. The cotyledons penetrate very extensively into the caruncles which greatly increases the surface area of contact between the fetus and the mother (Davies *et al.*, 2000).

The placenta not only acts as the site for exchange of nutrients between mother and fetus, but it is also a metabolically active organ in its own right. It secretes various hormones, growth factors and cytokines that regulate development of feto-placental unit and alter maternal physiology to support fetal growth (Gootwine, 2004). The ovine uteroplacental tissue also has been shown to produce nutrients such as lactate and certain amino acids, which are released in the fetal circulation during late gestation and thus provide nutrition to the fast-growing fetus (Bell and Ehrhardt, 1998). In fact, most of the requirements of the fetus for amino acids are met by the placenta and, thus are not taken from the maternal circulation (Harding, 2001). Various studies have suggested a regulatory role of the placenta for fetal IGF-1, such that the placenta can modulate the release or uptake of IGF-1 to or from the fetus to maintain the adequate levels of IGF-1 in the fetal circulation (Bauer *et al.,* 1998). It has been further reported that, in event of intrauterine growth retardation, placental secretion of growth hormone and IGF-1 is reduced, and fetal growth is thereby altered (McIntyre *et al.,* 2000). Owens *et al.* (1987) performed carunclectomy in sheep before pregnancy to restrict placental growth retardation. The fetuses from

these sheep had retarded growth compared to controls. The authors concluded that reduced availability of oxygen to both the pregnant uterus and the fetus due to poor placental development might result in the onset of intrauterine growth retardation. To meet with increasing fetal demands, the rate of transfer of nutrients across placenta increases with increase in the fetal growth (Sibley *et al.,* 1998; Sibley *et al.*, 2002).

Various placental parameters such as placental area, weight, size, number and weight of caruncles and mid-gestational placental volume have been shown to be related to fetal development and birthweight (Alexander, 1974; Kinare *et al.*, 2000; Wilsher and Allen, 2002). Placental morphology and histology are modified during conditions of impaired uterine capacity and vary according to the maternal nutrient supply (Mellor, 1983; Giussani *et al.*, 2002; Wilsher and Allen, 2002; MacLaughlin *et al.*, 2005). Vonnahme *et al.* (2006) reported not only the birth of lighter lambs in the nutrient restricted group compared to the control; but also a reduction in the placental efficiency among the nutrient restricted placentas. These authors found a preponderance of type B, C and D placentomes over type A placentomes in the nutrient restricted group. These studies thus indicate that, in a nutrient restricted environment, the interdigitation of maternal and fetal villi increases; this increase in villous surface area enhances the transfer of nutrients from maternal compartment to fetal compartment to support the adequate fetal growth. This suggests compensatory role of the placenta to support fetal growth under adverse uterine conditions. Moreover, given that the placenta produces substrates such as lactate, amino acids etc, therefore any alternation in the placental morphology might alter its synthetic activity, which further can also modulate fetal growth.

In an experiment with large Thoroughbred and small pony horses, Allen *et al.* (2002b) reported the highest birthweight when a Thoroughbred foal was born from a

Thoroughbred dam and the lowest birthweight when a pony foal was born from the pony dam. The birthweights of Thoroughbred-in-pony foals were significantly less than Thoroughbred-in-Thoroughbred foals. Similarly, the placental mass, gross area of placenta, gross area of allantochorion, volume of allantochorion and total microscopic area of the feto-maternal contact were also higher in Thoroughbred-in-Thoroughbred placentas compared to pony-in-pony placentas. On the other hand, the pony-in-Thoroughbred foals were heavier than pony-in-pony foals and all the above placental parameters were also higher in pony-in-Thoroughbred placentas compared to pony-in-pony placentas. Further, the birthweights of Thoroughbred-in-pony foals and pony-in-Thoroughbred foals did not differ from each other and occupied an intermediate position; interestingly the parameters of their placenta also did not differ from each other and occupied the intermediate position. Clearly, these results suggest that a bigger placenta with greater contact surface area resulted in development of bigger foals and vice versa; but these findings also indicate the role of placenta to support the growth of fetus in both the reciprocal maternal environments. In a similar study, Wilsher and Allen (2002) reported that, with advancing gestation, the chorionic villi of Thoroughbred-in-pony placenta become longer, probably to meet with the escalating demands of large genotype Thoroughbred fetus from a small genotype pony dam. The findings of Giussani *et al.* (2002) in an another experiment with the horse, were also similar. Such studies are thus indicative of a compensatory growth of the placenta to support fetal growth in restricted environment. Thus, it appears that the placenta is an important compartment in itself and has the potential to support fetal growth to an extent during adverse uterine conditions.

Ovine placental lactogen

Ovine placental lactogen is a nonglycosylated, single-chain, 23 kDa protein molecule comprising of 198 amino acids and has three S–S bonds (Gertler and Djiane, 2002). It binds with both prolactin receptors and growth hormone receptors and thereby stimulates growth (Anthony *et al.*, 1995a). Although placental lactogen is present in membrane bound granules from at least Day 16 of pregnancy, the sensitivity of current assays mean that it cannot be detected in maternal peripheral blood until Day 60 of gestation. Both maternal peripheral oPL concentrations and oPL mRNA concentration increase as gestation progresses and reach peak values during late-gestation, whereas the fetal serum oPL concentration reach a maximum during mid-gestation (Kappes *et al.*, 1992).

The concentration of placental lactogen (both maternal and fetal) is directly proportional to the placental mass, number of fetuses and fetal weight (Schoknecht *et al.*, 1991; Kappes *et al.*, 1992; Gootwine, 2004). Recent studies suggest that oPL has a direct luteotrophic effect on corpus luteum particularly until Day 45 of gestation and forms a part of the placental complex which maintains progesterone synthesis to support pregnancy in later periods of gestation (Gregoraszczuk *et al.*, 2000).

There is also evidence to suggest that oPL regulates fetal growth by influencing maternal and fetal metabolism, acting as a partitioning agent to regulate nutrient supply to the fetus and modulating fetal metabolism to use substrates (Anthony *et al.*, 1995b). oPL also stimulates the production of insulin-like growth factors (Handwerger and Freemark, 2000) which are implicated in fetal growth. Moreover, oPL stimulates amino acid transport in fetal rat muscles and inhibit glycogen breakdown in fetal rat hepatocytes (Freemark and Handwerger, 1983; Freemark and

Handwerger 1985) and increases glycogen synthesis in sheep hepatocytes (Freemark and Handwerger, 1986). An enhanced glycogen synthesis would require more fetal glucose and thus will result in net increased demand of fetal glucose from maternal compartment.

Waters *et al.* (1985) reported that infusing antibodies to neutralise circulating oPL caused a decline in plasma non-esterified fatty acids and an increase in insulin concentrations, although glucose levels remained unaltered. Similarly, Thordarson *et al*. (1987) found an increase in the concentrations of plasma non-esterified fatty acids and glucose, but no change in the levels of insulin, following administration of an intravenous infusion of placental extract, enriched in oPL. These studies suggest that oPL stimulates maternal lipolysis and causes maternal insulin resistance. Both of these factors further help to spare more glucose for the fetus. Earlier studies have also suggested that placental lactogen is an important regulator of maternal IGF. This partitioning of nutrients from the maternal to the fetal compartment during pregnancy might also be mediated through IGF-2, which is a potent lipolytic agent and augments glycogenogenesis (Gluckman and Barry, 1988). Therefore, it is plausible that placental lactogen partitions nutrients and substrates from the maternal compartment to the fetal compartment and, hence, acts as an important modulator of feto-maternal interaction.

The binding sites for oPL in fetal liver are reduced by 60% to 75% by maternal nutrient deprivation or fasting and are restored back to their original number when nutrition is restored (Freemark *et al.*, 1989). This suggests that the reduced binding of placental lactogen to fetal liver might be a cause of retarded fetal growth when the fetus develops in maternal nutrient restriction conditions.

In the study of Cheviot and Suffolk sheep by Jenkinson *et al.* (2007), concentrations of oPL, particularly on Days 90 and 110 of pregnancy, were positively correlated with birthweight, although no such relationship existed for concentrations on Day 130.

Therefore, placental lactogen appears as an essential humoral factor that is involved in the regulation of fetal development and maternal and fetal metabolism during pregnancy.

Progesterone

Progesterone is vital for the maintenance of pregnancy and is synthesised by the cells of corpus luteum during early pregnancy in sheep but, by Day 55 of gestation, the placenta becomes the major source of progesterone production (Harrison and Heap, 1978). Studies of ewes that were ovarectomised during first few days of pregnancy and supplemented with exogenous progesterone have shown that progesterone enhances the weight of the embryo (Foote *et al.*, 1957). Similarly, Bindon (1971) also reported positive effects of supplementation of exogenous progesterone upon embryonic development by Day 20 of gestation. Other studies in sheep have also demonstrated that progesterone is required for maintenance of the proportion of inner cell mass and trophectoderm, normal fetal development and growth along with the growth of some key vital fetal organs (Kleeman *et al.*, 1994; Hartwich *et al.*, 1995). A positive association between maternal progesterone concentration and fetal weight, head width, thoracic circumference, weight of all the major fetal organs such as fetal heart, brain, kidney, spleen and total gut and birthweight has been reported (Manalu and Sumaryadi, 1998; Kleemann *et al.*, 2001). Exogenous progesterone supplementation to overnourished adolescent ewes during the first third of gestation

has been shown to increase the birthweight of lambs without increasing either total fetal placental mass or cotyledon mass (Wallace *et al.*, 2003). On the other hand, other studies have failed to support the notion that exogenous administration of progesterone for first few days of pregnancy significantly increases embryo survival (Diskin and Niswender, 1989) or birthweight in sheep (Kenyon *et al.*, 2005).

Studies in rats have shown that progesterone provides trophic support to both the placenta and fetus, since a complete progesterone withdrawal during mid-pregnancy results in reduction in placental protein synthesis, placental weight and fetal weight (Ogle *et al.*, 1990; Mark *et al.*, 2006). Similarly, the anabolic effects of progesterone have also been shown in earlier *in vitro* studies using the rat (Manzi *et al.*, 1994), inasmuch as that the supplementation of progesterone was associated with an increase in DNA and protein synthesis and cell replication.

In dam-size model in horse, (Allen *et al.*, 2002a) maternal plasma progesterone concentration was considered to be controlled by the breed of the fetus rather than the breed of the dam. These authors found the highest mean plasma progesterone concentration in Thoroughbred-in-pony dams and lowest in pony-in-Thoroughbred dams, whereas the Thoroughbred-in-Thoroughbred and pony-in-pony occupy intermediate positions, which is also indicative of its probable role to support fetus under impaired uterine conditions.

Insulin-like growth factor-1

Insulin-like growth factor-1 is 70 amino acid polypeptide with a molecular weight of 7 kDa. It is structurally similar to insulin and is a component of IGF system, which include IGF-1 and IGF-2, their receptors (namely IGF-1R and IGF-2R), a family of six IGF-binding proteins and IGFBP proteases (Allan *et al.*, 2001). IGFs are

produced almost by every cell of the body and affect cell metabolism, cell growth and proliferation via various endocrine, paracrine and autocrine pathways (Jones and Clemmnos, 1995). IGF-1 exerts its mitogenic effects through IGF-1R which is the physiological receptor and has high affinity for IGF-1 (Nissley and Lopaczynski, 1991).

The role of IGF-1 as a modulator of embryonic, fetal and post-natal growth has been reported by many earlier studies. Disruption of the IGF-1 production results in delayed embryonic development, fetal growth retardation, reduction in the body size, severe muscular dystrophy, neonatal mortality and retarded post-natal growth (Baker *et al.*, 1993; Powell-Braxton *et al.*, 1993; Gicquel and Le Bouc, 2006). IGF-1 is also responsible for modulating growth in adults and a cause for the divergent growth in males compared to females (Roberts *et al.*, 1990). IGF-1 is associated with size at birth and catch-up growth during post-natal life in the human (Fail *et al.*, 1995).

Studies on mice have thrown considerable light on the role of IGF-1 during gestation. Blair *et al.* (1988) not only demonstrated the role of IGF-1 in somatic cell growth but also its involvement as a mediator of the genetics of growth. Following a crossbreeding between high and low IGF-1 lines in mice and subsequently supplementing exogenous IGF-1 to the dams during gestation, Gluckman *et al.* (1992) reported that IGF-1 on its own is adequate to modulate the mean fetal weight, since supplementation of IGF-1 nullified the inverse relationship between mean fetal weight and the number of fetuses in the litter. However, by transplanting high or low IGF-1 line mouse embryos to a normal line of mouse, the same authors reported that although the inverse relation between the mean fetal weight and the litter size was re-established, the embryos of the high IGF-1 line always remained heavier. These data suggest that maternal IGF-1 acts as a promoter of fetal growth possibly by altering

the nutrient delivery across the placenta or influencing placental metabolism. Similarly, Morel *et al.* (1994) reported that fetuses developing in dams with high maternal IGF-1 concentration grow more rapidly than those in dams with low IGF-1. IGF-1R receptors have been localised in the sheep placenta, which indicates that IGF-1 exerts its effects on the growth of fetus probably via alteration in the placental function (Bassett *et al.*, 1990). IGF-1 is detectable in many fetal tissues during the first trimester of pregnancy and its concentration increases with a parallel increase in the gestation (Gluckman, 1997). *In vitro* studies have also shown the anabolic and growth promoting effects of IGF-1 on fetal tissues such as increased protein, glycogen and DNA synthesis and promotion of cell differentiation (Hill *et al.*, 1987; Fowden, 1995).

Oliver *et al.* (1993) reported that the concentration of fetal IGF-1 is regulated by fetal glucose concentration. Harding *et al.* (1994) further proposed that a state of high maternal glucose results in high fetal glucose, which results in production of more fetal IGF-1. This increased fetal IGF-1 concentration enhances the uptake of glucose from systemic circulation of fetus, which consequently results in increased fetal growth. Both maternal and fetal IGF-1 has been reported to influence feto-placental metabolism (Gluckman and Harding, 1997). Moreover, IGF-1 has been reported to influence the transfer of nutrients across the placenta, particularly during the second half of gestation. Exogenous administration of IGF-1 during mid-gestation has been associated with an increase in mRNA for Glucose Transporter 1 (GLUT1; which is associated with transfer of glucose from dam circulation to placenta) and GLUT3 (implicated in glucose transport from placenta to fetal circulation) (Zhou and Bondy, 1993). Thus, it appears that fetal glucose is a significant regulator of fetal IGF-1

concentrations and that fetal IGF-1 enhances fetal development by promoting the glucose supply to the fetus.

Glucose

Glucose is the main source of fuel for metabolism and energy in the cells for most of the animals (Herdt, 1997) including fetus and placenta (Hay and Sparks, 1985; Boden, 1996; Scholl *et al.*, 2001). Developing fetus requires nutritional substrates to maintain its metabolism and to meet with the increasing demands of its growth. However, the fetus itself produces very little glucose; therefore the fetal demands for glucose are met by its supply from the mother. Glucose is transferred across the placenta by facilitated diffusion and by involvement of active transporter systems present on the fetal and maternal trophoblast (Marconi *et al.*, 1996). The fetal glucose concentrations depend upon maternal, whilst fetal glucose concentrations also regulate fetal concentration of IGF-1 which, in turn, regulates the transfer of substrates across the feto-placental unit and uptake of glucose from the fetal circulation to its tissues (Bassett *et al.*, 1990; Oliver *et al.*, 1993). Fetal growth retardation in the human is associated with a low maternal glucose concentration (Marconi *et al.*, 1996). Twofold higher glucose consumption in the preterm growth-restricted placentas compared to normally grown preterm placentas has been reported by Challis *et al.* (2000), thereby suggesting that placental consumption of glucose might be responsible for the alterations in the maternal to fetal differences of glucose concentration. The net transfer of the glucose to the fetus across placenta is highly dependent upon the maternal plasma glucose concentrations (Jones *et al.*, 2007).

Free fatty acids

The fetal demands for glucose increase with advancing gestation and, therefore, to maintain normal concentrations of glucose in the circulation, the pregnant ewe starts mobilising her body fat reserves, which results in an increased concentration of circulating FFA (also called as non esterified fatty acids) (Russel *et al.*, 1967). The transfer of fatty acids across the placenta is much less efficient than that of glucose; however, the increased lipolysis results in utilisation of fatty acids as source of energy for mother, and thus more glucose is spared to be used as a fuel source for fetus (Herrera, 2002). Moreover, an increase in the plasma FFA concentration also results in inhibition of insulin-dependent glucose uptake by the body cells to about 30% (Sivan *et al.*, 1997) which further helps in sparing glucose for fetal development. Therefore, a higher concentration of FFA in the circulation of the pregnant ewe dams would indicate an increased substrate demand from fetus and vice versa. Thus, evaluation of plasma FFA concentration would be a good indicator of feto-maternal dialogue.

Overall conclusion

Epidemiological studies in the human suggest that of all the factors that affect fetal development, birthweight, metabolism and post-natal health of an individual; maternal uterine environment is one of the more critical. However, few studies have examined the influence of the uterine environment on long-term health and productivity in livestock. Earlier investigations have suggested that the embryo is sensitive to the surrounding environment, but paradoxically, none have examined the effects of uterine environment upon morphogenesis of the early conceptus. Moreover the stage of gestation at which such effects might be introduced to the development

of conceptus is not fully known. Hence, the present study investigated the effects of maternal uterine environment upon morphogenesis of peri-implantation Day 19 embryos and their trophoblasts with the hypothesis that the uterine environment introduces its effects to the development of conceptus at an early embryonic stage and, which are mediated through trophoblast.

It is recognised that the onset of metabolic and developmental disturbances in animals could result in serious consequences on health and productivity. However, it is still not fully known whether such defects that arise during the antenatal period, could persist into post-natal life of lambs. Although, earlier studies have assessed the birthweight of lambs in relation to various maternal factors, only very few have incorporated other body dimensions which are important to fully assess the effects of antenatal environment upon neonatal and post-natal health status. Overall, there is a paucity of literature on the effects of maternal environment upon post-natal growth. Surprisingly, no dam-size studies in sheep have investigated the glucose metabolism of post-natal lambs. The present study thus postulated that the impairment set-up during conceptus development would persist at birth and affect subsequent post-natal development and glucose metabolism of lambs.

Earlier studies have implicated the placenta and various humoral and metabolic factors such as oPL, progesterone, IGF-1, glucose and FFA in feto-maternal dialogue. Based on this information, the present study investigated various parameters of mid-gestational and full term placentas to examine their involvement as mediators of the feto-maternal dialogue in different uterine environments in sheep. Similarly, aforementioned humoral and metabolic factors were evaluated at different stages of gestation to further understand the mechanistic basis of feto-maternal interaction under different uterine conditions.

Thus, the present study was planned to investigate the effects of uterine environments, in a sheep dam-size model (within and reciprocal transfer of pure-breed embryos between large and small size dams) upon embryonic, fetal, neonatal and postnatal development of lambs with the hypothesis that such effects are introduced to the development of the conceptus during the early embryonic period and that they persist during subsequent, fetal, neonatal and post-natal development.

Chapter 3 - The effects of maternal uterine environment upon embryonic development in sheep

Abstract

Pure-breed embryos were transferred within and reciprocally between large (Suffolk: S) and small (Cheviot: C) breeds of sheep to establish four treatment groups; SinS (large control), SinC (restricted environment), CinS (luxurious environment) and CinC (small control). Recipient ewes were euthanased on Day 19 of gestation. Conceptuses were recovered and immediately dissected into embryos and trophoblast. Embryo morphology was examined on whole-mount preparations. SinC embryos were smaller ($P<0.05$) than SinS, whereas, CinS were bigger ($P<0.05$) than CinC (e.g. length: SinC: 11.04 ± 0.57 mm, SinS: 13.42 ± 0.53 mm, CinS: 15.23 ± 0.68 mm, CinC: 12.85 ± 0.53 mm). A total of ten random sections of trophoblast tissue were stained. Of these, five were stained with Periodic Acid Schiff's (PAS) stain (with haematoxylin as counterstain) to reveal total PAS positive cells and binucleate cells. The remaining five sections were stained with haemotoxylin and eosin for assessing development of the epithelium. The number of total PAS positive cells or binucleate cells was less in SinC than SinS trophoblasts (both $P<0.05$), however there tended ($P=0.07$) to be more binucleate cells in CinS than CinC. The binucleate cells tended to be larger ($P=0.07$) in SinS than SinC trophoblasts, and were larger ($P<0.05$) in CinS than CinC. The height of epithelial cells was greater ($P<0.05$) in CinS than in CinC, whereas no such difference ($P>0.05$) was observed between SinC and SinS trophoblasts. Thus, a restricted uterine environment resulted in development of smaller embryos on Day 19 whereas a luxurious uterine environment permitted development of bigger embryos by Day 19. The differences in the number and characteristics of binucleate cells and trophoblast epithelium

suggest the involvement of the trophoblast as a mediator of effects of maternal uterine environment upon embryonic development by Day 19 of gestation. These results also indicate that such effects of uterine environment are introduced on the conceptus at a very early stage of development even when the physical space of the uterus is not a constraint and hence suggesting the potential involvement of various pathways of maternal epigenetics.

Introduction

Recent epidemiological studies of human populations have indicated that the uterine environment in which a fetus develops has a substantial influence upon its post-natal health status. These epidemiological findings have indicated that the insult to the conceptus during critical stages of development might lead to small size and weight at birth, which is subsequently, linked with long-term post-natal health consequences such as cardiac disorders, Type 2 diabetes and hypertension (Barker, 1995; Godfrey and Barker, 2000; Symonds *et al.*, 2004; Gluckman and Hanson, 2004b). Earlier studies in animals (Walton and Hammond, 1938; Dickinson *et al.*, 1962; Cowley *et al.*, 1989) have also reported the effects of maternal environment on fetal development and consequently on birthweight and post-natal growth. Moreover, many important production traits of livestock, such as milk, wool production and carcass characteristics, are directly related to birthweight and post-natal growth of animals (Greenwood *et al.*, 1998; Bell 2006). Thus the knowledge of effects of maternal uterine environment on early embryos is not only important for improvement of health in human but also to enhance productivity in livestock.

The fetal growth is a complex process that depends on genetic, cellular, nutritional and various other growth and hormonal factors of fetal and maternal origin (Eisen and Durrant, 1980; Dziuk, 1992; Anthony *et al.*, 1995b; Brooks *et al.*, 1995; Cogswell and Yip, 1995; Fowden, 1995; Bell, 2006; Blum *et al.*, 2007; Gardner *et al.*, 2007). The primary factors that appear to regulate fetal growth and, consequently, birthweight of an individual are its genotype and the maternal environment that it experiences *in utero.* Studies have shown that the maternal uterine capacity has a significant effect upon the birthweight and post-natal growth (Walton and Hammond, 1938; Dickinson *et al.*, 1962; Cowley *et al.*, 1989; Allen *et*

al., 2002b; Giussani *et al.*, 2002; Allen *et al.*, 2004; Gardner *et al.*, 2007; Gootwine *et al.*, 2007; Van Der Linden *et al.*, 2007; Blair *et al.*, 2010). Similarly, some nutrition studies have also reported the impairment in the visceral organs along with reduced longevity in animals born from a restricted uterine environment, despite a catch-up post-natal growth (Vehaskari *et al.*, 2001; Ozanne and Hales, 2005). Thus, various earlier maternal environment models have shown the effects of uterine environment on fetal growth, birthweight, organ development and post-natal growth, but to date the stage at which maternal environment introduces its effects to the conceptus development is not clearly known.

The interaction between the conceptus and the mother that can potentially result in impaired fetal development starts at a very early stage of pregnancy (Gaviria and Hernandez, 1994; Goff, 2002; Spencer *et al.*, 2004a). Following fertilisation, the zygote undergoes a cascade of cell divisions (cleavage) which results in the formation and compaction of blastomeres. These blastomeres are surrounded by a superficial layer of cells (trophectoderm, later known as trophoblast). In the ovine conceptus, cleavage divisions up to the 16-cell stage are regulated by maternal gamete mRNA and thereafter by the embryonic genome. The early embryonic period around Day 19 corresponds to the stage of rapid cell division and organogenesis (Dziuk, 1992) and, hence, it is a critical period of development. Moreover, recent studies have indicated that after formation of the blastocyst, subsequent development of the cells of inner cell mass largely depends on the surrounding environment (McGeady *et al.*, 2006).

The formation of the trophoblast starts, at least in part, as a response to inter-cellular contact patterns during cleavage. After elongation, from around Day 16, binucleate cells start to appear in the ovine trophoblast and are randomly scattered as clusters

throughout the trophoblast (Boshier, 1969). The number of binucleate cells increases as gestation progresses and the process of migration and fusion of binucleate cells with uterine epithelium ensures the delivery of binucleate cell granules to the maternal compartment (Wooding, 1984; Rodriguez *et al.*, 2000). The role of binucleate cells in the early embryonic development seems to be crucial, since their granules contain placental lactogen which is a hormone believed to regulate fetal growth by way of its diverse biological functions (Anthony *et al.*, 1995a). During early stages of pregnancy, trophoblast development can be assessed by observing its epithelial layers. Formation of two layers of cuboidal or columnar cell layers is indicative of good development. On the other hand, the presence of a single cuboidal layer indicates an impaired development of the trophoblast. Both, the number of binucleate cells and the degree of trophoblast epithelial development have been reported as reliable indicators for embryonic viability (Rodriguez *et al.*, 2000). Thus, the trophoblast is the interface between the embryo and its maternal environment and, therefore, is a key tissue through which the maternal environment can influence early embryonic development.

Implantation starts around Day 15 of pregnancy in sheep (Noakes, 2001), but the conceptus is not firmly attached until around Day 21. Therefore, Day 19 embryonic stage allowed sufficient time (13 days) of embryonic development either in the recipient uterine environment, along with a feasibility to collect embryos by flushing without any likelihood of damage. Thus, the Day 19 embryonic stage was considered suitable for examining the effects of uterine environments upon morphogenesis of conceptuses.

Hence, the present study was undertaken to investigate the effects of maternal uterine environment upon peri-implatnation embryos and their trophoblasts on Day 19 of

gestation. The hypothesis is that the maternal uterine environment would affect both conceptus and trophoblast developments to this stage such that a restricted uterine environment would reduce the development of embryo and trophoblasts, whereas a luxurious uterine environment would enhance their development. In the present study, the model of the nutrient restriction to the fetus was achieved using a physiologically restricted uterine environment.

Materials and Methods

Experimental animals and design

The study was undertaken using ewes of dissimilar mature body size (Cheviot and Suffolk), in a previously-established model for modifying uterine environment (Jenkinson *et al.*, 2007).

The experimental model was achieved by transferring pure-breed embryos from Suffolk (large breed; mean (± SD) pre-mating liveweight: 74.3 ± 9.2 kg) and Cheviot (small breed; mean (± SD) pre-mating liveweight: 57.9 ± 7.3 kg) ewes into recipients of each breed, to establish four groups of pregnancies: SinS (large fetal genotype in large uterus; large control), SinC (large fetal genotype in small uterus; restricted uterine environment), CinS (small fetal genotype in large uterus; luxurious uterine environment) and CinC (small fetal genotype in small uterus; small control). All animals were managed at Keeble Farm, Massey University, New Zealand under standard lowland commercial farm conditions. Animals were provided with a generous allowance of ryegrass (*Lolium sp*.) white clover (*Trifolium repens*) pastures and had *ad libitum* access to water.

The average body condition score of the ewes used in the experiment was 2.6 (range 1.5 to 4.0). All donors were four year-old ewes, in order to avoid introducing any

variation in embryonic quality due to age of donors. A total of 9 Suffolk and 13 Cheviot donors were used to transfer embryos to 58 Suffolk and 52 Cheviot recipient ewes both within and reciprocally between the breeds. The recipients were ewes of mixed age (3 to 6 years old) and parity, and were balanced between treatment groups for age and condition score. These recipient ewes were randomly divided into two groups, so that one group was implanted with embryos of its own breed and the other group with embryos of the opposite breed. Thus, a total of 29 in SinS, 25 in SinC, 29 in CinS and 27 in CinC embryo transfers were performed resulting in 25 SinS, 22 SinC, 25 CinS and 24 CinC pregnancies based on the detection of non-return to oestrus by Day 17, as detected by vasectomised rams (see below for detailed methodology). Ten recipients from each treatment group were used to recover Day 19 conceptuses, with the remaining ewes from each group were used for recovery of Day 90 fetuses (not reported in this thesis).

Oestrus synchronisation, superovulation, Day 6 embryo recovery and transfer

Oestrus in both donors and recipients was synchronised by placement of intravaginal progesterone-releasing devices (Eazi-breed CIDR; Pharmacia; Auckland, New Zealand) for 13 days. Superovulation of donor ewes was achieved by injecting a total of 216 mgNIH of porcine FSH (Folltropin-V; Bioniche Animal Health; Ontario, Canada) administered intramuscularly in seven tapering doses (48, 48, 28, 28, 24, 20, 20 mgNIH/dose) at 12 h intervals, beginning at 60 h prior to CIDR removal (Table 3.1). At the time of CIDR removal, each donor or recipient was injected with 200 IU of serum gonadotrophin (Pregnecol; Horizon Technology Pty Ltd; NSW, Australia). Semen was collected by electroejaculation from 2 rams per breed and donor ewes of each breed were randomly inseminated laparoscopically with 0.5 mL semen of the ram of the same breed, 32 h after CIDR removal. Six days after insemination,

embryos were recovered via midline laparotomy. This was performed under general anaesthesia using thiopentone sodium (Bomathal, Bomac Laboratories Ltd; Auckland, New Zealand) administered intravenously at the dose rate of 5 mg/kg bodyweight, followed by endotracheal intubation. Subsequent adequate maintenance of the anaesthesia was achieved by 2% halothane in oxygen. The animals were placed in dorsal recumbency. After surgical preparation of the site, a small midline incision was made in the caudal abdomen to allow visualisation and access to the uterus. After exteriorisation of the genitalia, a Foley catheter was placed in the caudal portion of the uterine horn via a stab incision and a flushing catheter was placed into each utero-tubular junction.

Table 3.1. Superovulatory protocol used for Suffolk and Cheviot donors in sheep

Days	Drug and Dosage	Time of administration	Route of administration
0	0.3 g CIDR	Morning	Intravaginal
10	48 mg NIH Folltropin –V	7PM	Intramuscular
11	48 mg NIH Folltropin –V	7AM	Intramuscular
	28 mg NIH Folltropin –V	7PM	Intramuscular
12	28 mg NIH Folltropin –V	7AM	Intramuscular
	24 mg NIH Folltropin –V	7PM	Intramuscular
13	Removal of CIDR	7AM	
	20 mg NIH Folltropin –V	7 AM	Intramuscular
	200 IU Pregnecol	7 AM	Intramuscular
	20 mg NIH Folltropin –V	7PM	Intramuscular

Each horn was flushed with 50 mL of a commercially prepared embryo flushing solution (Complete Ultra, Emcare Flush; ICP Bio Limited; Henderson, Auckland, New Zealand) which was collected in a petri dish. The surgical wound was closed and ewes received post-operative procaine penicillin (Duplocillin LA; Intervet Ltd; Wellington, New Zealand: 10.5 mg/kg bodyweight subcutaneously) and flunixin meglumine (Flunixin; Norbrook NZ Ltd; Auckland, New Zealand: 2.2 mg/kg body weight) as prophylactic measures to prevent infection and to provide analgesia.

The flushings so collected in the petri dish were immediately examined via a light microscopy (25x magnifications) to observe and morphologically evaluate the embryos. Based on this morphological evaluation, embryos were categorised as transferable (late morulae to early blastocyst) or non-transferable.

Following selection of the embryos, they were immediately transferred to the recipients by laparoscopy, at the rate of single embryo per recipient. Prior to embryo transfer, the recipient ewes were sedated with acepromazine (Acezine, Ethical agents Ltd.; Auckland, New Zealand: 0.1 mg/kg bodyweight intramuscularly). These ewes were then restrained in the dorsal recumbency in an artificial insemination crate. Three laparoscopic sites were prepared, one to the right and two to the left of the midline (cranial and caudal to umbilicus) and these sites were anaesthetised by infiltration of 1mL of 2% lignocaine (Nopaine, Phoneix Pharm Distributors Ltd; Auckland, New Zealand). A laparoscope was introduced by making a stab puncture at one lateral site of the abdomen, and the ovary with the corpus luteum was located by visualizing through the laparocope. A small portion of the ipsilateral uterine horn was exteriorised by making a small incision on the other lateral abdominal area. The transferable embryo was then loaded in holding medium (Complete Ultra, Emcare Embryo Holding Medium; ICP Bio Limited; Henderson, Auckland, New Zealand) in

a tom cat catheter (11.4 cm, 3.5 Fr.; Sovereign Kendall, The Kendall Company; Mansfield, MA, USA) and transferred by a stab puncture into the tip of the uterine horn ipsilateral to the ovary with corpus luteum. After transfer, the uterine horns were placed back and the wound was closed.

The required number of pregnancies for each group was achieved by undertaking two successive synchronisation, superovulation, and embryo transfer sessions four days apart. The pregnant ewes from all the treatment groups in both the embryo transfer session were maintained together under farm conditions. Crayon-harnessed vasectomised rams were used to detect pregnancy status of the recipient ewes after embryo transfer. Ewes that did not return to first oestrus (detected by vasectomised rams) by Day 11 post embryo transfer were considered to be pregnant.

Collection of genitalia and recovery of conceptuses

On Day 19 of gestation, the pregnant recipient dams (n=10 per group) were euthanased via captive bolt and exsanguination. The uterus was removed immediately and placed on ice. The excess tissue was removed from the genitalia. The cervical tissue was cut from the uterocervical junction in such a way that the internal os was visible as a patent opening. The uterine horn ipsilateral to the ovary with a corpus luteum was then flushed with 50 mL of 0.9% saline (0.9% sodium chloride, Baxter Healthcare Pty Ltd.; NSW, Australia) for recovery of the conceptus. A similar procedure was then repeated on the other uterine horn, to recover any residual trophoblast tissue. The embryo was dissected free from the extraembryonic membranes and was preserved in 5 mL vials filled with 10% buffered formal saline. The trophoblast tissue was placed in the histology cassettes and preserved in 10% buffered formal saline. All procedures were performed on ice.

Measurement of uterine horns

The length of uterine horn ipsilateral to the ovary with a corpus luteum (pregnant uterine horn) was measured from the utero-cornual junction to the tip of horn using a cotton thread and then calibrating the thread with a measuring scale. A similar procedure was repeated on the uterine horn contralateral to the corpus luteum (non-pregnant uterine horn).

Weighing of conceptus

After trimming the excess connective tissue, the genitalia were weighed using a table-weighing balance and, following the recovery of conceptus and wiping out any excess fluid (until no fluid was apparent on the surface of genetalia), the genitalia were again weighed. Thus, the weight of conceptus was calculated using the following equation:

Weight of conceptus = weight of genitalia before flushing – weight of genitalia after flushing.

This method has limitations as the conceptus was not weighed directly and hence the conceptus weight so obtained might not be the fully correct weight. However, it is emphasised that the priority was to collect the conceptus without any damage and then dissect embryo out from the extraembryonic membranes. This study was focussed on assessing the morphology and size of embryos and different histological characteristics of the trophoblast. Therefore, it was decided to weigh the conceptus using above method so as to keep it intact for assessing the development of embryo and trophoblast and therefore to achieve the objectives of the present study.

Measurement of embryo morphology

Several techniques were investigated to evaluate embryo morphology. The first method explored was to make three-dimensional images of the embryos by using microcomputed tomography, but this was not viable because embryos at this stage did not have enough contrast and as such they were soft tissues. To overcome this problem, embryos could have been incubated for one or two weeks with heavy metals such as osmium tetraoxides but because of the toxic nature of the Osmium, doing so would have rendered the embryos unsuitable for any further investigations such as histology, if thought to be planned later on these embryos.

The other technique tried was that of confoccal microscopy. Though this achieved the required resolution of the embryonic tissues, it was not possible to cover the whole embryo as one image, thereby making the morphological measurement of embryos a cumbersome procedure.

Light microscopy was found to be more suitable for measuring the morphological characteristics of the Day 19 embryos. Light microscopy not only provided sufficient resolution but, by using this technique, it was also possible to examine and capture the whole embryo as one image. The embryos at this stage are translucent, and the internal structures can be visualised by external observation. The morphological features of Day 19 embryos include a pronounced C-shape (dorso-ventral curvature), distinct mesonephric, cardiac and optic regions and the length of these embryos can be calculated by crown-rump length of the curvature and their width can be measured around the heart bulge (Green and Winters, 1945; Wales and Cuneo, 1989).

The whole embryos were photographed using a stereomicroscope (Leica MZ12 fitted with DFC 320 camera; Leica Microsystems**,** Heerbrugg, Switzerland) at a

magnification of 1.6x (Figure 3.1). A 1 mm scale was used to calibrate the software. Subsequently, embryo length, embryo width, heart bulge width and length of neural tube differentiation were measured using Metmorph software (Metmorph 7.5.6.0; Molecular Devices, California, USA) (Figure 3.2). Embryo length was defined as the distance between the medial aspect of the head of embryo to the tip of embryonic tail with the line passing through the greater curvature of embryo. Embryo width was defined as the distance between two widest points of embryos with the line passing through the mid section of heart bulge and including the somites. Heart bulge width was defined as the distance between the two widest points of the heart bulge with the line passing through the mid-section of heart bulge but excluding somites. The length of neural tube differentiation was the distance between two widest points of differentiating neural tube parallel to the longitudinal axis of the embryo.

Figure 3.1. Whole mount embryo photograph of Day 19 embryo in sheep photographed using a stereomicroscope (Leica MZ12 fitted with DFC 320 camera; Leica Microsystems**,** Heerbrugg, Switzerland).

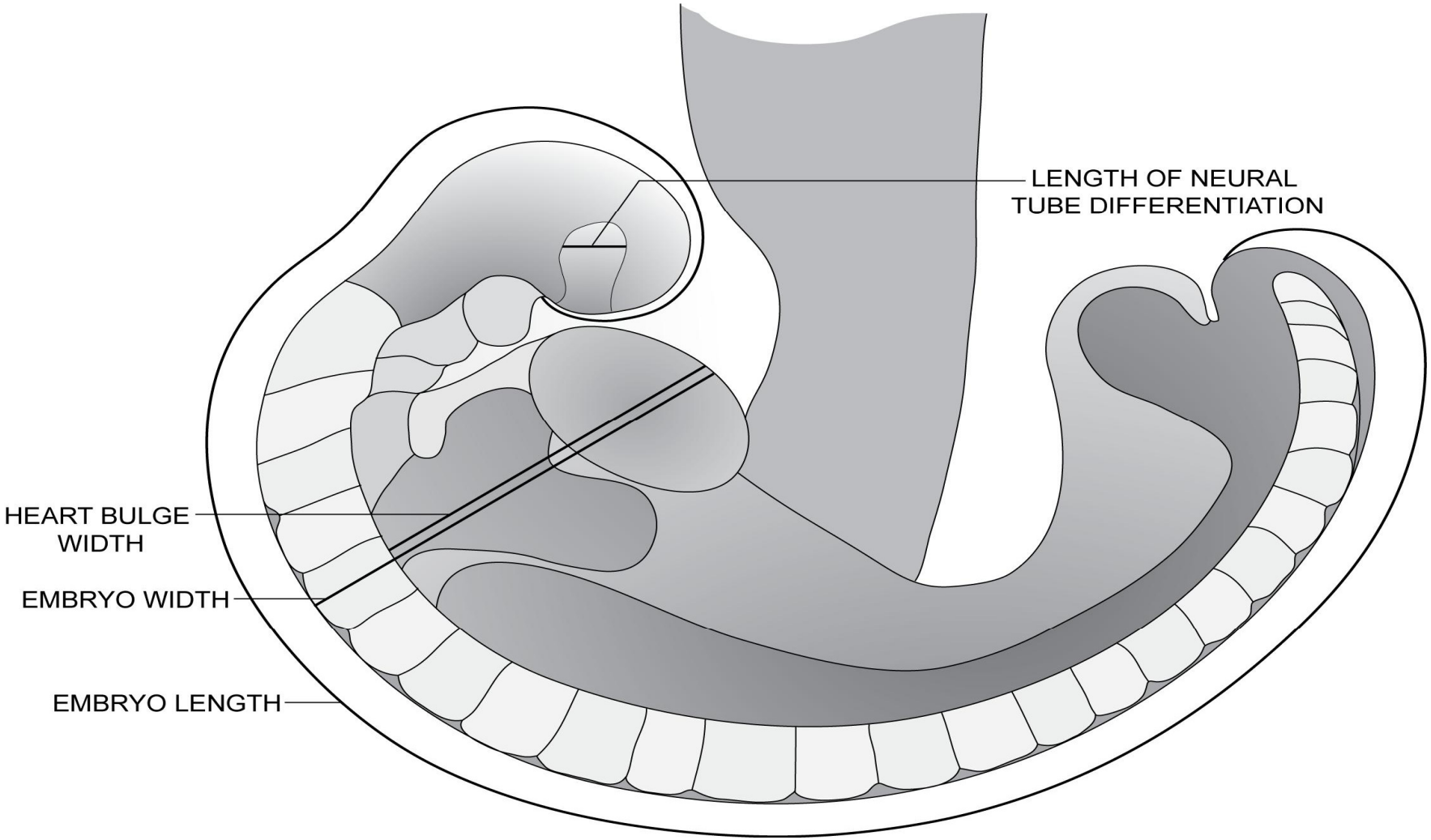

Figure 3.2. Line diagram of Day 19 embryo in sheep showing the measurements of embryo length, embryo width, heart bulge width and length of neural tube differentiation, measured using Metmorph software (Metmorph 7.5.6.0; Molecular Devices, California, USA).

Evaluation of trophoblast characteristics

Following routine paraffin wax embedding, trophoblast tissues were sectioned to a thickness of 2µm. A total of ten random sections of trophoblast tissue were taken per animal. Of these, five were stained with Periodic Acid Schiff's stain (with haematoxylin as counterstain) to reveal binucleate cells. The binucleate cells have dark-stained cytoplasm due to the presence of numerous cytoplasmic ribosomes, widespread arrangement of rough endoplasmic reticulum and well-developed large Golgi bodies (Wango *et al.,* 1990). These Golgi bodies give rise to large, membrane-bound granules which occupy almost half of the cell volume (Wooding *et al.*, 1980; Igwebuike, 2006). These granules are Periodic Acid Schiff (PAS) positive and hence the binucleate cells can be visualised and identified by PAS staining (Boshier, 1969).

The remaining five sections were stained with haemotoxylin and eosin for assessing the epithelial development. A total of fifteen microscopic images were captured for each stain type using a compound light microscope (Zeiss Axiophot with 40x objective and fitted with DFC 320 camera; Carl Zeiss, Germany). The counting of PAS positive cells or binucleate cells and measurement of the diameter of binucleate cells was performed only on those images in which trophoblast tissues comprised at least 80% of their total area (Braun *et al.*, 2007). Cells were defined as PAS positive if at least 30% of the cytoplasm was visibly stained (Ward *et al.*, 2002). The number of PAS positive cells per 1000 epithelial cells and the number of binucleate cells per 1000 epithelial cells was counted. Cells were only counted as binucleate cells if they had two nuclei and also fulfilled the above criterion for cytoplasm staining. Only those trophoblast sections were used to measure height of epithelial cells that were covered with at least 60% of the trophoblast tissue and had single layers of trophoblast cells. The diameter of binucleate cells and the height of trophoblast epithelial cells were measured using

ImageJ software (ImageJ 1.40g, NIH, USA). Epithelial cell height was defined as the distance between the basement membrane and the apex of a cell in its vertical axis. Total area of binucleate cells of trophoblast tissue was calculated using following equation:

Total area of binucleate cells = Total number of binucleate cells x average area of binucleate cell

Ethical approval

All the experimental work was conducted with approval of Massey University Animal Ethics Committee.

Statistical analysis

Data were subjected to analysis of variance using the Generalised Linear Model procedure with respect to the main effects of breed of ewe, breed of embryo, age of recipient and embryo transfer session (i.e. first vs second). Post-hoc differences between the groups were detected using least significant differences. Statistical analysis was undertaken using SAS statistical package (SAS, 2005; SAS 9.1.3, SAS Institute, North Carolina, USA). All two-way and three-way interactions were included in the initial model, but were removed if found non-significant ($P>0.05$) and model was then re-fitted. The interaction between the breed of ewe and breed of lamb was always kept in the model irrespective of whether it was significant ($P<0.05$) or not ($P>0.05$), since this interaction defines the different maternal environments.

Results

The overall pregnancy rate of the recipient ewes was 86.4%. There were no significant (all P>0.1) differences due to breed of donor, breed of recipient or embryo transfer period on number or quality of Day 6 embryos or on pregnancy rate (data not shown). There was also no significant (P>0.1) effect of embryo transfer period on any parameters of embryo or trophoblast morphology. The proportion of the transferable embryos was similar from the two breeds of donor (data not shown).

Uterine and conceptus parameters

There was no difference (P>0.05) in the length of the pregnant or non-pregnant uterine horn between SinC and SinS or between CinS and CinC groups. Similarly, the weight of conceptus did not differ (P>0.05) between either of the treatment groups (Table 3.2).

Table 3.2. The effects of maternal uterine environment on the length of pregnant or non-pregnant uterine horn and the weight of conceptus on Day 19 of gestation in sheep (Mean ± SE). Differences between the treatment groups are non-significant (P>0.05).

Treatment groups	n	Pregnant uterine horn length, cm	Non-pregnant uterine horn length, cm	Conceptus weight, g
SinS[1] (large control)	9	16.52 ± 0.97	15.58 ± 0.86	1.72 ± 0.43
SinC[2] (restricted environment)	10	17.28 ± 0.92	15.95 ± 0.82	2.00 ± 0.41
CinS[3] (luxurious environment)	10	15.48 ± 0.92	14.81 ± 0.82	2.29 ± 0.41
CinC[4] (small control)	10	15.53 ± 0.92	15.27 ± 0.82	2.24 ± 0.41

[1]Suffolk in Suffolk, [2]Suffolk in Cheviot, [3]Cheviot in Suffolk, [4]Cheviot in Cheviot.

Embryo morphology

Embryonic length, embryonic width, heart bulge width and length of neural tube differentiation of SinC embryos were significantly (P<0.05) smaller than SinS embryos (Table 3.3). On the other hand, embryonic length, embryonic width and heart bulge width of CinS embryos were significantly (P<0.05) greater than in CinC embryos. However, there was no difference (P>0.05) in the length of neural tube differentiation between these latter two groups.

Table 3.3. The effects of maternal uterine environment upon embryo morphometry on Day 19 of gestation in sheep (Mean ± SE). Within a column, means without a common superscript are different from each other (P<0.05).

Treatment groups	n	Embryo length, mm	Embryo width, mm	Heart bulge width, mm	Length of neural tube differentiation, mm
SinS[1] (large control)	9	13.42^{c} ± 0.53	2.44^{bc} ± 0.08	1.95^{b} ± 0.08	0.29^{b} ± 0.02
SinC[2] (restricted environment)	8	11.04^{a} ± 0.57	2.00^{a} ± 0.08	1.51^{a} ± 0.09	0.23^{a} ± 0.02
CinS[3] (luxurious environment)	5	15.23^{d} ± 0.68	2.66^{c} ± 0.11	1.99^{b} ± 0.11	0.29^{b} ± 0.02
CinC[4] (small control)	8	12.85^{bc} ± 0.53	2.21^{ab} ± 0.08	1.66^{a} ± 0.09	0.26^{ab} ± 0.02

[1]Suffolk in Suffolk, [2]Suffolk in Cheviot, [3]Cheviot in Suffolk, [4]Cheviot in Cheviot.

Trophoblast parameters

The total number of PAS positive cells or binucleate cells (Figures 3.3 & 3.4) was significantly higher ($P<0.05$) in SinS trophoblasts than in SinC trophoblasts, whereas no such difference ($P>0.05$) was observed in PAS positive cells between CinS and CinC groups (Table 3.4). The number of binucleate cell tended to be higher ($P=0.07$) in CinS than in CinC trophoblasts. The binucleate cells tended to be larger ($P=0.07$) in SinS trophoblasts than in SinC trophoblasts; whereas they were significantly larger ($P<0.05$) in CinS compared to CinC trophoblasts. There was no significant ($P>0.05$) difference in the mean epithelial cell height between SinC and SinS groups; however the mean epithelial cell height was greater ($P<0.05$) in CinS trophoblasts compared to CinC trophoblasts (Table 3.4). The total area of binucleate cells in trophoblast tissue was less ($P<0.05$) in SinC compared to SinS (8.3 ± 3.6 mm^2 vs 26.0 ± 3.8 mm^2 respectively), whereas it was higher ($P<0.05$) in CinS than CinC (26.5 ± 3.6 mm^2 vs 13.0 ± 3.8 mm^2, respectively) (Figure 3.5).

Linear regression analysis of binucleate cell number with the embryonic length partitioned by breed of dam showed a significant correlation (embryo length (mm) = 11.75 ± 0.019 BNC (cells/1000), $P<0.05$) between binucleate cell number and embryonic length. However, no such relationship was observed for epithelial cell height (embryo length (mm) = 11.55 ± 0.088 epithelial height (µm), $P>0.5$).

Table 3.4. The effects of maternal uterine environment on trophoblast characteristics on Day 19 of gestation in sheep (Mean ± SE).Within a column, means without a common superscript are different from each other (P<0.05).

Treatment groups	n	Total PAS[1] positive cells per1000 epithelial cells	BNC[2] per1000 epithelial cells	Average binucleate cell area, μm^2	Epithelial cell height, µm
SinS[3] (large control)	9	249.3^{c} ± 31.0	89.1^{c} ± 13.1	293.4^{bc} ± 10.4	17.49^{ab} ± 0.90
SinC[4] (restricted environment)	10	96.3^{a} ± 29.5	31.1^{a} ± 12.3	266.9^{ab} ± 9.9	15.79^{a} ± 0.85
CinS[5] (luxurious environment)	10	205.1^{bc} ± 29.5	84.0^{bc} ± 12.3	318.7^{c} ± 9.9	19.52^{b} ± 0.85
CinC[6] (small control)	10	141.4^{ab} ± 31.1	50.1^{ab} ± 13.0	261.6^{a} ± 9.9	16.61^{a} ± 0.85

[1]Periodic Acid Schiff's, [2] Binucleate cells, [3]Suffolk in Suffolk, [4]Suffolk in Cheviot, [5]Cheviot in Suffolk, [6]Cheviot in Cheviot.

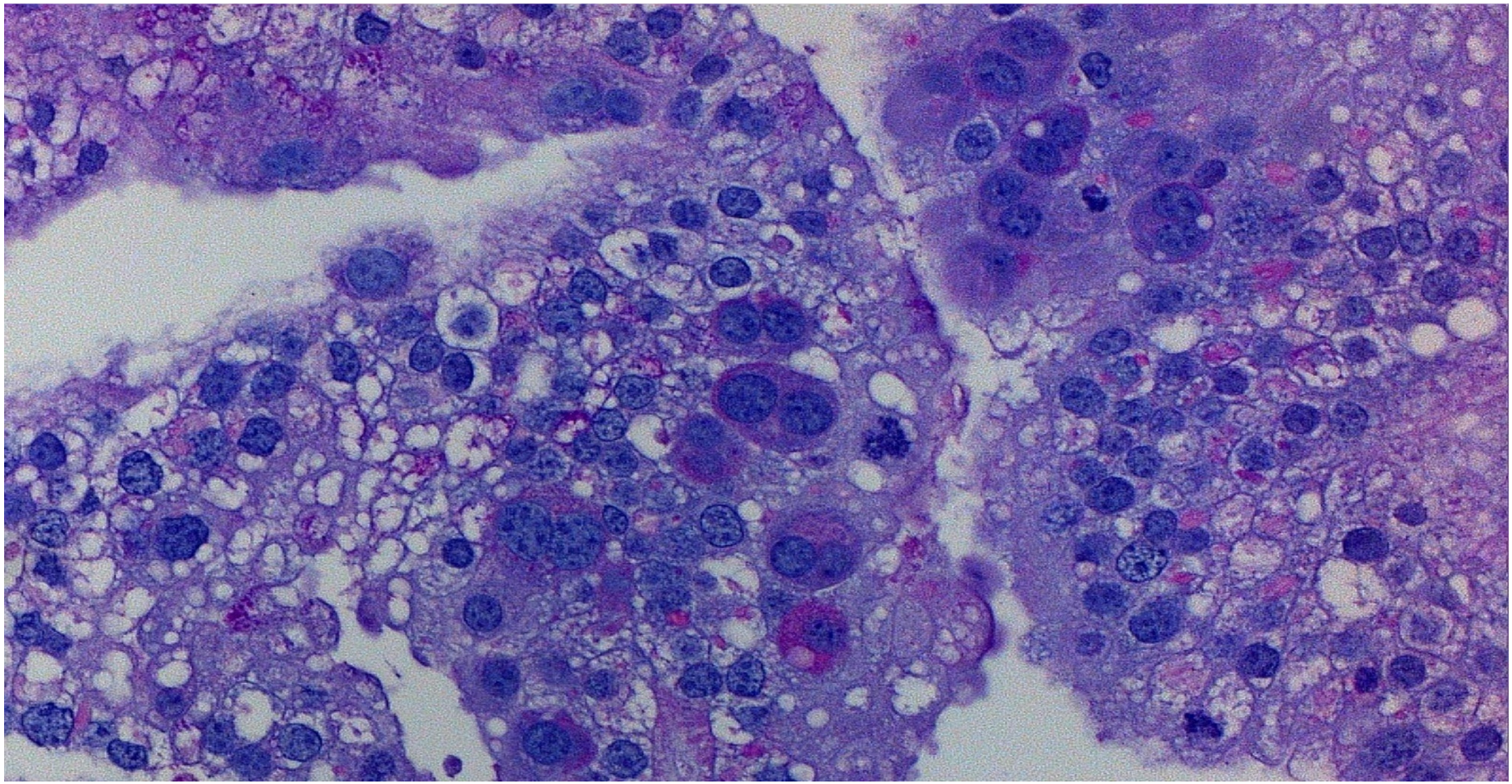

Figure 3.3. Trophoblast tissue sections stained with hematoxylin and Periodic Acid Schiff's (PAS) stain and photographed with compound light microscope (Zeiss Axiophot with 40x objective and fitted with DFC 320 camera; Carl Zeiss, Germany) to reveal PAS positive binucleate cells on Day 19 of gestation in sheep.

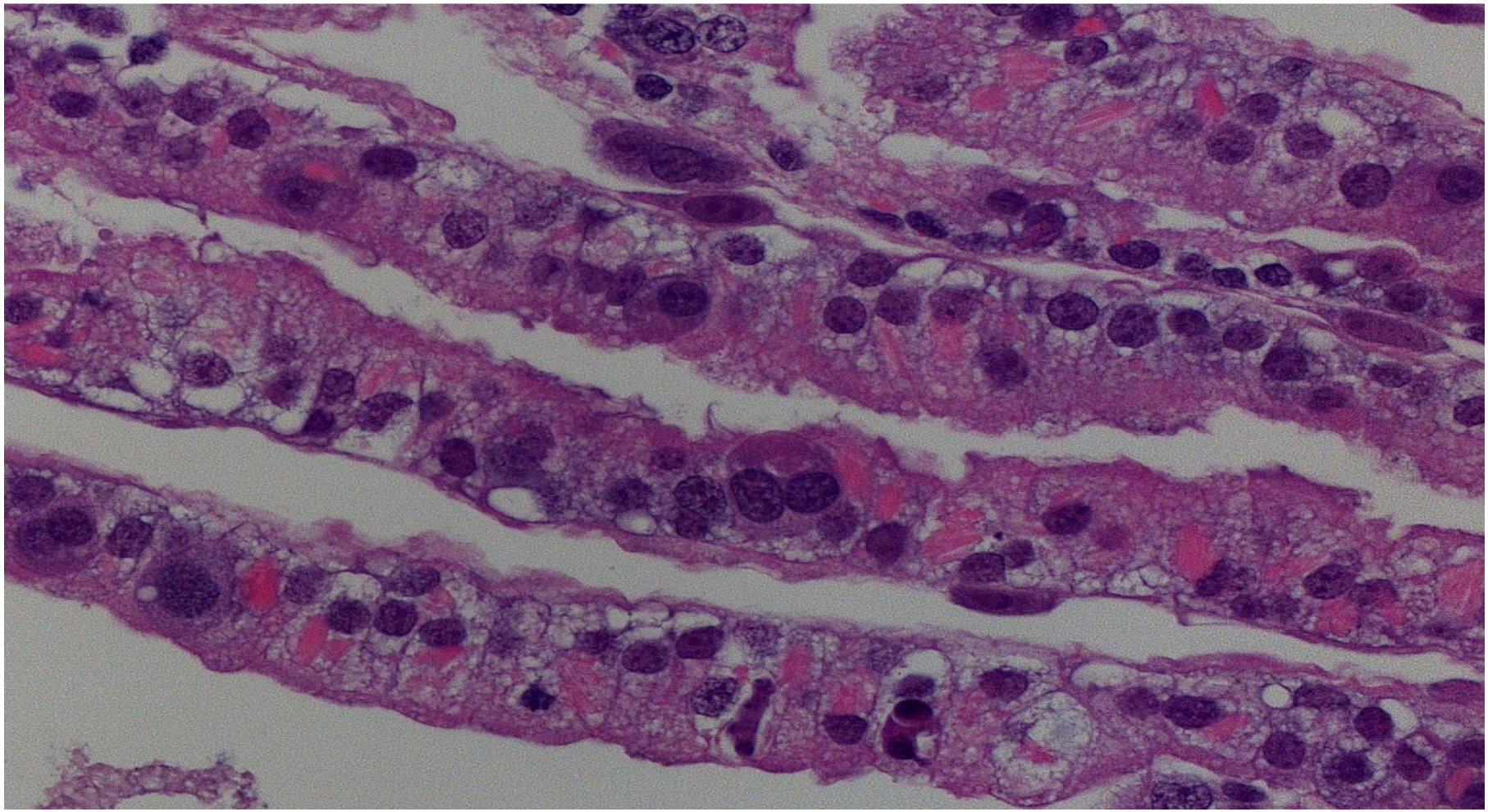

Figure 3.4. Trophoblast tissue sections stained with hematoxylin and eosin stain and photographed with compound light microscope (Zeiss Axiophot with 40x objective and fitted with DFC 320 camera; Carl Zeiss, Germany) to reveal epithelial cells on Day 19 of gestation in sheep.

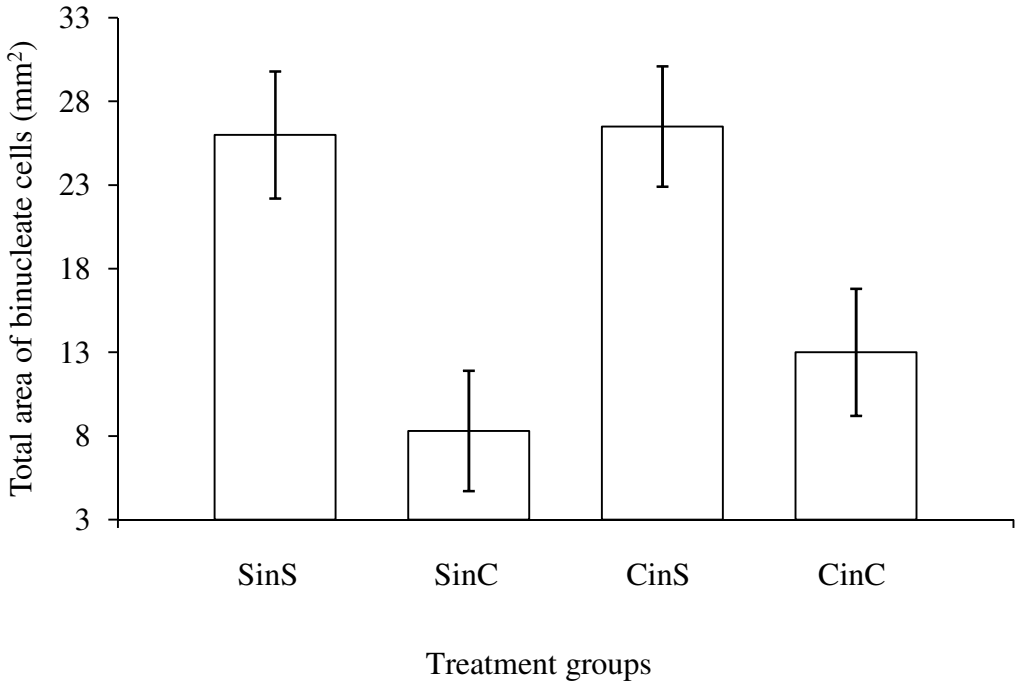

Figure 3.5. The effect of maternal uterine environment on total area of binucleate cells in different treatment groups on Day 19 of gestation in sheep (Mean ± SE). Bars that are labelled with different superscripts are significantly different from each other ($P<0.05$) S: Suffolk, C: Cheviot.

Discussion

The key findings of the present study were that a restricted uterine environment (SinC compared to SinS) resulted in development of smaller embryos on Day 19, whereas a luxurious uterine environment (CinS compared to CinC) permitted development of bigger embryos on Day 19. This clearly suggests that the maternal uterine environment of the early stage of pregnancy affects development of peri-implantation embryos. This effect of maternal uterine environment was so marked that it appears to have overridden the genetic potential of these embryos. Thus the Suffolk embryos, which are genetically programmed for bigger size, could not achieve this size in a restricted maternal uterine environment (SinC) compared to control (SinS). Conversely, the small genotype Cheviot embryos grew bigger than their genetic potential when allowed to develop in the luxurious uterine environment (CinS) compared to the control (CinC). This is the first study to show the effect of maternal uterine environment on the morphogenesis of peri-implantation embryos (Day 19) and their trophoblast characteristics. These results suggest that the early embryo is susceptible to the surrounding external environment and perhaps has sufficient developmental plasticity to adjust with the uterine environment resulting in alteration to their development.

That the peri-implantation embryos are sensitive to the surrounding environmental conditions has also been indicated by a few *in vitro* or *in vivo* studies using bovine, equine or ovine models (Bertolini *et al.*, 2002; Tremoleda *et al.*, 2003; Nyborg *et al.*, 2005). Recent studies have also suggested that environmental effects on critical mechanisms of early development are mediated through various maternal epigenetic mechanisms such as DNA methylation and histone modification, which result in the development of abnormal or impaired patterns of gene expression in the phenotypes (Wolff *et al.*, 1998; Cooney *et al.*, 2001; Jaenisch and Bird, 2003). The significance of

these epigenetic events in the early embryonic stage upon subsequent embryonic and post-natal development is now increasingly recognised, and there is a growing body of evidence that such epigenetic modifications can produce permanent non-genomic effects (Jaenisch and Bird, 2003; Symonds *et al.*, 2007). It might be possible that the uterine environment altered the embryo-maternal interaction and introduced epigenetic modification to embryonic genome and thus affected development of embryos. It is also plausible that the embryos in the present study might have adjusted their development using their developmental plasticity to adjust with the new environment of maternal-embryonic interface, which resulted in their altered morphogenesis; (predictive adaptive hypothesis (Gluckman and Hanson, 2004a)). However, a clear understanding of this mechanism awaits future research.

In the sheep conceptus, trophoblast binucleate cells appear from Day 16 of pregnancy (Boshier, 1969). From Day 16 onwards, these cells contribute about 15 to 20% of the total trophectodermal cell mass in both sheep and cows (Wooding and Wathes, 1980; Wooding, 1984). The number of binucleate cells increases with advancing gestation and growth of placentomes (Lee *et al.*, 1985). The total number of binucleate cells in the present study was consistent with those reported by Wooding and Wathes (1980) and Wooding (1984), although the numbers of both total PAS positive cells and binucleate cells varied between treatment groups. The number of PAS positive cells, and particularly that of binucleate cells in the trophoblast, was correlated with the length and degree of development of the embryos from which they were derived. Therefore, it appears that one of the mechanisms by which the maternal environment might have caused the altered embryonic development was via regulation of the formation of binucleate cells. It is plausible that the restricted maternal environment (SinC) retarded embryonic morphogenesis by suppressing the formation of trophoblast binucleate cells.

On the other hand, the tendency towards a higher number of binucleate cells in a luxurious uterine environment (CinS) might explain the enhanced development of embryos in this group. This notion is supported by earlier studies that suggest differences in the endometrium during the early period of pregnancy might be involved in the formation of binucleate cells and trophoblast development (Reynolds and Redmer, 1992; Zheng *et al.*, 1998; Rodriguez *et al.*, 2000). An alteration in the development pattern at such an early stage, when the critical mechanisms of developmental are active, perhaps results in permanent non-genomic effects which consequently lead to impaired birthweight and size which was evident in earlier studies that involved maternal uterine environment models (Walton and Hammond, 1938; Dickinson *et al.*, 1962; Cowley *et al.*, 1989; Giussani *et al.*, 2002; Allen *et al.*, 2004; Gardner *et al.*, 2007; Gootwine *et al.*, 2007).

Binucleate cells secrete placental lactogen which regulates conceptus growth by its diverse biological functions such as modulating the metabolism of the fetus to use substrates, and by acting as a partitioning agent for nutrient supply between mother and the fetus (Anthony *et al.*, 1995a). Moreover, oPL also exert its effects on the uterine milk protein secretion, which is a trophic factor for early embryonic growth (Stewart *et al.*, 2000). Around implantation, the process of migration and fusion of binucleate cells with the uterine endometrium ensures the delivery of binucleate cell granules (that contain placental lactogen) to the maternal compartment (Wooding, 1984). In future studies thought should be given to measure the ovine placental lactogen at this stage of gestation to further investigate this mechanism.

It is conceivable that an inadequate number of the binucleate cells might result in a lowered production of placental lactogen, which in turn could constrain early embryonic growth. It is equally interesting to note that differences in the characteristics of

binucleate cells (such as in the diameter; Table 3.4) were observed in the luxurious uterine environment (CinS) compared to its control (CinC), even though such differences were absent in the restricted environment (SinC) versus its control (SinS). It is unclear why the number of binucleate cells differed between the restricted and the control group compared with the diameter of the binucleate cells that differed between the luxurious and control groups. Perhaps the total area of binucleate cells would probably be a more reliable estimate of the potential to produce placental lactogen as it is reflective of total surface area of the binucleate cells. In the present study the area differences between the comparable groups (i.e. restricted (SinC) versus its control (SinS) and between the luxurious (CinS) and its control (CinC)) indicate a potential underlying mechanism through which the embryonic development might have been regulated by the maternal uterine environment. Further studies are required to investigate this mechanism and in particular to evaluate the functional capability of the trophoblast in terms of production of placental lactogen and consequently its role in conceptus-maternal interaction in various uterine environments. This can be achieved by evaluating the expression of placental lactogen in trophoblast tissue and/or measuring the plasma placental lactogen concentration by collecting the blood via intubation of utero-ovarian vein.

Whether enhanced trophoblast development could also have augmented the embryo growth is less clear; however, adequate trophoblast development and its invasion into uterine tissue have been suggested as critical factors for a successful pregnancy outcome (Younis and Samueloff, 2003). Likewise, the degree of trophoblast epithelial development has been suggested as a reliable indicator for embryonic viability (Rodriguez *et al.*, 2000). In the present study, the mean trophoblast epithelial cell height was greater in CinS embryos (luxurious uterine environment) compared to the control

(CinC) and thus these findings are consistent with the previous above study. The present results thus suggest that an increase in the trophoblast epithelial cell height might result in an enhancement of the embryonic development.

The trophoblast is the precursor of the placenta and hence any alteration in trophoblast characteristics such as in the number of binucleate cells or epithelial development might affect the subsequent placental development or perhaps function. The placenta is the site for exchange of nutrients between the mother and fetus (Gootwine, 2004). So, any alteration in the placental characteristics could affect fetal development. The present results suggest that the maternal uterine environment during the early stage of pregnancy affects the development of the trophoblast and this alteration of trophoblast subsequently might affect development of placenta. An alteration in the placental development could affect subsequent feto-maternal dialogue, consequently leading to altered fetal growth, birthweight and birth-size, hence, following-up the placental characteristics during later in gestation in equally important. No difference was found between the lengths of pregnant or non-pregnant uterine horns. Thus, the differences in the embryonic morphogenesis at such an early stage of development can not be directly attributed to a difference in the uterine space.

Conclusion

The present study has shown an effect of maternal environment on the development of early embryos and the involvement of the trophoblast in conceptus-maternal interaction, and thus, describing the cellular mechanism to a larger extent. It was found that the maternal uterine environment alters the development of early embryos apparently modulating their genetic potential for growth. It is likely that these effects are mediated through the trophoblast during early conceptus-maternal intraction. However, the

underlying mechanism of this interaction further needs to be investigated at molecular levels. Similarly, the involvement of various endocrinological and metabolic factors later in gestation needs to be investigated to gain further understanding of this feto-maternal dialogue.

Chapter 4 - The effects of uterine environment upon fetal and placental development during gestation: an ultrasonographic study

Abstract

Pure-breed single embryos were transferred within and reciprocally between large (Suffolk: S) and small (Cheviot: C) breeds of sheep to establish four treatment groups; SinS (large control), SinC (restricted environment), CinS (luxurious environment) and CinC (small control). Recipient ewes carried single fetuses to full term. During gestation, fetal head width and fetal head length were measured on Day 55, and cross-sectional area of placentomes evaluated on Day 110. The fetal head length of SinC fetuses was less ($P<0.05$) than SinS fetuses (2.84 ± 0.09 cm vs 3.16 ± 0.09 cm, respectively), whereas no such difference ($P>0.05$) was observed between CinS and CinC fetuses. There were no differences ($P>0.05$) in the fetal head width between SinC and SinS or between CinS and CinC fetuses. Placentomal cross-sectional area did not differ ($P>0.05$) between SinC and SinS placentomes or between CinS and CinC placentomes. It was concluded that the fetal development is impaired in a restricted uterine environment by Day 55 of gestation but is not enhanced in a luxurious uterine environment; and that sonography can be used to detect such differences in the fetal development in sheep by Day 55 of pregnancy.

Introduction

A large body of epidemiological studies of human (Barker, 1995; Kirchengast and Hartmann, 1998; Godfrey and Barker, 2000; Robinson *et al.*, 2000a; Godfrey and Barker, 2001; Gluckman and Hanson, 2004a) and a few studies of animals (Walton and Hammond, 1938; Cowley *et al.*, 1989; Gardner *et al.*, 2007; Gootwine *et al.*, 2007; Long *et al.*, 2009) have suggested that maternal uterine environment affects the fetal development which consequently leads to alteration in birthweight and post-natal growth. The placenta is the site of exchange of nutrients and waste products between mother and fetus. It is also an active metabolic organ which secretes various hormones, growth factors and cytokines that act as modulators of feto-maternal dialogue (Gootwine, 2004). Various parameters of placental morphology and/or histology have been shown modified to support adequate fetal growth under impaired uterine conditions (Giussani *et al.*, 2002; Wilsher and Allen, 2002; Vonnahme *et al.*, 2006). The rate of transfer of nutrients across placenta increases with increasing fetal growth rate (Sibley *et al.*, 1998; Sibley *et al.*, 2002).

In Chapter 3, the morphological characteristics of the Day 19 embryos and their trophoblasts (a placental precursor) in different maternal uterine environments were investigated. Those results suggested that, in a restricted uterine environment (SinC), the development of embryos were impaired compared to control environment (SinS); whereas in the luxurious environment (CinS) the embryonic development was enhanced than in the control environment (CinC). Further the trophoblast parameters were suppressed in SinC trophoblasts than in SinS, as shown by a lower binucleate cell number and a tendency of smaller binucleate cells in SinC compared to SinS trphoblasts. On the other hand, the development of CinS trophoblasts was enhanced compared to CinC trophoblasts, since the number of binucleate cells tended to be higher

and both the binucleate cells and trophoblast epithelial cells were bigger in CinS than in CinC trophoblasts. Therefore, an evaluation of the fetal and placental development with advancing gestation would help to understand whether any alteration in the development of conceptus in early stages of gestation (Day 19) would result in any observable changes in its development later in pregnancy. Such a study might also help to unravel the involvement of the placenta in the feto-maternal dialogue (Wilsher and Allen, 2002; Vonnahme *et al.*, 2006). Hence the effects of maternal uterine environment upon subsequent development of conceptus were investigated in this chapter.

Real-time ultrasound imaging is a reliable, rapid, accurate and non-invasive method for early pregnancy diagnosis in sheep (Buckrell *et al.*, 1986). Besides its use in pregnancy diagnosis, sonography has also been used in sheep and cattle to measure fetal body parameters, such as biparietal diameter, occipito-snout length or placentomal diameter, to predict the age of the fetus and to estimate its growth (De Bulnes *et al.*, 1998; Breukelman *et al.*, 2004). Until Day 80 of pregnancy in sheep, sonography has been suggested as a reliable tool to measure fetal head length and width, and a consistent relationship has been reported between the age of the fetus and dimensions of its head width or head (Kelly and Newnham, 1989; Sergeev *et al.*, 1990). In sheep, the biparietal diameter and the other parameters of the skull can be measured as early as Day 38 of gestation (Kaulfuss *et al.*, 1999). However, it is not always possible to obtain good symmetrical images of the skull before Day 50 of pregnancy, and measurement of fetal head length is more cumbersome compared to that of fetal head width (Kelly and Newnham, 1989). Sonography has been widely used in humans to assess placental development and to diagnose placental abnormalities (Abramowicz and Sheiner, 2006; Sodowski *et al.*, 2007; Alhamdan *et al.*, 2009). Real-time ultrasound measurement of mean placentomal diameter has been used to evaluate placental development in sheep

around Day 110 of pregnancy (Kelly *et al.*, 1987; Kaulfuss *et al.*, 1998). The measurement of the fetal head is impossible after Day 95 of pregnancy because the dimensions of the fetal head almost equate to the width of the transducer and, hence, it is not possible to obtain a complete linear image of the skull in the sonographic field (Kelly *et al.*, 1987; De Bulnes *et al.*, 1998).

Earlier studies have suggested that by Day 100 of gestation, the placental morphology is well established to support fetal growth and the diameter of the placentomes is a consistent and apparently reliable indicator of placental development (Ehrhardt and Bell, 1995; Kaulfuss *et al.*, 1998). Moreover, the rate of transfer of the nutrients across placenta increases with increases in the fetal growth rate (Sibley *et al.*, 1998; Sibley *et al.,* 2002). Studies have also shown an alteration in the morphology of placentomes to support fetal growth in a nutrient restricted environment (Vonnahme *et al.*, 2006). Therefore, the size of placentome on Day 110 might be an indicator of the nutrient transfer to fetus and thus any difference in the cross-sectional areas of placentome might reflect an alteration in the development of placenta to support fetal growth.

Hence, in the present study real-time ultrasound imaging was used to evaluate the fetal and placental development by measuring the width and length of fetal head on Day 55, and the cross-sectional area of placentome on Day 110 of gestation in different uterine environments.

Materials and methods

Experimental design and animals

Four groups of pregnancies were established viz. Suffolk in Suffolk (SinS; large control), Suffolk in Cheviot (SinC; restricted environment), Cheviot in Suffolk (CinS; luxurious environment) and Cheviot in Cheviot (CinC; small control) by transferring

purebred single embryos per recipient within and reciprocally between large Suffolk (S) and small Cheviot (C) breed of sheep. The synchronisation, superovulation, embryo recovery and embryo transfer was undertaken using similar protocols and conditions as explained in the previous experiment conducted during previous breeding season (Chapter 3). In the present experiment the recipient ewes were allowed to carry fetuses until term.

In this study, a total of 13 Suffolk and 13 Cheviot donors were used to transfer embryos to 91 Suffolk and 85 Cheviot recipients, both within and reciprocally between the breeds. The average body condition score of the ewes used in the experiment was 2.6 (range 1.5 to 4.0). The recipient ewes were of mixed ages (3 to 6 years old) and were randomly divided into two groups; so that one group was implanted with the embryos of its own breed and the other group with embryos of the opposite breed. The recipient ewes were balanced for age and condition score within the treatment groups. A total of 49 in SinS, 47 in SinC, 42 in CinS and 38 in CinC single embryo were transferred and a total of 33 SinS, 29 SinC, 24 CinS and 24 CinC pregnancies were maintained to full term. The recipient ewes used for measuring the fetal and placentomal characteristics were selected randomly from each treatment group from the first embryo transfer session only and included 14 SinS, 14 SinC, 14 CinS and 12 CinC pregnancies. To achieve the required number of pregnancies in each group, two successive synchronisation, superovulation, and embryo transfer sessions were undertaken 37 days apart, such that the recipient ewes that failed to conceive to the first embryo implantation (return to first oestrus based on the detection by vasectomised rams) were mixed again and re-used as recipients in the second embryo transfer session. The pregnant ewes from all treatment groups in both the embryo transfer sessions were maintained together at Keeble Farm, Massey University, New Zealand under the

standard lowland commercial farm conditions. Animal were provided with generous allowance of ryegrass (*Lolium L.*) and white clover (*Trifolium repens L.*) pastures and had *ad libitum* access to water.

Measurement of fetal and placental parameters

The ewes used for sonography were restrained in the standing position and transabdominal scanning was done using a real-time, B-mode veterinary portable ultrasound scanner equipped with 5.0 MHz sector transducer (Pie Medical 240 Parus; Pie Medical; Limburg, Netherland). Transabdominal scanning is achieved by manipulating the scanner over hairless skin in the lower abdomen and inguinal sites (Fowler and Wilkins, 1984) using the same plane in all the ewes.

Fetal head width and lengths were measured on Day 55 of gestation on the symmetric images of the skull (Figure 4.1), whereas the cross-sectional area of the placentomes was measured on Day 110 of pregnancy. Sonographic images of skulls or placentomes were recorded on VHS videotapes. The images from these videotapes were converted to digital images using Adobe Premier Software (Adobe Premiere 1.5; Adobe, New York, USA). Still frames from these digital images were then analysed in ASUSDVD (ASUSDVD 6.0; ASUSTek; Sydney, Australia) to measure the fetal and placentomal parameters using a manual scale.

Fetal head width was defined as the difference between the two orbital sockets when they were visible in the sonographic field. Fetal head length was defined as the distance between the occipit and the tip of nasal bones and (also referred as occipito-snout length) (Figure 4.2) (Kelly and Newnham, 1989). For each of these fetal parameters two replicate measurements were taken for each fetus and the average values of the replicates were calculated prior to the statistical analysis.

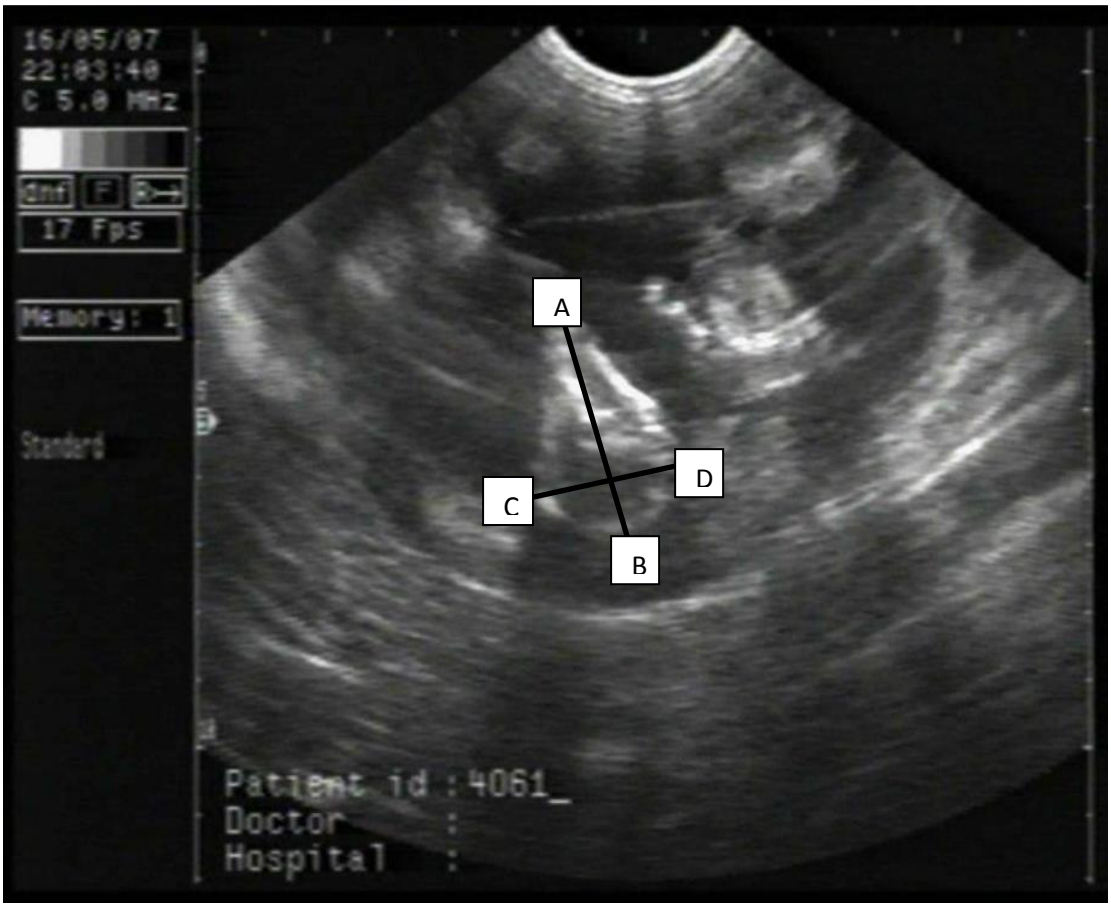

Figure 4.1. Sonographic image of fetal skull on Day 55 of gestation in sheep; obtained by B-mode veterinary portable ultrasound scanner equipped with 5.0 MHz sector transducer (Pie Medical 240 Parus; Pie Medical; Limburg, Netherland). Line from point A to Point B represents the length of head and line from Point C to Point D represents the width of head.

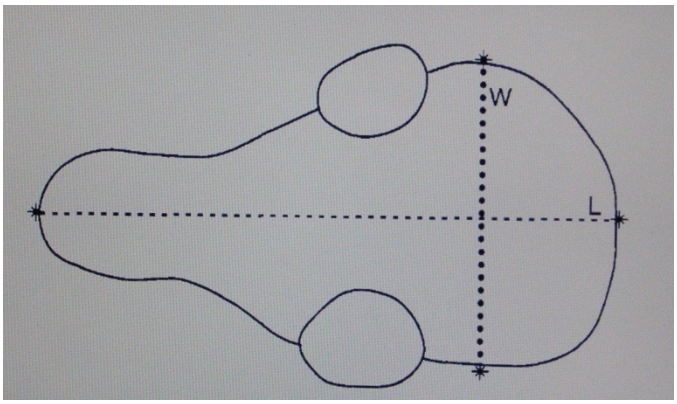

Figure 4.2. Schematic diagram of measurements of fetal head width and length (Kelly and Newnham, 1989) on Day 55 of gestation in ewes; W (fetal head width), L (fetal head length).

Only those placentomes which appeared as complete circular structures in the sonographic field and had a central anechoic cavity (Figure 4.3) were selected for measurement. For each placenta, the diameter of ten placentomes was measured. To obtain the cross-sectional area of the placentome, the diameter of each placentome was divided by two to calculate its radius. The cross-sectional area of placentome was then calculated by the following equation:

Cross-sectional area of placentome (cm^2) = Πr^2

Where: r = radius of the placentome

Ethical approval

All the experimental procedure was carried out with approval of Massey University Animal Ethics Committee.

Statistical analysis

The data were subjected to analysis of variance using Generalised Linear Model procedure from the SAS statistical package (SAS, 2005; SAS 9.1.3, SAS Institute, North Carolina, USA). Post-hoc differences between the groups were detected using least significant differences. The main effects of breed of ewe, breed of lamb, age of recipient and sex of lamb and all two-way and three-way interactions were included in the initial model, but were removed if found non-significant ($P>0.05$) and the model was then re-fitted. The interaction between the breed of ewe and breed of lamb was always kept in the model irrespective of whether it was significant ($P<0.05$) or not ($P>0.05$), since this interaction defines the different maternal environments.

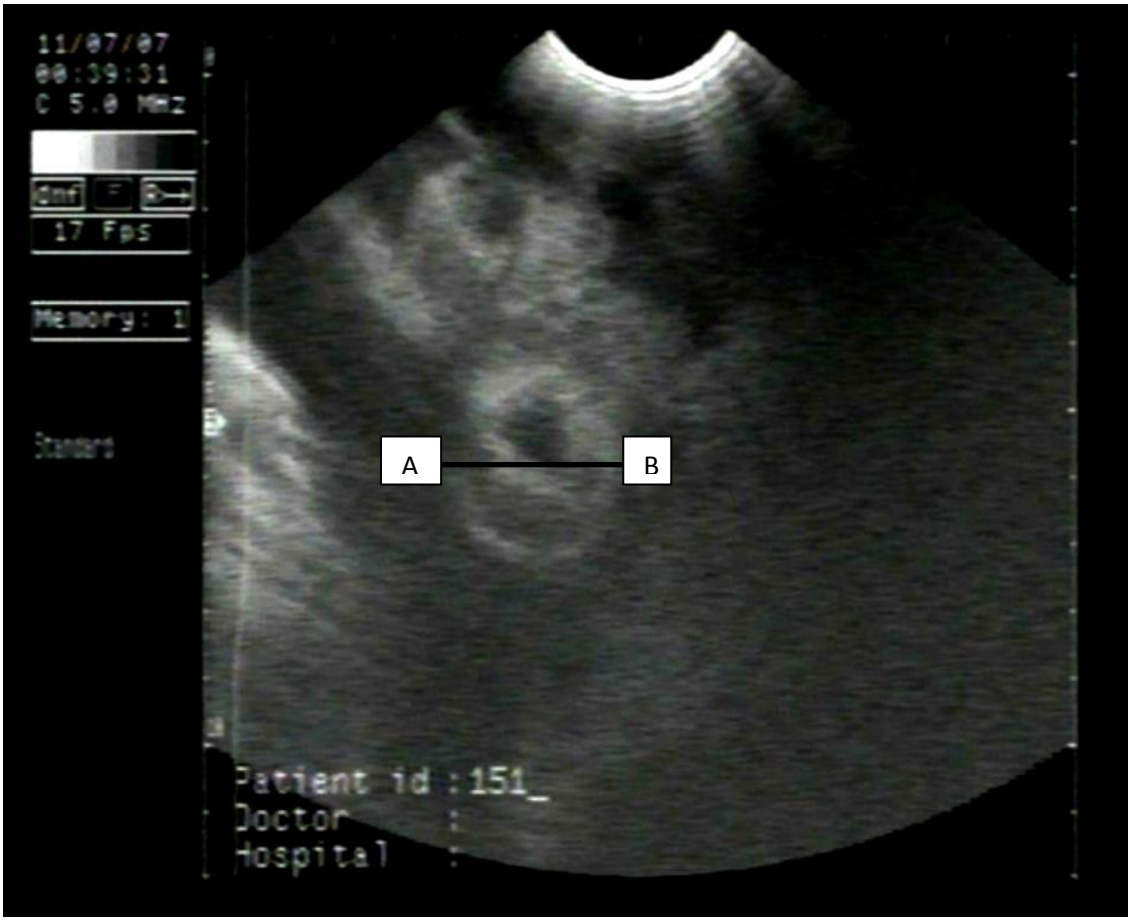

Figure 4.3. Sonographic image of placentomes on Day 110 of gestation in sheep obtained by B-mode veterinary portable ultrasound scanner equipped with 5.0 MHz sector transducer (Pie Medical 240 Parus; Pie Medical; Limburg Netherland). Line from Point A to Point B represents the diameter of the placentome.

Results

The overall pregnancy rate of the recipient ewes was 86.4%. There were no (P>0.1) differences due to breed of donor, breed of recipient or embryo transfer period on number or quality of Day 6 embryos or pregnancy rate of recipient ewes (data not shown).

Fetal head width did not differ (P>0.05) between SinC and SinS or between CinS and CinC fetuses (Table 4.1). Fetal head length in SinC fetuses was less (P<0.05) than SinS fetuses, however no difference (P>0.05) was observed between CinS and CinC fetuses.

Table 4.1. The effects of maternal uterine environment on fetal head width and head length on Day 55 of gestation in sheep (Mean ± SE). Within a column, means without a common superscript are significantly different from each other (P<0.05).

Treatment Groups	n	Fetal head width, cm	Fetal head length, cm
SinS[1] (large control)	14	1.94[ab] ± 0.06	3.16[b] ± 0.09
SinC[2] (restricted environment)	14	1.85[a] ± 0.07	2.84[a] ± 0.09
CinS[3] (luxurious environment)	14	1.97[ab] ± 0.06	2.89[a] ± 0.08
CinC[4] (small control)	12	2.06[b] ± 0.07	2.82[a] ± 0.10

[1]Suffolk in Suffolk, [2]Suffolk in Cheviot, [3]Cheviot in Suffolk, [4]Cheviot in Cheviot.

There was no difference (P>0.05) in the cross-sectional area of placentomes between SinC and SinS placentas (4.65 ± 0.44 cm^2 vs 4.23 ± 0.44 cm^2, respectively) or between CinS and CinC placentomes (5.55 ± 0.46 cm^2 vs 4.77 ± 0.50 cm^2, respectively) (Figure 4.4).

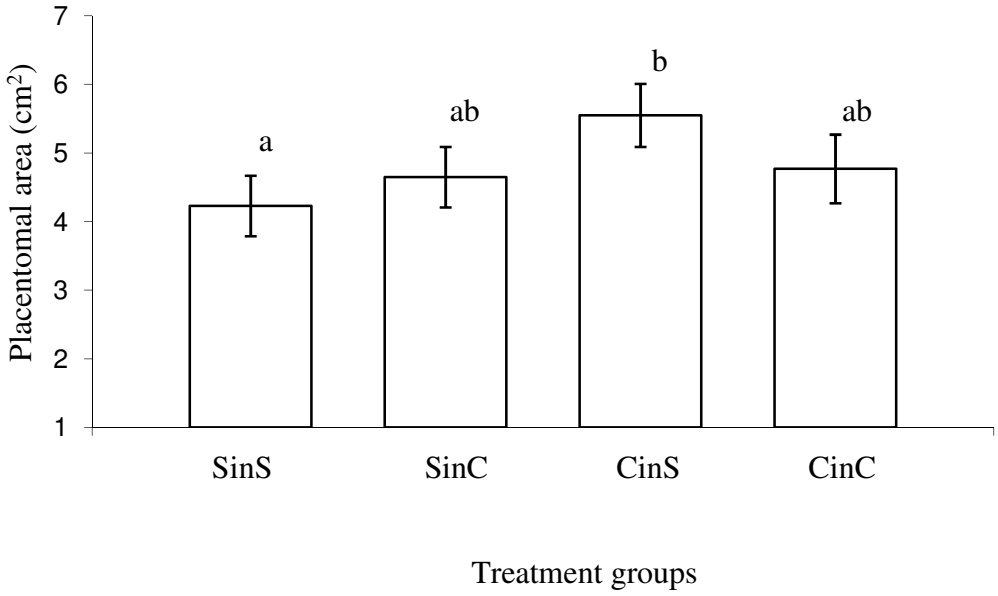

Figure 4.4. The effect of maternal uterine environment upon cross-sectional area of placentomes in different treatment groups on Day 110 of pregnancy in sheep. (Mean ± SE). Bars that are labelled without a common superscript are significantly different from each other ($P<0.05$); S: Suffolk, C: Cheviot.

Discussion

The maternal uterine environment is increasingly recognised as an important factor that regulates the development of the fetus during the antenatal period and which has long-term consequences on post-natal health and productivity. Hence, it is of interest to study the development of the fetus during gestation in different uterine environments to detect any differences in its morphology at an early stage of development.

The key result of the present study was that the fetal development in the restricted uterine environment group (SinC) was impaired compared to control animals (SinS), as shown by the smaller fetal head dimensions in SinC fetuses. These findings are akin to the results of Chapter 3 that SinC embryos were smaller than SinS embryos. These results indicate that the impairment in the development of the conceptus, which first appeared in the embryonic period in a restricted environment, also persists to Day 55 of gestation. This supports the current notion that the fetus 'senses' its *in utero* conditions as a predictor of its future nutritional environment enabling the fetus to adjust its development accordingly (Gluckman and Hanson, 2004a; Kuzawa, 2004). A few studies have also reported the development of smaller lambs when have grown in a restricted maternal environment (Cowley *et al.*, 1989; Gardner *et al.*, 2007; Gootwine *et al.*, 2007), although none of these studies measured the dimensions of the fetuses *in utero.*

It is interesting to note that there were no differences in the fetal dimensions between the luxurious (CinS) and control (CinC) groups. This is in contrast to the results in the Chapter 3, in which CinS embryos were bigger than CinC embryos on Day 19. Thus, it appears that with advancing gestation the fetuses in the luxurious uterine environment were unable to derive benefit from their privileged uterine environment. This suggests

that once the metabolic demands of the smaller genotype (Cheviot) fetuses were met; they did not exert any incremental demand on the luxurious uterine environment of the bigger genotype (Suffolk) mother. These results demonstrated intriguing differences from similar studies in horses (Giussani *et al.*, 2002; Allen *et al.*, 2004), that showed that the luxurious uterine environment increased the birthweight and size of foals. Perhaps, such differences could be due to a much larger difference in dam-size (Thoroughbred versus pony compared to Suffolk versus Cheviot) or the difference in placentation in the species (sheep versus horse) (Noakes, 2001) or perhaps even to the stage of gestation at which expansion of the extra-embryonic membranes occurs (Betteridge, 2000). Until further research unravels the mechanisms in sheep and horses, it will not be possible to explain these differences.

The cross-sectional area of the placentomes did not differ between SinC and SinS placenta on Day 110. This is at variance with the dimensions of the trophoblast in Chapter 3, where trophoblasts of SinC embryos were smaller (binucleate cell number and total PAS positive cell number lower and the area of binucleate cells tended to be smaller) than that of SinS embryos. This perhaps indicates a compensatory growth of the SinC placenta with advancing gestation, possibly coinciding with the parallel increase in the metabolic demands of a bigger genotype Suffolk fetus from a smaller genotype Cheviot mother. Moreover, the placental morphology can be modified if the fetus is developing under impaired uterine conditions (Vonnahme *et al.*, 2006) and since the rate of transfer of nutrients across the placenta increases with increases in the fetal growth (Sibley *et al.*, 1998; Sibley *et al.*, 2002). This might explain the differences in the trophoblast vs placental characteristics between SinC and SinS groups with advancing gestation. Measurements of the birthweight of such newborn lambs will show whether such compensation augmented the fetal growth of SinC fetuses.

The absence of a significant difference in the cross-sectional area of placentomes between the luxurious (CinS) and control (CinC) groups suggests there is no incremental demand form CinS fetuses. This is at variance with the findings in Chapter 3, where the trophoblasts were bigger (area of binucleate cells and height of epithelial cells was higher and number of binucleate cells tended to be higher) in CinS conceptuses compared to CinC conceptuses. This again suggests that with advancing gestation, genetically smaller Cheviot fetuses did not exert any increased incremental demand on the bigger Suffolk mother. However, it is difficult to just explain these differences based only on the cross-sectional area of placentomes. Further, placental parameters, such as weight of fetal membranes, surface area of placentomes, number of cotyledons, and weight of cotyledons and volume of placenta need to be evaluated to elucidate these differences. It was not possible to assess these parameters using sonography, requiring that future studies should collect mid-gestational or full-term placentas to enable such investigations.

The present study applied sonography as a non-invasive tool to measure fetal structures to describe the fetal development in various maternal uterine environments. Previous studies have shown that in sheep, fetal head width and head lengths are reliable measurements to estimate fetal age and growth, particularly before Days 80 or 90 of gestation (Kelly and Newnham, 1989; De Bulnes *et al.*, 1998). The use of sonography as a tool to measure fetal growth or to detect abnormalities in human pregnancies (Robinson and Fleming, 1975; Cullen *et al.*, 1990; Hotchin *et al.*, 2000) has been in clinical use over many years. However, such studies in the livestock are limited, with only a few reporting measurement of various dimensions of the fetus as indicators of its *in utero* development (Curran *et al.*, 1986; Kahn, 1989; Kaulfuss *et al.*, 1999).

Conclusion

The current findings indicate that fetal development is impaired in a restricted uterine environment, which is in accord with results obtained in the Chapter 3. These results further suggest that fetal development was not enhanced in a luxurious uterine environment suggesting an inability of genetically small fetuses to benefit from their environment with advancing gestation. The absence of differences in the cross-sectional area of placentomes between the comparable groups, (i.e SinC versus SinS and CinS versus CinC) with advancing gestation was in contrast to the differences in the trophoblast characteristics between these groups during early embryonic period (Chapter 3). This might be indicative of a role of the placenta as a mediator of these differential effects of the restricted and luxurious uterine environments. However, evaluation of other placental characteristics is required to examine mechanisms by which this might be regulated.

Chapter 5 - Maternal environment as a regulator of birthweight and body dimensions of newborn lambs

Abstract

Achieving an optimal birthweight is essential to achieve high perinatal survival and consequently to maximise productivity in livestock. Pure-breed embryos were transferred within and reciprocally between large (Suffolk: S) and small (Cheviot: C) breeds of sheep to establish four treatment groups; SinS (large control), SinC (restricted environment), CinS (luxurious environment) and CinC (small control). The recipient ewes carried single fetuses to term. The maternal plasma concentrations of ovine placental lactogen (oPL), progesterone, insulin like growth factor 1 (IGF-1), free fatty acid (FFA) and glucose were measured on Days 50, 90, 120 and 140 of pregnancy. A glucose tolerance test was undertaken on pregnant ewes on Day 120 of gestation. Birthweight, body dimensions and placental characteristics of lambs were recorded at birth. There were no differences ($P>0.05$) in oPL and progesterone concentrations between SinC and SinS ewes. The concentrations of FFA on Days 120 and 140, and that of glucose at Days 50 and 120 were higher ($P<0.05$) in SinC ewes than SinS ewes. On the other hand, CinS ewes had lower FFA concentration ($P<0.05$) at all time points than CinC ewes. The concentrations of IGF-1 on Days 50 and 90 were higher ($P<0.05$) in CinS ewes than CinC ewes, whereas the concentrations of glucose at Days 50 and 140 were lower ($P<0.05$) in CinS ewes compared to CinC ewes. SinC ewes had a higher area under curve of glucose concentrations and clearance half-time of glucose (both $P<0.05$) than SinS ewes. The birthweight of SinC lambs (5.04 ± 0.20 kg) was significantly lower than ($P<0.05$) SinS lambs (5.94 ± 0.19 kg) and body dimensions of SinC lambs were smaller ($P<0.05$) than SinS lambs. On the other hand, neither the

birthweight nor the body dimensions of CinS lambs differed ($P>0.05$) from CinC lambs. Cotyledon number was significantly ($P<0.05$) lower in CinS (57.5 ± 6.3) than SinS group (74.2 ± 5.9), whereas, mean cotyledon weight was higher ($P<0.05$) in CinS (2.42 ± 0.20 g) than SinS (1.74 ± 0.21 g). These results indicate that a restricted uterine environment reduces birthweight and body dimensions of newborn lambs, whereas a luxurious uterine environment did not enhance either of them. It appears that uterine environment regulates the development of fetus by altering the concentrations of plasma oPL, progesterone, IGF-1, FFA and glucose.

Introduction

Chapters 3 and 4 of this thesis described the differential effects of restricted and luxurious uterine environments upon the morphology of embryos and trophoblasts, and on the subsequent development of fetuses and their placentas with advancing gestation. It is of interest to find out whether these effects persist at birth. The major factors that regulate birthweight of an individual are its genotype and the environment that it experiences *in utero* (Polani, 1974; Eisen and Durrant, 1980; Bell, 2006; Gardner *et al.*, 2007). Recent epidemiological studies of human populations have indicated that the uterine environment in which a fetus develops is linked with its birthweight and lifetime health status (Barker, 1995; Godfrey and Barker, 2000; Gluckman and Hanson, 2004a). Maternal uterine capacity has a marked influence upon birthweight and size in horses (Walton and Hammond, 1938; Giussani *et al.*, 2002; Allen *et al.*, 2004), mice (Cowley *et al.*, 1989) and sheep (Gardner *et al.*, 2007; Gootwine *et al.*, 2007). However, birthweight *per se* is a relatively crude indicator of the development of a newborn (Barker, 1998), and recent epidemiological studies in humans have also indicated a relationship between stunted development or thinness at birth and subsequent post-natal health status (Martyn *et al.*, 1996; Forsen *et al.*, 1997). Hence, other body dimensions (e.g. head circumference) should be incorporated to provide a more reliable evaluation of the developmental status of an individual at birth.

As mentioned earlier, the placenta is not only a site for exchange of nutrients between mother and fetus, but it is also a metabolically active organ. It secretes various hormones, growth factors and cytokines that regulate development of feto-placental unit and alters maternal physiology to support adequate fetal growth (Gootwine, 2004). Placental characteristics such as placentomal type, gross area of allantochorion, microscopic area of feto-maternal contact, mass of allantochorion and chorionic volume

have been shown associated with fetal growth, when the fetus develops in an impaired uterine environment (Giussani *et al.*, 2002; Wilsher and Allen, 2002; Vonnahme *et al.*, 2006).

Various humoral and metabolic factors have also been implicated in the feto-maternal dialogue. Placental lactogen regulates fetal growth by influencing maternal and fetal metabolism, acting as a partitioning agent to regulate nutrient supply to the fetus (Anthony *et al.*, 1995a; Anthony *et al.*, 1995b) and stimulating the secretion of insulin-like growth factors (Handwerger and Freemark, 2000). Progesterone is required for normal maintenance of pregnancy (Bindon, 1971) and for providing trophic support to the placenta and fetus (Ogle *et al.*, 1990; Kleeman *et al.*, 1994; Mark *et al.*, 2006). Maternal IGF-1 has fetal growth promoting action in sheep and mice (Blair *et al.*, 1988; Gluckman *et al.*, 1992; Morel *et al.*, 1994); and in the human, plasma IGF-1 concentration is related to size at birth (Fail *et al.*, 1995).

Glucose is the main source of energy for the fetus and the placenta (Hay and Sparks, 1985; Boden, 1996; Scholl *et al.*, 2001). Maternal plasma glucose concentration is directly related to fetal glucose concentration which consequently regulates secretion of fetal IGF-1 such that a high fetal glucose concentration stimulates the production of IGF-1 and *vice versa* (Oliver *et al.*, 1993; Gluckman and Harding, 1997). The efficiency of glucose metabolism of the mother has also been shown to influence the weight of both placenta and fetus and body dimensions of the newborn, whilst impaired maternal glucose metabolism is associated with various adverse neonatal outcomes (Lao *et al.*, 1997; Bo *et al.*, 2004; Lampl and Jeanty, 2004). With advancing gestation, fetal demands for glucose increases. Therefore, to spare more glucose for the fetus, the pregnant ewe mobilises its body fat reserves, resulting in an increased concentration of FFA in maternal circulation (Herrera, 2002). This increase in the plasma FFA

concentration also inhibits insulin-dependent glucose uptake by body cells (Sivan *et al.*, 1997), which further spares more glucose for fetal development. Hence, the maternal plasma FFA concentration is a potentially useful indicator of the effectiveness of the feto-maternal dialogue. The aforegoing studies indicate that the maternal uterine environment is a key factor that regulates birthweight and fetal size at birth. Various factors such as placenta, maternal glucose metabolism, and maternal plasma concentrations of oPL, progesterone, IGF-1, glucose and FFA are implicated in the feto-maternal dialogue. However, few studies of livestock have described the effects of uterine environment upon neonatal development, and no studies have investigated the mechanism by which the uterine environment regulates birthweight and birth-size in a dam-size model (within and reciprocal transfer of purebred embryos between large and small size dams) in sheep.

The present study was undertaken to investigate the effects of maternal uterine environment upon birthweight and body dimensions of newborn lambs and to understand the role of the placenta and various humoral and metabolic factors in feto-maternal dialogue in different maternal environments in sheep.

Results of this study (birthweight, body dimensions of newborn lambs and placental characteristics) have been reported in part elsewhere (Sharma *et al.*, 2009).

Materials and Methods

Experimental design and animals

Purebred single embryos were transferred within and reciprocally between large Suffolk (S) and small Cheviot (C) breeds of sheep to establish four groups of pregnancies viz. Suffolk in Suffolk (SinS; large control), Suffolk in Cheviot (SinC; restricted environment), Cheviot in Suffolk (CinS; luxurious environment) and Cheviot in Cheviot

(CinC; small control), as described in Chapter 3. In the present experiment, the recipient ewes carried a single fetus to term.

A total of 13 Suffolk and 13 Cheviot donors were used to transfer embryos to 91 Suffolk and 85 Cheviot recipients, both within and reciprocally between the breeds. The recipient ewes were of mixed ages (3 to 6 years old) and were randomly divided into two groups; so that one group was implanted with the embryos of its own breed and the other group with embryos of the opposite breed. Thus, a total of 49 in SinS, 47 in SinC, 42 in CinS and 38 in CinC single embryos were transferred. The recipient ewes were balanced for age and condition score within the treatment groups. A total of 33 SinS, 29 SinC, 24 CinS and 24 CinC single bearing pregnancies were maintained to full term. To achieve the required number of pregnancies in each group, two successive synchronisation, superovulation, and embryo transfer sessions were undertaken 37 days apart, such that the recipient ewes that failed to conceive their first embryo implantation (return to first oestrus based on the detection by vasectomised rams) were re-used as recipients in the second embryo transfer session.

The pregnant ewes from all treatment groups in both the embryo transfer sessions were maintained together as one flock. All the animals used in the experiment were managed at Keeble Farm, Massey University, New Zealand under standard lowland commercial farming conditions. Animal were provided with generous allowance of ryegrass (*Lolium L.*) and white clover (*Trifolium repens L.*) pastures and had *ad libitum* access to water.

Metabolic studies of pregnant ewes

Sampling throughout pregnancy

Blood samples (5 mL; Lithium heparin anticoagulant) were collected from each pregnant ewe by jugular venipuncture into heparinized (BD Vacutainer Systems, UK) vials on Days 50, 90, 120 and 140 of gestation. After collection, blood samples were immediately centrifuged at 1000 g (3000 rpm, Heraeus Labofuge 200 centrifuge; Heraeus, Buckinghamshire, UK) for 15 min to separate plasma. Plasma samples were stored at -20^{0}C for later measurement of concentrations of oPL, progesterone, IGF-1, glucose and FFA.

Glucose challenge

A glucose tolerance test was undertaken on Day 120 of gestation using 8 pregnant ewes per treatment group, randomly selected from the first embryo transfer session. On the day before the glucose challenge, both jugular veins were catheterized with indwelling polyvinyl catheters by threading through a 12-gauge needle after infiltration of local anaesthetic (Nopamine, Lignocaine Hydrochloride U.S.P. 20 ng/mL, Ethical Agents Ltd, Auckland, New Zealand). The catheters were secured to the neck with a tape and placed on the back of the animal under a meshed stocking. One catheter was used for glucose administration and the other catheter was used for blood collection. Ewes were fasted overnight (12 h period), but had access to *ad libitum* water. Glucose-D (Dextrose 40%, Bomac Laboratories Ltd, Auckland, New Zealand) was administered intravenously at the rate of 0.17 g/kg live weight. Blood samples (5 mL; Lithium heparin anticoagulant) on ice were taken from each ewe in heparinised vacutainers (BD Vacutainer Systems, UK) at -5, 0, +5, +10, +20, +30 and +60 min in respect to glucose administration. After completion of sampling, the catheters were removed and the

animals were returned to pasture. Blood samples were immediately centrifuged following collection at 1000g (3000 rpm, Heraeus Labofuge 200 centrifuge; Heraeus, Buckinghamshire, UK) for 15 min at 4^0C and the plasma was stored at -20^0C.

Insulin and glucose concentrations from the glucose challenge were measured in all of the plasma samples, whereas cortisol was measured in -5 min plasma sample to estimate the basal cortisol levels.

In the present study, a glucose tolerance test was preferred over a euglycemic-hyperinsulinemic clamp approach, as the later is a very cumbersome procedure and therefore could render more stress to the pregnant ewes compared to a glucose tolerance test. Adopting more stressful procedures might increase the secretion of cortisol, which itself interferes with the secretion and action of insulin (Holmang and Bjorntorp, 1992; Lambillotte *et al.*, 1997). Thus, to minimise the stress on the dams and to avoid introducing variation in the results, the euglycemic- hyperinsulinemic clamp approach was not used.

Measurement of oPL, Progesterone, IGF-1, glucose and FFA concentrations

Ovine placental lactogen concentrations in the plasma were measured in duplicate by radioimmunoassay (RIA), using a monoclonal antibody, which had been established and validated for maternal and fetal sheep plasma (Oliver *et al.*, 1992). The limit of sensitivity, as determined by twice the standard deviation of blank values (representing the least amount of hormone which could cause a significant displacement of radiolabelled hormone from the antibody), was 0.2 ng/mL. The intra- and inter-assay coefficients of variation were 5.23% and 11.7%, respectively.

Progesterone concentration in plasma was measured in duplicate via RIA (^{125}I-progesterone and antiserum ImmuChem^{TM} Double Antibody Progesterone ^{125}I RIA kit

for *in vitro* diagnostic use, MP Biomedicals, USA). The limit of sensitivity was 0.0025 ng/mL. The intra-assay coefficients of variation for progesterone were 16.1% (n = 10), 8.4% (n = 15) and 9.9% (n = 10) respectively for solutions containing low, medium and high concentrations of the steroid. Inter-assay coefficients of variation were calculated from duplicates of the solutions included at the beginning and end of each assay. The inter-assay coefficients of variation for fourteen assays were 16.5%, 14.6% and 18.6% for low, medium and high solutions, respectively.

The concentration of IGF-1 was measured in duplicate using an IGFBP-blocked RIA previously established and validated (Blum and Brier, 1994; Vickers *et al.*, 1999)**.** The limit of sensitivity was 0.7 ng/mL and the intra- and inter-assay coefficients of variation were 5.24% and 12.2%, respectively.

Insulin concentrations were measured in duplicate 20 µL aliquots of plasma by radioimmunoassay with iodinated insulin as the tracer (I^{125} labelled insulin in a protein based buffer with sodium azide as preservative, Diagnostic Systems Laboratories Inc., USA). The limit of sensitivity was 0.98 µIU/mL; intra- and inter-assay coefficients of variation were 11.9% and 14.7%, respectively (n=4). The standard insulin antibody had 100% cross reactivity with human insulin, 99% with porcine insulin, but less than 0.1% with glucagon, somatostatin, pancreatic polypeptide and IGF-1.

Cortisol concentrations were measured in duplicate 25 µL aliquots of plasma by RIA. The assay used iodinated (^{125}I) cortisol prepared in human serum matrix with sodium azide and gentamicin sulphate as preservative as the tracer (ImmuChem^{TM} Coated Tube cortisol ^{125}I RIA kit for *in vitro* diagnostic use, MP Biomedicals, USA). The cross reactivity of the antibody was 100% with cortisol, 45.6% with prednisolone, 5.5% with corticosterone, 2.7% with prednisone, 25% with cortisone and less than 0.1% with progesterone, dexamethasone, dihydrotestosterone and testosterone. The limit of

sensitivity was 0.42µg/dL, and the intra-assay coefficients of variation were 23.5%, 14.5% and 12.1% for low, medium and high solutions respectively (n=1).

Concentrations of glucose and FFA were measured in duplicate using a Hitachi 902 autoanalyser (Hitachi High Technologies Corporation, Tokyo, Japan) by enzymatic colorimetric assay, using commercial kits (glucose kits from Roche, Mannheim, Germany and FFA kits from Randox Laboratories Ltd, Ardmore, Crumlin, UK).

Studies of newborn lambs

Lambs

The recipient ewes were allowed to lamb spontaneously in separate but contiguous paddocks. Each lamb was weighed within 12 to 18 h of birth. Head width, head length, crown-rump length (CRL), heart girth circumference, foreleg length, hind leg length and femur length of the lambs were measured at the same time using a standard flexible measuring tape. Head length was defined as the distance between the tip of nose and the central point of the caudal margins of attachment of both ears (occipital). Head width was defined as the distance between the lateral canthus of one eye to the lateral canthus of the other eye passing through the crown. Crown-rump length was measured from the crown to the base of tail passing through loin and rump. Heart girth circumference was measured parallel to the 4^{th} rib passing through caudal proximity of scapulae. Foreleg length was defined as the straight distance between the caudal aspect of the scapula to the hoof. Hind leg length was the straight distance between the greater trochanter of the femur and the hoof. Femur length was the straight length of the femur from its head to condyles.

Body mass index of newborn lamb was calculated by dividing the birthweight of a lamb by the square root of its crown-rump length. The ratio between the birthweight and head

width of a lamb was also calculated. The birthweight to head circumference is used in human epidemiological studies (Martyn *et al.*, 1996) to understand the effects of impaired antenatal environments upon the growth of head or to find out the pattern of fetal growth such as 'brain sparing'. Hence, birthweight to head width ratio was calculated in the present study to assess where any such effects occurred in sheep.

Placentas

Placentas were collected in clean plastic bags within 6 to 12 h of lambing. After dissection, fetal membranes weight (with and without cotyledons), number of cotyledons and total weight of cotyledons were measured. Mean cotyledon weight was calculated by dividing the weight of cotyledons for each placenta by the total number of cotyledons in that placenta.

Ethical approval

All experimental procedures were carried out with the approval of Massey University Animal Ethics Committee.

Analysis of data

Glucose tolerance test

Basal glucose concentration was calculated as the average of glucose concentrations at -5 and 0 min prior to glucose infusion. The concentration of glucose at each subsequent time of sample collection was defined as adjusted glucose concentration and was calculated by subtracting the basal glucose concentration from the glucose concentration at a given time point. The peak glucose concentration was defined as its highest concentration in plasma after its administration, and the time at which peak concentration was reached was recorded as time to peak glucose. Glucose tolerance was measured as the area under glucose curve between the basal glucose concentration and

the concentration of +60 min sample. The logarithmic decay rate of glucose was calculated between the peak concentration of glucose to the +60 min sample by using Graph Pad Prism 4 software (Graph Pad, California, USA) and was defined as the difference between the adjusted peak glucose concentration and the adjusted glucose concentration of the last sample, divided by the difference between the time at which peak glucose concentration reached and the time at which last blood sample was collected. The clearance half-time of glucose was defined as the time in which the adjusted concentration of glucose remains half of its peak concentration in the circulation and was calculated by using the following equation:

Clearance half-time (min): Glucose = (0.693 /logarithmic decay rate of glucose) X 100 (Kaneko, 1997).

Basal insulin concentration was calculated as the average of insulin concentrations at -5 and 0 min prior to glucose infusion. The concentration of insulin at each subsequent time of sample collection was defined as the adjusted insulin concentration and was calculated by subtracting the basal insulin concentration from the insulin concentration at a given time point. Insulin secretion was estimated using Graph Pad Prism 4 software as the area under the plasma insulin curve in response to glucose challenge between the basal insulin concentration and the concentration of +60 min sample. The highest concentration of insulin was defined as peak insulin concentration and the time at which the peak insulin was reached was recorded as time to peak insulin secretion. The ratios between the basal glucose concentration and basal insulin concentration and between the area under curve of glucose concentrations and the area under curve of insulin concentrations were also calculated.

Statistical analysis

Data were subjected to analysis of variance with respect to breed of recipient ewes, age of recipients, breed of lamb, sex of lamb and embryo transfer session. Hormone and metabolite data were also analysed using repeat measures of analysis of variance. However, this procedure did not allow the inclusion of all data values, as several ewes were not blood sampled at every time point. Nonetheless, this test was performed to determine whether it added any additional information to the results such as the differences in concentrations of these hormones and metabolites between the treatment groups, with respect to time and age. Given the similarity of results, repeat measures of analysis are not represented in the Results but are shown in Appendix 1. Regression analysis of hormones and metabolites upon birthweight or CRL was performed. Residual correlations between the hormones and metabolites that had significant regression coefficients with either birthweight or crown-rump length was also calculated. All the statistical procedures were performed using SAS statistical package (SAS, 2005; SAS 9.1.3, SAS Institute, North Carolina, USA). Three-way or four-way interactions were not included in the model as the number of ewes in these subcells did not enable a valid test. All other interactions and effects were included in the initial model, but removed if found non-significant ($P>0.05$) and model was then re-fitted. The interaction between the breed of ewe and breed of lamb was always kept in the model irrespective of whether it was significant ($P<0.05$) or not ($P>0.05$), since this interaction defines the different maternal environments. Post-hoc differences between groups were detected using least significant differences.

Results

The overall pregnancy rate was 74.4%. There were no differences (all $P>0.1$) in the pregnancy rate, the number or quality of Day 6 embryos or sex ratio of lambs due to

breed of donor, breed of recipient, age of recipient or embryo transfer period (i.e. first vs second) between groups (data not shown). All ewes lambed at full term after more than 144 days of gestation (data not shown).

Ewe hormone and metabolite measures in pregnancy

Plasma oPL concentration

Between SinC and SinS ewes, concentration of oPL did not differ (P>0.05) either on Days 90 or 120. On the other hand, on Day 120, oPL concentration in CinS ewes tended (P=0.06) to be lower than CinC ewes (Table 5.1).

Plasma progesterone concentration

Plasma progesterone concentrations did not differ (P>0.05) between SinC and SinS or between CinS and CinC ewes on either Days 50, 90, 120 or 140 of gestation (Table 5.2), although, there was a tendency (P=0.06) for CinS ewes to have lower progesterone than CinC ewes on Day 120.

Plasma IGF-1 concentration

Plasma IGF-1 concentrations did not differ (P<0.05) between SinC and SinS ewes on Days 50, 90, 120 or 140 of gestation (Table 5.3), although SinC ewes tended to have lower concentration than SinS ewes on Day 50 (P=0.09), Day 120 (P=0.07) and Day 140 (P=0.07), however there was no difference (P>0.05) on Day 90. On the other hand, plasma IGF-1 concentration in CinS ewes were higher (P<0.05) than CinC ewes on Days 50 and 90, however there were no differences (P>0.05) on Days 120 and 140.

Plasma glucose concentration

SinC ewes had higher ($P<0.05$) concentrations of plasma glucose than SinS ewes on Days 50 and 120 of gestation, but there were no differences ($P>0.05$) on Days 90 and 140 (Table 5.4). On the other hand, CinS ewes had lower ($P<0.05$) plasma glucose concentrations on Days 50 and 140; however, no differences ($P>0.05$) could be observed on Days 90 and 120 between these two groups.

Plasma FFA concentration

Between SinC and SinS ewes, no difference ($P>0.05$) could be observed on Days 50 and 90 in respect of FFA concentrations (Table 5.5). However, on Days 120 and 140, SinC ewes had higher ($P<0.05$) plasma FFA than SinS ewes. On the other hand, CinS ewes had lower ($P<0.05$) FFA concentrations than CinC ewes on Days 50, 90, 120 and 140 of gestation.

Table 5.1. Plasma oPL[1] concentration in different uterine environments at various stages of gestation in pregnant ewes (ANOVA; Mean ± SE). Within a column, means without a common superscript are different from each other ($P<0.05$).

Treatment groups	n	Day 90 oPL, ng/mL	n	Day 120 oPL, ng/mL
SinS[2] (large control)	17	32.61[b] ± 3.45	17	109.77 ± 13.55
SinC[3] (restricted environment)	19	30.14[ab] ± 3.18	15	102.40 ± 14.43
CinS[4] (luxurious environment)	18	21.66[ab] ± 3.38	16	102.64 ± 13.99
CinC[5] (small control)	15	22.73[a] ± 3.54	16	141.29 ± 13.99

[1] Ovine placental lactogen, [2]Suffolk in Suffolk, [3]Suffolk in Cheviot, [4]Cheviot in Suffolk, [5]Cheviot in Cheviot.

Table 5.2. Plasma progesterone concentration in different uterine environments at various stages of gestation in pregnant ewes (ANOVA; Mean ± SE). Within a column, means without a common superscript are different from each other (P<0.05).

Treatment groups	n	Day 50 progesterone, ng/mL	n	Day 90 progesterone, ng/mL	n	Day 120 progesterone, ng/mL	n	Day 140 progesterone, ng/mL
SinS[1] (large control)	21	3.76 ± 0.26	19	4.54 ± 0.31	17	8.75[a] ± 0.84	22	7.99[ab] ± 0.93
SinC[2] (restricted environment)	19	3.59 ± 0.28	16	5.20 ± 0.36	15	10.56[ab] ± 0.90	21	10.14[b] ± 0.95
CinS[3] (luxurious environment)	21	3.49 ± 0.27	16	4.59 ± 0.37	13	9.88[ab] ± 0.96	22	7.36[a] ± 0.93
CinC[4] (small control)	17	3.45 ± 0.29	13	4.93 ± 0.39	16	12.43[b] ± 0.87	18	9.39[ab] ± 1.02

[1]Suffolk in Suffolk, [2]Suffolk in Cheviot, [3]Cheviot in Suffolk, [4]Cheviot in Cheviot.

Table 5.3. Plasma IGF-1[1] concentration in different uterine environments at various stages of gestation in pregnant ewes (ANOVA; Mean ± SE). Within a column, means without a common superscript are different from each other (P<0.05).

Treatment groups	n	Day 50 IGF-1, ng/mL	n	Day 90 IGF-1, ng/mL	n	Day 120 IGF-1, ng/mL	n	Day 140 IGF-1, ng/mL
SinS[2] (large control)	21	60.45[ab] ± 2.59	17	66.76[bc] ± 3.95	17	83.00 ± 4.41	21	85.41 ± 4.95
SinC[3] (restricted environment)	19	53.96[a] ± 2.72	19	58.88[ab] ± 3.63	15	70.99 ± 4.69	21	72.42 ± 4.95
CinS[4] (luxurious environment)	21	64.23[b] ± 2.64	18	73.14[c] ± 3.87	15	78.28 ± 4.55	22	81.05 ± 4.89
CinC[5] (small control)	17	53.47[a] ± 2.88	15	54.74[a] ± 4.04	16	76.23 ± 4.55	18	76.76 ± 5.33

[1]Insulin-like growth factor-1, [2]Suffolk in Suffolk, [3]Suffolk in Cheviot, [4]Cheviot in Suffolk, [5]Cheviot in Cheviot.

Table 5.4. Plasma glucose concentration in different uterine environments at various stages of gestation in pregnant ewes (ANOVA; Mean ± SE). Within a column, means without a common superscript are different from each other (P<0.05).

Treatment groups	n	Day 50 glucose, mmol/L	n	Day 90 glucose, mmol/L	n	Day 120 glucose, mmol/L	n	Day 140 glucose, mmol/L
SinS[1] (large control)	20	3.76[a] ± 0.12	17	3.81 ± 0.16	17	3.68[a] ± 0.20	22	3.67[a] ± 0.17
SinC[2] (restricted environment)	19	4.14[b] ± 0.13	19	4.08 ± 0.15	16	4.36[b] ± 0.21	21	3.87[ab] ± 0.17
CinS[3] (luxurious environment)	20	3.54[a] ± 0.13	18	3.77 ± 0.15	15	3.47[a] ± 0.21	21	3.44[a] ± 0.17
CinC[4] (small control)	17	4.32[b] ± 0.13	14	3.98 ± 0.16	16	3.91[ab] ± 0.21	17	4.17[b] ± 0.19

[1]Suffolk in Suffolk, [2]Suffolk in Cheviot, [3]Cheviot in Suffolk, [4]Cheviot in Cheviot.

Table 5.5. Plasma FFA[1] concentration in different uterine environments at various stages of gestation in pregnant ewes (ANOVA; Mean ± SE). Within a column, means without a common superscript are different from each other (P<0.05).

Treatment groups	n	Day 50 FFA, mmol/L	n	Day 90 FFA, mmol/L	n	Day 120 FFA, mmol/L	n	Day 140 FFA, mmol/L
SinS[2] (large control)	21	0.42^{ab} ± 0.04	16	0.46^{ab} ± 0.07	16	0.65^{a} ± 0.07	21	0.75^{a} ± 0.06
SinC[3] (restricted environment)	18	0.45^{bc} ± 0.04	18	0.60^{bc} ± 0.06	15	0.90^{b} ± 0.07	21	1.15^{b} ± 0.06
CinS[4] (luxurious environment)	20	0.31^{a} ± 0.04	17	0.41^{a} ± 0.06	16	0.55^{a} ± 0.07	21	0.65^{a} ± 0.06
CinC[5] (small control)	16	0.54^{c} ± 0.04	15	0.70^{c} ± 0.07	16	0.95^{b} ± 0.07	18	1.09^{b} ± 0.07

[1]Free fatty acid, [2]Suffolk in Suffolk, [3]Suffolk in Cheviot, [4]Cheviot in Suffolk, [5]Cheviot in Cheviot.

Hormone and metabolites regression upon birthweight or crown-rump length

Simple regression analysis of measured hormones and metabolites on birthweight showed the following significant effects.

Birthweight = (7.03 -0.0377 x Day 90 oPL) kg; P=0.03

Birthweight = (5.36 + 0.0047 x Day 120 oPL) kg; P= 0.04

Birthweight = (4.68 + 0.1407 x Day 140 progesterone) kg; P<0.001

Birthweight = (5.35kg + 0.7852 x Day 120 FFA) kg; P=0.007

The residual correlation coefficients between the hormones and metabolites are shown in Table 5.6.

Similarly, simple regression analysis of measured hormones and metabolites on crown-rump length showed the following significant effects.

CRL = (62.57 - 0.1157 x Day 90 oPL) cm; P=0.006

CRL = (56.55 + 0.0191 x Day 120 oPL) cm; P=0.05

CRL = (54.57 + 0.4608 x Day 120 progesterone) cm; P=0.0003

CRL = (56.39 + (3.40 x Day 120 FFA) cm; P=0.005

CRL = (56.00 + 4.1467 x Day 140 FFA) cm; P= 0.02

CRL = (62.56 - 1.0685 x Day 120 glucose) cm; P=0.03

The residual correlation coefficients between the hormones and metabolites are shown in Table 5.7.

Table 5.6. Residual correlation coefficients[1] with their significance values of hormones and metabolites that were individually significant (P<0.05) upon regression analysis with birthweight in different maternal environments in sheep.

	Day 120 oPL[2]	Day 140 progesterone	Day 120 FFA[3]
Day 90 oPL	0.35	-0.21	-0.18
Significance value	0.01	0.09	0.22
Day 120 oPL		0.30	0.05
Significance value		0.01	0.68
Day 140 progesterone			0.28
Significance value			0.03

[1] After adjustment for breed of ewe, breed of lamb and breedewe.breedlamb, [2] Ovine placental lactogen, [3] Free fatty acid.

Table 5.7. Residual correlation coefficients[1] with their significance values of hormones and metabolites that were individually significant (P<0.05) upon regression analysis with crown rump length in different maternal environments in sheep.

	Day 120 oPL[2]	Day 120 progesterone	Day 120 FFA[3]	Day 140 FFA	Day 120 glucose
Day 90oPL	0.35	-0.06	-0.18	-0.40	-0.20
Significance value	0.01	0.70	0.23	0.0007	0.17
Day 120 oPL		0.47	0.05	0.06	-0.28
Significance value		0.0001	0.68	0.62	0.02
Day 120 progesterone			0.45	0.40	-0.25
Significance value			0.0003	0.002	0.05
Day 120 FFA				0.40	-0.05
Significance value				0.001	0.71
Day 140 FFA					-0.009
Significance value					0.95

[1]After adjustment for breed of ewe, breed of lamb and breedewe.breedlamb, [2]Ovine placental lactogen, [3] Free fatty acid.

Metabolic measures of glucose and insulin in response to glucose challenge in pregnant ewes

The area under the curve of glucose concentrations was significantly ($P<0.05$) greater in SinC ewes than in SinS ewes (Table 5.8; Figure 5.1). Similar differences ($P<0.05$) were also observed in respect of the clearance half-time of glucose between these two groups. However, none of the other measures of glucose metabolism differed ($P>0.05$) between SinC and SinS or between CinS and CinC treatment groups.

The ratio of area under the curve for glucose concentration to area under the curve for insulin concentration was higher ($P<0.05$) in SinC ewes than in SinS ewes (Table 5.9). However, no differences ($P>0.05$) were observed for any other metabolic measures of insulin between either of the treatment groups. The area under the curve of insulin concentrations is shown in Figure 5.2.

Basal cortisol concentrations (Table 5.9) were significantly ($P<0.05$) lower in CinS than in CinC ewes (1.52 ± 0.68 µg/dL vs 3.65 ± 0.68 µg/dL, respectively) but did not differ ($P>0.05$) between SinC and SinS ewes.

Table 5.8. Plasma glucose measures in different maternal environments following a glucose challenge on Day 120 of gestation in pregnant ewes (Mean ± SE). Within a column, means without a common superscript are different from each other (P<0.05).

Treatment groups	n	Basal glucose concentration, mmol/L	Area under curve of glucose concentrations, min mmol/L	Logarithmic decay rate of glucose	Clearance half-time glucose, min	Peak glucose concentration, mmol/L	Time to peak glucose, min
SinS[1] (large control)	8	2.71[ab] ± 0.21	191.92[b] ± 7.29	0.022 ± 0.003	32.27[a] ± 3.98	5.63 ± 0.16	5
SinC[2] (restricted environment)	8	3.18[b] ± 0.21	216.16[a] ± 7.29	0.017 ± 0.004	45.49[b] ± 3.98	5.68 ± 0.16	5
CinS[3] (luxurious environment)	8	2.94[ab] ± 0.21	210.76[ab] ± 7.29	0.019 ± 0.004	37.45[ab] ± 3.98	5.80 ± 0.16	5
CinC[4] (small control)	8	2.56[a] ± 0.21	196.77[ab] ± 7.29	0.024 ± 0.004	35.54[ab] ± 3.98	5.44 ± 0.16	5

[1]Suffolk in Suffolk, [2]Suffolk in Cheviot, [3]Cheviot in Suffolk, [4]Cheviot in Cheviot.

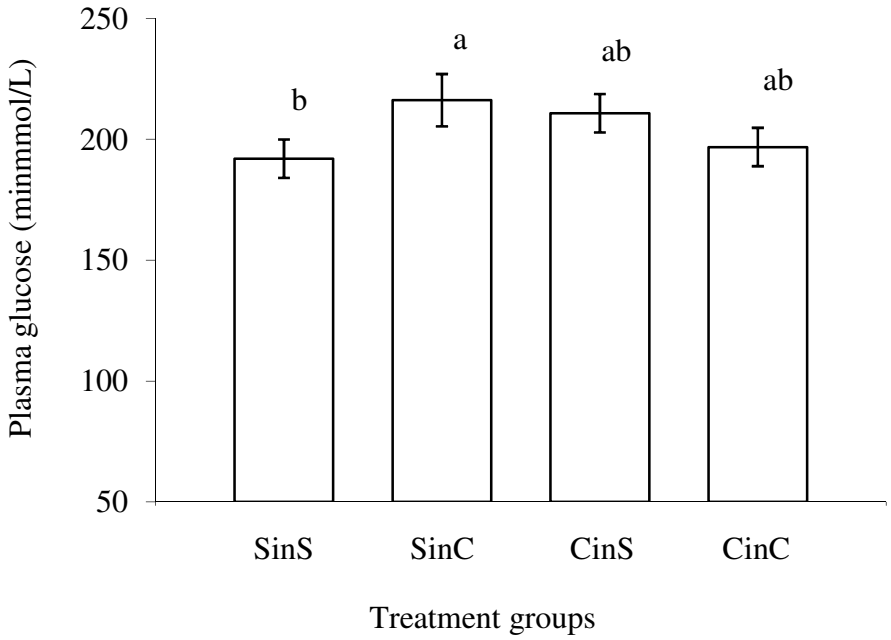

Figure 5.1. Area under curve of glucose concentrations in different maternal environments following glucose challenge on Day 120 of gestation in ewes (Mean ± SE). Bars without a common superscript are different from each other ($P<0.05$). S: Suffolk, C: Cheviot.

Table 5.9. Plasma insulin and other metabolic measures in different maternal environments following a glucose challenge on Day 120 of gestation in pregnant ewes (Mean ± SE). Within a column, means without a common superscript are different from each other (P<0.05).

Treatment groups	n	Basal insulin concentration, µIU/L	Area under curve of insulin concentrations, min µIU/L	Peak insulin concentration, µIU/L	Time to peak insulin, min	Basal glucose to basal insulin concentration ratio	Area under glucose to area under curve insulin concentrations ratio	Basal cortisol concentration, µg/dL
SinS[1] (large control)	8	8.65 ± 0.74	354.17 ± 52.51	9.33 ± 1.45	11.25 ± 1.67	0.41 ± 0.03	0.62[a] ± 0.16	1.65[b] ± 0.68
SinC[2] (restricted environment)	8	7.24 ± 0.74	246.31 ± 52.51	6.97 ± 1.45	12.50 ± 1.67	0.45 ± 0.03	1.15[b] ± 0.16	2.84[ab] ± 0.68
CinS[3] (luxurious environment)	8	7.43 ± 0.74	303.97 ± 52.51	7.22 ± 1.45	10 ± 1.67	0.42 ± 0.03	0.79[ab] ± 0.16	1.52[b] ± 0.68
CinC[4] (small control)	8	6.25 ± 0.74	210.63 ± 52.51	5.89 ± 1.45	13.75 ± 1.67	0.42 ± 0.03	1.11[b] ± 0.16	3.65[a] ± 0.68

[1]Suffolk in Suffolk, [2]Suffolk in Cheviot, [3]Cheviot in Suffolk, [4]Cheviot in Cheviot.

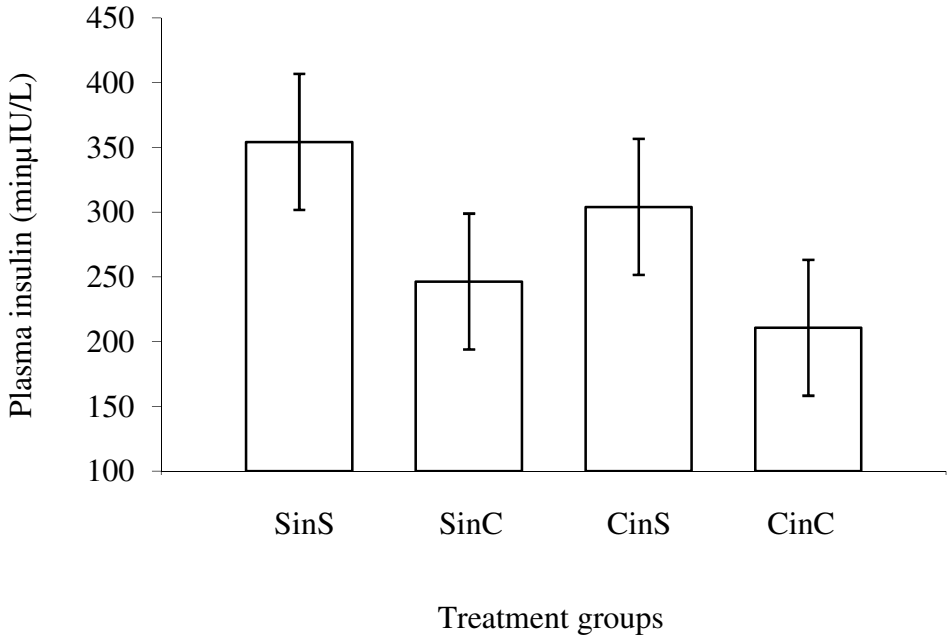

Figure 5.2. Area under curve of insulin concentrations in different maternal environments following glucose challenge on Day 120 of gestation in ewes (Mean ± SE). Differences between the treatment groups are non-significant (P>0.05). S: Suffolk, C: Cheviot.

Newborn lambs

The proportion of male and female lambs born was similar within and between groups. Male lambs were heavier ($P<0.05$) than female lambs (5.67 ± 0.15 kg vs 5.16 ± 0.4 kg, respectively) regardless of the treatment group. Males lambs were also larger ($P<0.05$) than females lambs regardless of ewe group (data not shown).

SinC lambs were lighter ($P<0.05$) than SinS lambs (5.04 ± 0.20 vs 5.94 ± 0.19 kg, respectively; Figures 5.3 and 5.4). All of the body dimensions of SinC lambs were smaller ($P<0.05$) than SinS lambs. Birthweight to head width ratio and the body mass index of SinC lambs were less ($P<0.05$) than in SinS lambs (Table 5.10).

Conversely, the birthweight of CinS lambs (5.54 ± 0.23 kg) did not differ significantly ($P>0.05$) from that of CinC lambs (5.16 ± 0.22 kg) (Figures 5.3 and 5.5). No differences ($P>0.05$) were observed in the body dimensions, ratio of birthweight to head width or body mass index between CinS and CinC lambs (Table 5.10).

Placental parameters

There were significantly ($P<0.05$) fewer cotyledons (Figure 5.6) in CinS and CinC placentas (57.5 ± 6.3 and 55.4 ± 4.6, respectively) than in SinS placentas (74.2 ± 5.9), whilst SinC (64.9 ± 4.6) occupied an intermediate position. The mean cotyledon weight was significantly ($P<0.05$) greater in CinS than in SinS placentas; however, none of the other placental parameters differed ($P>0.05$) between any of the groups (Table 5.11).

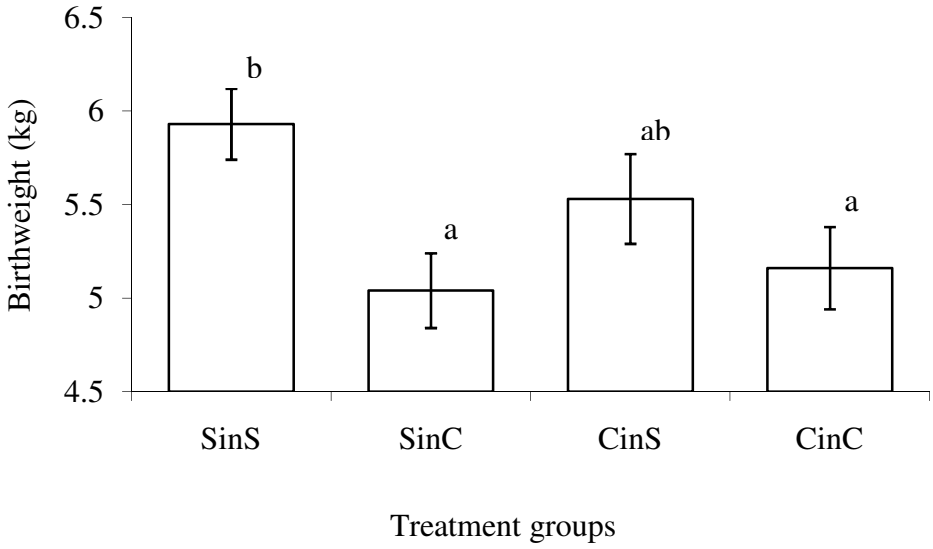

Figure 5.3. The effect of maternal uterine environment upon birthweight of newborn lambs (Mean ± SE). Bars that are labelled with different superscripts are significantly different from each other ($P<0.05$). S: Suffolk, C: Cheviot.

Table 5.10. The effects of maternal uterine environment upon body dimensions and body characteristics of newborn lambs (Mean ± SE). Within a column, means without a common superscript are different from each other (P<0.05).

Treatment groups	n	Head width, cm	Head length, cm	Crown-rump length, cm	Heart girth, cm	Foreleg length, cm	Hind leg length, cm	Femur length, cm	Birthweight to head width ratio	Body mass index, kg/m^2
SinS[1] (large control)	31	12.87^{b} ± 0.15	17.62^{b} ± 0.22	59.25^{b} ± 0.76	42.08^{b} ± 0.66	41.84^{c} ± 0.47	40.53^{c} ± 0.44	12.67^{b} ± 0.24	0.46^{b} ± 0.01	16.81^{b} ± 0.34
SinC[2] (restricted environment)	29	11.96^{a} ± 0.15	16.84^{a} ± 0.23	56.35^{a} ± 0.78	39.86^{a} ± 0.68	39.93^{b} ± 0.48	38.92^{b} ± 0.46	11.57^{a} ± 0.25	0.42^{a} ± 0.01	15.59^{a} ± 0.35
CinS[3] (luxurious environment)	21	12.55^{b} ± 0.18	17.68^{b} ± 0.27	56.89^{ab} ± 0.92	41.56^{ab} ± 0.80	39.15^{ab} ± 0.57	37.25^{a} ± 0.54	11.41^{a} ± 0.29	0.44^{ab} ± 0.02	16.96^{b} ± 0.41
CinC[4] (small control)	23	12.50^{b} ± 0.17	17.64^{b} ± 0.27	56.18^{a} ± 0.90	39.69^{a} ± 0.78	38.40^{a} ± 0.55	36.88^{a} ± 0.52	11.22^{a} ± 0.28	0.41^{a} ± 0.02	16.27^{ab} ± 0.40

[1]Suffolk.in Suffolk, [2]Suffolk in Cheviot, [3]Cheviot in Suffolk, [4]Cheviot in Cheviot.

Figure 5.4. Photographs of newborn lambs born from restricted uterine environment (SinC; left) and large control (SinS; right).

Figure 5.5. Photographs of newborn lambs born from small control (CinC; front) and luxurious environment (CinS; back).

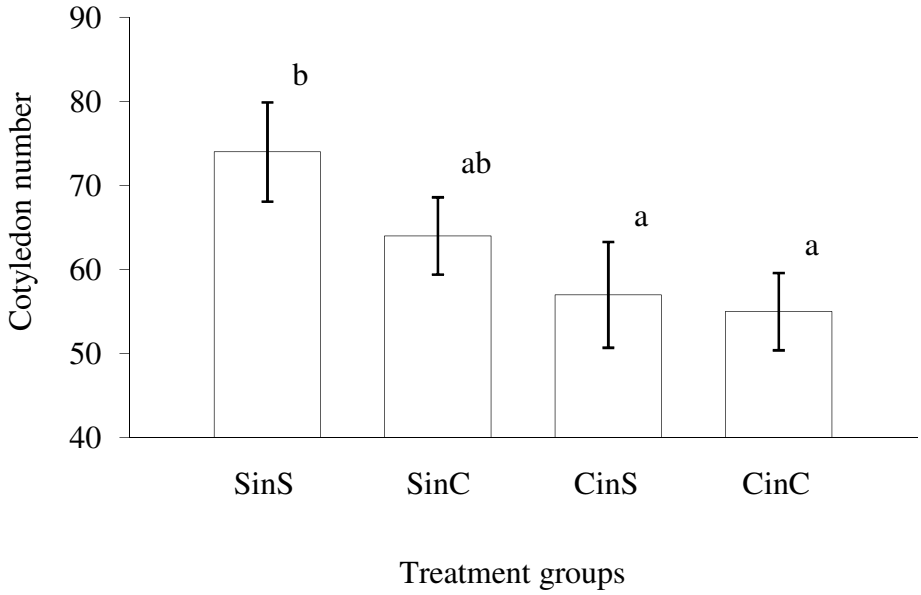

Figure 5.6. The effect of maternal uterine environment upon cotyledon number in sheep (Mean ± SE). Bars that are labelled with different superscripts are significantly different (P<0.05). S: Suffolk, C: Cheviot.

Table 5.11. The effects of maternal uterine environment upon various placental parameters (Mean ± SE). Within a column, means without a common superscript are different from each other (P<0.05).

Treatment groups	n	FMWC[1], (g)	FMWOC[2], (g)	CW[3], (g)	MCW[4], (g)
SinS[5] (large control)	15	372.7 ± 29.5	232.8 ± 18.3	116.3 ± 12.1	1.7[b] ± 0.2
SinC[6] (restricted environment)	21	336.0 ± 24.9	213.2 ± 15.5	106.4 ± 10.2	1.7[b] ± 0.2
CinS[7] (luxurious environment)	15	385.0 ± 24.5	232.5 ± 18.3	129.5 ± 12.1	2.4[a] ± 0.2
CinC[8] (small control)	21	362.7 ± 24.9	229.6 ± 15.5	113.3 ± 10.2	2.1[ab] ± 0.2

[1]Fetal membranes weight with cotyledons, [2]Fetal membranes weight without cotyledons, [3]Cotyledon weight, [4]Mean cotyledon weight, [5]Suffolk in Suffolk, [6]Suffolk in Cheviot, [7]Cheviot in Suffolk, [8]Cheviot in Cheviot.

Discussion

The key findings of this study were that the birthweight and body dimensions of lambs were reduced when they were born from a restricted uterine environment (SinC), but were not enhanced when born from a luxurious environment (CinS). The results of restricted uterine environment are in accord with the findings of Chapters 3 and 4, in which both embryonic and fetal development was impaired in a restricted uterine environment (SinC) compared to control (SinS). The findings of the present chapter suggest that the impairment in development that is created in a restricted uterine environment during the early embryonic period persists in the remainder of gestation and results in significant reduction in birthweight and body dimensions of such new born. These results are consistent with earlier studies that suggest important implications for animals' subsequent productivity and health. Earlier studies have shown a linkage between birthweight and subsequent health in the human (Barker, 1995; Greenwood *et al.*, 1998; Godfrey and Barker, 2000; Gluckman and Hanson, 2004a) and production traits of the animals (Greenwood *et al.*, 1998; Bell, 2006; Blair *et al.*, 2010). The present findings emphasise the importance of the early embryonic period in the programming of the animal during fetal life which establishes the potential for later life health and productivity.

Interestingly, the results of present chapter of the luxurious environment are contrary to the findings of Chapter 3, which showed embryonic development was enhanced in a luxurious uterine environment (CinS) compared to control animals (CinC). However, the present results are akin to those of Chapter 4, in which there were no differences in fetal dimensions between CinS and CinC fetuses. Furthermore, Jenkinson *et al.* (2007) also showed that the birthweight of the crossbred lambs was greater when born to a Suffolk than a Cheviot breed of ewe. Taken together these

data suggest that CinS fetuses were unable to utilise the privilege of their luxurious uterine environment with advancing gestation. Hence, the results of luxurious environment in present study suggest that besides the early embryonic period, the feto-maternal dialogue during subsequent gestation is also important in determining the final outcome of the gestation at birth.

Similar research in the horse (Walton and Hammond, 1938; Allen *et al.*, 2002b; Giussani *et al.*, 2002) has produced some intriguing similarities but also differences from the present study. In the equine studies, a restricted uterine environment significantly reduced the development at birth, whereas, by contrast, a luxurious uterine environment allowed a significant augmentation of birthweight. Allen *et al.* (2002b) reported that the birthweight of large genotype Thoroughbred foals was significantly reduced when born from small-sized pony dams (restricted uterine environment); a similar effect to that which was exerted upon the Suffolk fetus by the Cheviot dam. However, the percentage reduction in the birthweight (18%) of Suffolk lambs born from Cheviot dams was less to that of Thoroughbred foals born from pony dam (61%). On the other hand, the birthweight of small genotype pony foals was enhanced in the luxurious uterine environment of the large Thoroughbred dams, whereas no such effect was observed for Cheviot lambs born from Suffolk dams. However, there was a modest percentage increase in the birthweight (7%) of CinS lambs in the present study, albeit not statistically significant, and markedly less than that of pony foals born to Thoroughbred dams (58%). The reasons for these differences between species are unclear; however, it is relevant to note that the dissimilarity between the size of pony and Thoroughbred dams is very much greater than that between Cheviot and Suffolk ewes. Given that the relationship between dam-size to birthweight is not linear (Dickinson *et al.*, 1962; Robbins and Robbins,

1979) a lamb is about 6% of ewe weight, whereas a foal is about 10% of dam weight, might also help explain these variances between two studies. This difference could also be attributed to the different types of placentation in equine and ovine species or the different developmental mechanisms during early gestation (Betteridge, 2000). Until further research unravels the mechanisms in sheep vs horses, it will not be possible to explain these differences.

The differences in the birthweight were reflected in differences in body dimensions between the groups of lambs. Thus, body dimensions of lambs born to a restricted uterine environment (SinC) were smaller than in control (SinS) lambs. This may be of some importance in terms of the individuals' future health, since skeletal muscles are the main site for utilisation of fatty-acids and glucose (Petersen and Shulman 2002). In humans such impaired muscle development caused by fetal under-nutrition, may contribute to the metabolic dysfunction which underlies the increased incidence of metabolic syndrome, Type 2 diabetes and obesity during adulthood (Ozanne *et al.* 2005). Following the post-natal development and health of the lambs born in the present study would help to understand whether such impairment occurred in these animals.

Under adverse uterine conditions, the development of fetal brain is privileged over skeletal muscles and other vital organs such as liver, kidney or viscera (Barker, 1998); a phenomenon known as 'brain sparing'. However, this might also have longer-term serious health consequences such as morbidity and poor post-natal productivity. A lowered birthweight to head width ratio in SinC lambs compared to SinS lambs suggests that perhaps a 'brain sparing' has occurred in SinC lambs. Similarly, thinness at birth reflects fetal adaptations to adverse *in utero* conditions such as under-nutrition or hypoxia etc and recent epidemiological studies in human

have indicated a linkage between thinness at birth and the serious health outcomes during adult life (Martyn *et al.*, 1996). In this context it is important to note that the lower body mass index in SinC lambs compared to SinS lambs is indicative of a thinness of the lambs in the former group. Thus, these findings suggest a probable *in utero* adaptation by SinC lambs to their impaired antennal environment, which consequently might affect their growth potential in later life.

It is probable that the placenta is a significant mediator of the effects of under-nutrition upon fetal development, since it is not only responsible for nutrient exchange, but is also an active autocrine, paracrine and endocrine organ in its own right (Gootwine, 2004). An alteration in placental morphology is evident to support fetal growth under impaired uterine conditions (Mellor, 1983; Giussani *et al.*, 2002; Wilsher and Allen, 2002; Vonnahme *et al.*, 2006). The differences in the placental characteristics between different maternal environments were also evident in the present study. There were fewer cotyledons in CinS than SinS placentas, suggesting an inability of Cheviot genotype lambs to colonise an entire population of caruncles of Suffolk dams. Perhaps this might help to explain why the Cheviot lambs appeared unable to benefit from the luxurious uterine environment of the Suffolk dam. However, the total weight of cotyledons was unaffected by treatment group, so it is unclear how differences in intrauterine growth can be attributed to the cotyledon number *per se*. On the other hand, mean cotyledon weight was higher in CinS than in SinS placentas, suggesting that compensatory growth of placenta had occurred to counter the fewer cotyledons present in CinS placentas; although the lack of enhancement in the birthweight of lambs born from this group indicates that this compensation was inadequate.

Here, it is interesting to note that the trophoblast of CinS embryos were bigger than CinC embryos during the embryonic stage (Chapter 3), whereas the placentas of CinS fetuses did not differ than those of CinC fetuses at term. This indicates that, once the Cheviot fetuses achieved the growth for which they are genetically programmed; they did not exert any incremental demand on large Suffolk dams.

The placenta also secretes hormones such as placental lactogen and progesterone, both of which are able to regulate fetal development. There is good evidence to suggest that oPL has a significant role in augmenting fetal and placental development and birthweight (Butler *et al.*, 1981; Kappes *et al.*, 1992; Anthony *et al.*, 1995b). The growth promoting action of placental lactogen has also been supported by recent studies in which it was found to stimulate DNA synthesis and amino acid transport in fibroblasts and myoblasts of the human fetus and in heaptocytes of the rat and sheep fetus, along with increased glycogen synthesis in fetal sheep liver (Freemark and Handwerger, 1984; Freemark and Handwerger, 1986; Handwerger and Freemark, 2000). The secretion of oPL increases with increase in gestation (Kappes *et al.,* 1992). In the present study, oPL concentration increased with gestational stage, regardless of group, and a strong positive correlation was observed between Day 90 and Day 120 oPL concentrations, probably coinciding with an increase in fetal growth.

The results of present study are partially in accord with the earlier findings of Jenkinson *et al.* (2007), who reported a direct relationship between the concentrations of oPL on Days 90 or 120 of gestation with the birthweight of lambs. In the present study, the concentration of Day 120 oPL was positively associated with birthweight; paradoxically, however, the concentration on Day 90 was negatively correlated. The reason for the negative correlation between

Day 90 oPL concentration and birthweight cannot be explained. However, this might be due to that Day 90 is prior to the rapid growth of fetus.

Progesterone is also involved in embryonic development, providing trophic support to the placenta and influencing fetal and placental weight (Bindon, 1971; Ogle *et al.*, 1990; Kleeman *et al.*, 1994; Manalu and Sumaryadi, 1998; Mark *et al.*, 2006). *In vitro* studies in rats have suggested anabolic and growth promoting effects of progesterone (Manzi *et al.*, 1994), and showed that supplementation of progesterone during pregnancy was associated with an increase in DNA and protein synthesis and cell replication in the bone tissue.

In the present study, CinS ewes tended to have lower progesterone concentration than control ewes (CinC). Perhaps this may help to understand why there was no increase in the birthweight of CinS lambs compared to CinC lambs. Recent studies have suggested that oPL forms a part of the placental complex which maintains adequate progesterone synthesis during later period of gestation (Gregoraszczuk *et al.*, 2000). This is also supported by the present results as a strong positive correlation was observed between Day 120 oPL concentration and subsequent progesterone concentrations. An increase in 10 ng/mL of plasma oPL concentration is associated with 47g increase in birthweight, whereas 1 ng/mL increase in plasma progesterone concentration is associated with 140g increase in birthweight. Thus, tendencies of low concentrations of oPL and progesterone in CinS ewes indicate the possible mechanistic basis of a failure to find an increase in the birthweight and body dimensions of CinS lambs compared to CinC lambs, despite the fact that the former were in a luxurious environment. Conversely, the lack of any difference in oPL or progesterone concentrations between SinC and SinS ewes might indicate an increased substrate demand by the larger genotype Suffolk fetus from the smaller

genotype Cheviot dam with advancing gestation. This is also supported by the concentrations of oPL and progesterone in different maternal environments.

These above findings suggest that one of the mechanisms by which the maternal uterine environment regulates fetal development is alteration in the concentrations of oPL and progesterone, both of which not only affect fetal growth individually but also synergistically. This notion is supported by the positive correlation of the concentrations of oPL and progesterone.

There is a significant body of evidence to show that IGF-1 is a key regulator of embryogenesis, feto-maternal interactions and fetal, placental and somatic growth throughout gestation (Blair *et al.*, 1988; Powell-Braxton *et al.*, 1993; Gicquel and Le Bouc, 2006; Watson *et al.*, 2006; Blum *et al.*, 2007). IGF-1 acts as promoter of fetal growth by modulating the transfer of nutrients across placenta via an alteration in the placental metabolism (Gluckman *et al.*, 1992); thus, fetuses developing in a maternal environment with high IGF-1 concentrations have higher growth rates than fetuses developing in environment with lower maternal IGF-1concentrations (Morel *et al.*, 1994). Differences in IGF-1 concentrations between groups of ewes in the present study appear to be in accord with the aforegoing studies. This is evident by the tendency of restricted environment ewes (SinC) to have lower IGF-1 concentration than ewes of control environment (SinS) throughout the gestation. This not only helps in explaining the reduced fetal development in SinC ewes but also suggests that such a reduction in fetal development might have started in early stages of pregnancy. Thus, the high concentration of IGF-1 in the luxurious group (CinS) during early pregnancy (compared to CinC) might suggest an early enhancement of fetal development in CinS ewes, but the lack of differences in the maternal IGF-1 concentrations between CinS and CinC ewes with advancing gestation might indicate

of a lack of nutrient demand from Cheviot fetuses. Therefore, these findings are compatible with the notion that IGF-1 modulates fetal growth both in restricted and luxurious uterine environments.

Besides hormones and growth factors, the developing fetus requires nutritional substrates to maintain its metabolism and to meet the demands of its growth. Glucose is the main source of energy for both the fetus and placenta (Hay and Sparks, 1985) and, with advancing gestation, the fetal demands for glucose increases. Therefore, to maintain normal concentrations of glucose in circulation, pregnant ewes mobilise body fat reserves to provide an alternative substrate for her own energy metabolism (Russel *et al.*, 1967; Dunshea *et al.*, 1988). The placentas of growth-restricted fetuses have been shown to consume a twofold higher glucose than normally grown preterm placentas, suggesting a lack of transfer from the maternal to the fetal compartment in growth-restricted fetuses (Challis *et al.*, 2000). In the present study, higher plasma glucose concentrations in SinC ewes than SinS ewes might indicate a lack of transfer of sufficient glucose to fetal compartment. The higher area under the curve of glucose concentrations, clearance half-time of glucose and the ratio of area under the curve of glucose concentrations to area under the curve of insulin concentrations in SinC ewes than SinS ewes is also compatible with this suggestion. Moreover, a high fetal glucose concentration stimulates IGF-1 production from the fetus which then promotes fetal growth (Oliver *et al.*, 1993; Harding *et al.*, 1994). Hence, it is conceivable that an inadequate transfer of glucose to SinC fetuses probably would have resulted in less production of fetal IGF-1 and consequently poor fetal growth.

A higher concentration of FFA in SinC ewes compared to SinS ewes suggests an effort by a large genotype Suffolk fetus to exert incremental demand of substrates from small genotype Cheviot dam. This notion is supported by the trends of these

differences between these two groups such that the differences were non-significant during the early period of gestation; whereas they were apparent with advancing gestation. This strongly suggests the 'pull' of nutrients by the large genotype Suffolk fetus, since the fetal demands increases with advancing gestation (Sibley *et al.*, 1998; Sibley *et al.*, 2002). The lower concentration of FFA in CinS ewes compared to CinC ewes throughout gestation strongly suggest that small genotype Cheviot fetuses were unable to exert an incremental demand upon large genotype Suffolk dams and hence no enhancement was observed in their birthweight or body dimensions compared to CinC lambs.

Conclusion

The present study suggested that a restricted uterine environment reduces the birthweight, body dimensions and other body characteristics of newborn lambs, whereas a luxurious environment does not enhance the development of lambs at the time of birth. It was concluded that the uterine environment modulates fetal growth by an alteration in the concentrations of oPL, progesterone and IGF-1. This relationship between concentrations of oPL and progesterone is synergistic, such that both act together as regulators of fetal development. Another potential mechanism by which uterine environment regulates fetal growth is probably the regulation of supply of glucose to developing fetus directly and the control of its utilisation by IGF-1, indirectly. The concentration of FFA in maternal circulation might be a useful indicator of the feto-maternal dialogue, such that an increased maternal plasma FFA concentration is perhaps associated with increased fetal substrate demand and vice versa.

Chapter 6 - The effects of antenatal uterine environment upon post-natal glucose metabolism in female lambs

Abstract

Pure-breed single embryos were transferred within and reciprocally between large (Suffolk: S) and small (Cheviot: C) breeds of sheep to establish four different maternal environments (SinS (large control), SinC (restricted environment), CinS (luxurious environment) and CinC (small control)). These recipient ewes were allowed to lamb spontaneously at full term and each newborn lamb was identified at birth. A glucose tolerance test using 8 female lambs from each treatment group was undertaken at six-months and at one-year-of-age. The metabolic measures of glucose or insulin or the basal cortisol concentration did not differ ($P>0.05$) between SinC and SinS groups either at six months or at one year of age. At six months of age, differences between CinS and CinC were not significantly ($P<0.05$) different, but several tended ($P<0.10$) towards significance. Area under the curve of glucose concentrations and clearance half-time of glucose tended (both $P=0.07$) to be lower in CinS lambs than CinC lambs, whereas the logarithmic decay rate of glucose tended ($P=0.07$) to be higher in CinS lambs than CinC lambs. The peak insulin concentration tended ($P=0.09$) to be higher in CinS lambs than CinC lambs. At one year of age, the logarithmic decay rate of glucose was higher ($P<0.05$) in CinS lambs compared to CinC lambs. However none of the metabolic measures of insulin or basal cortisol concentration differed ($P>0.05$) between CinS and CinC lambs. Thus, it appeared that the lambs born from a luxurious uterine environment (CinS vs CinC) had faster uptake of glucose from the circulation. At six months, this was evident by an increase in the glucose tolerance and the response of insulin to glucose, whereas at one year of age, the improvement in glucose metabolism was due to an increase in

the glucose tolerance but without any apparent increase in the response of insulin to glucose. On the other hand, a restricted uterine environment (SinC vs SinS) had no effect upon the post-natal glucose metabolism of female lambs either at six months or at one-year-of-age.

Introduction

The vast majority of recent human epidemiological studies indicate that the uterine environment that an individual experiences, regulates its fetal growth and birthweight and has a linkage with onset of adulthood diseases such as non-insulin-dependent diabetes, coronary-heart-disease, hypertension and/or obesity (Barker and Clark, 1997; Forsen *et al.*, 1997; Godfrey and Barker, 2000; Harding, 2003; Gluckman and Hanson, 2004b; Symonds *et al.*, 2007). An impaired uterine environment also adversely affects vital physiological and metabolic pathways of the fetus, such as the cardiovascular system, the hypothalamic-pituitary-adrenal axis and its glucose metabolism (Barker *et al.*, 1993; Giussani *et al.*, 2002; Bloomfield *et al.*, 2004; Bo *et al.*, 2004), which are probably the means by which such individuals are more susceptible to adulthood diseases.

Studies in livestock suggest that the uterine environment affects birthweight and the size and development of skeletal muscles in various species of animals (Walton and Hammond, 1938; Cowley *et al.*, 1989; Allen *et al.*, 2002b; Giussani *et al.*, 2002; Zhu *et al.*, 2006; Gardner *et al.*, 2007; Gootwine *et al.*, 2007). Moreover, an impaired uterine environment results in insulin resistance in the skeletal muscles in rats (Ozanne *et al.*, 2003). Since the skeletal muscles are the main sites for utilisation of glucose (Petersen and Shulman, 2002), any disruption in the development during the fetal stage has the potential to render individuals susceptible to disorders in glucose metabolism (Ozanne *et al.*, 2005).

Oliver *et al.* (2001) showed that maternal nutrient restriction from 60 days before mating to 30 days post mating increases the insulin response to glucose in the fetuses during the late gestation. Conversely, Gardner *et al.* (2005) found no such effect of

early gestation nutrient restriction; although late gestation nutrient restriction was associated with glucose intolerance and insulin resistance in post-natal lambs; whilst Clarke *et al.* (2000) found that birthweight was unrelated to subsequent glucose-insulin relationships. Moreover the glucose/insulin relationships change with time after birth. For example, Holness *et al.* (1996) found that the rat pups born from nutrient-restricted dams had greater insulin response to glucose at 4 weeks than did controls. Conversely Petry *et al.* (1997) found that rat pups born from nutrient-restricted dams had a poorer insulin response to glucose than did controls, particularly when nutrient-restricted pups were subsequently fed a high quality diet *ad libitum*. Ozanne and Hales (2002) summarised such data by noting that the both the stage of gestation at which nutrient restriction occurs and the post-natal stage at which it is assessed affects the insulin response to glucose, and that abnormal glucose-insulin relationships are more likely to occur where there is a mis-match between the gestational and post-natal nutrient environments.

The above studies indicate that the uterine environment has the potential to affect key metabolic pathways in the offspring. It appears that not only the stage of gestation at which fetus is exposed to an inadequate uterine environment is of significance in determining the alteration to its metabolic pathways, but also that the manifestation of this effect during the post-natal life can be age dependent. Only a few studies in livestock have reported the effects of uterine environment upon post-natal glucose metabolism in the offspring and it is apparent that none have studied such effects in a sheep dam-size model (within and reciprocal transfer of pure-breed embryos between large and small size dams). Hence, the present chapter investigated the effects of whole gestational restricted or luxurious uterine environment upon glucose metabolism of female lambs at six months and one year of age.

Materials and Methods

Experimental design and animals

Purebred embryos were transferred within and reciprocally between ewe breeds of dissimilar mature body size (small Cheviot (C) and large Suffolk (S)), to establish different maternal uterine environments (Suffolk in Suffolk (SinS; large control), Suffolk in Cheviot (SinC; restricted environment), Cheviot in Suffolk (CinS; luxurious environment) and Cheviot in Cheviot (CinC; small control)), as described in Chapters 3 and 5. In the present experiment the recipient ewes were allowed to lamb spontaneously at full term. Each treatment group was kept in separate but contiguous paddocks during the lambing period and each newborn lamb was identified at birth. The effects of maternal uterine environment upon birthweight and body dimensions of newborn lambs have been described in Chapter 5. Ewes and lambs born from all treatment groups were managed together as one group until three months of age. The lambs were docked at three weeks of age and weaned from their mothers at three months of age. After weaning, all of the lambs were managed together as one group until six months of age. At six months of age, all male lambs of all treatment groups were removed from the experiment. Female lambs from all of the treatment groups continued to be managed together until one year of age. All animals (ewes and lambs) used in the present study were managed at Keeble Farm, Massey University, New Zealand, under standard lowland commercial farm conditions. Animals were provided with generous allowances of ryegrass (*Lolium L.*) and white clover (*Trifolium repens L.*) pastures and had *ad libitum* access to water.

Collection of blood samples in post-natal lambs

A glucose tolerance test using 8 female lambs from each treatment group (i.e. from SinS, SinC, CinS and CinC) was undertaken at six months and at one year of age. However at one year of age, two lambs from CinS group were removed from the study due to accidental subcutaneous administration of a part volume of glucose. Male lambs could not be included in the study due to the logistic reasons. Prior to the glucose challenge, lambs were fasted overnight (12 h), but had *ad libitum* access to water. Glucose-D (Dextrose 40%, Bomac Laboratories Ltd, Auckland, New Zealand) was administered into the jugular vein at a dose of 0.17 g/kg live weight. Blood samples (5 mL; Lithium heparin anticoagulant) were collected by venepuncture, from the jugular vein opposite to that used for glucose administration, into heparinised vacutainers (BD Vacutainer Systems, UK) at -5, 0, +10, +20, +30 and +60 min relative to glucose administration. At one year of age an additional blood sample was taken at +5 min. After completion of the sampling, the animals were returned to pasture. Blood samples were immediately centrifuged at 1000 g (3000 rpm, Heraeus Labofuge 200 centrifuge; Heraeus, Buckinghamshire, UK) for 15 min and the plasma samples were stored at -20^{0}C.

Measurement of insulin, glucose and cortisol concentrations

Insulin and glucose concentrations were measured at all time points in both glucose challenges, and cortisol concentration was measured only in the -5 min sample.

Glucose concentrations were measured using a Hitachi 902 autoanalyser (Hitachi High Technologies Corporation, Tokyo, Japan) by enzymatic colorimetric assay using commercial kits (kits from Roche, Mannheim, Germany).

Insulin concentrations were measured in duplicate 20 µL aliquots of plasma by radioimmunoassay (RIA) with iodinated insulin as the tracer (I^{125} labelled insulin in a protein based buffer with sodium azide as preservative, Diagnostic Systems Laboratories Inc., USA). The limit of sensitivity as determined by twice the standard deviation of blank values (representing the least amount of hormone which could cause a significant displacement of radiolabelled hormone from the antibody), was 0.98 µIU/mL; intra- and inter-assay coefficients of variation were 11.9% and 14.7%, respectively (n=4). The standard insulin antibody had 100% cross reactivity with human insulin, 99% with porcine insulin, but less than 0.1% with glucagon, somatostatin, pancreatic polypeptide and IGF-1.

Cortisol concentrations were measured in duplicate 25 µL aliquots of plasma by RIA. The assay used iodinated (^{125}I) cortisol prepared in human serum matrix with sodium azide and gentamicin sulphate as preservative as the tracer (ImmuChem™ Coated Tube cortisol ^{125}I RIA kit for *in vitro* diagnostic use, MP Biomedicals, USA). The cross reactivity of the antibody was 100% with cortisol, 45.6% with prednisolone, 5.5% with corticosterone, 2.7% with prednisone, 25% with cortisone and less than 0.1% with progesterone, dexamethasone, dihyro-testosterone and testosterone. The limit of sensitivity was 0.42µg/dL, and the intra-assay coefficients of variation were 23.5%, 14.5% and 12.1% for low, medium and high solutions respectively (n=1).

Ethical approval

All experimental procedures were undertaken with approval of the Massey University Animal Ethics Committee.

Analysis of data

Glucose tolerance test

In response to the glucose challenge, the following metabolic measures of glucose were calculated: basal glucose concentration, peak glucose concentration, area under curve of glucose concentrations, time to peak glucose, logarithmic decay rate of glucose and clearance half-time of glucose (described in detail in Chapter 5). The following metabolic measures of insulin were calculated: basal insulin, area under curve of insulin concentrations, peak insulin concentration, time to peak insulin and the ratios between basal glucose and basal insulin concentration and between area under curve of glucose concentrations and area under curve of insulin concentrations. Details of these calculations are described in Chapter 5.

Statistical analysis

Data were subjected to analysis of variance using the Generalised Linear Model procedure from the SAS statistical package (SAS, 2005; SAS 9.1.3, SAS Institute, North Carolina, USA). Post-hoc differences between the groups were detected using least significant differences. The main effects of breed of ewe, breed of lamb and embryo transfer session (i.e. first and second) and all two-way interactions between these parameters were included in the initial model, but were removed if found non-significant ($P>0.05$) and the model was then re-fitted. Three-way interactions were not included in the model as the number of ewes in these subcells did not enable a valid test. The interaction between the breed of ewe and breed of lamb was always kept in the model irrespective of whether it was significant ($P<0.05$) or not ($P>0.05$), since this interaction defines the different maternal environments. For analysing the

logarithmic decay rate of glucose, data were transformed by calculating natural logarithm of each adjusted glucose concentration.

Results

The effects of maternal uterine environment upon birthweight and body dimensions of both male and female newborn lambs have been described in Chapter 5 and also reported elsewhere (Sharma *et al.*, 2009).

Metabolic measures of glucose and insulin in response to glucose challenge in lambs at six months of age

The majority of the metabolic measures for the glucose did not differ significantly (P>0.05) between the SinC and SinS groups at six months of age (Table 6.1), although SinC lambs tended (P=0.09) to have lower basal glucose concentration than SinS lambs. Whilst differences between CinS and CinC were not significant (P>0.05), several parameters tended towards significance. CinS lambs tended (P=0.07) to have lower area under the curve of glucose concentrations than CinC lambs. The logarithmic decay rate of glucose concentration tended (P=0.07) to be higher in CinS lambs than CinC lambs, whereas the clearance half-time of glucose tended (P=0.07) to be less in CinS lambs than CinC lambs. The effects of uterine environment on area under the curve of glucose concentrations are shown in Figure 6.1.

No differences (P>0.05) were observed between SinC and SinS or between CinS and CinC lambs in respect of any of the measures of insulin, although several tended to be different between CinS and CinC lambs (Table 6.2). The peak insulin concentration tended (P=0.09) to be higher in CinS lambs than CinC lambs whereas the ratio of basal glucose concentration to basal insulin concentration tended

(P=0.06) to be lower in CinS lambs than CinC lambs. The effect of uterine environment on area under the curve of insulin concentrations is shown in Figure 6.2.

No differences (P>0.05) were found in basal cortisol concentrations between SinC and SinS lambs or CinS and CinC lambs (Table 6.2).

Table 6.1. The effects of uterine environment on metabolic measures of plasma glucose following glucose challenge in female lambs at 6 months of age (Mean ± SE). Means within a column are not different from each other (P>0.05).

Treatment groups	n	Basal glucose concentration, mmol/L	Area under curve of glucose concentrations, min mmol/L	Logarithmic decay rate of glucose	Clearance half time glucose, min	Peak glucose concentration, mmol/L	Time to peak glucose, min
SinS[1] (large control)	8	3.45 ± 0.15	204.20 ± 15.65	0.021 ± 0.004	38.92 ± 20.28	5.66 ± 0.44	10.00 ± 1.63
SinC[2] (restricted environment)	8	3.09 ± 0.15	215.34 ± 15.65	0.019 ± 0.004	40.23 ± 20.28	5.24 ± 0.41	10.00 ± 1.52
CinS[3] (luxurious environment)	8	3.12 ± 0.16	201.48 ± 15.65	0.023 ± 0.004	40.23 ± 20.28	4.97 ± 0.41	12.50 ± 1.52
CinC[4] (small control)	8	3.24 ± 0.15	242.28 ± 15.65	0.012 ± 0.004	95.06 ± 20.28	5.68 ± 0.41	12.50 ± 1.52

[1]Suffolk in Suffolk, [2]Suffolk in Cheviot, [3]Cheviot in Suffolk, [4]Cheviot in Cheviot.

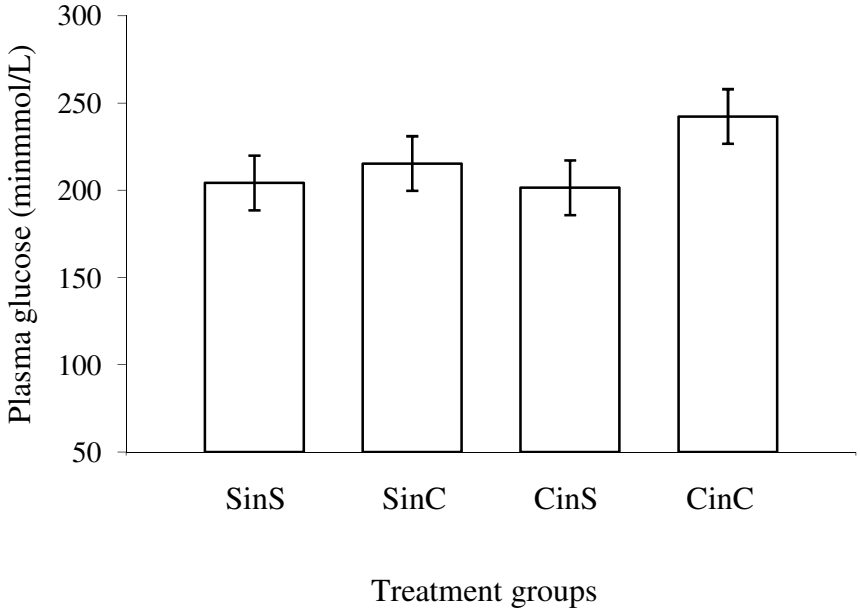

Figure 6.1. The effect of maternal uterine environment on the area under curve of glucose concentrations in female lambs at six months of age (Mean ± SE). Differences between the treatment groups are non-significant (P>0.05). S: Suffolk, C: Cheviot.

Table 6.2. The effects of uterine environment on metabolic measures of plasma insulin and other measures following glucose challenge in female lambs at 6 months of age (Mean ± SE). Within a column, means without a common superscript are different (P<0.05).

Treatment groups	n	Basal insulin concentration, µIU/L	Area under curve of insulin concentrations, min µIU/L	Peak insulin concentration, µIU/L	Time to peak insulin, min	Basal glucose to basal insulin concentration ratio	Area under curve glucose to area under curve insulin concentrations ratio	Basal cortisol concentration, µg/dL
SinS[1] (large control)	8	5.77[ab] ± 0.51	327.41[b] ± 41.02	15.00 ± 3.29	15.00 ± 4.71	0.61[ab] ± 0.04	0.76[a] ± 0.43	1.26 ± 0.29
SinC[2] (restricted environment)	8	4.97[a] ± 0.51	243.63[ab] ± 38.37	7.49 ± 3.29	16.25 ± 4.71	0.63[b] ± 0.04	0.90[ab] ± 0.40	0.60 ± 0.29
CinS[3] (luxurious environment)	8	6.73[b] ± 0.51	212.96[ab] ± 41.02	14.07 ± 3.37	10.00 ± 4.71	0.50[a] ± 0.04	1.21[ab] ± 0.43	0.49 ± 0.29
CinC[4] (small control)	8	5.60[ab] ± 0.51	171.95[a] ± 38.37	5.82 ± 3.29	16.25 ± 4.71	0.60[ab] ± 0.04	2.19[b] ± 0.40	1.12 ± 0.29

[1]Suffolk in Suffolk, [2]Suffolk in Cheviot, [3]Cheviot in Suffolk, [4]Cheviot in Cheviot.

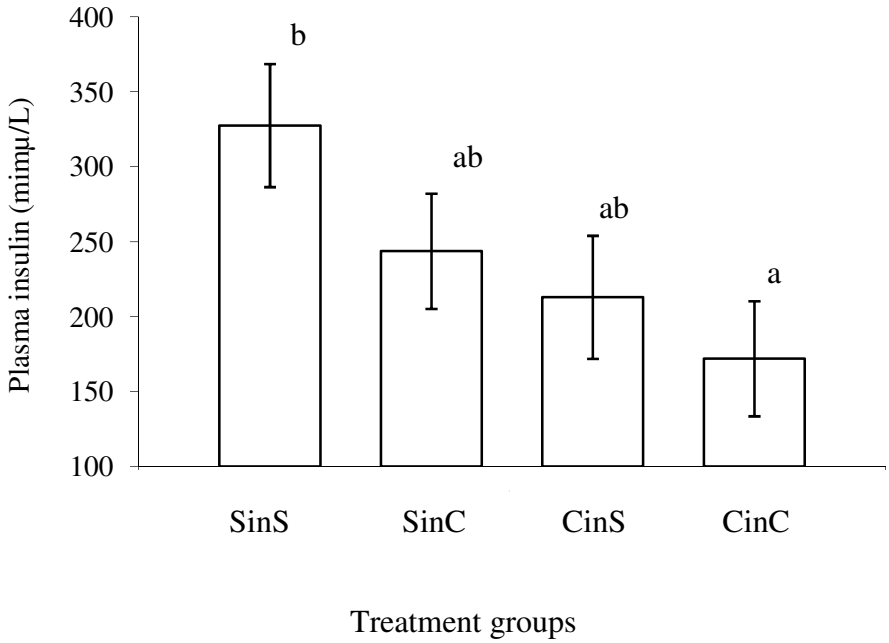

Figure 6.2. The effect of maternal uterine environment on the area under curve of insulin concentrations in female lambs at six months of age (Mean ± SE). Bars that are labelled with different superscripts are significantly different from each other ($P<0.05$). S: Suffolk, C: Cheviot.

Metabolic measures of glucose and insulin in response to glucose challenge in lambs at one year of age

The logarithmic decay rate of glucose was higher ($P<0.05$) in CinS lambs compared to CinC lambs, whereas the peak glucose concentration tended ($P=0.06$) to be lower in CinS lambs than CinC lambs (Table 6.3). None of the measures of glucose metabolism differed ($P>0.05$) between SinC and SinS lambs. There was no difference ($P>0.05$) in any of the metabolic measures of insulin between CinS and CinC and between SinC and SinS lambs (Table 6.4). The effects of uterine environment on area under the curve of glucose concentrations and area under the curve of insulin concentrations are shown in Figures 6.3 and 6.4, respectively.

No differences ($P>0.05$) were found in basal cortisol concentration between SinC and SinS lambs or CinS and CinC lambs (Table 6.4).

Table 6.3. The effects of maternal uterine environment on metabolic measures of plasma glucose following glucose challenge in female lambs at 1 year of age (Mean ± SE). Within a column, means without a common superscript are different from each other (P<0.05).

Treatment groups	n	Basal glucose concentration, mmol/L	Area under curve of glucose concentrations, min mmol/L	Logarithmic decay rate of glucose	Clearance half time glucose, min	Peak glucose concentration, mmol/L	Time to peak glucose peak, min
SinS[1] (large control)	8	3.44 ± 0.08	193.48 ± 13.60	0.019[a] ± 0.005	39.08 ± 19.33	5.25[ab] ± 0.56	5.00 ± 2.62
SinC[2] (restricted environment)	8	3.43 ± 0.08	183.06 ± 13.39	0.017[a] ± 0.005	55.58 ± 19.33	4.69[b] ± 0.56	8.13 ± 2.62
CinS[3] (luxurious environment)	6	3.25 ± 0.10	176.47 ± 15.86	0.049[b] ± 0.008	26.64 ± 27.33	4.61[ab] ± 0.64	12.50 ± 3.70
CinC[4] (small control)	8	3.31 ± 0.08	208.51 ± 13.65	0.019[a] ± 0.005	68.02 ± 19.33	6.31[a] ± 0.56	8.13 ± 2.62

[1]Suffolk in Suffolk, [2]Suffolk in Cheviot, [3]Cheviot in Suffolk, [4]Cheviot in Cheviot.

Table 6.4. The effects of maternal uterine environment on metabolic measures of plasma insulin and other following glucose challenge in female lambs at 1 year of age (Mean ± SE). Within a column, means without a common superscript are different from each other (P<0.05).

Treatment groups	n	Basal insulin concentration, µIU/L	Area under curve of insulin concentrations, min µIU/L	Peak insulin concentration, µIU/L	Time to peak insulin, min	Basal glucose to basal insulin concentration ratio	Area under glucose to area under curve insulin concentrations ratio	Basal cortisol concentration, µg/dL
SinS[1] (large control)	8	5.34 ± 0.65	441.96 ± 72.84	14.18 ± 2.90	6.88 ± 0.82	0.65 ± 0.05	0.47[a] ± 0.12	0.50[b] ± 0.17
SinC[2] (restricted environment)	8	5.73 ± 0.65	385.59 ± 72.84	12.57 ± 2.90	5.63 ± 0.82	0.63 ± 0.05	0.68[ab] ± 0.12	0.64[ab] ± 0.18
CinS[3] (luxurious environment)	6	7.00 ± 0.75	216.88 ± 84.11	7.55 ± 3.35	6.67 ± 0.95	0.53 ± 0.06	1.00[b] ± 0.14	0.82[ab] ± 0.20
CinC[4] (small control)	8	5.96 ± 0.65	321.46 ± 72.84	10.95 ± 2.90	8.75 ± 0.82	0.58 ± 0.05	0.76[ab] ± 0.12	1.13[a] ± 0.17

[1]Suffolk in Suffolk, [2]Suffolk in Cheviot, [3]Cheviot in Suffolk, [4]Cheviot in Cheviot.

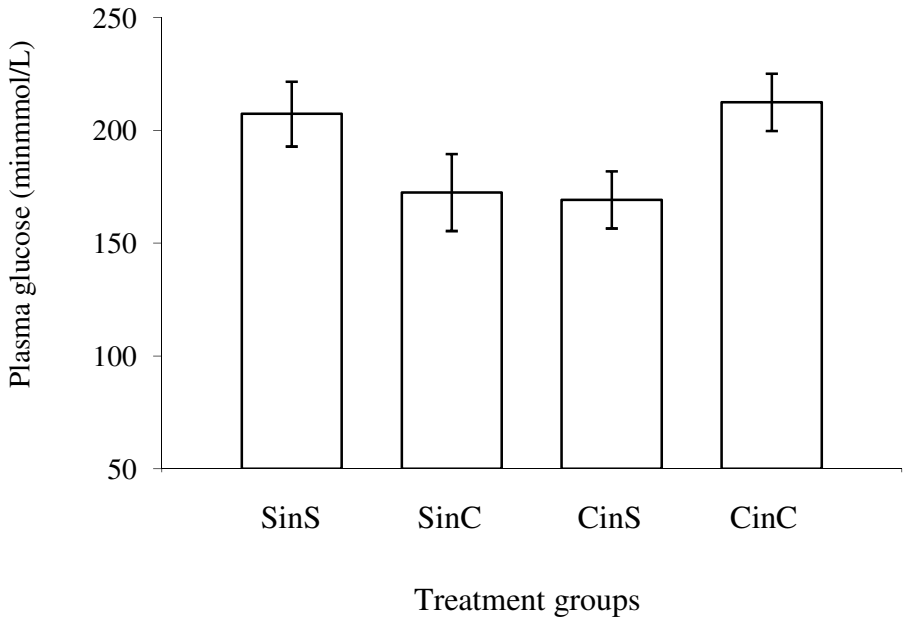

Figure 6.3. The effect of maternal uterine environment on the area under curve of glucose concentrations in female lambs at one year of age (Mean ± SE). Differences between the treatment groups are non-significant (P>0.05). S: Suffolk, C: Cheviot.

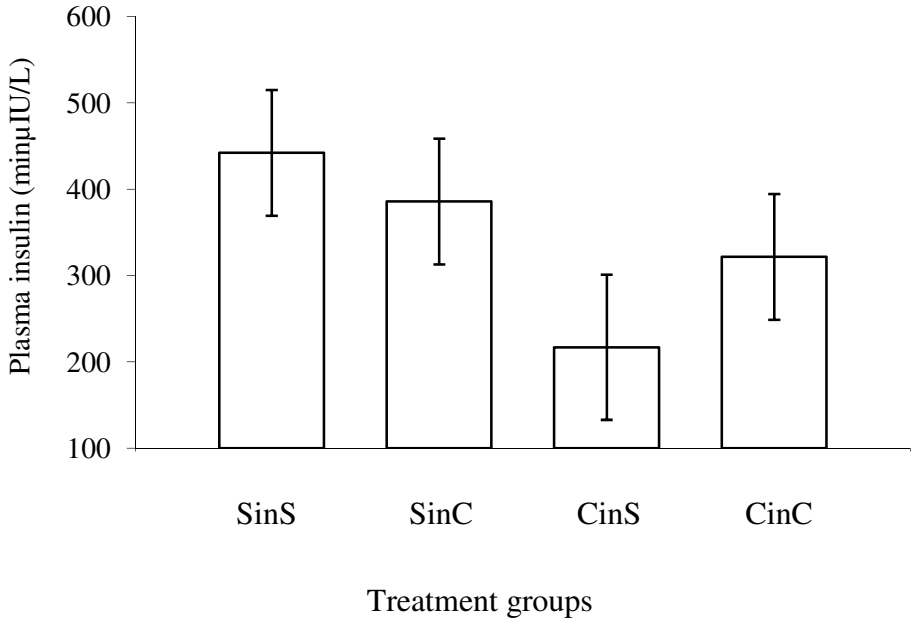

Figure 6.4. The effect of maternal uterine environment on the area under curve of insulin concentrations in female lambs at one year of age (Mean ± SE). Differences between the treatment groups are non-significant (P>0.05). S: Suffolk, C: Cheviot.

Discussion

In the present study, no significant differences were found in any of the metabolic measures of glucose or insulin between restricted uterine environment (SinC) and control (SinS) lambs either at six months or at one year of age. This suggests that the glucose metabolism of SinC and SinS lambs did not differ. These two groups however differed in birthweight from each other such that SinC lambs were significantly ($P<0.05$) lighter than SinS lambs (described in Chapter 5). The present results are in accord with the previous studies in sheep which have reported no difference in the glucose metabolism by one year of age between lambs that had normal or low birthweights (Clarke *et al.*, 2000).

Studies in humans and livestock have indicated that the impairment exhibited in the glucose metabolism during post-natal life occurs as a consequence of a perturbation of fetal development during later stages of gestation (Ravelli *et al.*, 1998; Gardner *et al.*, 2005). In the present study, the conditions of restricted uterine environment were maintained throughout the whole gestation (large genotype Suffolk lambs in small sized Cheviot dams). Hence, the lack of any difference in the glucose metabolism of SinC lambs compared to SinS lambs is indicative that SinC lambs might have experienced some abnormality of their development in the very earliest stage of pregnancy and subsequently adjusted their development accordingly; such that their later fetal development was a continuation of the early impaired development. This notion is also supported by the results of Chapter 3 and 4. The results of Chapter 3 suggested that SinC embryos were impaired in their development on Day 19 of gestation compared to SinS embryos since the morphological dimensions of SinC embryos were significantly ($P<0.05$) smaller than SinS embryos. Further, this idea is also sustained by the results of Chapter 4, in which the dimensions of SinC fetuses

were found smaller than that of SinS fetuses. This suggests that the impairment in the development of SinC fetuses was set-up at a very early stage of pregnancy and continued the same during the subsequent gestation. In other words, the impairment of *in utero* development of the lambs was present throughout the present study, whereas a perturbation of post-natal glucose metabolism requires that the impairment of fetal development is confined to late gestation. Hence, this might explain why the glucose metabolism of SinC and SinS lambs did not differ during post-natal life. This notion might also be supported by the earlier findings in rodents that the expression of glucose transporters (GLUTs) and particularly that of GLUT4 in the skeletal muscles of fetuses is not evident until mid-gestation, and increases rapidly towards later-gestation probably coinciding with placental and fetal growth (Castello *et al.*, 1993; Zhou and Bondy, 1993). These glucose transporters are implicated in the uptake of glucose from circulation and its uptake by different body cells and tissues (Watson and Pessin, 2001). Therefore, it is very unlikely that a developmental disruption of the conceptus during early period of gestation, when metabolic pathways of glucose have not been fully developed, would contribute towards any impairment in its subsequent glucose homeostasis. Epidemiological studies in the human have reported impaired glucose metabolism and prevalence of Type 2 diabetes in the post-natal life of individuals that were born with low birthweight (Barker *et al.*, 1993; Hales and Barker, 2001; Ozanne and Hales, 2002; Harding, 2003) and thus have intriguing differences from the results of the present study which could not be explained. However, earlier studies have suggested that individuals born with low birthweight as a result of maternal nutrient restriction develop disturbances in glucose metabolism, when they have access to high quality *ad libitum* nutrition (Petry *et al.,* 1997; Gluckman *et al.*, 2005a; Gluckman and

Hanson, 2006). However, in the present study, the lambs born from a restricted environment were managed on a common plane of nutrition and were not provided with different (such as high vs low quality) rations during post-natal life. Moreover, the alteration in glucose metabolism during post-natal life is an age-dependent phenomenon (Holness, 1996; Petry *et al.,* 1997; Ozanne and Hales, 2002). Therefore, future studies might be aimed to evaluate the glucose metabolism beyond one year of age in the lambs that were born from a restricted uterine environment.

The present results suggest a more rapid uptake of glucose in CinS lambs compared to CinC lambs both at six months and at one year of age. At six months of age, this was evident by the tendency of CinS lambs to have a lower area under the curve for glucose concentrations and a faster decline of glucose concentration (i.e. a higher logarithmic decay rate) than CinC lambs following a glucose challenge. Both these factors indicate a more rapid uptake of glucose from the circulation into tissues in CinS than CinC lambs. Tendencies for an enhanced insulin to glucose response and lower basal glucose to basal insulin concentration ratios in CinS lambs compared to CinC lambs also reflects an improved glucose homeostasis in the former. The glucose to insulin ratio is a reliable predictor of insulin resistance in the human (Ducluzeau *et al.*, 2003). Precise evaluation of glucose to insulin ratios requires that they are evaluated in euglycemic hyperinsulinemic clamp experiments rather than experiments that evaluate fasting glucose and insulin concentrations (Quon, 2001): nonetheless, a decreased ratio of basal glucose to basal insulin concentration in CinS lambs compared to CinC lambs is considered a good indicator of the increased glucose tolerance in the former as these findings also coincide well with other results from these two groups of lambs.

These findings suggest a faster insulin production in response to glucose and enhanced insulin activity in CinS lambs compared to CinC lambs. Insulin is an important molecule in glucose metabolism and is required to ensure the uptake of glucose from circulation (Guyton, 1991; Plum *et al.*, 2006). Therefore, a higher plasma insulin concentration and a lower glucose concentration in CinS lambs compared to CinC lambs, along with no difference in the area under the curve of insulin concentrations between the two groups is indicative of an improved glucose metabolism in CinS lambs. Similarly, at one year of age, a higher logarithmic decay rate of glucose in CinS lambs compared to CinC lambs, suggest fast uptake of glucose from the circulation to the body tissues in the former. Thus, both at six months and at one year of age, the glucose metabolism of CinS lambs appears to be improved compared to CinC lambs.

The mechanistic basis of improved glucose metabolism in CinS lambs cannot be entirely explained based on the present results. It is relevant to note that CinS fetuses remained in a luxurious environment during the whole gestation. Since the expression of glucose transporters which are required to take glucose from the circulation to the tissues, increases with advancing gestation (Castello *et al.*, 1993; Zhou and Bondy, 1993) and the onset of disturbances in post-natal glucose metabolism is a late-gestation phenomenon, (Ravelli *et al.*, 1998; Gardner *et al.*, 2005), it is plausible that in response to a luxurious environment the expression of these glucose transporters in the tissues of CinS fetuses might have increased. An increased expression of these glucose transporters in various body tissues for example adipose, skeletal muscles, endothelial cells, is implicated in enhanced disposal of glucose from the circulation and its uptake in body cells and tissues (Wallberg-Henriksson and Zierath, 2001; Watson and Pessin, 2001). Thus, an

enhanced glucose uptake in CinS lambs also suggests the involvement of various members of glucose transporters. Similarly, an improved insulin response in CinS lambs in present study is also indicative of a role of insulin receptors, since a down-regulation of insulin receptors is associated with insulin resistance in the human (Krook *et al.*, 2000). Therefore, future investigations might study the expression of various glucose transporters and insulin receptors in different tissues (particularly skeletal muscles and adipose tissue) of these lambs. However, until future research unravels these phenomena in sheep, it will not be possible to explain the precise mechanistic basis of improved glucose homeostasis in CinS lambs at this stage. However, earlier studies have suggested that the fetus adjusts its development according to its surrounding environment by considering it as a cue of its future environment: the so called 'predictive adaptive hypothesis' (Gluckman and Hanson, 2004a). The present results support the notion that a luxurious environment might have long-term health benefits during adulthood.

The differences in the metabolic measures of glucose and insulin between CinS and CinC lambs were more apparent with advancing age. At six months of age the metabolic measures of glucose or insulin between these two groups of lambs tended to be different ($P<0.10$), whereas at one year of age, these were significantly different ($P<0.05$). This is interesting because such trends are indicative of potential long-term health benefits to be born from a luxurious environment (CinS) which might have an impact on subsequent productivity. However, this can only be confirmed by undertaking future studies that evaluate the health and productivity of these lambs over a longer period of life. It could be speculated that such effects might have arisen due to differential muscular development or growth rates, since skeletal muscles are the main sites of glucose metabolism (Petersen and Shulman,

2002). Hence evaluation of body dimensions or growth rates of these lambs with advancing age could further help explain the mechanistic basis of this altered glucose metabolism. Such studies might also help to understand whether the efficient glucose metabolism in CinS lambs would help to augment their subsequent long-term post-natal growth or not.

Conclusion

A luxurious uterine environment (CinS vs CinC) improves the glucose metabolism in female lambs both at six months and at one year of age. It appears that at six months, the luxurious environment increases both the glucose tolerance and the response of insulin to glucose whereas, at one year of age, the glucose tolerance is increased without any apparent increase in the response of insulin to glucose. Given the findings of human literature, these results are surprising and needs further research. However, a restricted uterine environment (SinC vs SinS) has no effect upon the post-natal glucose metabolism of female lambs either at six months or at one year of age. Additional studies are required to further understand the mechanistic basis of these effects and their subsequent impact upon health and productivity in sheep.

Chapter 7 - The effects of antenatal environment upon post-natal growth in sheep

Abstract

Pure-breed single embryos were transferred within and reciprocally between large (Suffolk: S) and small (Cheviot: C) breeds of ewes to establish four different maternal environments (SinS (large control), SinC (restricted environment), CinS (luxurious environment) and CinC (small control)). Recipient ewes were allowed to lamb spontaneously at full term and each newborn lamb was identified at birth. Liveweight and body dimensions (head width and length, heart girth, crown-rump length, foreleg and hind leg length and femur length) were measured at birth, 3 weeks, 6 weeks, 9 weeks, 12 weeks, 6 months and 1 year of age and the growth of each lamb was also calculated at these time points. At birth, the lambs born from restricted environment (SinC) were lighter and smaller (both $P<0.05$) than SinS lambs, whereas CinS and CinC lambs did not differ from each other. During subsequent post-natal life, there were no differences ($P>0.05$) in the growth rates between SinC and SinS or between CinS and CinC lambs. The liveweight and most of the body dimensions of SinC lambs were lower ($P<0.05$) than SinS lambs in the first few months of age. No differences ($P>0.05$) were observed in liveweight or any body dimensions between CinS and CinC lambs. It was concluded that the effects of restricted uterine environment remain consistent during post-natal development of lambs; whereas a luxurious uterine environment has no effect upon post-natal growth.

Introduction

Previous chapters have described that the maternal uterine environment affects embryonic development, fetal growth, birthweight and birth size in sheep. It is now of interest to understand whether these effects persist during the post-natal life of lambs. Epidemiological studies in the human have shown that the post-natal growth of an individual is linked with its birthweigtht and the type of uterine environment that it experiences during gestation (Barker and Clark, 1997; Godfrey and Barker, 2000; Harding, 2003; Garite *et al.*, 2004; Gluckman and Hanson, 2004b; Symonds *et al.*, 2007).

Birthweight of animals is similarly affected by maternal size or uterine environment (Walton and Hammond, 1938; Dickinson *et al.*, 1962; Cowley *et al.*, 1989; Allen *et al.*, 2002b; Giussani *et al.*, 2002; Gootwine, 2005; Gardner *et al.*, 2007; Gootwine *et al.*, 2007). Production traits of livestock, such as milk, wool production, survival and carcass characteristics, are related to birthweight (Greenwood *et al.*, 1998; Bell, 2006). Moreover, growth rate of an animal is a reliable indicator of its future productivity (Kuzawa, 2007).

In studies of laboratory animals, the effects of uterine genotype upon liveweight and tail development has been reported in mice until few months after birth. The liveweight and tail lengths were found lower in mouse pups that were born from small-sized dam strain compared to those born from large sized-dam strain, and the uterine genotype influenced the post-natal development of pups for most of the period of study i.e. until 70 days after birth (Cowley *et al.*, 1989).

There have been relatively few studies in livestock which have reported similar patterns of post-natal growth. Studies of sheep have shown that reduced post-natal growth is

related to placental restriction and reduced size at birth; and that skeletal muscles remain smaller in post-natal lambs that had low birthweights (Greenwood *et al.*, 2000; De Blasio *et al.*, 2007). Greenwood *et al.* (1998) reported similar rates of growth in post-natal life between low- and normal-birthweight lambs. However, the low birthweight lambs always remain fatter than normal birthweight lambs and had limited capacity for bone and muscle growth. However, in this study there was no experimental maternal restriciton and the offspring used were either spontaneously of low birthweight or high birthweight. On the other hand, increased maternal nutrition to dams between Days 25 to 70 of pregnancy has no effect upon the number of muscle fibres, area of muscle fibre or overall quality of meat of post-natal piglets (Nissen *et al.*, 2003).

Using embryo transfer between large-sized Thoroughbred and small-sized pony horses, Allen *et al.* (2002b) reported a reduction in the birthweight and size of larger genotype Thoroughbred foals when born to a samller pony dam and, conversely, an increase in the birthweight of small genotype pony foals when born to a large Thoroughbred dam. These authors further reported no difference in the post-natal growth rates between Thoroughbred-in-pony and Thoroughbred-in-Thoroughbred or between pony-in-Thoroughbred and pony-in-pony foals (Allen *et al.*, 2004).

There is a relative paucity of literature on the effects of maternal uterine environment upon post-natal growth and, interestingly, no studies of these effects upon post-natal growth of lambs appear to have been so far undertaken. Hence, the present study was undertaken to investigate the effects of different uterine environments upon the post-natal growth of lambs.

Materials and Methods

Experimental design and animals

Four different maternal environments viz. Suffolk in Suffolk (SinS; large control), Suffolk in Cheviot (SinC; restricted environment), Cheviot in Suffolk (CinS; luxurious environment) and Cheviot in Cheviot (CinC; small control), were established as described in Chapters 3 & 5. In this study, recipient ewes carried lambs until full term. A total of 33 SinS, 29 SinC, 24 CinS and 24 CinC single-bearing pregnancies were maintained through to the end of gestation. The pregnant ewes from all treatment groups were maintained for the duration of pregnancy as one group under commercial farming conditions.

Recipient ewes were allowed to lamb spontaneously. Each group was kept in separate, but contiguous paddocks during the lambing period. Each lamb was identified at birth and within 24 h of birth it was weighed. Its head width, head length, crown-rump length, heart girth, foreleg length, hind leg length and femur length were measured at the same time (as described in Chapter 5). Subsequently, liveweight and all the aforementioned body dimensions were measured at 3, 6, 9, and 12 weeks, and 6 months, of average age. At 1 year of age, only the female lambs were available and they were weighed and their body dimensions were measured.

The growth rate of each lamb was calculated at each of these time points. Growth of lambs was calculated as the difference between the liveweight at a given time point and the liveweight at the preceding time point, divided by the number of days.

Lambs born from all treatment groups were managed along with their dams as one group until three months of age. The lambs were docked at 3 weeks of age and weaned at 3 months of age. After weaning, all lambs from the four treatment groups were

managed together as a single group until 6 months of age. At 6 months of age, all male lambs were removed from the experiment. Female lambs were then managed as one group until one year of age. All animals (ewes and lambs) used in the present study were managed at Keeble Farm, Massey University, New Zealand under the standard lowland commercial farm conditions. Animals were provided with generous allowance of ryegrass (*Lolium L.*) and white clover (*Trifolium repens L.*) pastures and had *ad libitum* access to water.

Ethical approval

All of the experimental procedures were carried out with approval of Massey University Animal Ethics Committee.

Statistical analysis

Data for lamb growth rates until one year of age were subjected to analysis of variance with respect to breed of ewe, breed of lamb and sex of lamb. Date of birth of lamb was fitted as a covariate in all of the statistical models. In addition, the growth rates of lambs at a given time point were also analysed by analysis of variance using the liveweight at the previous weighing as a covariate. Data for lamb liveweight and body dimensions from birth to 6 months were subjected to repeat measures of analysis of variance however the data of liveweights and body dimensions at one year of age were not analysed as at one year only females lambs were present and therefore the number of animals in treatment groups were low (SinS, n=11; SinC, n=9; CinS, n=6 and CinC n=8).

All two-way and three-way interactions were included in the initial model, but removed if found non-significant ($P>0.05$) and the model was then re-fitted. The interaction between the breed of ewe and breed of lamb was always kept in the model irrespective

of whether it was significant (P<0.05) or not (P>0.05), since this interaction defines the different maternal environments. For repeat measures of analysis, besides the interaction between the breed of ewe and breed of lamb, the interaction between breed of ewe, breed of lamb and time was also kept in the model irrespective of whether it was significant (P<0.05) or not (P>0.05), for similar reasons. All statistical analyses were performed using (SAS, 2005; SAS 9.1.3, SAS Institute, North Carolina, USA). Post-hoc differences between the groups were quantified using least significant differences.

Results

All ewes lambed at full term after more than 144 days of gestation (data not shown). The mortality percentage during first week of life in SinS, SinC, CinS and CinC groups was 3.23%, 20.68%, 21.73% and 18.18%, respectively, however due to low number of animal in subcells, these data were not tested statistically. There was no effect (P>0.05) of sex of lamb upon growth rate at any of the measured time points, except at 9 weeks of age when the growth rate of male lambs was more (P>0.05) than female lambs irrespective of the treatment group. After the growth of lamb at a given time point was analysed by fitting the liveweight of lamb at the previous weighing as a covariate; the effect of sex of lamb was found non-significant (P>0.05) at all the measured time points. At all time points, males lambs remained heavier and larger (P<0.05) than female lambs irrespective of the treatment group. The interaction between breed of ewe, breed of lamb and time was non-significant (P>0.05) for liveweight and all the body dimensions.

Liveweight

At birth

SinC lambs were lighter (P<0.05) at birth than SinS lambs, whereas the birthweight of CinS lambs did not differ (P>0.05) from CinC lambs (Table 7.1).

Post natal

The liveweight of SinC lambs was lower (P<0.05) than that of SinS lambs at 6 weeks and at 9 weeks of age, however, no such difference (P>0.05) was observed between these groups at 3 weeks, 12 weeks or 6 months of age (Table 7.1). On the other hand, the liveweight of CinS lambs and CinC lambs did not differ (P>0.05) at any of the time points.

Growth rate

There was no difference (P>0.05) in the growth rates between SinC and SinS or between CinS and CinC groups at any time point (3 weeks, 6 weeks, 9 weeks, 12 weeks, 6 months and 1 year of age) (Table 7.2). Likewise, there was no difference (P>0.05) in the growth rates between SinC and SinS or between CinS and CinC groups when the preceding liveweight was fitted as a covariate to the growth rate at a given time point, however, the growth rate in CinC lambs tended (P=0.08) to be higher than CinS lambs at 12 weeks of age (Table 7.3).

Table 7.1. The effects of maternal uterine environment upon liveweight of post-natal lambs up to six months of age (Mean ± SE). Within a column, means without a common superscript are significantly different from each other (P<0.05).

		Weight (kg)					
Treatment groups	n	Birth	3 weeks	6 weeks	9 weeks	12 weeks	6 months
SinS[1] (large control)	23	6.3[b] ± 0.3	14.1[b] ± 0.4	19.7[b] ± 0.5	25.5[c] ± 0.7	28.8[b] ± 1.0	37.0[b] ± 1.0
SinC[2] (restricted environment)	18	5.4[a] ± 0.3	13.2[ab] ± 0.5	17.8[a] ± 0.6	23.1[b] ± 0.8	26.6[b] ± 1.1	35.3[b] ± 1.1
CinS[3] (luxurious environment)	16	6.0[ab] ± 0.3	12.9[a] ± 0.5	17.4[a] ± 0.6	22.1[ab] ± 0.8	23.7[a] ± 1.2	30.0[a] ± 1.2
CinC[4] (small control)	13	5.3[a] ± 0.4	12.4[a] ± 0.6	16.0[a] ± 0.7	20.7[a] ± 0.9	22.8[a] ± 1.3	30.2[a] ± 1.3

Breedewe.breedlamb.time; P= 0.9, [1]Suffolk in Suffolk, [2]Suffolk in Cheviot, [3]Cheviot in Suffolk, [4]Cheviot in Cheviot.

Table: 7.2. The effects of maternal uterine environment upon the growth rate of post-natal lambs from birth to one year of age (Mean ± SE). Within a column, means without a common superscript are different from each other (P<0.05).

						Growth rate (kg/day)						
Treatment groups	n	At 3 weeks of age,	n	At 6 weeks of age,	n	At 9 weeks of age,	n	At 12 weeks of age,	n	At 6 months of age,	n	At one year of age,
SinS[1] (large control)	29	0.38^{b} ± 0.02	28	0.24 ± 0.02	26	0.26 ± 0.01	25	0.15^{b} ± 0.02	23	0.09^{b} ± 0.006	11	0.06 ± 0.02
SinC[2] (restricted environment)	22	0.36^{ab} ± 0.02	22	0.21 ± 0.02	22	0.25 ± 0.02	22	0.16^{b} ± 0.02	18	0.09^{b} ± 0.007	9	0.07 ± 0.02
CinS[3] (luxurious environment)	18	0.32^{a} ± 0.02	18	0.22 ± 0.02	18	0.22 ± 0.02	17	0.08^{a} ± 0.02	16	0.07^{a} ± 0.007	6	0.02 ± 0.03
CinC[4] (small control)	16	0.33^{ab} ± 0.02	16	0.18 ± 0.02	16	0.23 ± 0.02	16	0.12^{ab} ± 0.02	13	0.07^{ab} ± 0.008	8	0.08 ± 0.03

[1]Suffolk in Suffolk, [2]Suffolk in Cheviot, [3]Cheviot in Suffolk, [4]Cheviot in Cheviot.

Table: 7.3. The effects of maternal uterine environment on the growth rate of post-natal lambs, with preceding liveweight fitted as covariate, from birth to one year of age (Mean ± SE). Within a column, means without a common superscript are different from each other (P<0.05).

						Growth rate (kg/day)						
Treatment groups	n	At 3 weeks of age,	n	At 6 weeks of age,	n	At 9 weeks of age,	n	At 12 weeks of age,	n	At 6 months of age,	n	At one year of age,
SinS[1] (large control)	29	0.36[b] ± 0.02	28	0.23 ± 0.02	26	0.24 ± 0.01	25	0.13[b] ± 0.02	23	0.09[b] ± 0.006	11	0.07 ± 0.02
SinC[2] (restricted environment)	22	0.38[ab] ± 0.02	22	0.21 ± 0.02	22	0.25 ± 0.01	22	0.16[ab] ± 0.02	18	0.09[b] ± 0.007	9	0.09 ± 0.03
CinS[3] (luxurious environment)	18	0.31[a] ± 0.02	18	0.23 ± 0.02	18	0.23 ± 0.01	17	0.09[a] ± 0.02	16	0.06[a] ± 0.007	6	-0.0002 ± 0.03
CinC[4] (small control)	16	0.34[ab] ± 0.02	16	0.19 ± 0.02	16	0.25 ± 0.01	16	0.14[ab] ± 0.02	13	0.07[a] ± 0.008	8	0.06 ± 0.03

[1]Suffolk in Suffolk, [2]Suffolk in Cheviot, [3]Cheviot in Suffolk, [4]Cheviot in Cheviot.

Lamb body dimensions

At birth

All parameters of body morphology viz. lamb head width, head length, crown-rump length, heart girth, foreleg length, hind leg length and femur length, were smaller ($P<0.05$) in SinC lambs than in SinS lambs. On the other hand, the body dimensions of CinS and CinC lambs did not differ ($P>0.05$) from each other (described in detail in Chapter 5).

Post-natal

Head width (Table 7.4), head length (Table 7.5) and crown-rump length (Table 7.6) of SinC lambs remained lower ($P<0.05$) than SinS lamb until 12 weeks of age. However no such differences ($P>0.05$) were present in these body parameters between SinC and SinS lambs at 6 months of age. Heart girth of SinC lambs tended ($P=0.08$) to be lower than SinS at 3 weeks of age and was lower ($P<0.05$) at 6 weeks of age; however, no differences ($P>0.05$) were observed at 9 weeks, 12 weeks or at 6 months of age (Table 7.7). Foreleg (Table 7.8) and hind leg (Table 7.9) lengths of SinC lambs did not differ ($P<0.05$) from those of SinS lambs at any time. Femur length of SinC lambs was less ($P<0.05$) than that of SinS lambs up to 9 weeks of age but such differences were not apparent ($P>0.05$) after 12 weeks of age (Table 7.10).

None of the body dimensions differed ($P>0.05$) between CinS and CinC lambs at any time, however, the heart girth of CinS lambs tended ($P=0.07$) to be higher than CinC lambs at birth (data not shown).

Table 7.4. The effects of maternal uterine environment upon head width of postnatal lambs up to six months of age (Mean ± SE). Within a column, means without a common superscript are different from each other (P<0.05).

		Head width (cm)					
Treatment groups	n	Birth	3 weeks	6 weeks	9 weeks	12 weeks	6 months
SinS[1] (large control)	23	12.96[b] ± 0.15	14.86[b] ± 0.18	16.09[b] ± 0.21	17.05[b] ± 0.21	17.27[b] ± 0.19	18.03[b] ± 0.24
SinC[2] (restricted environment)	18	11.96[a] ± 0.18	14.16[a] ± 0.21	15.37[a] ± 0.24	16.21[a] ± 0.24	16.64[a] ± 0.22	17.74[ab] ± 0.27
CinS[3] (luxurious environment)	16	12.64[b] ± 0.19	14.30[a] ± 0.22	15.46[ab] ± 0.26	16.27[a] ± 0.25	16.33[a] ± 0.24	17.14[a] ± 0.28
CinC[4] (small control)	13	12.71[b] ± 0.21	14.24[a] ± 0.25	15.24[a] ± 0.29	16.06[a] ± 0.28	15.94[a] ± 0.27	17.41[ab] ± 0.32

Breedewe.breedlamb.time; P= 0.8, [1]Suffolk in Suffolk, [2]Suffolk in Cheviot, [3]Cheviot in Suffolk, [4]Cheviot in Cheviot.

Table 7.5. The effects of maternal uterine environment upon head length of postnatal lambs up to six months of age (Mean ± SE). Within a column, means without a common superscript are different from each other (P<0.05).

		Head length (cm)					
Treatment groups	n	Birth	3 weeks	6 weeks	9 weeks	12 weeks	6 months
SinS[1] (large control)	23	17.77[b] ± 0.23	21.16[b] ± 0.26	22.75[b] ± 0.24	24.53[b] ± 0.24	24.66[b] ± 0.25	27.03 ± 0.27
SinC[2] (restricted environment)	18	16.77[a] ± 0.27	20.35[a] ± 0.30	21.63[a] ± 0.28	23.69[a] ± 0.27	23.55[a] ± 0.29	26.52 ± 0.32
CinS[3] (luxurious environment)	16	17.95[b] ± 0.28	20.54[ab] ± 0.32	21.98[a] ± 0.29	23.98[ab] ± 0.29	23.64[a] ± 0.31	26.33 ± 0.33
CinC[4] (small control)	13	17.68[b] ± 0.32	20.95[ab] ± 0.36	21.65[a] ± 0.33	23.84[ab] ± 0.32	23.65[a] ± 0.34	26.18 ± 0.37

Breedewe.breedlamb.time; P= 0.9, [1]Suffolk in Suffolk, [2]Suffolk in Cheviot, [3]Cheviot in Suffolk, [4]Cheviot in Cheviot.

Table 7.6. The effects of maternal uterine environment upon CRL[1] of post-natal lambs up to six months of age (Mean ± SE). Within a column, means without a common superscript are significantly different from each other (P<0.05).

		CRL (cm)					
Treatment groups	n	Birth	3 weeks	6 weeks	9 weeks	12 weeks	6 months
SinS[2] (large control)	23	60.26 ± 0.88	80.24[c] ± 0.91	88.13[c] ± 1.18	96.07[c] ± 1.07	100.92[c] ± 1.30	110.79[b] ± 1.13
SinC[3] (restricted environment)	18	58.24 ± 1.02	76.79[b] ± 1.06	84.63[a] ± 1.35	91.21[b] ± 1.23	95.93[b] ± 1.49	108.60[b] ± 1.30
CinS[4] (luxurious environment)	16	58.22 ± 1.07	73.35[a] ± 1.11	79.60[b] ± 1.43	86.41[a] ± 1.30	88.63[a] ± 1.57	100.10[a] ± 1.37
CinC[5] (small control)	13	57.40 ± 1.22	74.36[ab] ± 1.26	78.83[b] ± 1.60	86.40[a] ± 1.47	88.83[a] ± 1.76	99.44[a] ± 1.54

Breedewe.breedlamb.time; P= 0.7, [1] Crown-rump length, [2]Suffolk in Suffolk, [3]Suffolk in Cheviot, [4]Cheviot in Suffolk, [5]Cheviot in Cheviot.

Table 7.7. The effects of maternal uterine environment upon heart girth of postnatal lambs up to six months of age (Mean ± SE). Within a column, means without a common superscript are different from each other (P<0.05).

		Heart girth (cm)					
Treatment Groups	n	Birth	3 weeks	6 weeks	9 weeks	12 weeks	6 months
SinS[1] (large control)	23	42.99[b] ± 0.68	58.68 ± 0.72	65.36[b] ± 0.83	71.07[b] ± 0.84	74.27[b] ± 0.93	76.81[b] ± 0.80
SinC[2] (restricted environment)	18	40.93[a] ± 0.79	56.76 ± 0.83	62.87[a] ± 0.96	62.23[ab] ± 0.96	72.82[ab] ± 1.06	75.76[b] ± 0.93
CinS[3] (luxurious environment)	16	43.04[ab] ± 0.83	56.85 ± 0.87	62.51[a] ± 1.01	69.13[ab] ± 1.02	71.63[ab] ± 1.12	71.45[a] ± 0.98
CinC[4] (small control)	13	40.78[ab] ± 0.94	56.36 ± 0.99	62.28[a] ± 1.14	68.05[a] ± 1.14	70.05[a] ± 1.26	71.62[a] ± 1.10

Breedewe.breedlamb.time; P= 0.9, [1]Suffolk in Suffolk, [2]Suffolk in Cheviot, [3]Cheviot in Suffolk, [4]Cheviot in Cheviot.

Table 7.8. The effects of maternal uterine environment upon foreleg length of post-natal lambs up to six months of age (Mean ± SE). Within a column, means without a common superscript are significantly different from each other (P<0.05).

		Foreleg length (cm)					
Treatment groups	n	Birth	3 weeks	6 weeks	9 weeks	12 weeks	6 months
SinS[1] (large control)	23	42.54[c] ± 0.45	51.04[b] ± 0.43	53.26[b] ± 0.53	56.71[c] ± 0.62	61.73[b] ± 0.57	61.56[b] ± 0.51
SinC[2] (restricted environment)	18	40.59[b] ± 0.52	50.09[b] ± 0.50	52.09[b] ± 0.62	55.65[bc] ± 0.71	61.15[b] ± 0.66	62.40[b] ± 0.59
CinS[3] (luxurious environment)	16	39.98[ab] ± 0.55	46.98[a] ± 0.52	49.76[a] ± 0.65	53.70[ab] ± 0.75	57.88[a] ± 0.69	56.51[a] ± 0.62
CinC[4] (small control)	13	38.91[a] ± 0.62	46.83[a] ± 0.60	48.95[a] ± 0.73	51.75[a] ± 0.84	57.18[a] ± 0.78	55.83[a] ± 0.70

Breedewe.breedlamb.time; P= 0.6, [1]Suffolk in Suffolk, [2]Suffolk in Cheviot, [3]Cheviot in Suffolk, [4]Cheviot in Cheviot.

Table 7.9. The effects of maternal uterine environment upon hind leg length of post-natal lambs up to six months of age (Mean ± SE). Within a column, means without a common superscript are significantly different from each other (P<0.05).

		Hind leg length (cm)					
Treatment groups	n	Birth	3 weeks	6 weeks	9 weeks	12 weeks	6 months
SinS[1] (large control)	23	41.25[c] ± 0.40	47.29[b] ± 0.36	50.83[b] ± 0.43	54.61[b] ± 0.46	58.81[b] ± 0.47	60.03[b] ± 0.44
SinC[2] (restricted environment)	18	39.67[b] ± 0.46	46.75[b] ± 0.42	50.17[b] ± 0.50	53.75[b] ± 0.53	58.36[b] ± 0.53	61.00[b] ± 0.51
CinS[3] (luxurious environment)	16	38.42[ab] ± 0.48	44.33[a] ± 0.45	47.54[a] ± 0.53	50.42[a] ± 0.56	54.45[a] ± 0.56	54.83[a] ± 0.53
CinC[4] (small control)	13	37.61[a] ± 0.55	43.61[a] ± 0.50	46.92[a] ± 0.59	49.69[a] ± 0.63	53.23[a] ± 0.63	54.50[a] ± 0.60

Breedewe.breedlamb.time; P= 0.7, [1]Suffolk in Suffolk, [2]Suffolk in Cheviot, [3]Cheviot in Suffolk, [4]Cheviot in Cheviot.

Table 7.10. The effects of maternal uterine environment upon femur length of post-natal lambs up to six months of age (Mean ± SE). Within a column, means without a common superscript are significantly different from each other (P<0.05).

		Femur length (cm)					
Treatment groups	n	Birth	3 weeks	6 weeks	9 weeks	12 weeks	6 months
SinS[1] (large control)	23	12.96[b] ± 0.28	16.68[b] ± 0.30	19.01[c] ± 0.27	21.64[c] ± 0.34	22.11[b] ± 0.36	24.18[b] ± 0.35
SinC[2] (restricted environment)	18	11.63[a] ± 0.33	15.77[a] ± 0.34	17.91[b] ± 0.32	20.27[b] ± 0.39	21.61[b] ± 0.41	24.30[b] ± 0.40
CinS[3] (luxurious environment)	16	11.80[a] ± 0.34	14.87[a] ± 0.36	16.74[a] ± 0.34	19.24[ab] ± 0.42	20.34[a] ± 0.44	21.96[a] ± 0.42
CinC[4] (small control)	13	11.66[a] ± 0.39	14.89[a] ± 0.41	16.78[a] ± 0.38	18.74[a] ± 0.47	19.78[a] ± 0.49	22.24[a] ± 0.47

Breedewe.breedlamb.time; P= 0.8, [1]Suffolk in Suffolk, [2]Suffolk in Cheviot, [3]Cheviot in Suffolk, [4]Cheviot in Cheviot.

Discussion

The key finding of this study was that the post natal growth rate of low birthweight (SinC) and high birthweight (SinS) lambs did not differ ($P>0.05$) from each other. The liveweight and body dimensions of SinC lambs remained lower ($P<0.05$) than SinS lambs until a few months of age. The average liveweight of SinC lambs was 900g lower than SinS lambs at birth, whereas at six months of age, this difference was 1.7 kg. However, no significant difference ($P<0.05$) was observed in the liveweight between these two groups at 12 weeks and 6 months of age, but liveweight of SinC lambs were consistently lower than SinS lambs. This is due in part to the increasing variance associated with the increase in mean such that no statistical difference could be detected. The findings of the present experiment indicate that the effects of restricted uterine environment remain consistent during post-natal growth in lambs. This also suggests that SinC lambs had become unable to attain the growth rates or carcass characteristics of SinS lambs at least up to one year of age, despite very similar nutritional environments. If so, this would imply that a restricted uterine environment has had a consistent (but non-significant) impact upon the subsequent growth and productivity of lambs that develop in such an environment.

A difference of 1.7 kg liveweight between SinC and SinS lambs could be of considerable economical importance. Broadly, this would mean nearly 1 kg of difference in carcass weight. This value might appear numerically small per lamb; however, for a flock of 1000 lambs, this would mean a deficit of 1000 kg of carcass, which is significant, indicating potential economical importance of the effects of uterine environment upon lamb production.

The consistent difference of liveweight between SinC and SinS lambs from birth to 6 months of age might also be of relevant biological importance. This is indicative of some permanent changes in the major tissues (e.g. muscles and bone) of lambs born from a restricted uterine environment. Interestingly, studies of muscle devlopment in post-natal lambs (Tulloh *et al.*, 1986; Greenwood *et al.*, 2000) indicate that low birthweight lambs have a limited capacity to utilise available nutrients. This is plausible because such lambs have less muscle mass. Since, the skeletal muscles are the main sites for the utilisation of fatty acids and glucose (Petersen and Shulman, 2002); fewer muscular cells could mean a lower capacity and hence a lower growth. If so, undertaking a study with large sample size might actually reveal the overt differences between the liveweight of SinC and SinS lambs with advancing age.

It could be argued that the low weight gain in SinC lambs might be due to Cheviot ewes providing insufficient milk to the larger genotype Suffolk lambs. However, the findings that the liveweight of SinC lambs was equal to that of SinS lambs at 3 weeks of age does not support this notion. Moreover, it is also relevant to note that even CinS lambs did not have significantly higher rates of weight gain than CinC lambs. Therefore, if the milk supply from the dam was to be a major factor for early post-natal growth, then a higher growth rate would have been expected in Cheviot lambs born to Suffolk dams than in Cheviot lambs born to Cheviot dams. Based on these observations, it would seem that milk production was unlikely to cause a differential growth rate between the breed combinations.

Earlier studies of the effects of the size of the dam upon post-natal growth (Allen *et al.*, 2004) have also reported no difference in the growth rates between the low birthweight foals born from restricted (Thoroughbred-in-pony) and those born from control (Thoroughbred-in-Thoroughbred) environments. Moreover, with advancing

age, the patterns of differences in liveweight, and body dimensions observed in the above study between (Thoroughbred-in-Thoroughbred) and (Thoroughbred-in-pony) were similar to that between SinC and SinS lambs in the present study. The results of the present study are also in accord with earlier findings in mice (Cowley *et al.*, 1989), in which lower liveweights and tail lengths were present in pups that were born to a small-sized dam strain compared to control.

The pattern of post-natal growth in SinC lambs might be explicable in terms of predictive adaptive response, such that the fetus perceives its *in utero* conditions as a reliable indicator for its subsequent environment to which it might be exposed during adulthood and hence adjusts its development accordingly (Gluckman and Hanson, 2004a). If so, this would suggest that SinC lambs might have adjusted their development according to their restricted uterine environment during their fetal stage and when reared under optimal nutrient conditions after birth, these lambs grew at an equal rate to that of control (SinS). This notion is also supported by the results of Chapter 3 and 4, in which the embryonic and fetal development of SinC fetuses were found impaired compared to SinS animals.

No statistically significant differences were observed between the growth rates, liveweight or body dimensions between CinS and CinC lambs. The present study thus has some intriguing differences compared to the earlier investigation by Allen *et al.* (2004). At three years of age, pony-in-Thoroughbred horses (luxurious uterine environment) were heavier and most of their body dimensions were bigger compared to pony-in-pony horses (control), which is in contrast to the differences, between CinS (luxurious uterine environment) and CinC lambs (control) in the present investigation. The differences in these two studies could be attributed to the differences in the birthweight between the corresponding treatment groups. Pony-in-

Thoroughbred foals were heavier and larger compared to pony-in-pony foals at birth unlike CinS and CinC lambs, which did not differ from each other at birth (Table 7.1).

Improved glucose homeostasis was observed in CinS lambs compared to CinC lambs at six months and more clearly at one year of age (Chapter 6). To understand, whether this would assist CinS lambs to augment their growth and productivity at later stages of life in comparison to CinC lambs and consequently long-term health benefits from being born from a luxurious environment, needs to be investigated in future studies.

Conclusion

To date no studies have reported the effects of uterine environment, as determined by the relative sizes of dam and fetus, upon post-natal development in sheep. The present study shows that lambs born from a restricted uterine environment grow at an equal rate to those born from a control. That is there is no catching-up of impaired body dimensions of an animal born from a restricted uterine environment. A restricted uterine environment affects post-natal development of lambs in only a few parameters at one year of age. A luxurious uterine environment did not have any beneficial effect upon the post-natal growth of lambs.

Chapter 8 - General discussion

The studies presented in this thesis were based upon the hypothesis that the effects of uterine environment upon the development of lambs are initiated during the early embryonic stage and persist through birth into subsequent post-natal life. This hypothesis was investigated by examining the effects of uterine environment upon embryonic, fetal, neonatal and postnatal development and glucose metabolism of lambs by using a model based on dams of genetically dissimilar mature body size. To accomplish this objective, different uterine environments were established by transferring pure-breed Suffolk or Cheviot embryos, both within and reciprocally between large Suffolk and small Cheviot ewes resulting in: restricted environment (large fetal genotype in small uterus, Suffolk in Cheviot: SinC), luxurious enviromnent (small fetal genotype in large uterus Cheviot in Suffolk: CinS) and large (SinS) and small (CinC) control environments.

In this thesis a method to collect and morphologically evaluate Day 19 embryos was developed. Both restricted and luxurious uterine environments affected morphogenesis of Day 19, peri-implantation, embryos and their trophoblasts. Development was impaired in a restricted (SinC) compared to control (SinS) uterine environment, and was enhanced in a luxurious environment (CinS) compared to its control (CinC). This suggest that the uterine environment can override the genetic potential for development of these embryos. Moreover, the findings that trophoblasts of SinC embryos were smaller than their control (SinS) and that the trophoblasts of CinS embryos were bigger than their control (CinC), and that the differences in the number of binucleate cells varied in parallel with the differences in the size of the embryo strongly indicated that the trophoblast is the key tissue which is involved in

this early embryo-maternal interaction. The smaller size of SinC embryos observed on Day 19 continued to be present at birth and into subsequent post-natal life. This was evident by the small body dimensions of SinC lambs at birth and their lower birthweight compared to SinS lambs. The findings of lower body mass index and birthweight to head width ratio of SinC lambs compared to SinS lambs supports the idea that the development of SinC fetuses was adversely affected by their restricted uterine environment. There was no difference in post-natal growth rate between these two groups of lambs. This, together with the lower liveweight of SinC lambs (significantly lower up to 9 weeks age and consistently lower, albeit non-significantly, up to the end of study period at 6 months of age) compared to SinS lambs further suggests that the effects of a restricted uterine environment that were evident at birth were perpetuated into subsequent post-natal life. Conversely, the fetuses that developed in a luxurious uterine environment (CinS) appeared to have not benefitted from their luxurious environment. Thus, whilst CinS fetuses were larger than controls on Day 19, by birth there were no differences, in birthweight or body dimensions between the two groups of lambs. Likewise, the equal growth rates of CinS and CinC lambs during post-natal life further suggests that a luxurious antenatal environment did not augment their potential for growth.

Earlier studies in horses (Allen *et al.*, 2002b; Allen *et al.*, 2004) have reported that whilst the birthweight and body dimensions of foals were reduced when born to a restricted uterine environment compared to the control, these traits were enhanced when foals were born from a luxurious uterine environment compared to control. The present studies thus have some intriguing similarities and differences with earlier experiments upon the effects of dam-size in the horse. This further suggests that in different species, the effects of uterine environment upon development of offspring

are not exactly the same and thus indicate a need to investigate such effects in each individual species until the reasons for these differences are understood.

There were no differences in placental characteristics between SinC and SinS placentas or between CinS and CinC placentas on Day 120 of gestation (assessed by sonography) or at birth, whereas there were substantial differences between the trophoblasts on Day 19. Thus, binucleate cell number was lower in SinC than in SinS, but higher in CinS than in CinC; binucleate cell area tended to be lower in SinC than SinS, but was higher in CinS than in CinC; and epithelial cell height was higher in CinS than CinC. It seems that there had been compensatory growth of SinC placentas to support fetal growth in a restricted uterine environment (SinC vs SinS). Conversely, the placenta of CinS and CinC did not differ with advancing gestation, possibly due to a lack of incremental substrate demand by the fetuses in a luxurious uterine environment (CinS). This notion is supported by the concentrations of FFA in the dams of the different groups, such that Cheviot ewes with a Suffolk fetus (SinC ewes) had higher FFA concentrations than did SinS ewes with advancing gestation; whereas CinS ewes always had lower FFA concentration than CinC ewes. An increase in maternal plasma FFA concentration is indicative of an increased utilisation of her body fat reserve to meet with her own energy requirements and to spare glucose for fetal growth (Herrera, 2002). Taken together, the present study plus the work of Allen *et al.* (2002a), Wilsher and Allen (2002), Vonnahme (2006) highlighted the importance of the placenta as a mediator of feto-maternal dialogue in growth regulation.

The effects of uterine environment upon the post-natal glucose metabolism of lambs were interesting. A restricted uterine environment had no effect upon the glucose metabolism. This was surprising when compared to the human literature, in which

there is a clear link between an impaired uterine environment and subsequent glucose metabolism of the offspring (Barker *et al.*, 1993; Ozanne and Hales, 2002; Harding, 2003). The present study supports the notion that besides the type of uterine environment, stage of gestation and a perhaps a mismatch between the antenatal and post-natal environment are some critical factors that determine the outcome of the uterine environment upon the metabolism of post-natal lambs (as shown by no differences in glucose metabolism between SinC and SinS lambs). However, more studies are required to further investigate the relationship between post-natal development and the timing of the insult to the conceptus during gestation and the mechanistic basis of these effects. On the other hand, a luxurious uterine environment improved the glucose metabolism of lambs both at six months and at one year of age. No previous studies were found that had investigated the effects of luxurious uterine environment upon post-natal glucose metabolism of lambs.

The results of these studies are of some significance to animal husbandry. For example, the average weight of a lamb born from a restricted uterine environment (SinC) was 900 g less than that of a control (SinS). The birthweight of an individual is related to its subsequent growth and productivity (Greenwood *et al.*, 1998; Bell, 2006). Thus, a difference in birthweight of this magnitude is likely to be of some economical importance. Moreover, with advancing gestation and at least until one year of age (the end period of present study) the growth of SinC and SinS lambs did not differ. The average difference in the bodyweight between SinC and SinS lambs at six months of age was 1.7 kg, which was nearly the twice of the difference at birth. This not only suggests that the effects of uterine environment that were evident at birth perpetuated in subsequent post-natal development of lambs but, potentially, towards lowered productivity of such lambs and hence, economic losses to the

farming sector. This is also indicative of an underlying biological impairment of SinC lambs. Thus, it appears that early embryonic period might be of significant importance in determining subsequent development of lambs. This suggests a need for re-consideration of the current practices of animal husbandry, where the general tendency is to maintain the animals on a maintenance ration after conception and starting the pregnancy allowance only in the later gestation. However, the approach should perhaps be to start supplementing the pregnancy ration from the early embryonic stage (Barry *et al.,* 2004), and continue the same throughout the subsequent gestation. These results thus raise important questions about the benefit-cost ratio of current practices of commercial farming to start supplementing pregnancy ration to dams at later stages of gestation. The question that appears to be carefully considered is whether to refrain from starting the pregnancy ration in the early stage of pregnancy to avoid extra costs of nutrition and then bear losses due to low birthweight and mediocre productivity of offspring, or to supplement pregnancy ration during the early embryonic period and avoid long-term economic losses by achieving good birthweight and subsequent productivity.

The glucose metabolism results suggest that there might be long-term health benefits to being born from a luxurious environment, which might have consequent implications upon productivity particularly with advancing age. However, a lack of enhancement in the neonatal and post-natal development in lambs born from a luxurious environment perhaps indicates that rearing of small genotype fetuses in large genotype dams might not be a beneficial livestock practice. Understanding the cost-effectiveness of these regimens in livestock industries necessitates that the growth of such offspring should be evaluated for a longer-term beyond one year of age. Moreover, maintaining animals in luxurious environment cannot generally be

adopted as an economical beneficial practice, unless the benefits from the productivity outweigh the costs of rearing animals luxuriously. However, if these results could be applied to the human, it seems that a luxurious uterine environment might have long-term health benefits, as an improved glucose metabolism would help reduce the onset of glucose intolerance and consequently lower risk of Type 2 diabetes. No doubt further studies are requires to investigate such effects.

It would be interesting to see whether similar responses to those of the current experiments could also be obtained from cattle. The current practices of cattle production or implementation of breeding policies in cattle usually do not involve taking into consideration the uterine environmental effects upon fetal development. Moreover, in the majority of countries cows are inseminated and conceive when still in negative energy balance. This means that their uterine environment is physiologically impaired for a developing embryo. It is plausible that this would affect not only the development of embryo and fetus, but the concentration of hormones such as placental lactogen, progesterone, insulin and IGF-1 would be altered, which could also lead to impairment in development of mammary tissues, milk yield and composition (Schmidt, 1966; Erb, 1976; Griinari *et al.*, 1997; Taylor *et al.*, 2004). This could result in double disadvantage through economic losses due to poor milk yield per cow and birth of smaller and lighter calves. Furthermore, poor development of calves might be perpetuated during subsequent post-natal development and passed to the next generation. This could result in the onset of a cycle of low birthweight, impaired health and mediocre productivity (Van Der Linden *et al.*, 2009; Koch, 1972). It could be possible that most of the current problems of post-natal productivity and reproduction in cows might have actually originated during their fetal stage depending upon the uterine environmental

conditions. Moreover, a general prevailing practice in the farming community to rear animals as non-pregnant until pregnancy is confirmed means that the critical period of early embryonic development is passed without supplementing animals with pregnancy ration. These results thus also indicate a simultaneous need to develop quick and reliable methodologies of early pregnancy diagnosis, so that farmers can be made aware of the pregnancy status of their animals at the earlier possible after insemination.

An understanding of the detailed mechanistic basis of the effects of uterine environment upon development of the peri-implantation conceptus awaits future research. However, the present results suggest that one mechanism by which uterine environment could affect development of the conceptus is by regulating the formation of binucleate cells. Binucleate cells produce placental lactogen (Wooding *et al.*, 1980) which acts as a trophic factor for early embryonic growth (Stewart *et al.*, 2000) and regulates fetal growth by influencing maternal and fetal metabolism, acting as a partitioning agent to regulate nutrient supply to the fetus and modulating fetal metabolism to use substrates and affecting mammogenesis (Anthony *et al.*, 1995b; Kann *et al.*, 1999). Therefore, it is conceivable that the number of binucleate cells would affect production of oPL, such that in a restricted environment, the production of oPL might be inadequate and *vice versa*. Hence, future studies should be aimed at evaluating concentrations of oPL in restricted, luxurious and control environments during the peri-implantation period. This, however, was not done in the present study, since in the early gestation the concentration of oPL in peripheral circulation is extremely low and undetectable by current assay methods. It might be possible to collect blood samples from utero-ovarian vein, but it is a very cumbersome procedure. However, other *in vitro* methods such as evaluating

expression of oPL in trophoblast tissues could have been used, but without prior knowledge of the effects of uterine environment upon embryonic and trophoblast development, doing so was not considered cost-effective or practical. Hence, it was decided to first examine whether the uterine environment affects morphogenesis of peri-implantation conceptuses or not.

In very early gestation, it is exceedingly unlikely that the metabolic demands of the conceptus or the physical space of the uterus directly limit conceptus development. In other words, the restriction of embryonic development that is imposed by a restricted uterine environment is dependent upon factors other than direct metabolic or space limitations. Differences in the development are more likely to reflect differential involvement or expression of pregnancy signal molecules such as interferon tau, lactoferrin, IGFs, interleukins and prostaglandins in different uterine environments (Thatcher *et al.*, 1984; Flint, 1995; Roberts *et al.*, 1996; Bazer *et al.*, 1997; Leung *et al.*, 2000; Robinson *et al.*, 2000b; Yanaihara *et al.*, 2000; Spencer *et al.*, 2004a). The findings that the uterine environment appears to have overridden the genetic potential of embryos for development indicate that the genome of these embryos might have been modified to survive and develop under impaired uterine conditions. Therefore, the other plausible mechanisms that require investigation in future studies are maternal epigenetic mechanisms. Earlier studies with laboratory animals have suggested possibility of modifications to DNA in response to external stimuli, or an alteration or absence of imprinting of genes, as a potential mechanism that interfere with conceptus development (Frank *et al.*, 2002; Jaenisch and Bird, 2003). These modifications can pass from one generation of cells to another and thus involve the long-term silencing of gene expression (Wolff *et al.*, 1998; Bergh *et al.*, 1999; Cooney *et al.*, 2001; Futscher *et al.*, 2002). This suggests that once set-up,

such alterations could persist permanently during subsequent life. Therefore, it is conceivable that such modifications might have led to the altered development of embryos in the present study, but its detailed understanding requires further research.

One approach to start understanding the aforementioned mechanisms would be to investigate the protein profile of trophoblasts and surrounding medium in different uterine environments. These changes should be identifiable in the trophoblast tissue, since the present study and few earlier studies have suggested the trophoblast as the key mediator of embryo-maternal interaction. Since, proteins are directly encoded by nucleic acids (Marth, 2008); any epigenetic modifications will result in an altered expression of protein molecules. Moreover, studies of proteins have the advantage of allowing investigation of any post-translational modification(s). The identification of proteins implicated in conceptus-maternal interactions might even help in developing interventions to overcome the adverse effects of uterine environment upon fetal development.

It may be that one of the mechanisms by which uterine environment modulates fetal development is by altering mid-gestation concentrations of oPL and progesterone, since these were both positively correlated with birthweight and crown-rump length. It is also possible that fetal development was affected by alterations in the supply of glucose from the mother to the fetus. Moreover, there was no difference in the concentration of IGF-1 in maternal plasma between SinC and SinS ewes; it was higher in CinS ewes than CinC ewes during early and mid-gestation but did not differ later in gestation. Thus, whether the fetal development was affected by the supply to glucose as a direct effect, or whether it was mediated through changes in the concentrations of IGF-1 in the fetus are not clear.

The measurement of fetal dimensions and placental parameters during mid-gestation (Chapter 4) was limited by the use of sonography. In future studies, examination of the mid-gestational fetal body dimensions, key fetal organs and their cellular details, expression of various humoral, growth and metabolic factors in fetal tissues and evaluation of additional placental characteristics (weight of placentomes, type of placentomes, placental volume, placental surface area, weight of fetal membranes, area of feto-maternal contact and histological evaluation) and functions might help to understand the differential effects of uterine environment upon fetal growth and its mechanistic basis. The placenta is a key tissue of feto-maternal interaction and it is plausible that in order to regulate fetal development, uterine environment first might regulate the placental structure or function. Future studies might also be aimed at measuring hormones such as oPL, IGF, insulin and glucose in fetal and maternal circulations, along with measurement of placental lactate and amino acids, as this would help to understand in depth the regulatory role of these hormones in feto-maternal interaction. Given that the placenta secretes these various anabolic hormones and substrates; measurement of these factors across both side of the placenta would also help to examine the biochemical compensatory role of the placenta to support fetal growth. Similarly, evaluating the expression of glucose transporters (GLUTs) in placental cells would help to understand the role of placenta as a mediator of onset of glucose metabolic pathway of the fetus under different uterine conditions.

Further, examining the vital organs, assessing carcass characteristics and composition and evaluation of humoral and metabolic profile and assessment of reproductive and lactation performance of post-natal lambs would help to further understand the effects of uterine environment upon lamb growth along with their

possible mechanistic basis. This is also important in the context of earlier studies which have also shown that the nutritional restriction around conception or very early in gestation might result in disturbances in the metabolic and cardiovascular pathways during the post-natal life of offspring, with or without having an effect on the birthweight and size (Ravelli *et al.,* 1998; Vehaskari *et al,* 2001; Oliver *et al.,* 2005). Hence, it would be interesting to understand any such metabolic defects in such post-natal lambs.

Conclusion

New results in this thesis indicate that the uterine environment controls early embryonic development of the conceptus even when the physical space of the uterus or the metabolic demands of developing embryo are not constraints for conceptus development. However, with advancing gestation, the role of fetus becomes equally important. It was concluded that the effects of uterine environment upon fetal development are mediated through the trophoblast in the early embryonic period and by the placenta during subsequent gestation, along with the involvement of oPL, progesterone, IGF-1 and glucose. The effects of a restricted uterine environment that are evident at birth also perpetuate and remain consistent during subsequent post-natal development and thus have implications for productivity. These studies emphasise the importance of the early embryonic period in determining subsequent health status and growth and suggest a re-consideration of the current practices of animal production.

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Appendix

APPENDIX 1: Hormones and metabolites concentrations in different uterine environments at various stages of gestation in pregnant ewes analysed using repeat measures of analysis of variance (Mean ± SE).

Appendix Table 1.1. Plasma oPL[1] concentration in different uterine environments at various stages of gestation in pregnant ewes using repeat measures of analysis of variance (Mean ± SE). Within a column, means without a common superscript are different from each other (P<0.05).

Treatment groups	n	Day 90 oPL, ng/mL	Day 120 oPL, ng/mL
SinS[2] (large control)	12	31.62^{b} ± 3.81	131.69^{ab} ± 15.87
SinC[3] (restricted environment)	11	27.87^{ab} ± 3.73	107.03^{ab} ± 15.85
CinS[4] (luxurious environment)	12	19.73^{a} ± 3.81	94.99^{a} ± 15.87
CinC[5] (small control)	13	22.69^{ab} ± 3.61	149.33^{b} ± 15.24

Breedewe.breedlamb.time; P = 0.03, [1]Ovine placental lactogen, [2]Suffolk.in Suffolk, [3]Suffolk in Cheviot, [4]Cheviot in Suffolk, [5]Cheviot in Cheviot.

Appendix Table 1.2. Plasma progesterone concentration in different uterine environments at various stages of gestation in pregnant ewes using repeat measures of analysis of variance (Mean ± SE). Within a column, means without a common superscript are different from each other (P<0.05).

Treatment groups	n	Day 50 progesterone, ng/mL	Day 90 progesterone, ng/mL	Day 120 progesterone, ng/mL	Day 140 progesterone, ng/mL
SinS[1] (large control)	11	4.14 ± 0.39	4.31 ± 0.40	10.05[a] ± 1.08	9.82 ± 1.15
SinC[2] (restricted environment)	8	3.95 ± 0.46	4.42 ± 0.47	10.37[ab] ± 1.27	11.51 ± 1.35
CinS[3] (luxurious environment)	7	4.42 ± 0.49	4.36 ± 0.50	10.71[ab] ± 1.35	8.88 ± 1.44
CinC[4] (small control)	10	3.45 ± 0.41	4.64 ± 0.42	14.05[b] ± 1.13	8.67 ± 1.20

Breedewe.breedlamb.time; P= 0.4, [1]Suffolk.in Suffolk, [2]Suffolk in Cheviot, [3]Cheviot in Suffolk, [4]Cheviot in Cheviot.

Appendix Table 1.3. Plasma IGF-1[1] concentration in different uterine environments at various stages of gestation in pregnant ewes using repeat measures of analysis of variance (Mean ± SE). Within a column, means without a common superscript are different from each other (P<0.05).

Treatment groups	n	Day 50 IGF-1, ng/mL	Day 90 IGF-1, ng/mL	Day 120 IGF-1, ng/mL	Day 140 IGF-1, ng/mL
SinS[2] (large control)	10	59.40 ± 4.54	70.13[ab] ± 5.23	79.43 ± 5.30	85.92 ± 6.73
SinC[3] (restricted environment)	10	56.54 ± 4.55	66.68[ab] ± 5.23	70.35 ± 5.30	83.07 ± 6.73
CinS[4] (luxurious environment)	11	59.18 ± 4.33	77.78[b] ± 4.98	75.87 ± 5.05	83.50 ± 6.41
CinC[5] (small control)	12	50.73 ± 4.15	57.64[a] ± 4.77	70.43 ± 4.84	73.11 ± 6.14

Breedewe.breedlamb.time; P= 0.6, [1]Insulin-like growth factor-1, [2]Suffolk.in Suffolk, [3]Suffolk in Cheviot, [4]Cheviot in Suffolk, [5]Cheviot in Cheviot.

Appendix Table 1.4. Plasma glucose concentration in different uterine environments at various stages of gestation in pregnant ewes using repeat measures of analysis of variance (Mean ± SE). Within a column, means without a common superscript are different from each other (P<0.05).

Treatment groups	n	Day 50 glucose, mmol/L	Day 90 glucose, mmol/L	Day 120 glucose, mmol/L	Day 140 glucose, mmol/L
SinS[1] (large control)	11	3.77[a] ± 0.17	3.86[ab] ± 0.18	3.40[bc] ± 0.31	3.83[ab] ± 0.27
SinC[2] (restricted environment)	10	4.34[b] ± 0.18	4.30[b] ± 0.19	4.55[c] ± 0.33	3.95[ab] ± 0.28
CinS[3] (luxurious environment)	11	3.49[a] ± 0.17	3.47[a] ± 0.18	3.09[a] ± 0.31	3.45[a] ± 0.27
CinC[4] (small control)	12	4.29[b] ± 0.16	4.00[b] ± 0.17	3.46[ab] ± 0.30	4.25[b] ± 0.25

Breedewe.breedlamb.time; P = 0.4, [1]Suffolk.in Suffolk, [2]Suffolk in Cheviot, [3]Cheviot in Suffolk, [4]Cheviot in Cheviot.

Appendix Table 1.5. Plasma FFA[1] concentration in different uterine environments at various stages of gestation in pregnant ewes using repeat measures of analysis of variance (Mean ± SE). Within a column, means without a common superscript are different from each other (P<0.05).

Treatment groups	n	Day 50 FFA, mmol/L	Day 90 FFA, mmol/L	Day 120 FFA, mmol/L	Day 140 FFA, mmol/L
SinS[2] (large control)	11	0.45^{bc} ± 0.05	0.29^{a} ± 0.11	0.77^{ab} ± 0.12	0.73^{a} ± 0.09
SinC[3] (restricted environment)	10	0.45^{ac} ± 0.05	0.52^{ab} ± 0.12	0.90^{ab} ± 0.13	1.20^{b} ± 0.10
CinS[4] (luxurious environment)	11	0.31^{a} ± 0.05	0.26^{a} ± 0.12	0.68^{a} ± 0.12	0.62^{a} ± 0.09
CinC[5] (small control)	12	0.59^{b} ± 0.05	0.72^{b} ± 0.11	1.06^{b} ± 0.12	1.13^{b} ± 0.09

Breedewe.breedlamb.time; P= 0.7, [1]Free fatty acid, [2]Suffolk.in Suffolk, [3]Suffolk in Cheviot, [4]Cheviot in Suffolk, [5]Cheviot in Cheviot.

APPENDIX 2: Autopsies of lambs that were either stillborn or died within 3 days of birth

Appendix Table 2.1. Autopsies of lambs in Suffolk in Suffolk treatment group (large control).

Lamb tag	Died or still born	Sex	Body weight before autopsy, kg	Liver		Kidney				Heart		Spleen		Thyroid		Pancreas
				wt, g	appear--ance	right		left		wt, g	appear--ance	wt, g	appear--ance	wt, g	appear--ance	appear--ance
						wt, g	appear--ance	wt, g	appear--ance							
7087	Died	F	4.8	127	Normal	13	Normal	13	Normal	44	Normal	8.5	Normal	2	Normal	Normal

Appendix Table 2.2. Autopsies of lambs in Suffolk in Cheviot treatment group (restricted uterine environment).

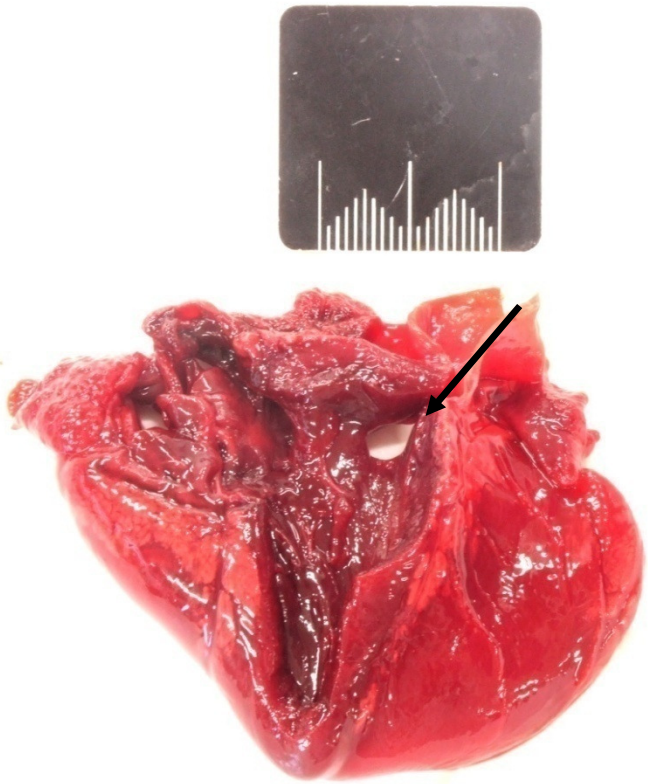
Lamb tag	Died or still born	Sex	Body weight before autopsy, kg	Liver		Kidney				Heart		Spleen		Thyroid		Pancreas
				wt, g	appear--ance	right		left		wt, g	appear--ance	wt, g	appear--ance	wt, g	appear--ance	appear--ance
						wt, g	appear--ance	wt, g	appear--ance							
7051	Died	M	2.40	36	Normal	6	Normal	6	Normal	27	Ventricular septal defect	4	Normal	0.5	Normal	Normal
7060	Died	M	4.85	85	Normal	13	Normal	12	Normal	41	Normal	6	Normal	0.8	Normal	Normal
7056	Died	M	5.83	137	Normal	14	Normal	16	Normal	53	Normal	9	Normal	0.8	Normal	Normal
Not tagged	Still birth	F	6.47	106	Normal	15	Normal	14	Normal	46	Normal	13	Normal	0.8	Normal	Normal
7083	Died	F	3.4	124	Normal	10.8	Normal	11	Normal	39	Normal	5.1	Normal	-	Normal	Normal

Appendix Table 2.3. Autopsies of lambs in Cheviot in Suffolk treatment group (luxurious uterine environment).

Lamb tag	Died or still born	Sex	Body weight before autopsy, kg	Liver		Kidney				Heart		Spleen		Thyroid		Pancreas
				wt, g	appear--ance	right		left		wt, g	appear--ance	wt, g	appear--ance	wt, g	appear--ance	appear--ance
						wt, g	appear--ance	wt, g	appear--ance							
Not tagged	Still birth	F	3.3	70	Normal	9	Normal	9	Normal	27	Normal	3	Normal	3	Enlarged	Normal
7094	Died	M	6.5	168	Normal	19.7	Normal	21	Normal	82	Normal	7.2	Normal	2	Normal	Normal
Not tagged	Still birth	M	3.3	54.3	Normal	8.8	Normal	8.5	Normal	26	Normal	2.8	Normal	1	Normal	Normal

Appendix Table 2.4. Autopsies of lambs in Cheviot in Cheviot treatment group (small control).

Lamb tag	Died or still born	Sex	Body weight before autopsy, kg	Liver		Kidney				Heart		Spleen		Thyroid		Pancreas
				wt, g	appear--ance	right		left		wt, g	appear--ance	wt, g	appear--ance	wt, g	appear--ance	appear--ance
						wt, g	appear--ance	wt, g	appear--ance							
7049	Died	F	5.9	120	Normal	9	Normal	16	Normal	53	Normal	6	Normal	3	Enlarged	Normal
7072	Died	M	3.2	61	Normal	10.1	Normal	10	Normal	30	Normal	3.3	Normal	1	Normal	Normal
7081	Died	M	5.2	122	Normal	16.9	Normal	16	Normal	43	Normal	8.3	Normal	2	Normal	Normal
Not tagged	Still birth	M	5.6	138	Normal	18.6	Normal	18	Normal	50	Normal	9.1	Normal	2	Normal	Normal
Not tagged	Still birth	F	5.9	197	Normal	18.6	Normal	18	Normal	44	Normal	10.8	Normal	2	Normal	Normal



Appendix Figure 2.1. Ventricular septal defect observed during autopsy (indicated by arrow) in the heart of a lamb born from restricted uterine environment (SinC); this lamb died within 24 h of birth.