Ewe nutrition during pregnancy:

Effects on the development of twin fetuses

A thesis presented in partial fulfilment of the requirements for the degree of

Master of AgriScience in Agriculture

At Massey University, Palmerston North

New Zealand

Natalia de la Paz Martín

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ABSTRACT

Martín, N.P. (2011). Ewe nutrition during pregnancy: Effects on the development of twin fetuses. A thesis presented in partial fulfilment of the requirements for the degree of Master of AgriScience in Agriculture. At Massey University, Palmerston North, New Zealand.

This study set out to investigate the effects of dam nutrition during pregnancy on the anatomical development of twin fetuses, with particular focus on the fetal mammary gland. Ewes were fed at 3 different levels in early pregnancy (day 21 to 50, Low (L_{D21-50}) vs. Medium (M_{D21-50}) vs. High (H_{D21-50})) and 2 different levels in mid- to late-pregnancy (day 50 to 140, Medium ($M_{D50-140}$) vs. High ($H_{D50-140}$)). At D140, 58 twin-bearing ewes were euthanised, and dam and fetal organs were collected and weighed.

H_{D21-50} ewes were heavier than L_{D21-50} and M_{D21-50} ewes at D50. At D140, $H_{D50-140}$ ewes were heavier, in better condition score and gained more weight than $M_{D50-140}$. Ewe nutrition in either period had no effect on the total placental membranes weight, gravid uterus weight, total placentome number or their level of eversion at D140. Nutritional treatments in both early and mid- to late-pregnancy failed to affect fetal weight or general size measurements (crown-rump length, girth circumference, femur or fore-leg length). The *semitendinosus* muscles from L_{D21-50}-$H_{D50-140}$ fetuses were heavier than L_{D21-50}-$M_{D50-140}$ and H_{D21-50}-$H_{D50-140}$ after adjustment for fetal weight. Fetuses from L_{D21-50} dams had lighter mammary glands compared to the M_{D21-50} and H_{D21-50} fetuses, and these differences remained after adjustment for fetal weight. Maternal nutrition affected other organs and glands, including thyroids, liver, brain and ovaries.

The results indicate a critical window of early mammary gland development between days 21 to 50 of gestation, as the fetal mammary glands for the group restricted in early gestation remained lighter, independent of fetal weight or size. A larger cohort of these animals has been kept to monitor their lifetime performance. This work has the potential to change current farming practices and possible review of the fundamentals of human nutrition and health.

ACKNOWLEDGEMENTS

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CHAPTER 1 INTRODUCTION

The amount and composition of dam nutritional regimen during pregnancy plays a fundamental role in regulating fetal and placental development in sheep (Heasman *et al.*, 1999; Wu *et al.*, 2006; Symonds *et al.*, 2007) and it can have fetal programming effects (Heasman *et al.*, 1999). The fetal programming theory suggests that the fetus makes predictive adaptations according to the intrauterine environment, so that the organism will be adequately prepared for a similar postnatal environment (Gluckman & Hanson, 2004). However, if there is a mismatch between the predicted and actual mature environment, the developmental adaptations may end up being harmful (Gluckman & Hanson, 2004). Such is the case of intrauterine growth restriction (IUGR). IUGR is the impaired growth and development of the mammalian embryo/fetus or its organs during gestation, and it may result in developmental adaptations that permanently change the structure, physiology, metabolism and postnatal growth of the offspring (Wu *et al.*, 2006). Consequently, IUGR is a major concern in domestic animal production because it reduces neonatal survival, efficiency of feed utilisation, negatively affects body composition and meat quality, and impairs long-term health and athletic performance (Wu *et al.*, 2006). In humans, IUGR may be the origin of a number of diseases in later life, including coronary heart disease, diabetes and hypertension (Barker, 1998).

During recent years, New Zealand farmers have increased their lambing percentage through selection of twin-born ewes as replacements, because this improves the farm productive efficiency (Kenyon, 2008). However, a twin pregnancy has higher nutritional demand (Rattray *et al.*, 1974; Cleal *et al.*, 2007; Kenyon *et al.*, 2009) and twin fetuses are more sensitive to maternal constraint (Gootwine *et al.*, 2007) than singles. As a result, the level of the maternal nutrition during pregnancy generally has a greater effect on twin-born lambs than their single-born counterparts. Additionally, gestation typically occurs during winter in extensive grazing farming systems and late winter is commonly the period of greatest feed shortage (Mathews *et al.*, 1999) because the low grass growth rates often does not meet the animal demand. Under these circumstances, IUGR is common in animals carrying multiple fetuses (Gootwine *et al.*, 2007). Thus, there is significant potential to influence the offspring in both the short- and long-term, e.g. survival, growth, body composition and future production, and these changes can be of great economic importance.

The effect of nutrition on fetal mammary gland development and its later functionality is of special interest due to its implications for growth, development and survival of the next generation of offspring (van der Linden *et al.*, 2009). Previous studies (Jenkinson, 2003; van der Linden *et al.*, 2009; Blair *et al.*, 2010) have shown contrasting effects of maternal nutrition on the weight, total duct area and number of ducts of the fetal mammary gland. The disparities were partially attributed to different feeding levels and timing of the nutritional insults. Due to the design of those studies, with only two feeding treatments from day 19-21 until day 140 of pregnancy (Jenkinson, 2003; Kenyon *et al.*, 2009; van der Linden *et al.*, 2009), it was not known if the maternal maintenance diet enhanced the mammary gland development or if the ad-lib diet reduced it. The only way of determining this is by imposing a third diet (restricted). A further limitation was the impossibility to identify the most important stage of gestation influencing the development of the fetal mammary gland, although it appeared to be sensitive to maternal nutritional status from early stages of fetal life (Jenkinson, 2003). The organogenesis of the mammary gland starts early in embryogenesis (Robinson *et al.*, 1999a) and ductular development, where secretory cells will proliferate during lactation, also occurs in early fetal life (Knight & Sorensen, 2001). Thus, it is likely that the development of the fetal mammary gland is more sensitive to under-nutrition in early gestation than in later stages.

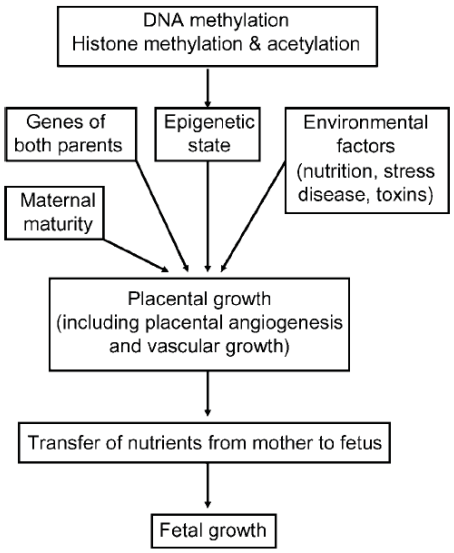
Therefore, the aim of this research was to investigate the effects of early (day 21 to 50) and mid- to late- (day 50 to 140) dam pregnancy nutrition on the anatomical development of twin-fetuses, with particular focus on the fetal mammary gland.

CHAPTER 2 LITERATURE REVIEW

2.1 Factors influencing the fetal development

The fetal growth in the uterus is a complex biological event influenced by genetic, epigenetic, physiological, maternal maturity and environmental factors (Wu *et al.*, 2006; Kenyon, 2008), summarised in Figure 2-1. There is an 'optimum' birth weight in which an uncomplicated natural delivery can occur and neonatal survival is maximised (Gardner *et al.*, 2007). A deviation from this 'optimum' or perturbed fetal development may be apparent as altered fetal or birth weight, length, girth or disproportionate tissue growth, because substrates tend to be directed to maintain essential tissues and organs at the expense of less vital ones (Quigley *et al.*, 2008). These alterations may result in permanent changes to the structure, physiology and metabolism of the offspring (so called intrauterine growth restriction (IUGR) effects), and even when there are no apparent changes in fetuses size, effects may emerge as altered neonatal survival or potentially body composition and health in post-natal life (Wu *et al.*, 2004; Bloomfield *et al.*, 2006; Wu *et al.*, 2006; Kenyon, 2008; Quigley *et al.*, 2008; Symonds *et al.*, 2010).

Figure 2-1: Regulation of mammalian fetal growth. Intrauterine growth is regulated by genetic, epigenetic, and environmental factors. These factors affect placental growth and therefore the availability of nutrients for fetal growth. Source: Wu *et al.* (2006)



The aim of this literature review is to summarise maternal nutritional effects on embryonic and fetal development. Many other factors which also affect fetal growth or have consequences for postnatal life will be mentioned early in the review, but not in detail.

2.1.1 Dam live weight and body condition

Both live weight and body condition of the mother prior to and at breeding, as well as the parity, age and history of barrenness, can affect fetal development.

Changes in maternal live weight prior to mating have been associated with altered utero-placental weights in early-mid gestation (MacLaughlin *et al.*, 2005) and changed gestational length (Bloomfield *et al.*, 2003). Mating live weight can explain a high proportion of the variation in singleton lamb birth weights (Figure 2-2, Gardner *et al.* (2007)), particularly when ewes are offered low nutritional treatments or fed below maintenance (Russel *et al.*, 1981). However, little effect is seen in those singleton (Russel *et al.*, 1981) or twin (Kenyon *et al.*, 2004) lambs born to ewes fed maintenance levels (Figure 2-3).

Ewe live weight is repeatedly confounded with ewe size. The body size is a measurement of the animal's mean width, length and depth (Ducker & Boyd, 1977), and it has genetic causes, it can be influenced by early nutrition and does not vary to any significant extent in adult life (Doney & Gunn, 1981; Doney *et al.*, 1982). Lambs born to small framed ewes are lighter than those born to large ewes on maintenance feeding, and these differences can remain until weaning (Kenyon *et al.*, 2009).

Figure 2-2: Relationship between the weight (kg) of Welsh Mountain (○, n=59) and Mule (●, n=34) ewes and the birth weight (kg) of their singleton offspring. Linear regression indicated a significant effect of ewe weight on weight of the lamb (F=100, P<0.001, R^2=0.20). Source: Gardner *et al.* (2007).

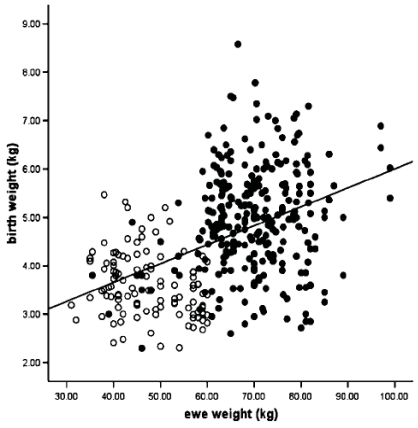
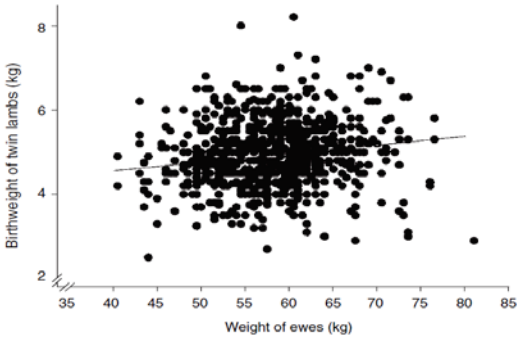


Figure 2-3: Relationship between the weight (kg) of ewes at mating and birth weight (kg) of twin lambs (R^2=0.012; y=3.76 + 0.0201x ± 0.005). Source: Kenyon *et al.* (2004).



The body condition score of the ewe at mating is one of the most important determinants of lamb birth weight (Gardner *et al.*, 2007). Heavier lambs are born from heavier ewes with higher body condition scores (Adalsteinsson, 1979; Kenyon *et al.*, 2009). Moreover, ewe live weight and condition score at mating are highly correlated (Kenyon *et al.*, 2004; Quigley *et al.*,

2008). For mixed-aged Romney ewes, 1 point of body condition is associated with an increase of 7.32 kg of live weight (Kenyon *et al.*, 2004). In singleton lambs, the higher the condition of the ewe at mating, the lower the fetal/maternal weight ratio (Figure 2-4, Gardner *et al.* (2007)). However, birth weights of twin lambs tended to increase with increasing body condition score, up to a maximum of 3.0; thereafter a decrease was reported (Kenyon *et al.*, 2004). Both the body composition of the mother and her ability to maintain a normal glucose supply to the fetus are major determinants of fetal growth and are themselves dependent on the stage of fetal development (Symonds *et al.*, 2007).

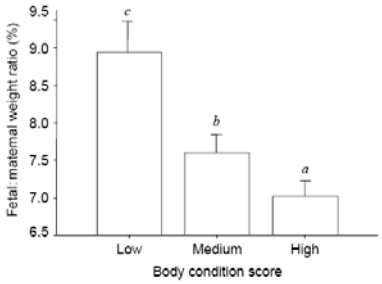


Figure 2-4: Fetal/maternal weight ratio (%) of lambs at term split according to low (<2), medium (2-3.5) or high (>3.5) body condition score ewes. Values are mean ± S.E.M. for individual data points for low (n=29), medium (n=122) or high (n=15) body condition score of ewes at mating. Differing superscripts indicate statistical difference at P<0.001. Source: Gardner *et al.* (2007).

2.1.2 Dam parity

Dam parity affects fetal growth with birth weight increasing up to the fourth pregnancy, though the greatest difference is between the first two pregnancies (Gardner *et al.*, 2007), Figure 2-5). This is probably an effect of an increased vascularisation of the uterus which facilitates greater fetal growth in subsequent pregnancies, and therefore the second born is heavier than first born (Gardner *et al.*, 2007). In addition, increasing parity is related to greater age and maturity of the dam as well as larger litter size (Gardner *et al.*, 2007; Gootwine *et al.*, 2007), which in turn affects birth weight and neonatal outcome *per se.* Primiparous ewes have usually not reached their mature body weight and therefore, fetal nutritional demands may be in conflict with maternal nutritional needs (Gootwine *et al.*, 2007). Litter size effects will be discussed in section 2.1.6.

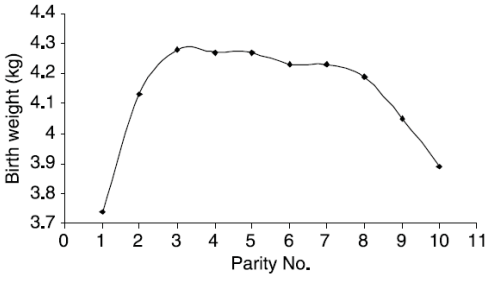


Figure 2-5: Least-squares means for birth weights of lambs relative to parity of ewes. After Gootwine and Rozov 2006. Source: Gootwine *et al.* (2007).

2.1.3 Environment

The environment or management system (Vonnahme *et al.*, 2006), climate (Gardner *et al.*, 2007), season (Gootwine *et al.*, 2007), heat and cold stress can all affect fetal growth, lamb birth weight and survival (Anthony *et al.*, 2003; Kenyon, 2008). For example, ewes adapted to harsh environments can maintain the normal nutrient delivery to their developing fetuses when subjected to an early- to mid-gestational nutrient restriction (Vonnahme *et al.*, 2006), and therefore, lamb birth weight and survival is not affected compared to lambs whose mothers were well fed. Thus, it is important to consider the environmental interaction with other stress factors.

2.1.4 Photoperiod

Sexual activity in sheep is strongly influenced by photoperiod, which is the main environmental factor responsible for the seasonality of reproduction that ensures that births occur at the optimal time of the year, usually spring, which allows the newborn to grow under favourable conditions of temperature and food availability in advance of the next winter (Forcada & Abecia, 2006). The pineal gland perceives the photoperiod, and releases melatonin (Forcada & Abecia, 2006). Melatonin has an important role on the function of the fetal brown adipose tissue (Symonds *et al.*, 2010). The photoperiod also affects plasma prolactin concentrations in both maternal and fetal circulation, which in turn promotes the generation of brown adipose tissue in the fetus that will enable enhanced heat production in the new born (Symonds *et al.*, 2010).

2.1.5 Shearing

Mid-pregnancy or winter shearing induces chronic maternal adaptations to the cold that increases the energy requirements and thus the voluntary food intake (Dyrmundsson, 1991). This enhances the secretion and action of thyroid hormones, noradrenaline and growth hormone, which promote the mobilisation and later utilisation of fat stores, which in turn prevent a decline in plasma glucose concentration in late pregnancy (Symonds *et al.*, 1988) and improves fetal growth (Kenyon *et al.*, 2003). The benefits for the fetus are an increase in brown fat, a larger liver with greater glycogen stores (Clarke *et al.*, 1997), improved thermal, thyroid and respiratory function (Clarke *et al.*, 1997), and a higher birth weight (Dyrmundsson, 1991; Kenyon *et al.*, 2003; Kenyon *et al.*, 2004).

2.1.6 Birth rank of the lamb

Litter size has the greatest influence on birth weight (Figure 2-6, Gardner *et al.* (2007)). Single born lambs are heavier at birth and weaning than twins which in turn are heavier than triplet born lambs (Morris & Kenyon, 2004; Gootwine *et al.*, 2007; Kenyon, 2008). This inverse relationship between litter size and birth weight (Figure 2-7) is due to the 'maternal constraint of fetal growth' (Gluckman & Hanson, 2004), with twin fetuses being more sensitive to maternal constraint than singleton fetuses (Gootwine *et al.*, 2007). The 'maternal constraint' includes the physiological capacity of the mother to supply metabolic substrates, the physical capacity of the mother to carry multiple fetuses, mechanical forces in the uterus and fetal genotypic effects (Gardner *et al.*, 2007). Therefore, IUGR occurs naturally in animals carrying multiple fetuses (Gootwine *et al.*, 2007). However, it has also been suggested that the fetal growth trajectory in twins is set in early gestation, determining a reduced growth trajectory by virtue of being a twin well before it is affected by uterine capacity, though it can be further modified by maternal nutritional status at this stage or in later stages by placental supply (Bloomfield *et al.*, 2006).

Twin-bearing ewes may also be under greater nutritional stress (Kenyon *et al.*, 2009), and their pregnancies have a higher nutritional demand compared to a singleton-bearing (Rattray *et al.*, 1974). Consequently, the level of the maternal nutrition during pregnancy will have a greater effect on twin-born lambs (Rattray *et al.*, 1974; Kenyon *et al.*, 2009), and can limit the fetal growth rate at the end of pregnancy (Figure 2-8, Rattray *et al.* (1974)). This will be discussed in later sections.

Figure 2-6: Fetal/maternal weight ratio (%) of lambs at term split according to singleton or twins. Values are mean ± S.E.M. for individual data points of singleton (n=95) and twin (n=50) lambs. Statistical differences are *P<0.001. Source: Gardner *et al.* (2007).

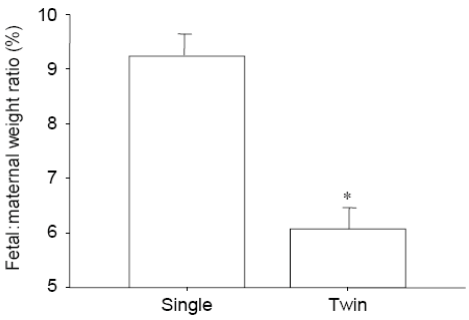


Figure 2-7: Birth weights and perinatal survival rates for lambs (n=4781) born to Afec-Assaf ewes (Volcani Center, Israel), according to litter size. After Gootwine and Rozov 2006. Source: Gootwine *et al.* (2007).

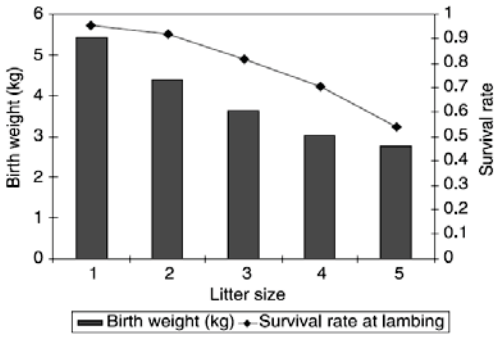
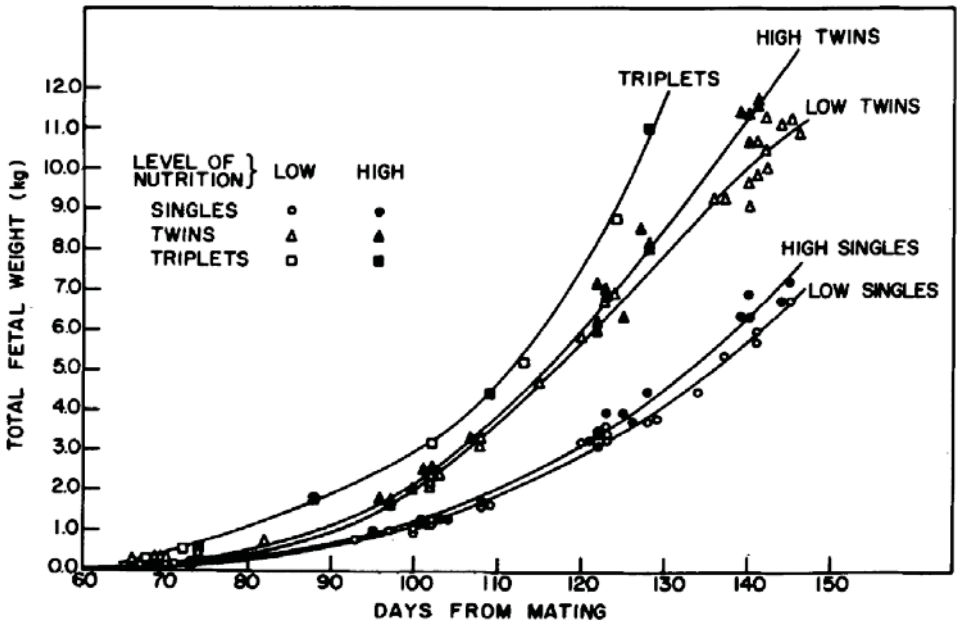


Figure 2-8: Relationships between fetal weight and day of gestation (based on a polynomial regression). Level of nutrition from day 70 to 140 are Low (1.5x maintenance) and High (2.0x maintenance). Source: Rattray *et al.* (1974).



2.2 Nutrition during pregnancy

The composition and level of maternal diet throughout pregnancy has consequences on the dam, placenta, embryo and resulting fetus (Robinson *et al.*, 2006; Symonds *et al.*, 2007), and therefore is an important modifiable factor that affects the ewe's productivity (Symonds *et al.*, 2010). In general terms, ewes which are well fed are heavier and have better body condition scores, which leads to a higher fecundity (more lambs) and greater lamb birth weights (Adalsteinsson, 1979) compared to poorly fed ewes. However, the timing, duration and severity of a nutritional insult, as well as the age of the dam and the long-term environment or management system, may all result in different effects, as previously described (Harding & Johnston, 1995; Robinson *et al.*, 1999b; Vonnahme *et al.*, 2006; Gootwine *et al.*, 2007; Quigley *et al.*, 2008).

Nutrition influences ruminant fertility directly by the supply of specific nutrients required for the establishment of pregnancy and building blocks for tissue growth (i.e., glucose, amino acids and essential nutrients), and indirectly through its impact on the circulating concentrations of the hormones and other nutrient-sensitive metabolites (e.g., progesterone and growth factors), that affect the interaction between fetus, placenta and mother (Harding & Johnston, 1995; Robinson *et al.*, 1999b; Robinson *et al.*, 2006). Slight alterations in nutrient supply during critical periods of embryonic and fetal life, in both plane of nutrition and specific

dietary nutrients, can impart different changes in growth and development that affect the neonatal survival and adult performance (Robinson *et al.*, 1999b; Bloomfield *et al.*, 2006). In this respect, this literature review will only examine the effects of differing levels of global caloric nutrition, but will not review studies in which the protein/energy ratio was manipulated.

Birth weight, an easy and simple measurement, is highly related to neonatal and adult health (Gardner *et al.*, 2007). However, birth weight is not always an appropriate measure of the quality of fetal growth (Harding & Johnston, 1995), as it is a summative at the end of a long period and it can be obtained by quite different intrauterine growth trajectories (Figure 2-9, Bloomfield *et al.* (2006)). Many studies have suggested that under or over-nutrition can have specific effects and alter the ratios of organ and tissue weights to total body weight (Robinson *et al.*, 1999b; Vonnahme *et al.*, 2003; Bloomfield *et al.*, 2006; Quigley *et al.*, 2008). Shifts in fetal development can be seen, from oversize of fetal lambs and hypertrophy of skeletal muscle fibres, to reduced fetal growth associated with alterations in muscle development (Robinson *et al.*, 1999b). The phenomenon of 'sparing effects' on vital organs, such as the brain, has been commonly observed in fetuses subjected to nutrient deprivation (Quigley *et al.*, 2008; Gao *et al.*, 2009) and reflects physiological adaptations that divert scarce resources at the expense of other tissues. Two fetal body components that are of particular interest in growth retarded fetuses (Robinson *et al.*, 1999b) are the skeleton, essential for functional competence, and the lipid reserve, key in thermogenic response and survival. Also, the fetal mammary gland has gained importance due to its impact on the milk production and growth of the following generation (van der Linden *et al.*, 2009). Consequently, this literature review will identify the effects of differing nutritional levels in various stages of gestation on the placenta and fetal body weight, dimension and organ sizes and development of the offspring in both the short and long-term. The effects on the post-natal performance of the offspring will not be reviewed.

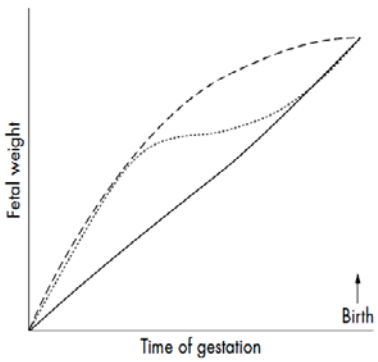
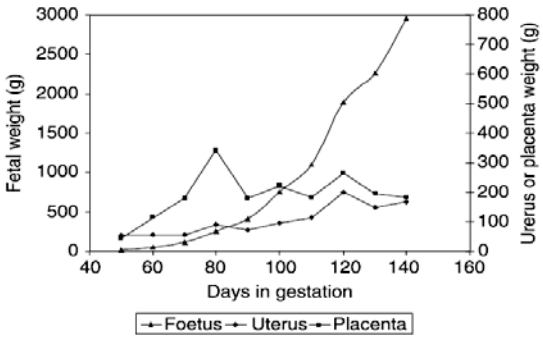


Figure 2-9: Differential fetal growth trajectories resulting in similar birth weights. The graph shows three exaggerated hypothetical growth trajectories: rapid growth in the firth half of pregnancy with slowing thereafter (---); a similar initial trajectory followed by a period of slow growth before there is intrauterine catch-up growth ·(··); initially slower growth with acceleration in the last half of gestation (–). Source: Bloomfield *et al.* (2006).

2.2.1 Stages of Gestation

The average gestation length in sheep is 147 days, and throughout this period, the growth patterns of the placenta and fetus differ (Figure 2-10, Gootwine *et al*. (2007)). The ovine pregnancy can be divided into three phases or trimesters, from both a nutritional and time point of view (Kenyon, 2008). The first period (day 0 to 50), coincides with embryogenesis, fetal organogenesis and tissue hyperplasia. Consequently, the energy requirements for the developing embryo and conceptus may be small, but the metabolic activity and specific growth rate of the fetus are high (Robinson *et al.*, 1999b). The second stage (day 50 to 100) is of maximal placental growth, peaking at around day 80 of gestation, and is followed by marked changes in structural properties and conformation to increase in functional capacity, in particular nutrient transport (Schneider, 1996). The last trimester (days 100 to 150), or fetal phase, is characterised by the maximal fetal growth and is associated with the highest nutritional demand (Rattray *et al.*, 1974; Kenyon & Webby, 2007; Symonds *et al.*, 2007).

Figure 2-10: Growth of the foetus and placenta during pregnancy in Florida Native ewes (n=4 per age group). After Bazer *et al.*, unpublished results. Source: Gootwine *et al.* (2007)



The long-term outcomes of maternal under-nutrition during different stages of pregnancy can be quite different and a summary is presented in Figure 2-11. Each tissue and organ have different critical windows where altered development can have long-term effects. Examples are shown in Figure 2-12 for the ovary, Figure 2-13 for the mammary gland and Figure 2-14 for muscle, brown and white adipose tissue. The assumption that the organs most affected by a nutrient insult are those that are rapidly growing at the time, is being increasingly challenged (Harding & Johnston, 1995). For that reason, this chapter will review the effects of dam nutrition during peri-conception and each of the three trimesters of gestation. Studies will be characterised based on when the nutritional treatment began even if it extended into later periods of pregnancy. All studies discussed include only mixed-aged ewes.

Figure 2-11: Summary of the main developmental windows, organs affected and different long-term effects in the offspring after maternal nutrient restriction at defined stages of the reproductive cycle in sheep. CV, cardiovascular system. Adapted from: Symonds *et al.* (2007; 2010).

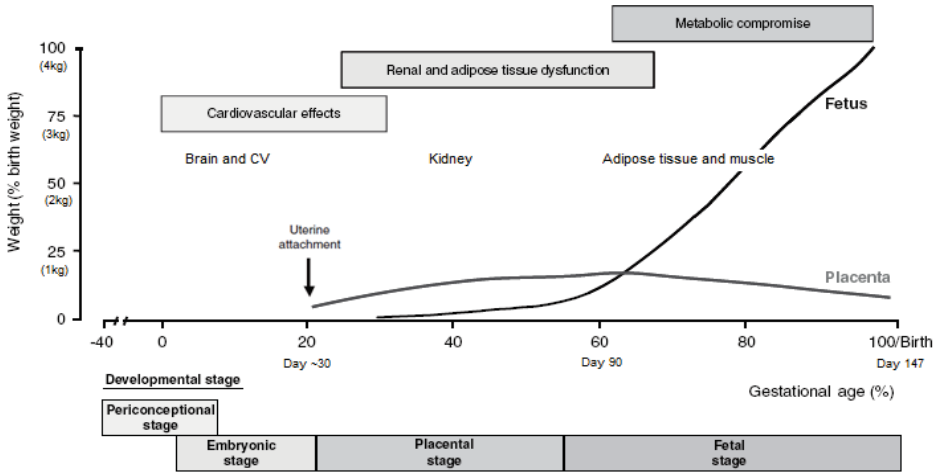


Figure 2-12: Critical periods during gestation in sheep for the expression of effects of maternal under-nutrition (0.5×maintenance vs. 1.0×maintenance) on fetal ovarian development. After McEvoy and Robinson 2002. Source: Robinson *et al.* (2006).

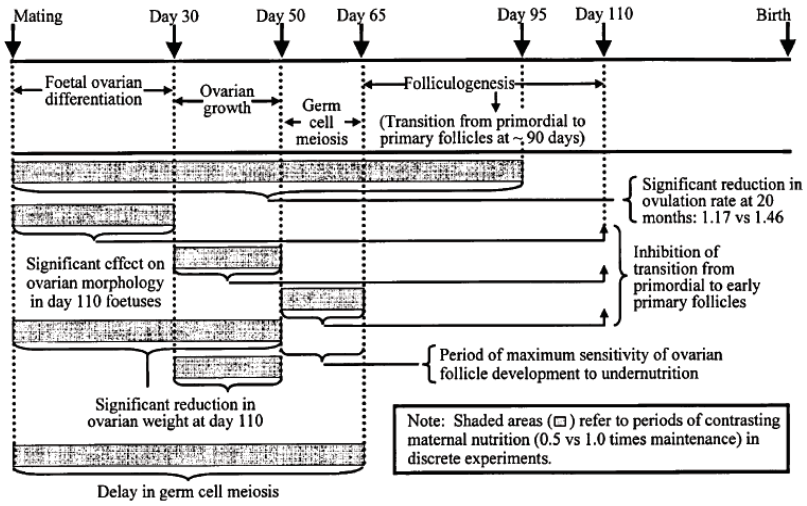


Figure 2-13: Schematic representation of lifetime mammary development. The width of the arrow represents qualitatively the size of the gland. Proposed critical windows, points at which altered development could have a long-term effect, are shown by text boxes. Source: Knight & Sorensen (2001).

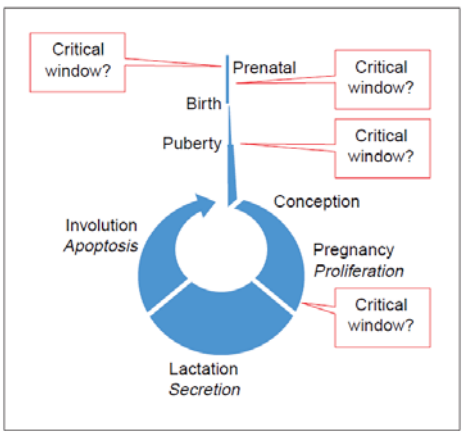
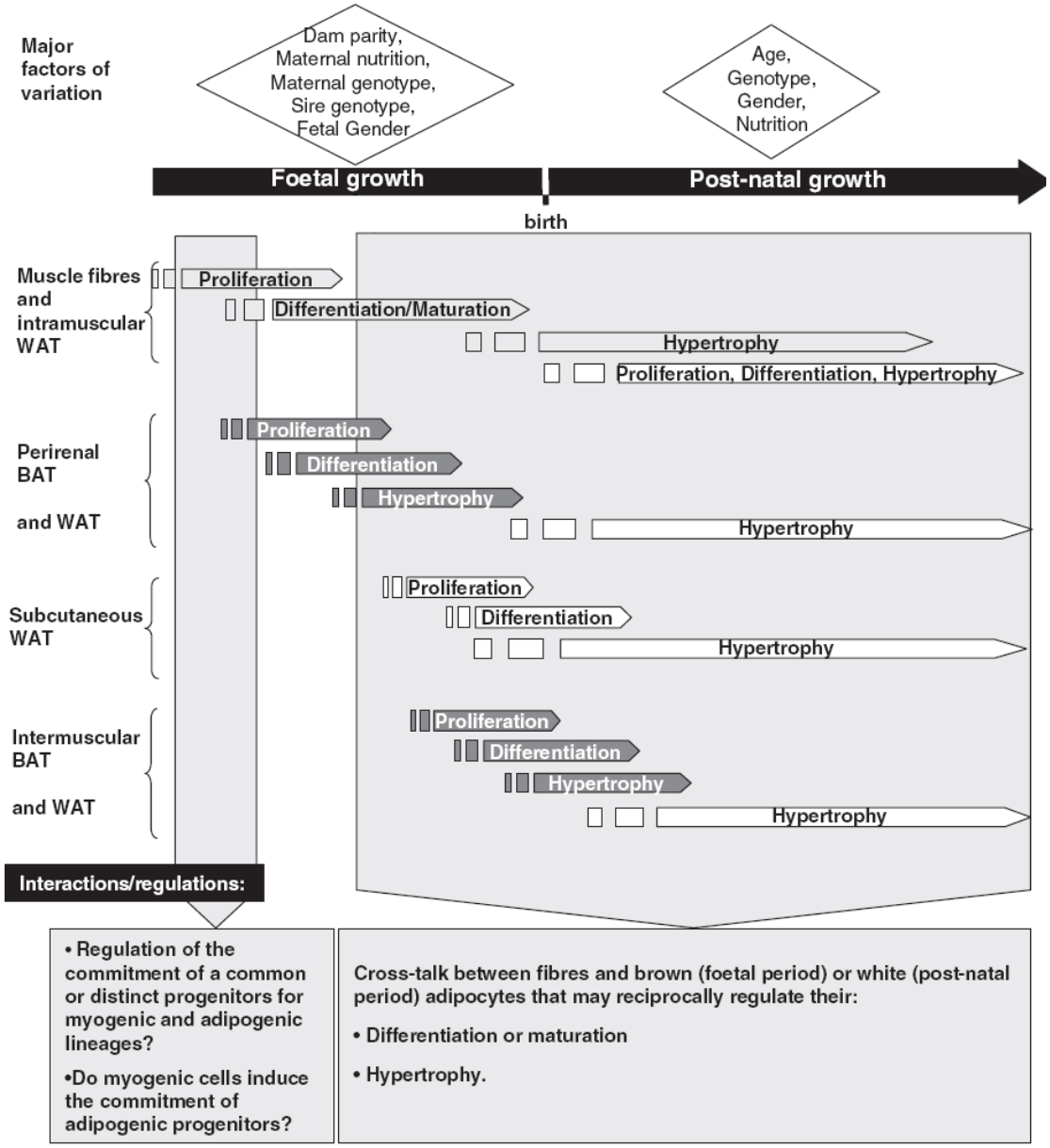


Figure 2-14: Depiction of the cellular events involved in the ontogenesis of muscle fibres (light grey), brown adipose tissues (BAT; dark grey) and white adipose tissues (WAT; white) in cattle. Major variation factors and putative interactions between tissues and/or regulation are indicated. Source: Bonnet *et al.* (2010).



2.2.2 Peri-conception

There is evidence that the fetal growth trajectory and metabolic responses in late gestation are determined much earlier, probably in the peri-conceptional period, though they may be further modified (Wu *et al.*, 2004; Bloomfield *et al.*, 2006). Moreover, maternal nutrition in this period has other effects on fetal development and even length of gestation (Bloomfield *et al.*, 2003; Bloomfield *et al.*, 2006).

Effects on fetal growth, body weight, dimensions, organs and placenta

When ewes are undernourished starting two months prior to mating until a month after (Oliver *et al.*, 2005), the fetal growth rate is slower during late gestation (Harding & Johnston, 1995). However, when these fetuses are exposed to a late-nutritional challenge, they keep on growing at the same slow rate (Harding & Johnston, 1995), somehow shifting to alternative fuel sources, suggesting a series of adaptations in both maternal and fetal metabolic and endocrine status that would allow fetal survival under such insult (Oliver *et al.*, 2005). On the contrary, fetuses from ewes well nourished in the peri-conceptional period that are growing quickly in late gestation, slow their growth when subjected to a restricted maternal feeding (Harding & Johnston, 1995), and the partial pressure of oxygen is raised, indicating a reduced oxidative demand (Oliver *et al.*, 2005).

The slow growth trajectory set by maternal under-nutrition in the peri-conceptional period may not be reflected in either fetal weight (Table 2-1), body proportions (Table 2-2) or placental weight (Table 2-4). Nevertheless, some tissues and organ weights such as heart, kidney and brain, can be altered (Table 2-3), which suggests a deviation in the sequence of growth and possibly the function of the organ (Harding & Johnston, 1995).

Effects on the placenta are also variable, although they seem to be greater in twin-bearing ewes (Table 2-4). For example, twin-bearing ewes under restricted feeding starting three months prior to conception, had greater proportion of everted placentomes than *ad lib* fed ewes in mid and late gestation (Quigley *et al.*, 2008). This effect is not seen in singleton-bearing dams (Oliver *et al.*, 2005; Quigley *et al.*, 2008).

Table 2-1: The effects of maternal nutritional manipulation starting prior to mating on fetal growth, weight and metabolism. Adapted from: Kenyon (2008) and van der Linden (2010).

Fetal Parameter	Period	Nutritional Treatments	Effects	Reference
Fetal Growth	-60 to 7 and 7 to 147	R (0.7M) vs. M (1.0M) each period (4 treatments)	Single- no effect. Twin- reduced in M followed by R (M-R group)	(Edwards and McMillen, 2002)
	-60 to 30 and 105 to 115	L (loss 10% BW) vs. H each period (4 treatments)	L at peri-conception slowed down growth at the end of pregnancy (programming effect) and showed much less change in growth rate when ewes were exposed to further under-nutrition (L-L).	(Harding & Johnston, 1995)
	-60 to 30	UN (1-2% BW/d; loss 10-15% BW) vs. N (3-4% BW/d). Fast D121-124	D111 and D132: Single and twin- no effect on growth rate (but differences between single vs. twin)	(Rumball *et al.*, 2008)
Fetal Weight	-89 to 133	R (0.6M), C (1.2M) vs. AL (1.8M)	D50,92: Single and twin- no effect. D133: Single- AL heavier than R. Twin- no effect.	(Quigley *et al.*, 2008)
	-60 to 7 and 7 to 147	R (0.7M) vs. M (1.0M) each period (4 treatments)	Birth: Single- no effect. Twin- lighter in M followed by R (M-R group)	(Edwards and McMillen, 2002)
	-60 to 30 and 105 to 115	L (loss 10% BW) vs. H each period (4 treatments)	D125: no effect.	(Harding & Johnston, 1995)
	-60 to 30	UN (1-2% BW/d; loss 10-15% BW) vs. N (3-4% BW/d). Fast D121-123.	D131 and Birth: Single- no effect.	(Oliver *et al.*, 2005)
	-60 to 30	UN (1-2% BW/d; loss 10-15% BW) vs. N (3-4% BW/d). Fast D121-124	D132: Single and twin- no effect.	(Rumball *et al.*, 2008)
	-45 to 7	C (1.0M) vs. UN (0.7M)	D53-56: Single and twin- no effect in fetal weight but altered relationship with placental weight.	(MacLaughlin *et al.*, 2005)
	-14 to 70	0.85 M vs. 1.0 M	Birth: no effect.	(Hawkins *et al.*, 2000)
Metabolism	-89 to 133	R (0.6M), C (1.2M) vs. AL (1.8M)	D92 and D133: R [NEFA] Twin>Single	(Quigley *et al.*, 2008)
	-60 to 30	UN (1-2% BW/d; loss 10-15% BW) vs. N (3-4% BW/d). Fast D121-123.	D131: Single- UN higher glucose, lactate and placental lactogen; lower amino nitrogen, oxygen concentration and pH.	(Oliver *et al.*, 2005)
	-60 to 30	UN (1-2% BW/d; loss 10-15% BW) vs. N (3-4% BW/d). Fast D121-124	D114 to 121: Single- UN lower fetal plasma glucose and insulin concentrations; no differences on IGF-1 or urea concentration. Twin- UN higher fetal plasma urea concentrations; no differences on glucose, insulin or IGF-1 concentrations. D124: Single- Fetal plasma glucose and insulin concentrations dropped more in response to maternal fasting in UN, and were lower through refeeding; no difference in IGF-1 concentrations between nutritional groups.	(Rumball *et al.*, 2008)

Treatments: R, restricted; M, maintenance; C, control; AL, *ad libitum*; L, low; H, high; UN, under-nutrition; N, normal nutrition. BW, body weight; d, day. Effects: D, day of measurement.

Table 2-2: The effects of maternal nutritional manipulation starting prior to mating on fetal dimensions.

Fetal Measurement	Period	Nutritional Treatments	Effects	Reference
Crown-rump length	-89 to 133	R (0.6M), C (1.2M) vs. AL (1.8M)	D50,92: Single and twin- no effect. D133: Single- control heavier than R (AL in between). Twin- no effect.	(Quigley *et al.*, 2008)
	-60 to 30 and 105 to 115	L (loss 10% BW) vs. H each period (4 treatments)	D125: no effect.	(Harding & Johnston, 1995)
	-60 to 30	UN (1-2% BW/d; loss 10-15% BW) vs. N (3-4% BW/d). Fast D121-123.	D131 or at birth: Single- no effect.	(Oliver *et al.*, 2005)
	-60 to 30	UN (1-2% BW/d; loss 10-15% BW) vs. N (3-4% BW/d). Fast D121-124	D132: Single and twin- no effect.	(Rumball *et al.*, 2008)
Girth	-89 to 133	R (0.6M), C (1.2M) vs. AL (1.8M)	D50: Single and twin- no effect. D92: Twin- AL greater abdominal girth than control (R in between) and AL greater thoracic girth than R and control. Single- no effect. D133: Single- AL greater abdominal and thoracic girth than R. Twin- no effect.	(Quigley *et al.*, 2008)
	-60 to 30	UN (1-2% BW/d; loss 10-15% BW) vs. N (3-4% BW/d). Fast D121-123.	D131 or at birth: Single- no effect, but slightly smaller chest girth at birth.	(Oliver *et al.*, 2005)
	-60 to 30	UN (1-2% BW/d; loss 10-15% BW) vs. N (3-4% BW/d). Fast D121-124	D111 and D132: Single and twin- no effect on chest girth.	(Rumball *et al.*, 2008)
Limb	-60 to 30	UN (1-2% BW/d; loss 10-15% BW) vs. N (3-4% BW/d). Fast D121-123.	D131 or at birth: Single- no effect.	(Oliver *et al.*, 2005)
	-60 to 30	UN (1-2% BW/d; loss 10-15% BW) vs. N (3-4% BW/d). Fast D121-124	D111 and D132: Single and twin- no effect on hind limb length.	(Rumball *et al.*, 2008)
Head	-89 to 133	R (0.6M), C (1.2M) vs. AL (1.8M)	D50: Single- R smaller skull length. D92: Single- R smaller skull length than AL. D133: Single and twin- No effect in skull width or length.	(Quigley *et al.*, 2008)

Treatments: R, restricted; M, maintenance; C, control; AL, *ad libitum*; L, low; H, high; UN, under-nutrition; N, normal nutrition. BW, body weight; d, day. Effects: D, day of measurement.

Table 2-3: The effects of maternal nutritional manipulation starting prior to mating on fetal organs. Adapted from: Brameld & Daniel (2008).

Tissue	Period	Nutritional Treatments	Effects	Reference
Kidney	-89 to 133	R (0.6M), C (1.2M) vs. AL (1.8M)	D133: Single- no effect. Twin- R relative weight heavier than AL.	(Quigley *et al.*, 2008)
	-60 to 30 and 105 to 115	L (loss 10% BW) vs. H each period (4 treatments)	D125: L-L or H-L larger.	(Harding & Johnston, 1995)
	-60 to 30	UN (1-2% BW/d; loss 10-15% BW) vs. N (3-4% BW/d). Fast D121-123.	D131: Single- no effect.	(Oliver *et al.*, 2005)
Lung	-89 to 133	R (0.6M), C (1.2M) vs. AL (1.8M)	D50,133: Single- R lighter. Twin- no effect.	(Quigley *et al.*, 2008)
	-60 to 30 and 105 to 115	L (loss 10% BW) vs. H each period (4 treatments)	D125: H-L reduced lung weight by 20%.	(Harding & Johnston, 1995)
	-60 to 30	UN (1-2% BW/d; loss 10-15% BW) vs. N (3-4% BW/d). Fast D121-123.	D131: Single- no effect.	(Oliver *et al.*, 2005)
Heart	-89 to 133	R (0.6M), C (1.2M) vs. AL (1.8M)	D133: Single and twin- no effect.	(Quigley *et al.*, 2008)
	-60 to 30 and 105 to 115	L (loss 10% BW) vs. H each period (4 treatments)	D125: L-L or H-L larger.	(Harding & Johnston, 1995)
	-60 to 30	UN (1-2% BW/d; loss 10-15% BW) vs. N (3-4% BW/d). Fast D121-123.	D131: Single- no effect on absolute weight but bigger relative weight.	(Oliver *et al.*, 2005)
Brain	-89 to 133	R (0.6M), C (1.2M) vs. AL (1.8M)	D92,133: Twin- R reduced brain/liver ratio. D133: Single- R relative weight larger than AL, and R greater brain/liver ratio than control.	(Quigley *et al.*, 2008)
	-60 to 30 and 105 to 115	L (loss 10% BW) vs. H each period (4 treatments)	D125: L-L reduced brain weight by 12%.	(Harding & Johnston, 1995)
	-60 to 30	UN (1-2% BW/d; loss 10-15% BW) vs. N (3-4% BW/d). Fast D121-123.	D131: Single- no effect.	(Oliver *et al.*, 2005)
Adrenal	-60 to 30	UN (1-2% BW/d; loss 10-15% BW) vs. N (3-4% BW/d). Fast D121-123.	D131: Single- no effect.	(Oliver *et al.*, 2005)
Muscle	-89 to 133	R (0.6M), C (1.2M) vs. AL (1.8M)	D133: Single and twin- R reduced absolute weight of *longissimus*, *semitendinosus* and *supraspinatus* (but unaffected relative weight).	(Quigley *et al.*, 2008)
	-18 to 6	0.5M vs. 1.5M	D75: reduced total number of 2 fibres and 2:1 fibre ratio of *semitendinosus* muscle. No differences in total number of 1 fibres and both 1 and 2 fibre diameters.	(Quigley *et al.*, 2005)
Adipose tissue	-30 to 100	R (0.7M) vs. C (1.0M)	Slaughter weight (58.5kg): no differences in adiposity.	(Nordby *et al.*, 1987)

Treatments: R, restricted; M, maintenance; C, control; AL, *ad libitum*; L, low; H, high; UN, under-nutrition; N, normal nutrition. BW, body weight; d, day. Effects: D, day of measurement.

Table 2-4: The effects of maternal nutritional manipulation starting prior to mating on the placenta.

Placenta	Period	Nutritional Treatments	Effects	Reference
Placenta Weight	-60 to 30 and 105 to 115	L (loss 10% BW) vs. H each period (4 treatments)	D125: no effect or slight increase.	(Harding & Johnston, 1995)
	-60 to 30	UN (1-2% BW/d; loss 10-15% BW) vs. N (3-4% BW/d). Fast D121-123.	D131: Single- no effect.	(Oliver *et al.*, 2005)
	-60 to 30	UN (1-2% BW/d; loss 10-15% BW) vs. N (3-4% BW/d). Fast D121-124	D132: Single and twin- no effect.	(Rumball *et al.*, 2008)
	-45 to 7	C (1.0M) vs. UN (0.7M)	D53-56: Single and twin- no effect on uterine, fetal membranes or total placental weight, but altered relationship with fetal weight.	(MacLaughlin *et al.*, 2005)
Placentome	-89 to 133	R (0.6M), C (1.2M) vs. AL (1.8M)	D50,92,133: Single and twin- No effect on total number of placentomes, total placentome weight, mean placentome weight or fetal/placentome weight ratio. D92,133: Type B,C,D placentomes heavier than A. Single- no effect on placentome morphology. Twin- R greater proportion of everted placentomes than AL fed ewes (D92- type B, C and D; D133- type C and D).	(Quigley *et al.*, 2008)
	-60 to 30	UN (1-2% BW/d; loss 10-15% BW) vs. N (3-4% BW/d). Fast D121-123.	D131: Single- no effect on placentome numbers.	(Oliver *et al.*, 2005)
	-60 to 30	UN (1-2% BW/d; loss 10-15% BW) vs. N (3-4% BW/d). Fast D121-124	D132: Single and twin- no effect on number or proportion of placentomes, but greater proportion of placentome D type.	(Rumball *et al.*, 2008)
	-45 to 7	C (1.0M) vs. UN (0.7M)	D53-56: Single and twin- no effect on placentome number or mean placentome weight.	(MacLaughlin *et al.*, 2005)

Treatments: R, restricted; M, maintenance; C, control; AL, *ad libitum*; L, low; H, high; UN, under-nutrition; N, normal nutrition. BW, body weight; d, day. Effects: D, day of measurement.

2.2.3 Early Pregnancy (days 0-50)

During early stages of pregnancy, the embryo nutrient demand is minute though very specific in qualitative and temporal requirements, and therefore fetal growth is vulnerable to maternal dietary deficiency in nutrients during the first trimester of gestation (Wu *et al.*, 2004; Bloomfield *et al.*, 2006). Changes that appear to be minor can cause major alterations in subsequent fetal development (Robinson *et al.*, 1999b) due to programming effects (Symonds *et al.*, 2007). During this phase, uterine attachment occurs at around days 22 and 28 of gestation, and placenta growth commences in both maternal caruncle and fetal cotyledon approximately at day 30 (Symonds *et al.*, 2010). Placentome number is generally established by day 40 of gestation (Schneider, 1996), and is not thought to change throughout pregnancy, although individual placentomes alter in size (Heasman *et al.*, 1999). The main dietary source to the embryo comes directly from uterine secretions that may act as a 'buffer' to any changes in the nutritional environment of the mother (Symonds *et al.*, 2007). The nutrient supply, in turn, interacts with maternal factors influencing the placental growth and setting the partition between the gravid uterus and maternal body (Robinson *et al.*, 1999b). Manipulating food intake during the first month of pregnancy can lead to abrupt changes in ewe live weight and body condition score, which can remain evident throughout the entire pregnancy, but may not affect the fertility and long-term productivity of mature ewes (Annett & Carson, 2006).

Effects on fetal growth and body weight

Generally under-nutrition imposed in the first month to 40 days of pregnancy has not had a negative effect on lamb birth weight (Table 2-5), suggesting that the growth potential of the fetus is not affected (Robinson *et al.*, 1999b; Annett & Carson, 2006; Gardner *et al.*, 2007; Symonds *et al.*, 2010). Any growth retardation effects can be reverted under the influence of mid-pregnancy diets and the fetuses can show compensatory growth, and end up actually being heavier at birth (Munoz *et al.*, 2008). This leads to the concept that under-nutrition in early pregnancy is less likely to negatively affect birth weight than under-nutrition in the later stages of pregnancy (Gardner *et al.*, 2007; Kenyon, 2008) and some potential effects on the fetal growth appear to be temporary and not detectable at birth (Robinson *et al.*, 1999b).

Nonetheless, the effects of under-nutrition on fetal growth later in pregnancy can be negative if the underfeeding period is extended until day 60 (Robinson *et al.*, 1999b). Restricted feeding throughout pregnancy results in significant reductions in fetal growth (Mellor & Murray, 1982; Anthony *et al.*, 2003; Wu *et al.*, 2004), and reduces weight of the offspring at birth (Wallace,

1948; Harding & Johnston, 1995). Similarly negative effects of under-nutrition are more often seen in the birth weight of twins, due to the greater nutritional demand of twin pregnancies compared to singletons (Rattray *et al.*, 1974), resulting in fetuses being nutritionally deprived (Blair *et al.*, In Press). However, chronic nutrient restriction may not result in fetal retardation (Vonnahme *et al.*, 2006), as the plasma concentration of catabolic hormones is reduced, glucose production is maintained and thus fetal growth is not compromised (Symonds *et al.*, 2010).

There is some evidence that restricted feeding in early pregnancy can cue the fetus in such a way to increase its chances of survival and that of its offspring. In one study, the immune status (measured through zinc sulphate turbidity (ZST) units, free tri-iodothyronine (T_3) and thyroxine (T_4) serum concentrations) of lambs born from ewes that were restricted in early pregnancy was higher, regardless of mid-pregnancy nutrition, and this was thought to be related to the higher efficiency of the new born to absorb colostrum (Munoz *et al.*, 2008). In another study, twin-born female lambs to maintenance fed dams throughout pregnancy, were lighter at birth and showed a reduced growth to weaning compared to those born from dams fed *ad libitum* (Kenyon *et al.*, 2009), but they could have an advantage in physiological stressful situations in life (van der Linden *et al.*, 2010). These offspring had an increased glucose metabolism from the liver (i.e., gluconeogenesis and/or glycogenolysis) and higher milk production in terms of litres, lactose percentage, lactose and crude protein yields (van der Linden, 2010), suggesting that they could be more efficient at producing offspring (van der Linden *et al.*, 2009).

Effects on fetal dimensions

Despite the absence of a general fetal growth response, there is evidence that development of specific fetal tissues, most notably the bones, are sensitive to nutrition in early pregnancy (Annett & Carson, 2006; Firth *et al.*, 2008). This coincides with the initial stages of limb bud development and differentiation (around day 25, Black (1983)) and results in a much-reduced potential for skeletal growth during the period of rapid fetal growth in late pregnancy (Annett & Carson, 2006). These effects were illustrated in different studies (Table 2-6) where the restricted feeding starting in early gestation reduced the length of limbs (Annett & Carson, 2006; Blair *et al.*, In Press), crown-rump length (CRL) (McCrabb *et al.*, 1992; Vonnahme *et al.*, 2003; Vonnahme *et al.*, 2006) and girth circumference (McCrabb *et al.*, 1992; Blair *et al.*, In Press). However, some of the effects can be overcome by mid- to late-pregnancy nutrition and

result in fetuses at term with no differences or even greater CRL (McCrabb *et al.*, 1991; McCrabb *et al.*, 1992; Heasman *et al.*, 1998; Annett & Carson, 2006; Vonnahme *et al.*, 2006; Munoz *et al.*, 2008), girth (McCrabb *et al.*, 1991; Heasman *et al.*, 1998; Annett & Carson, 2006; Munoz *et al.*, 2008) or limbs (Munoz *et al.*, 2008).

Table 2-5: The effects of maternal nutritional manipulation starting in early pregnancy on fetal weight, post-natal growth and metabolism. Adapted from: Kenyon (2008) and van der Linden (2010).

Parameter	Period	Nutritional Treatments	Effects	Reference
Fetal weight	0 to 39 (EP); 40 to 90 (MP)	EP- L (0.6M) vs. M (1.0M) vs. H (2.0M) ; MP- M (1.0M) vs. H (2.0M) - (6 treatments)	Birth: L-EP heavier. No effect of MP nutrition.	(Munoz *et al.*, 2008)
	0 to 95	0.5M vs. 1.0M	Birth: no effect.	(Gopalakrishnan *et al.,* 2004)
	0 to parturition	L vs. H	Birth: L lighter.	(Schinckel & Short, 1961)
	0 to 30; 110 to parturition	0.5M vs. 1.0M	Birth: no effect.	(Gardner *et al.,* 2005)
	1 to 31	L (0.6M) vs. M (1.0M) vs. H (2.0M)	Birth: no effect.	(Annett & Carson, 2006)
	1 to 35	0.5M vs. 1.5M	Birth: no effect.	(Parr *et al.,* 1986)
	1 to 70	R vs. C	Birth: no effect.	(Krausgrill *et al.,* 1999)
	1 to 95	0.5M vs. 1.0M	Birth: no effect.	(Rae *et al.,* 2002)
	1 to 90 and 91 to parturition	Loss vs. Gain 25% BW each period (4 treatments)	Birth: Loss in each period reduced birth weight.	(Everitt, 1967)
	21 to 140	AL vs. M	Twins. D65: M lighter in fetuses from Heavy ewes. D100: no effect. D140: M lighter.	(Blair *et al.*, In Press)
	21 to 140	AL vs. M	Birth: Twin- AL heavier.	(van der Linden, 2010)
	21 to 140	H vs. M	D140: Twin- H heavier.	(Firth *et al.*, 2008)
	28 to 77	R (0.5M) vs. C (2.0M)	D80 and D145: Single- no effect.	(Heasman *et al.*, 1998; Heasman *et al.*, 1999)
	28 to 78	R (0.5M) vs. C (1.0M)	D78: Single and twins- R lighter.	(Vonnahme *et al.*, 2003; Vonnahme *et al.*, 2006)
	28 to 78	R (0.5M) vs. C (1.0M)	D78: Single and twins from ewes adapted to harsh environments- no differences.	(Vonnahme *et al.*, 2006)
	28 to 78	0.5M vs. 1.0M	Birth: no effect.	(Gilbert *et al.*, 2005; Ford *et al.,* 2007)
	28 to 80	0.5M vs. 1.0M vs. 1.5M	Birth: no effect.	(Gopalakrishnan *et al.,* 2005)
	30 to 80	R (0.6M) vs. C (2.25M)	D80: Single- no effect.	(Clarke *et al.*, 1998)
	30 to 70 or 30 to 85	0.5M vs. 1.0M	Birth: no effect.	(Daniel *et al.,* 2007)
	30 to 96	C (gain 6.6kg) vs. R (loss 8.0kg)	D96 and D140: Single- no effect.	(McCrabb *et al.*, 1991)
	30 to 96	C (10 kg DM/week) vs. R (~3.4 kg DM/week)	D96, D140 and Birth: Single- no effect.	(McCrabb *et al.*, 1992)
	30 to parturition	0.9M vs. 1.1M	Birth: no effect.	(Deligeorgis *et al.,* 1996)
	35 to 142	R (~0.7M) vs. well fed	D142: R lighter.	(Mellor & Murray, 1982)
	45 to parturition	UN vs. H nutrition	Birth: UN reduced weight.	(Gunn *et al.*, 1995)

Treatments: R, restricted; M, maintenance; C, control; AL, *ad libitum*; L, low; H, high; UN, under-nutrition. BW, body weight; DM, dry matter. Effects: D, day of measurement. ZST, zinc sulphate turbidity units; T3, free tri-iodothyronine; T4, thyroxine.

Table 2-5 (cont.)

Parameter	Period	Nutritional Treatments	Effects	Reference
Post-natal growth	0 to 39 (EP); 40 to 90 (MP)	EP- L (0.6M) vs. M (1.0M) vs. H (2.0M) ; MP- M (1.0M) vs. H (2.0M) (6 treatments)	H-EP higher growth rate to 6 weeks and to weaning (probably better milk production of the dam), less mortality and heavier at weaning. No differences between MP nutrition though M-MP tended to have greater growth rate to weaning.	(Munoz *et al.*, 2008)
	0 to 30; 110 to parturition	0.5M vs. 1.0M	No effect at 1 year of age.	(Gardner *et al.*, 2005)
	1 to 35	0.5M vs. 1.5M	No effect on growth to weaning.	(Parr *et al.*, 1986)
	1 to 70	R vs. Control	No effect on growth to weaning.	(Krausgrill *et al.*, 1999)
	1 to 95	0.5M vs. 1.0M	No effect on growth to weaning.	(Rae *et al.*, 2002)
	1 to 90 and 91 to parturition	Loss vs. Gain 25% BW each period (4 treatments)	Loss in each period reduced weaning weight.	(Everitt, 1967)
	21 to 140	AL vs. M	M reduced growth rate up to weaning.	(van der Linden, 2010)
	28 to 78	0.5M vs. 1.0M	0.5M heavier at 280 days of age.	(Ford *et al.*, 2007)
	28 to 78	0.5M vs. 1.0M	No effect.	(Gilbert *et al.*, 2005)
	28 to 80	0.5M vs. 1.0M vs. 1.5M	No effect on growth to weaning.	(Gopalakrishnan *et al.*, 2005)
	30 to parturition	0.9M vs. 1.1M	0.9M lighter.	(Deligeorgis *et al.*, 1996)
	45 to parturition	UN vs. H nutrition	UN lighter until 18 months of age, but no differences thereafter.	(Gunn *et al.*, 1995)
Metabolism	0 to 39 (EP); 40 to 90 (MP)	EP- L (0.6M) vs. M (1.0M) vs. H (2.0M) ; MP- M (1.0M) vs. H (2.0M) (6 treatments)	Birth: L-EP higher free T3, and tended to have higher ZST units and T4 than H or M; no differences between MP nutrition.	(Munoz *et al.*, 2008)
	28 to 78	R (0.5M) vs. C (1.0M)	D78: R decrease in fetal blood glucose concentrations.	(Vonnahme *et al.*, 2003; Vonnahme *et al.*, 2006)
	21 to 140	AL vs. M	16-month-old: Twin-female offspring from M-dams showed increased glucose concentrations in response to ETT. No major effect on glucose metabolism, adrenal function, lipolysis or insulin resistance.	(van der Linden *et al.*, 2010)

Treatments: R, restricted; M, maintenance; C, control; AL, *ad libitum*; L, low; H, high; UN, under-nutrition. BW, body weight; DM, dry matter. Effects: D, day of measurement. ZST, zinc sulphate turbidity units; T3, free tri-iodothyronine; T4, thyroxine.

Table 2-6: The effects of maternal nutritional manipulation starting in early pregnancy on fetal dimensions.

Fetal Measurement	Period	Nutritional Treatments	Effects	Reference
Crown-rump length	0 to 39 (EP); 40 to 90 (MP)	EP- L (0.6M) vs. M (1.0M) vs. H (2.0M) ; MP- M (1.0M) vs. H (2.0M) (6 treatments)	Birth: no effect. M-MP longer.	(Munoz *et al.*, 2008)
	1 to 31	L (0.6M) vs. M (1.0M) vs. H (2.0M)	D40-83 and Birth: no effect.	(Annett & Carson, 2006)
	21 to 140	AL vs. M	Twin. D65: M tended to be shorter. D100 and 140: M shorter.	(Blair *et al.*, In Press)
	28 to 77	R (0.5M) vs. C (2.0M)	Single. D80: no effect. D145: R increase (longer).	(Heasman *et al.*, 1998; Heasman *et al.*, 1999)
	28 to 78	R (0.5M) vs. C (1.0M)	D78: Single- no effect. Twins- R shorter.	(Vonnahme *et al.*, 2003; Vonnahme *et al.*, 2006)
	28 to 78	R (0.5M) vs. C (1.0M)	D78: Single and twins from ewes adapted to harsh environment- no effect.	(Vonnahme *et al.*, 2006)
	30 to 80	R (0.6M) vs. C (2.25M)	D80: Single- no effect.	(Clarke *et al.*, 1998)
	30 to 96	C (gain 6.6kg) vs. R (loss 8.0kg)	D96 and D140: Single- no effect.	(McCrabb *et al.*, 1991)
	30 to 96	C (10 kg DM/week) vs. R (~3.4 kg DM/week)	Single. D96: R smaller. D140: no differences.	(McCrabb *et al.*, 1992)
	35 to 142	R (~0.7M) vs. well fed	D81: no effect. D142: R smaller.	(Mellor & Murray, 1982)
Girth	0 to 39 (EP); 40 to 90 (MP)	EP- L (0.6M) vs. M (1.0M) vs. H (2.0M) ; MP- M (1.0M) vs. H (2.0M) (6 treatments)	D57: H-EP greater abdominal diameter compared to M or L; no effect of MP nutrition. D68 and D80: no differences in abdominal diameter. Birth: no differences in thoracic circumference.	(Munoz *et al.*, 2008)
	1 to 31	L (0.6M) vs. M (1.0M) vs. H (2.0M)	D40-83 and Birth: no effect.	(Annett & Carson, 2006)
	21 to 140	AL vs. M	Twin. D65: no effect. D100 and 140: M smaller.	(Blair *et al.*, In Press)
	28 to 77	R (0.5M) vs. C (2.0M)	D80 and D145: Single- no effect on thoracic circumference.	(Heasman *et al.*, 1998; Heasman *et al.*, 1999)
	30 to 80	R (0.6M) vs. C (2.25M)	D80: Single- no effect.	(Clarke *et al.*, 1998)
	30 to 96	C (gain 6.6kg) vs. R (loss 8.0kg)	D96 and D140: Single- no effect.	(McCrabb *et al.*, 1991)
	30 to 96	C (10 kg DM/week) vs. R (~3.4 kg DM/week)	Single. D96: no effect. D140: R smaller thoracic girth.	(McCrabb *et al.*, 1992)
	35 to 142	R (~0.7M) vs. well fed	D81 and D142: R smaller girth.	(Mellor & Murray, 1982)

Treatments: R, restricted; M, maintenance; C, control; AL, *ad libitum*; L, low; H, high. DM, dry matter. Effects: D, day of measurement.

Table 2-6 (cont.)

Fetal Measurement	Period	Nutritional Treatments	Effects	Reference
Limb	0 to 39 (EP); 40 to 90 (MP)	EP- L (0.6M) vs. M (1.0M) vs. H (2.0M) ; MP- M (1.0M) vs. H (2.0M) (6 treatments)	Birth: no differences in right fore-limb but hind-limb shorter in M-EP; no effect of MP nutrition.	(Munoz *et al.*, 2008)
	1 to 31	L (0.6M) vs. M (1.0M) vs. H (2.0M)	D83 and Birth: L shorter limbs than H fetuses.	(Annett & Carson, 2006)
	21 to 140	AL vs. M	Twin. D65, 100 and 140: M tended to have shorter hind-legs. D100: M tended to have shorter fore-leg. D140: M shorter fore-leg. No differences after adjustment for fetal weight.	(Blair *et al.*, In Press)
	30 to 80	R (0.6M) vs. C (2.25M)	D80: Single- no effect.	(Clarke *et al.*, 1998)
Head	0 to 39 (EP); 40 to 90 (MP)	EP- L (0.6M) vs. M (1.0M) vs. H (2.0M) ; MP- M (1.0M) vs. H (2.0M) (6 treatments)	D57: L-EP smaller cranial diameter compared to M or H; no effect of MP nutrition. D68 and D80: no differences. At birth: no effect of EP on head length; M-MP longer head.	(Munoz *et al.*, 2008)
	1 to 31	L (0.6M) vs. M (1.0M) vs. H (2.0M)	D40-83: Head length- no effect.	(Annett & Carson, 2006)

Treatments: R, restricted; M, maintenance; C, control; AL, *ad libitum*; L, low; H, high. DM, dry matter. Effects: D, day of measurement.

Effects on organ size

Muscle mass is determined mainly by the number of fibres within a muscle and the sizes of those fibres (Swatland & Cassens, 1973; McCoard *et al.*, 1997; Maltin *et al.*, 2001). The formation of muscle fibres *in utero* takes place in two or three waves, with primary fibres forming first (starting at day 32), followed by the secondary fibres (day 38) which grow around these primaries, and tertiary fibres (day 62-76) which develop between the secondary ones (Maltin *et al.*, 2001; Brameld & Daniel, 2008). Maternal dietary intake is one of the most important in determining the lean mass in the fetus (Firth *et al.*, 2008). All studies showing an increase or decrease in number of fibres formed, involved nutritional manipulations in the first two-thirds of gestation, during myofibre hyperplasia (Swatland & Cassens, 1973; Robinson *et al.*, 1999b), in which secondary fibres are preferentially affected (Maltin *et al.*, 2001). Hence, this sets the maximum potential for the major formation of secondary and tertiary fibres (Brameld & Daniel, 2008). Consequently, nutrient restriction in early pregnancy can cause a permanent reduction in muscle fibre number (Swatland & Cassens, 1973; Brameld & Daniel, 2008) and this has been observed in different muscles (*semitendinosus, longissimus dorsi* and *vastus lateralis*) as summarised in Table 2-7. However, if the dietary restriction is removed before the cessation of the major proliferation of muscle fibres (day 80; Swatland and Cassens (1973)), some compensatory growth can occur (Brameld & Daniel, 2008) and an effect on the growth potential of lambs is unlikely, even on the post-natal performance in terms of lamb mortality or daily live weight gain to weaning (Annett & Carson, 2006).

Muscle and adipose cells seem to be linked by competition or prioritisation in their differentiation and metabolism of nutrients (Figure 2-14, Bonnet *et al.* (2010)). Unlike muscle fibres, the number of adipocytes is not fixed at birth (Brameld & Daniel, 2008; Bonnet *et al.*, 2010) and there is evidence that maternal nutrition can affect the amount and type of adipose tissue in the offspring (Table 2-7). The adipose tissue is classified into white (WAT) which is the primary site of energy storage, and brown (BAT), characterised by the expression of uncoupling protein-1 which allows energy expenditure through thermogenesis (Bonnet *et al.*, 2010). Under-nutrition during early pregnancy is associated with long-term increases in adipose tissue (particularly BAT) in the offspring, probably due to a reduction in muscle fibres (Bonnet *et al.*, 2010) and/or a reduction in basal metabolic rate with excess of energy that needs to be stored (Brameld & Daniel, 2008). Nevertheless, this increase in BAT not necessarily translates into a greater thermogenesis and neonatal viability (Budge *et al.*, 2000).

Long-term nutritional outcomes during the embryogenesis appear to have central effects within the brain and cardiovascular function (Symonds *et al.*, 2007), even though the actual brain substrate delivery is unlikely to be limited in early gestation (Harding & Johnston, 1995). Twin fetuses from ewes fed maintenance (Blair *et al.*, In Press), or fetuses from ewes restricted (Vonnahme *et al.*, 2003) had lighter hearts (Table 2-7), but the heart can be disproportionally bigger when adjusted for fetal weight, and this is associated with a higher placental vascular resistance (Vonnahme *et al.*, 2003).

Lungs, liver and kidneys can also be negatively affected if the dam is under restricted feeding starting in early pregnancy. However, it was seen that liver weight per unit of fetal weight is increased, as a mechanism to increase the metabolic activity, particularly gluconeogenesis, to provide glucose to the developing fetus (Vonnahme *et al.*, 2003) and to maximise the use of the reduced food resources (Blair *et al.*, In Press). Later in life, these animals could have an advantage in physiological stressful situations such as pregnancy and lactation, as their livers may be able to supply more glucose to support their growing conceptus and milk production to increase the chances of survival of their offspring (van der Linden *et al.*, 2010). A similar situation is found for the kidney (Blair *et al.*, In Press).

Adrenal glands, thyroids, thymuses and gastrointestinal tracts are also affected by low plane of nutrition starting in early gestation (Blair *et al.*, In Press). Lighter spleen and thymuses could affect the immune-competence of the offspring later in life, whereas lighter thyroids could affect the normal development, growth and maturation of organs and tissues and tissues that will determine the survival of the individual (Symonds, 1995).

Under-nutrition during early gestation can affect the normal development of the oogonia (Table 2-7) and therefore fetuses can have lighter ovaries (Blair *et al.*, In Press). This can have negative consequences on life time reproductive performance, such as reduction in ovulation rate (Figure 2-13).

Organogenesis of the mammary gland starts early in embryogenesis as derivates of the epidermis, and much of its development is autonomous, governed by interactions between epithelium and surrounding mesenchyme (Robinson *et al.*, 1999a). The embryonic mammary gland development has been studied mostly in mice but not to the same extent in sheep. In mice (Figure 2-15), the mammary bud starts to differentiate on embryonic days 10-11 and the ductal branching morphogenesis on day 16 (Robinson *et al.*, 1999a; Cowin & Wysolmerski, 2010). During these days, a ductal lumen is formed and the skin overlying the primary mammary mesenchyme is remodelled into the typical nipple structure (Cowin & Wysolmerski,

2010). At the conclusion of embryonic development (day 18-20), the mammary gland consists of a short primary duct ending in a small, branched ductal tree at one end of a larger mammary fat pad, that will further develop during puberty and pregnancy to ultimately produce the mature milk-producing glands found during lactation (Cowin & Wysolmerski, 2010). Although the mammary gland organogenesis is not likely to be influenced by external factors, the fetal gland shows endocrine sensitivity (Robinson *et al.*, 1999a). In addition, ductular development that occurs in early fetal life (one of the critical windows, as highlighted previously in Figure 2-13) may affect secretory tissue mass and thus milk production during lactation, because the secretory cells proliferate in the ducts (Knight & Sorensen, 2001). The effects of maternal nutrition on the mammary gland development in sheep fetuses have been inconsistent (Table 2-7), partially attributed to different feeding levels and timing of the nutritional insults. In one study (Jenkinson, 2003), ewes were fed either a maintenance, *ad libitum* or a combination of both diets throughout gestation and the weights of the fetal mammary glands were not affected at day 103 or 137 of gestation, but the fetuses from ewes fed a maintenance diet had a smaller duct area, less ducts and smaller secretion cell area than those in the *ad libitum* group (Blair *et al.*, 2010). In another study (van der Linden *et al.*, 2009), ewes were fed a maintenance or *ad libitum* diet as well, but found no effect on total duct area or total number of ducts at day 100 of gestation. However, the mammary glands of fetuses from ewes fed maintenance were heavier at day 100 (van der Linden *et al.*, 2009) and tended to be lighter at day 140 (Blair *et al.*, In Press) of gestation than those from the ewes fed *ad-libitum*. Paradoxically, these offspring from the maintenance group produced more milk and milk of altered composition at 18 months of age (van der Linden *et al.*, 2009). Although these results do contrast, it can be concluded that mammary gland development can be altered in utero by maternal nutrition and this can affect later milk production.

Figure 2-15: Overview of embryonic mammary development in mice. Source: Cowin & Wysolmerski (2010).

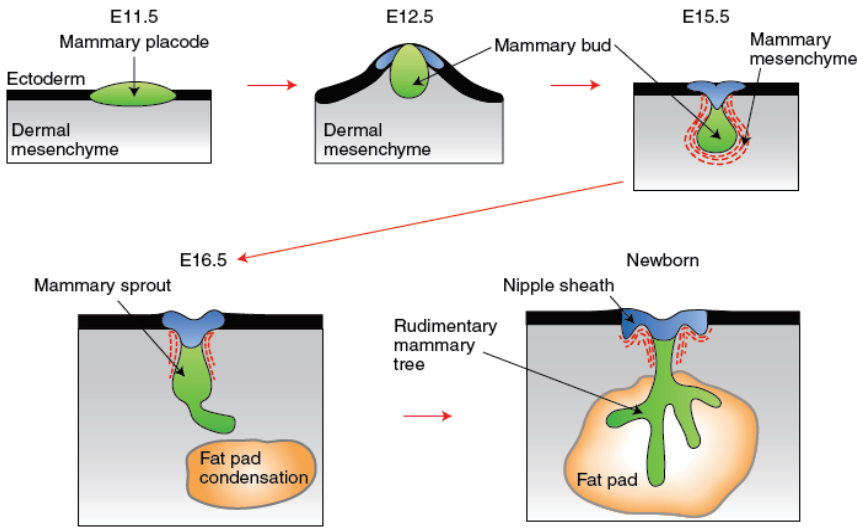


Table 2-7: The effects of maternal nutritional manipulation starting in early pregnancy on fetal organs. Adapted from: Kenyon (2008), Brameld & Daniel (2008) and van der Linden (2010).

Tissue	Period	Nutritional Treatments	Effects	Reference
Liver	21 to 140	AL vs. M	Twin. D65, 100 and 140: no effect. D140: M heavier as relative weight.	(Blair *et al.*, In Press)
	28 to 77	R (0.5M) vs. C (2.0M)	D145: Single- no effect.	(Heasman *et al.*, 1998)
	28 to 78	R (0.5M) vs. C (1.0M)	D78: R negative- smaller but greater as relative weight.	(Vonnahme *et al.*, 2003)
	30 to 80	R (0.6M) vs. C (2.25M)	D80: Single- no effect.	(Clarke *et al.*, 1998)
	30 to 96	C (gain 6.6kg) vs. R (loss 8.0kg)	D96: Single- no effect.	(McCrabb *et al.*, 1991)
Kidney	21 to 140	AL vs. M	Twin. D65, 100 and 140: no effect. D140: M heavier as relative weight.	(Blair *et al.*, In Press)
	28 to 77	R (0.5M) vs. C (2.0M)	D145: Single- no effect.	(Heasman *et al.*, 1998)
	30 to 80	R (0.6M) vs. C (2.25M)	D80: Single- no effect.	(Clarke *et al.*, 1998)
	30 to 96	C (gain 6.6kg) vs. R (loss 8.0kg)	D96: Single- no effect on any kidney.	(McCrabb *et al.*, 1991)
Lung	21 to 140	AL vs. M	Twin. D65: no effect. D100: M tended to be lighter.	(Blair *et al.*, In Press)
	28 to 78	R (0.5M) vs. C (1.0M)	D78: R negative.	(Vonnahme *et al.*, 2003)
	28 to 77	R (0.5M) vs. C (2.0M)	D145: Single- no effect.	(Heasman *et al.*, 1998)
	30 to 80	R (0.6M) vs. C (2.25M)	D80: Single- no effect.	(Clarke *et al.*, 1998)
	30 to 96	C (gain 6.6kg) vs. R (loss 8.0kg)	D96: Single- no effect on any kidney.	(McCrabb *et al.*, 1991)
Gastro-intestinal track	21 to 140	AL vs. M	Twin. D65 and 100: no effect. D140: M lighter.	(Blair *et al.*, In Press)
Heart	21 to 140	AL vs. M	Twin. D65 and 100: no effect. D140: M lighter.	(Blair *et al.*, In Press)
	28 to 77	R (0.5M) vs. C (2.0M)	D145: Single- no effect.	(Heasman *et al.*, 1998)
	28 to 78	R (0.5M) vs. C (1.0M)	D78: Twin- R negative, smaller but greater relative weight.	(Vonnahme *et al.*, 2003)
	30 to 80	R (0.6M) vs. C (2.25M)	D80: Single- no effect.	(Clarke *et al.*, 1998)
	30 to 96	C (gain 6.6kg) vs. R (loss 8.0kg)	D96: Single- no effect on any kidney.	(McCrabb *et al.*, 1991)
Brain	28 to 77	R (0.5M) vs. C (2.0M)	Single. D80: R smaller. D145: no effect.	(Heasman *et al.*, 1998; Heasman *et al.*, 1999)
	30 to 80	R (0.6M) vs. C (2.25M)	D80: Single- R smaller.	(Clarke *et al.*, 1998)
	30 to 96	C (gain 6.6kg) vs. R (loss 8.0kg)	D96: Single- no effect.	(McCrabb *et al.*, 1991)
Thyroid	21 to 140	AL vs. M	Twin. D65: no effect. D100 and 140: M lighter.	(Blair *et al.*, In Press)
Thymus	21 to 140	AL vs. M	Twin. D65: no effect. D100 and 140: M lighter.	(Blair *et al.*, In Press)

Treatments: R, restricted; M, maintenance; C, control; AL, *ad libitum*; L, low; H, high; UN, under-nutrition; N, normal nutrition. Effects: D, day of measurement.

Table 2-7 (cont.)

Tissue	Period	Nutritional Treatments	Effects	Reference
Adrenal gland	21 to 140	AL vs. M	Twin. D65 and 100: no effect. D140: M lighter.	(Blair *et al.*, In Press)
Spleen	21 to 140	AL vs. M	Twin. D65 and 100: no effect. D140: M lighter.	(Blair *et al.*, In Press)
Muscle	21 to 140	AL vs. M	Twin. D65 and 100: no effect. D140: M lighter *semitendinosus*.	(Blair *et al.*, In Press)
	21 to 140	H vs. M	D140: Twin- Lean Mass in fetus from Large dams in H was greater than any other twins (from Small dams or in M feeding).	(Firth *et al.*, 2008)
	28 to 78	0.5M vs. 1.0M	D78: decrease 2:1 fibre ratio of *longissimus dorsi* muscle.	(Zhu *et al.,* 2004)
	30 to 70	0.5M vs. 1.0M	After birth: decrease number of fibre/area and increase diameter of fast fibres of *longissimus dorsi, vastus lateralis* and *semitendinosus* muscle. Increase number of fibres/area with similar diameter of slow fibres, and decrease in fast: slow fibre ratio.	(Fahey *et al.,* 2005b)
Adipose tissue	1 to 110	R (0.5M) vs. C (1.0M)	D110: R increase in perirenal adipose tissue weight.	(Gopalakrishnan *et al.*, 2001)
	28 to 77	R (0.5M) vs. C (2.0M)	D145: Single- no effect on perirenal adipose tissue.	(Heasman *et al.*, 1998)
	28 to 80	R (0.5M) vs. C (1.0M)	D145: R increase in perirenal adipose tissue weight.	(Bispham *et al.,* 2002)
Ovary	0 to 47	R (0.5M) vs. 1.5M	R affected the normal development of the oogonia (fetal ovarian differentiation 0-30 and growth 30-50). D110: ovarian morphology affected and reduction in ovarian weight.	(Borwick *et al.*, 1997)
	0 to 30/50; 31 to 50/65; 66 to 110; 0 to 110	0.5M vs. 1.0M	0.5M delayed the fetal follicle development.	(Rae *et al.,* 2001)
	1 to 30/50; 31 to 50/65; 66 to 110; 0 to 110	0.5M vs. 1.0M	0.5M negatively affected ovarian development.	(Lea *et al.,* 2006)
	21 to 140	AL vs. M	Twin. D100: no effect. D140: M lighter.	(Blair *et al.*, In Press)
	30 to parturition	0.9M vs. 1.1M	No effect on female or male gonad weight at 55 days of age but 0.9M reduced response to GnRH.	(Deligeorgis *et al.,* 1996)
	45 to parturition and lactation	UN vs. H nutrition	UN negatively affected reproductive life time performance.	(Gunn *et al.*, 1995)
Mammary gland	19 to 140	AL (1.5M) vs. M	D100 and 140: AL greater duct area and more secretion cells area. No effect on the mammary weight.	(Jenkinson, 2003)
	21 to 140	AL vs. M	D140: Twin- M tended to be lighter.	(Blair *et al.*, In Press)
	21 to 140	AL vs. M	D100: Twin- AL lighter, no effect on total duct area or total number of ducts.	(van der Linden *et al.*, 2009)

Treatments: R, restricted; M, maintenance; C, control; AL, *ad libitum*; L, low; H, high; UN, under-nutrition; N, normal nutrition. Effects: D, day of measurement.

Effects on placenta

Placental growth commences approximately at day 30 (Symonds *et al.*, 2010) and it appears to be particularly sensitive to the ewe nutrition between days 30-50 (McCrabb *et al.*, 1992). In general terms, restricted feeding throughout pregnancy results in significant reductions in placental growth (Wallace, 1948; Mellor & Murray, 1982; Anthony *et al.*, 2003; Wu *et al.*, 2004). Poor nutrition from 28 days until 78-80 days of gestation, where maximal placental growth takes place, can also restrict the placental mass (Symonds *et al.*, 2010) or the placentome sizes (Clarke *et al.*, 1998), and generally reduces the placental efficiency (fetal weight/total placentomal weight, Vonnahme *et al.* (2003; 2006)). Thus, it is likely that a small placenta caused by the reduced nutrition during the first trimester, will aggravate the effects of continuing maternal under-nutrition, with the result of fetuses born small and of low viability (Mellor & Murray, 1982).

However, under-nutrition during early gestation only, has little or no effect on placental growth (Table 2-8), as the changes appear to be temporary and not visible at birth (Robinson *et al.*, 1999b). Ewes fed to meet their energy requirements for the remainder of pregnancy can have a larger placenta at term (McCrabb *et al.*, 1991; Heasman *et al.*, 1998; Symonds *et al.*, 2010), enhanced placental efficiency (Symonds *et al.*, 2010), higher everted placentomes (Heasman *et al.*, 1998) or increased vascularity due to a higher number of caruncular blood vessels (Vonnahme *et al.*, 2003). Consequently, the fetal growth is not affected negatively in the second half of gestation. Furthermore, some studies found that the nutrient transfer capacity of the placenta is not impaired by extremes of dietary intake from conception continuing up to the time with maximal fetal growth (day 110), as the lamb birth weight was not affected (Robinson *et al.*, 1999b; Annett & Carson, 2006; Gardner *et al.*, 2007; Symonds *et al.*, 2010).

Different mechanisms take place when ewes are adapted to harsh environments. After a restricted feed intake from 28 days until 78-80 days of gestation, ewes or their conceptuses initiate conversion of type A placentomes to other placentomal types (B, C and D), what may function to maintain normal nutrient delivery to their developing fetuses through an increase in size, vascularity and blood flow, and no differences in fetal weight can be seen. Such conversion fails to occur in ewes used to being well fed (Vonnahme *et al.*, 2006).

Table 2-8: The effects of maternal nutritional manipulation starting in early pregnancy on the placenta.

Placenta	Period	Nutritional Treatments	Effects	Reference
Placenta weight	28 to 77	R (0.5M) vs. C (2.0M)	Single. D80: R lighter. D145: R increase placental weight and decrease fetal/placental weight ratio.	(Heasman *et al.*, 1998; Heasman *et al.*, 1999)
	28 to 78	R (0.5M) vs. C (1.0M)	D78: R reduced placental efficiency (fetal weight/total placentomal weight)	(Vonnahme *et al.*, 2003; Vonnahme *et al.*, 2006)
	30 to 80	R (0.6M) vs. C (2.25M)	D80: Single- no effect.	(Clarke *et al.*, 1998)
	30 to 96	C (gain 6.6kg) vs. R (loss 8.0kg)	Single. D96: R increase 21%; D140: R increase 30%.	(McCrabb *et al.*, 1991)
	30 to 96	C (10 kg DM/week) vs. R (~3.4 kg DM/week)	D96 and D140: Single- R lighter.	(McCrabb *et al.*, 1992)
	35 to 142	R (~0.7M) vs. well fed	D142: Twin- R lighter.	(Mellor & Murray, 1982)
Placentome	0 to 39	EP- L (0.6M) vs. M (1.0M) vs. H (2.0M); MP- M (1.0M) vs. H (2.0M) (6 treatments)	D57, D68 and D80: no differences in cotyledon diameter.	(Munoz *et al.*, 2008)
	28 to 77	R (0.5M) vs. C (2.0M)	D145: Single- R increase number of placentomes and proportion of everted placentomes, and cotyledonary weight and number. No difference on caruncular weight.	(Heasman *et al.*, 1998)
	28 to 78	R (0.5M) vs. C (1.0M)	Ewes adapted to harsh environment- D78: R conversion of type A placentomes to other placentomal types (B, C and D)	(Vonnahme *et al.*, 2006)
	28 to 78	R (0.5M) vs. C (1.0M)	D78: Number, type or distribution of placentomes not affected; vascularity was increased due to a higher number of caruncular blood vessels	(Vonnahme *et al.*, 2003)
	28 to 78	R (0.5M) vs. C (1.0M)	D78: R reduced placental efficiency (fetal weight/total placentomal weight)	(Vonnahme *et al.*, 2003; Vonnahme *et al.*, 2006)
	30 to 80	R (0.6M) vs. C (2.25M)	D80: Single- no effect on proportion of everted placentomes. R reduced the placentome size due to a decrease in the fetal component, which had less DNA content and more haemoglobin concentration.	(Clarke *et al.*, 1998)
	30 to 96	C (gain 6.6kg) vs. R (loss 8.0kg)	Single. D96 and D140: no effect on number of cotyledons.	(McCrabb *et al.*, 1991)
	30 to 96	C (10 kg DM/week) vs. R (~3.4 kg DM/week)	D96: Single- no effect of functional placentome numbers.	(McCrabb *et al.*, 1992)

Treatments: R, restricted; M, maintenance; C, control; AL. DM, dry matter. Effects: D, day of measurement.

2.2.4 Mid (days 50-100) and Late (days 100-150) Pregnancy

The most profound effects of maternal under or over- nutrition on feto-placental growth and development seem to occur when nutritional challenges are applied during the rapid placental development period, approximately between days 40 and 80 of pregnancy (Robinson *et al.*, 1999b; Wu *et al.*, 2006). The total placental weight increases to a maximum value around 70 days of gestation (Schneider, 1996). Hence, placental growth appears to be more sensitive to maternal feed restriction during the earlier stages (days 30-50) and fetal growth during the latter stages (days 75-96) of mid-pregnancy (McCrabb *et al.*, 1992). However, the nutrient restriction during this stage does not necessarily result in functional placental insufficiency and fetal growth restriction, and therefore the outcomes are not straight forward. Furthermore, there have been different results reported and these may relate to differences in ewe breed, age, parity, maturity, size, body condition, as well as nutritional status or environmental exposures of the ewes during fetal or postnatal life (McCrabb *et al.*, 1991; Robinson *et al.*, 1999b; Anthony *et al.*, 2003; Vonnahme *et al.*, 2006).

During late pregnancy, most of the increase in the size of the fetus takes place (Rattray *et al.*, 1974; Robinson *et al.*, 1999b; Kenyon *et al.*, 2007; Symonds *et al.*, 2007), and the majority of the important fetal growth factors (e.g. IGF-I, insulin and thyroid hormones) are nutritionally regulated (Bloomfield *et al.*, 2006). Nutrient restriction during late gestation may result in placental or fetal growth restriction, depending on maternal nutrient reserves at the onset of restriction (Harding & Johnston, 1995; Anthony *et al.*, 2003), the length of the restriction (Mellor & Murray, 1982) and the severity of it (Gardner *et al.*, 2007). Ewes in moderate to high body condition (2.5-3) 8 weeks before lambing, can afford to lose a further 0.5 body condition without prejudicing their lambing performance (Russel, 1984) because the maternal body can adapt to alterations in nutritional status (Robinson *et al.*, 1999b), but every effort must be made to prevent the condition of the poorer ewes falling below body condition 2 at lambing (Russel, 1984).

Effects on fetal growth, body weight and dimensions

Energy intake during the last period of gestation has a significant influence on lamb birth weight (Gardner *et al.*, 2007). In general terms, an increased feeding from mid gestation enhances fetal growth (Symonds *et al.*, 2010) and therefore has a positive effect on lamb birth weight, particularly in ewes with low live weight, size and body condition at mating (Russel *et*

al., 1981). On the contrary, a nutrient restriction starting in mid (Table 2-9) or late (Table 2-10) pregnancy, reduces fetal growth and birth weight. An acute restriction at this stage (e.g. 50-60% of metabolisable energy requirements), reduces fetal growth drastically that cannot be compensated later on (Gardner *et al.*, 2007), and involves maternal ketosis and impaired liver function (Robinson *et al.*, 1999b). Nonetheless, the fetal response to such restriction may not be so extreme, as the fetal growth trajectory in late gestation can be determined in the peri-conceptional period (Bloomfield *et al.*, 2006).

In the case of chronic under-nutrition, the metabolism of mother, fetal organs and tissues have time to adapt to this condition. The fetal growth is desaccelerated gradually, and fetal cortisol concentrations are stimulated, which enhance the hepatic and renal glycogenic capability (Robinson *et al.*, 1999b). Maternal adaptations can include shifts in voluntary food intake, enhanced net uterine uptake of glucose through increases in glucose production from peripheral mobilisation and hepatic uptake of amino acids, increased placenta transport of glucose, augmented placental gene expression and maternal concentrations of lactogen to increase fetal IGF production, maternal insulin resistance to reduce glucose uptake by the maternal tissues, between many others (Robinson *et al.*, 1999b).

The effects of restricted feeding from mid gestation onwards are generally small on the fetal CRL or girth, depending on the severity of the restriction (Table 2-11).

Table 2-9: The effects of maternal nutritional manipulation starting in mid pregnancy on fetal weight and post-natal growth. Adapted from: Kenyon (2008).

Fetal Parameter	Period	Nutritional Treatments	Effects	Reference
Fetal weight	50 to 96	C (10 kg DM/week) vs. R (~3.4 kg DM/week)	D96, D140 and Birth: Single- no effect.	(McCrabb *et al.*, 1992)
	50 to 130	0.6M vs. 1.0M	D90 and D130: 0.6M lighter.	(Scheaffer *et al.*, 2004)
	50 to 140	Sub M vs. M	Birth: Sub M lighter.	(Kelly *et al.*, 1996)
	64 to 135	C (1.0M) vs. R (0.6M)	D135: R lighter.	(Lekatz *et al.*, 2010)
	64 to parturition	2, 4, 6 vs. 8 cm swards	Birth: 2 cm lighter.	(Morris & Kenyon, 2004)
	64 to parturition	2 vs. 6 cm swards	Birth: 2 cm lighter.	(Corner *et al.*, 2005)
	70 to 140	1.5M vs. 2.0M	Twin - 2.0M heavier (total 11.35kg vs. 10.29kg for 1.5M).	(Rattray *et al.*, 1974)
	70 to parturition	0.7M vs. 1.1M	Birth: 0.7M lighter.	(Bielli *et al.*, 2005)
	70 to 107 and 108 to 147	2cm vs. 4 cm sward each period (2-2, 2-4, 4-2 vs. 4-4 cm)	Birth: 2-2 and 4-2 lighter than 4-4.	(Corner *et al.*, 2008)
	75 to 96	C (10 kg DM/week) vs. R (~3.4 kg DM/week)	D96, D140 and Birth: Single- no effect.	(McCrabb *et al.*, 1992)
	80 to 144	C (1.0M) vs. well-fed (1.5M)	D141-144: Well-fed heavier.	(Budge *et al.*, 2000)
	90 to parturition	R1 (0.175), R2 (0.33) vs. C (AL) MJ ME $kgw^{-0.75}$/d	Birth: R1 lighter carcass weight but no differences in relative weight.	(Gao *et al.*, 2009)
	mid- to late-	1.0M vs. above M	Birth: above M increase.	(Bielli *et al.*, 2001)
Post-natal growth	mid- to late-	1.0M vs. above M	Above M increase growth to weaning.	(Bielli *et al.*, 2001)
	50 to 140	Sub M vs. M	Weaning: Sub M lighter.	(Kelly *et al.*, 1996)
	64 to parturition	2, 4, 6 vs. 8 cm swards	No effect on growth to weaning or survival.	(Morris & Kenyon, 2004)
	70 to 107 and 108 to 147	2cm vs. 4 cm sward each period (2-2, 2-4, 4-2 vs. 4-4 cm)	Weaning: 2-2 lighter than 4-4.	(Corner *et al.*, 2008)

Treatments: R, restricted; M, maintenance; C, control; AL, *ad libitum*. DM, dry matter; ME, metabolisable energy; MJ, mega joules; kgw, kilogram of body weight; d, day. Effects: D, day of measurement.

Table 2-10: The effects of maternal nutritional manipulation starting in late pregnancy on fetal growth, weight, post-natal growth and metabolism. Adapted from: Kenyon (2008) and van der Linden (2010).

Fetal Parameter	Period	Nutritional Treatments	Effects	Reference
Fetal weight	105 to 115 (UN10) vs. 105 to 125 (UN20)	0.3-0.5 vs. 13-15 MJ ME/d	Birth: UN20 lighter.	(Oliver *et al.,* 2001)
	110 to 147	R (0.5-0.6 ME req)	Birth: lighter. The effect is +207±3 g/1 MJ of energy intake	(Gardner *et al.*, 2007)
	100 to parturition	0.7M vs. 1.0M	Birth: 0.7M lighter.	(Borwick *et al.,* 2003)
	last 6 weeks	Sub M, 1.0M vs. AL	Birth: Sub M lighter than M and M lighter than AL.	(Wallace, 1948)
	last 6 weeks	0.6M vs. H	Birth: 0.6M lighter.	(Tygensen *et al.,* 2007)
	last 6 weeks	7 vs. 15 MJ ME/d	Birth: 7 MJ lighter.	(Husted *et al.,* 2008)
Post-natal growth	100 to parturition	0.7M vs. 1.0M	Weaning: 0.7M lighter (14 weeks of age).	(Borwick *et al.,* 2003)
	105 to 115 (UN10) vs. 105 to 125 (UN20)	0.3-0.5 vs. 13-15 MJ/d	Weaning: UN20 lighter. No effect at 30 months of age.	(Oliver *et al.,* 2001)
	last 6 weeks	Sub M, 1.0M vs. AL	Weaning: Sub M lighter.	(Wallace, 1948)
	last 6 weeks	0.6M vs. H	Weaning: 0.6M lighter. No effect at 145 days of age.	(Tygensen *et al.,* 2007)
	last 6 weeks	7 vs. 15 MJ ME/d	7 MJ lighter at 11 months of age. No effect thereafter.	(Husted *et al.,* 2008)

Treatments: R, restricted; M, maintenance; C, control; AL, *ad libitum*; L, low; H, high; UN, under-nutrition; N, normal nutrition. ME, metabolisable energy; MJ, mega joules; d, day. Effects: D, day of measurement.

Table 2-11: The effects of maternal nutritional manipulation starting in mid or late pregnancy on fetal dimensions.

Fetal Measurement	Period	Nutritional Treatments	Effects	Reference
Crown-rump length	50 to 96	C (10 kg DM/week) vs. R (~3.4 kg DM/week)	Single. D96: R smaller. D140: no effect.	(McCrabb *et al.*, 1992)
	50 to 130	0.6M vs. 1.0M	D90 and D130: no effect.	(Scheaffer *et al.*, 2004)
	64 to parturition	2, 4, 6 vs. 8 cm swards	Birth: no effect.	(Morris & Kenyon, 2004)
	75 to 96	C (10 kg DM/week) vs. R (~3.4 kg DM/week)	Single. D96: R smaller. D140: no effect.	(McCrabb *et al.*, 1992)
	90 to parturition	R1 (0.175), R2 (0.33) vs. C (AL) MJ ME $kgw^{-0.75}$/d	Birth: R1 smaller.	(Gao *et al.*, 2009)
Girth	50 to 96	C (10 kg DM/week) vs. R (~3.4 kg DM/week)	D96 and D140: Single- no effect.	(McCrabb *et al.*, 1992)
	64 to parturition	2, 4, 6 vs. 8 cm swards	Birth: 2 cm smaller.	(Morris & Kenyon, 2004)
	75 to 96	C (10 kg DM/week) vs. R (~3.4 kg DM/week)	D96 and D140: Single- no effect.	(McCrabb *et al.*, 1992)
	90 to parturition	R1 (0.175), R2 (0.33) vs. C (AL) MJ ME $kgw^{-0.75}$/d	Birth: R1 and R2 smaller thoracic girth. No effect on umbilical girth.	(Gao *et al.*, 2009)

Treatments: R, restricted; M, maintenance; C, control; AL, *ad libitum*. DM, dry matter; ME, metabolisable energy; MJ, mega joules; kgw, kilogram of body weight; d, day. Effects: D, day of measurement.

Effects on fetal organ size

The organs most affected by maternal malnutrition from mid gestation are the late-maturing organs like the kidney, lung, liver and spleen (Table 2-12). Lung growth is particularly susceptible to nutritional challenge in late gestation possibly because they are rapidly growing at this time (Harding & Johnston, 1995). The restricted growth is largely accounted by a decreased cell number and size per unit of tissue, and this can affect respiratory health and impair lung function permanently during postnatal life (Gao *et al.*, 2009). Similar effects are seen in the liver, where smaller cell hypertrophy results in a lighter organ; this in turn reduces the number of receptors for growth hormone and could lead to growth retardation of the fetus (Gao *et al.*, 2009). Moreover, to adapt to maternal under-nutrition, the fetus initiates hepatic gluconeogenesis that changes the fetal glucose homeostasis and this influences the fetal insulin and IGF-1 concentrations, making these hormones critical determinants of the response in fetal growth to alteration in the supply of glucose (Robinson *et al.*, 1999b). In the case of the spleen of nutrient restricted fetuses, there is mainly a cell proliferation reduction that markedly lowers the organ weight (Gao *et al.*, 2009). This can affect the immune-competence of the offspring, causing high neonatal morbidity and mortality (Gao *et al.*, 2009). The kidney is a sensitive organ to influences during placental development (Symonds *et al.*, 2007), although this may not be reflected in altered organ size at birth (Gao *et al.*, 2009).

The brain and heart have high priority and more nutrients are diverted to the their metabolism at the expense of less essential organs such as the trunk, limbs, abdominal viscera and skin (Bloomfield *et al.*, 2006; Gao *et al.*, 2009). However, the heart can be smaller due to severe maternal under-nutrition from day 90 of pregnancy onwards (Gao *et al.*, 2009). This redistribution of fetal cardiac output may play an important role in maintaining the relative growth and optimal function, but can also predispose to cardiovascular diseases, i.e. hypertension, coronary disease, stroke and metabolic disruption later in life, affecting production efficiency and health (Barker, 1998; Vonnahme *et al.*, 2003; Vonnahme *et al.*, 2006).

The gastro-intestinal tract is also sensitive to reduced nutrition from mid pregnancy (Gao *et al.*, 2009), and this may be an effect of reduced flow of nutrients across the placenta (Robinson *et al.*, 1999b). The gastrointestinal tissues are affected to different extents. The abomasum and jejunum can be lighter which contribute to a growth retardation of the stomach and intestine, therefore influencing the growth and viability of neonatal lambs (Gao *et al.*, 2009).

The reproductive organs may also be affected by maternal under-nutrition from mid gestation onwards. The fetal ovary has a nutritionally sensitive window during days 50-65 of gestation, with a direct effect on its development that can alter the germ cell meiosis and ovulation rate in adult life (Figure 2-12, Robinson *et al.* (2006)). In the embryonic mammary gland, the fat pad becomes a secondary stroma, supporting ductal morphogenesis in the late fetal life and leading to the establishment of a rudimentary epithelial system at birth (Robinson *et al.*, 1999a).

The fetal adipose tissue, or more specifically adipose metabolism, is very sensitive to maternal nutrition in mid- to late-gestation (Symonds *et al.*, 2007) because the number of adipocytes increases mainly before birth (brown pre-adipocytes) until 1 year of age (white pre-adipocytes), depending on the anatomical location of the adipose tissue (Bonnet *et al.*, 2010). The perirenal BAT constitutes up to 80 % of adipose tissue depots in the ovine fetus, and the increase in BAT weight plus specific enzymic and morphological changes, occur predominantly between 120 days of gestation and term (Clarke *et al.*, 1997). Thus, an increase in maternal nutrition from mid gestation promotes BAT growth and maturation, instead of muscle (Clarke *et al.*, 1997; Budge *et al.*, 2000). This brings clear benefits as BAT contains uncoupling protein-1 that enables the rapid generation of heat in the newborn and enhances neonatal viability (Clarke *et al.*, 1997; Budge *et al.*, 2000). Maternal prolactin may be the mediator of this mechanism (Figure 2-16, Symonds *et al.* (2010)). Opposite effects are seen in fetuses from mothers undernourished in late gestation (Table 2-12).

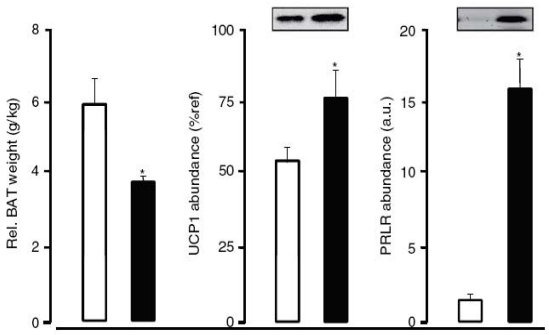


Figure 2-16: Effect of increased maternal food intake over the final half of gestation on relative fat mass, the brown adipose tissue specific uncoupling protein (UCP) 1 and the abundance of the prolactin receptor (PRLR) in the newborn sheep. Mothers were either fed to 100% of total metabolisable energy requirements (open boxes) or *ad libitum* (i.e. 150% of metabolisable energy requirements; closed boxes). After Budge *et al.* (2000). Source: Symonds *et al.* (2010).

Table 2-12: The effects of maternal nutritional manipulation starting in mid or late pregnancy on fetal organs. Adapted from: Brameld & Daniel (2008).

Tissue	Period	Nutritional Treatments	Effects	Reference
Liver	90 to parturition	R1 (0.175), R2 (0.33) vs. C (AL) MJ ME $kgw^{-0.75}$/d	Birth: R1 lighter, due to smaller cell hypertrophy. No differences in relative weight.	(Gao *et al.*, 2009)
Kidney	90 to parturition	R1 (0.175), R2 (0.33) vs. C (AL) MJ ME $kgw^{-0.75}$/d	Birth: no effect.	(Gao *et al.*, 2009)
Lung	90 to parturition	R1 (0.175), R2 (0.33) vs. C (AL) MJ ME $kgw^{-0.75}$/d	Birth: R1 smaller than R2, and R2 smaller than C, due to a decreased cell number and size per unit of tissue. No differences in relative weight.	(Gao *et al.*, 2009)
Gastro-intestinal track	90 to parturition	R1 (0.175), R2 (0.33) vs. C (AL) MJ ME $kgw^{-0.75}$/d	Birth: R1 reduced the weight of the abomasum and small intestine, but no differences in relative weight. No differences in rumen or reticulum-omasum.	(Gao *et al.*, 2009)
Heart	90 to parturition	R1 (0.175), R2 (0.33) vs. C (AL) MJ ME $kgw^{-0.75}$/d	Birth: R1 smaller but no differences in relative weight.	(Gao *et al.*, 2009)
Brain	90 to parturition	R1 (0.175), R2 (0.33) vs. C (AL) MJ ME $kgw^{-0.75}$/d	Birth: no effect as absolute weight but relative smaller in C.	(Gao *et al.*, 2009)
Spleen	90 to parturition	R1 (0.175), R2 (0.33) vs. C (AL) MJ ME $kgw^{-0.75}$/d	Birth: R1 and R2 lighter (absolute and relative weight), due to a cell proliferation reduction.	(Gao *et al.*, 2009)
Pancreas	90 to parturition	R1 (0.175), R2 (0.33) vs. C (AL) MJ ME $kgw^{-0.75}$/d	Birth: no effect.	(Gao *et al.*, 2009)
Muscle	55 to 95	0.5M vs. 1.0M (ME requirements)	After birth: increase diameter of fast fibres of *vastus lateralis* muscle. No effect on diameter of slow fibres of *vastus lateralis*, or any fibres (fast or slow) of *longissimus dorsi* and *semitendinosus* muscle. No effect on number of fibres/area (fast or slow) in any muscle.	(Fahey *et al.*, 2005b)
	85 to 115	0.5M vs. 1.0M (ME requirements)	After birth: no effect on number of fibre/area or diameter of fibres (fast or slow) of l*ongissimus dorsi, vastus lateralis* and *semitendinosus* muscle.	(Fahey *et al.,* 2005b)
Adipose tissue	80 to 144	C (1.0M) vs. well-fed (1.5M)	D141-144: Well-fed increase BAT, UCP-1 abundance and thermogenesis activity. C had higher BAT per kg of body weight.	(Budge *et al.*, 2000)
	115 to 145	R (0.5M) vs. C (1.0M)	D145: R decrease in perirenal adipose tissue (BAT) weight.	(Budge *et al.,* 2002)
Ovary	50 to 65	R (0.5M)	Negative- alteration in germ cell meiosis (D50-65) and ovulation rate in adult life	(Robinson *et al.*, 2006)

Treatments: R, restricted; M, maintenance; C, control; AL, *ad libitum*. ME, metabolisable energy; MJ, mega joules; kgw, kilogram of body weight; d, day. Effects: D, day of measurement.

Effects on placenta

After mid pregnancy, placental weight is maintained or may even decline close to term, but there is an extensive morphological remodelling and functional maturation throughout the second half of gestation to support the increasing metabolic demands of the growing fetus (Schneider, 1996). However, adverse conditions may accelerate this normal prepartum decline in placental weight (Gardner *et al.*, 2002) and alter the placentome weight, particularly the cotyledonary tissue (Lekatz *et al.*, 2010), and thus placentome distribution (Gardner *et al.*, 2002). Under-nutrition from mid-gestation can therefore decrease placental weight (Table 2-13), depending on ewe characteristics such as size, body condition, maturity and nutritional status (McCrabb *et al.*, 1991; Harding & Johnston, 1995; Robinson *et al.*, 1999b; Anthony *et al.*, 2003). Alterations in placental growth can result in low birth weight (Robinson *et al.*, 1999b) due to the high correlation between placental weight and fetal weight at the end of pregnancy (Mellor & Murray, 1982).

Table 2-13: The effects of maternal nutritional manipulation starting in mid or late pregnancy on the placenta.

Placenta	Period	Nutritional Treatments	Effects	Reference
Placenta weight	50 to 96	C (10 kg DM/week) vs. R (~3.4 kg DM/week)	D96 and D140: Single- no effect.	(McCrabb *et al.*, 1992)
	64 to 135	C (1.0M) vs. R (0.6M)	D135: R lighter.	(Lekatz *et al.*, 2010)
	70 to 140	1.5M vs. 2.0M	Twin- 2.0M heavier membranes plus cotyledons (total 1.35kg vs. 1.18kg for 1.5M)	(Rattray *et al.*, 1974)
	75 to 96	C (10 kg DM/week) vs. R (~3.4 kg DM/week)	D96 and D140: Single- no effect.	(McCrabb *et al.*, 1992)
Placentome	64 to 135	C (1.0M) vs. R (0.6M)	D135: no effect on placentome number, mass, caruncular or cotiledonary weight. R decrease cotiledonary protein concentration and cellular size, caruncular growth and placental vascularisation.	(Lekatz *et al.*, 2010)

Treatments: R, restricted; M, maintenance; C, control. DM, dry matter. Effects: D, day of measurement.

2.3 Summary and conclusions from the Literature Review

The amount and composition of dam nutrition during pregnancy plays a fundamental role in regulating fetal and placental development in sheep (Heasman *et al.*, 1999; Wu *et al.*, 2006; Symonds *et al.*, 2007) and it can have fetal programming effects (Heasman *et al.*, 1999). If there is a mismatch between the predicted and actual mature environment, the developmental adaptations may end up being pathogenic (Gluckman & Hanson, 2004). Such is the case in IUGR, which is a major concern in domestic animal production because it reduces neonatal survival, efficiency of feed utilisation, negatively affects body composition and meat quality, and impairs long-term health and athletic performance (Wu *et al.*, 2006). IUGR is common in animals carrying multiple fetuses (Gootwine *et al.*, 2007) because of the higher nutritional demand of a twin pregnancy compared to a singleton (Rattray *et al.*, 1974; Cleal *et al.*, 2007; Kenyon *et al.*, 2009). Additionally, gestation occurs during winter in extensive grazing farming systems, which is commonly the period of greatest feed shortage (Mathews *et al.*, 1999) because the low grass growth rates often do not meet animal demand. Thus, the potential to influence the offspring in both short- and long-term, e.g. survival, growth, body composition and future production, can be vast and of great economic importance.

The ovine pregnancy can be divided into three phases or trimesters: [1] early gestation or embryogenesis (day 0 to 50), [2] mid gestation or placental stage (day 50 to 100) and [3] late gestation or fetal phase (days 100 to 150). Maternal nutrition can have different effects depending on the stage of gestation. During early pregnancy, uterine attachment and placenta growth commences, and the fetal nutrient requirements are very specific as it is the period when organogenesis is occurring. Mid pregnancy is the time when the placenta is rapidly developing and maternal nutrition has the greatest effects on the feto-placental growth. During late pregnancy, most of the increase in the size of the fetus takes place and therefore, energy intake has a significant influence on lamb birth weight.

The effect of nutrition on fetal mammary gland development and later functionality is of special interest, due to its implications for growth, development and survival of the next generation of offspring (van der Linden *et al.*, 2009). Due to the design of the previous studies in sheep (Jenkinson, 2003; van der Linden *et al.*, 2009; Blair *et al.*, 2010), it was not known if the maternal maintenance diet enhanced fetal mammary gland development or if the *ad libitum* diet reduced it, as the offspring from the maintenance group produced more milk and milk of altered composition at 18 months of age (van der Linden *et al.*, 2009). The only way of determining this would be by imposing three levels of maternal nutrition, e.g. restricted,

maintenance and *ad libitum*. A further limitation in those studies was that they didn't identify when in pregnancy the maternal nutrition influenced the development of the fetal mammary gland. The organogenesis of the mammary gland starts early in embryogenesis (Robinson *et al.*, 1999a) and ductular development, where secretory cells proliferate during lactation, also occurs in early fetal life (Knight & Sorensen, 2001). Thus, it is likely that the development of the fetal mammary gland is more sensitive to under-nutrition in early gestation than in later stages.

Therefore, the aim of this research was to investigate the effects of early (day 21 to 50) and mid- to late- (day 50 to 140) dam pregnancy nutrition on the anatomical development of twin-fetuses. We used only twin-bearing ewes due to the economic importance of twins in New Zealand sheep production and their higher vulnerability to maternal under-nutrition. It was hypothesised that dams underfed during early pregnancy, will have fetuses with impaired mammary gland development. In addition, other organs may display altered growth.

CHAPTER 3 MATERIALS AND METHODS

3.1 Background information

A full description of the experiment has been reported by Kenyon *et al.* (2011). Briefly, a group of mixed-aged Romney ewes (n= 1169, average 66.3 ± 0.18 (s.e.) kg live weight and 2.96 ± 0.02 condition score), from a commercial flock were synchronised by vaginal insertion of a progesterone-controlled internal drug releasing device (CIDR, 0.3 g progesterone, Pharmacia & UpJohn, Auckland, New Zealand) for 14 days (Day -14 to 0, D-14 to D0). On D0 (14 April 2009), half of the ewes were artificially inseminated, via intra-uterine laparoscopy using semen randomly allocated from one of five Romney rams. The remaining ewes underwent the same procedure the following day (D1) and then both cohorts of ewes were merged. At D5, twelve crayon-harnessed, entire, Romney rams were introduced to the ewes. Ewes were managed under commercial conditions and were offered herbage with a minimum post-grazing mass of 1200 kg DM/ha until D20, to allow unrestricted feed intake (Kenyon & Webby, 2007).

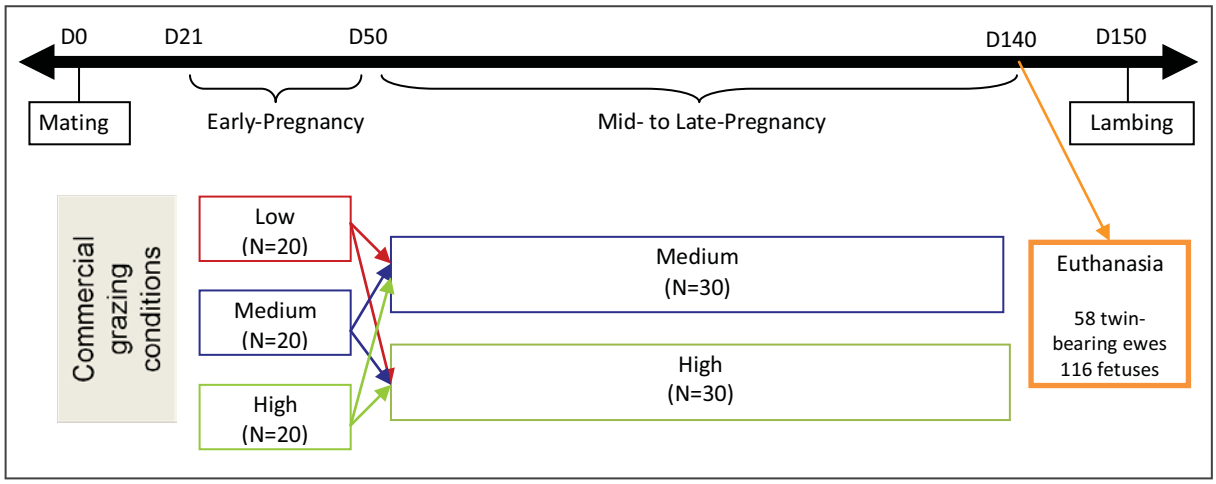
At D21, the entire rams were removed as were ewes which returned to service. The remaining ewes were randomly allocated to one of three nutritional regimens to D50 (period D21-50: Low (L_{D21-50}) vs. Medium (M_{D21-50}) vs. High (H_{D21-50})). The aim of the L_{D21-50} nutritional regimen was to achieve a loss in total ewe live weight of 100g/d, while the M_{D21-50} regimen was to ensure no change in total ewe live weight and the H_{D21-50} nutritional regimen purpose was to provide *ad libitum* grazing conditions and weight gain. To monitor the nutritional regimens, pasture measurements (pre- and post-grazing mass, plus quality analysis) were taken weekly and ewes were weighed and condition scored.

At D48, all ewes were pregnancy scanned via transabdominal ultrasonography. At D50, non-pregnant, single- and triple-bearing ewes, and ewes with incomplete data were removed and the remaining twin-bearing ewes were randomly allocated to one of two further nutritional treatments until D140 (period 50-140: Medium ($M_{D50-140}$) vs. High ($H_{D50-140}$)). In each of the two D50-140 nutritional regimens, animals from all three D21-50 nutritional regimens were included. The aim of the $M_{D50-140}$ nutritional regimen was to ensure that total ewe live weight increased at a level similar to that of the expected conceptus mass (Rattray *et al.*, 1974). The $H_{D50-140}$ nutritional regimen aim was to provide *ad libitum* grazing conditions.

3.2 The present study

The study utilised a 3x2 factorial design resulting in six nutritional groups, as described in Figure 3-1. During D138 to D141 (D140), 10 randomly selected twin-bearing ewes from each of the six nutritional groups (n=60 in total) were removed from the main study and euthanised by captive-bolt pistol and exsanguinations. Two ewes, one from $M_{D21\text{-}50}$-$M_{D50\text{-}140}$ and one from $H_{D21\text{-}50}$- $M_{D50\text{-}140}$, were removed from the final analysis as they were found not to be twin-bearing ewes. Consequently, there were 58 ewes included in the study together with their fetuses: $L_{D21\text{-}50}$-$M_{D50\text{-}140}$ (n=10), $L_{D21\text{-}50}$-$H_{D50\text{-}140}$ (n=10), $M_{D21\text{-}50}$-$M_{D50\text{-}140}$ (n=9), $M_{D21\text{-}50}$-$H_{D50\text{-}140}$ (n=10), $H_{D21\text{-}50}$-$M_{D50\text{-}140}$ (n=9) and $H_{D21\text{-}50}$-$H_{D50\text{-}140}$ (n=10).

Figure 3-1: Trial design and timeline



This study took place at Massey University Keeble Sheep and Beef farm (5 km south of Palmerston North, New Zealand) from March to September 2009. The study and all animal handling procedures were approved by the Massey University Animal Ethics Committee, Palmerston North, New Zealand.

3.3 Measurements

3.3.1 Pasture

As described by Kenyon *et al*. (2011), pre- and post-grazing herbage masses were measured weekly with a rising-plate meter (Ashgrove Pastoral Products, New Zealand; 50 readings per paddock). The herbage mass (kg DM/ha) was determined by using the formula: (158 × MR) + 200, where MR is the meter reading (Hodgson *et al.*, 1999). During the period D21 to D50 pre-grazing herbage plucks were taken at twice weekly intervals, and during D50 to D139 plucks

were taken fortnightly. Samples were immediately frozen at -20ºC and freeze dried prior to quality analysis. The levels of metabolisable energy (ME) were determined by near infrared reflectance (NIR) spectrometry and analysed using Optic User Software (OPUS) version 5.0.

3.3.2 Dam

Live weights of ewes were recorded on D-1, 21, 30, 40, 50, 69, 90, 113 and 137. Ewes were condition scored (scale 0-5 including half units, Jefferies (1961)) on D-1, 21, 50 and 137.

After euthanasia, carcass weight (kg) and soft tissue depth (mm) over the twelfth rib at 110 mm from the midline (GR point) were measured. The total gravid uterus weight (uterus, placenta and fetuses in kg), abdominal fat (kg), liver (g) and total and left mammary gland weight (g) were recorded.

3.3.3 Fetus

The fetuses were removed from the uterus and the umbilical cords ligated at the abdomen before being severed. Each fetus was gently squeezed to remove amniotic fluid from the wool. Fetal weight (kg) and sex were recorded, and the crown-rump length (CRL), thoracic girth circumference, left forelimb and hind limb length measured (cm). The brain and pineal gland, liver, kidneys (x2), total kidney fat, pancreas, spleen, heart, total heart fat, lungs (x2), thymus, thyroid gland (x2), adrenal glands (x2) and left *semitendinosus* muscle were weighed (g). The combined testes weight (g) was recorded for the male fetuses, whilst the mammary gland and combined ovaries weights (g) were recorded for the female fetuses.

3.3.4 Placenta

The myo-endometrium (including caruncles) and fetal membranes (including cotyledons) from each ewe were weighed (total weight of the placenta) before dividing the membranes per fetus and manual separation of the placentomes. The placentomes were classified into four types according to the level of eversion: A (inverted), B, C and D (everted) (Vatnick *et al.*, 1991). Then each type of placentome was counted and their total weights recorded. The total number of empty caruncles was also counted.

3.4 Statistical analysis

Complete ewe-fetal data were collected from 58 ewes and their 116 twin-fetuses and statistical analysis was only undertaken on these animals. All ewe, placental and fetal measurements were subjected to analysis of variance using the Generalised Linear Model procedure in Minitab (version 13.1, Minitab Inc, Pennsylvania, USA) and Tuckey-test was utilised for pairwise analysis. The models used to analyse ewe live weight, body condition score, ewe organs and placental measurements included the fixed effects of nutritional regimen during D21 to 50, D50 to 140 and their interaction. Non-significant two-way interactions between the nutrition periods were not removed to allow for testing of the study design. Days of artificial insemination and of euthanasia were fitted as fixed effects whilst sire was fitted as random effect. Sex of the fetus and the interactions between sex and nutritional regimens (two and three way interactions) were fitted as fixed effects in the models for fetal weight, body dimensions and organs weights. Non-significant two-way interactions between any of the nutritional treatments and sex of the fetus or three-way interactions between sex and the two nutritional regimens were removed. Fetal measurements were analysed with and without fetal weight as a covariate.

The changes in ewe live weights were calculated for the periods D21-50, D50-140 and D21-140. The data of maternal carcass, liver, mammary gland (total and left) and gravid uterus weights were not normally distributed so Log 10 was used for the analysis to achieve normal distribution. One H_{D21-50}-$H_{D50-140}$ ewe (#12) had no record for live weight or condition score on D137. One L_{D21-50}-$M_{D50-140}$ ewe (#24) was discarded for the analysis of gravid uterus as the record was wrong (1567 g).

The ratios between the weights of fetal brain:fetus, liver:fetus, liver:brain and fetus:placentomes were calculated for each fetus at D140. The data of the fetal thymus, thyroid, pineal and ovaries weights were not normally distributed so Log 10 was used to achieve a normal distribution for statistical analysis. The data of CRL, girth circumference, fore-leg, hind-leg, femur and adrenals could not be normalised using Log10, LogN, square root or square, so the analysis was done with the non-normal distributed raw data. Liver:brain ratio was not normally distributed either and analysis was performed on the abnormal distributed ratios. For the girth circumference, one male L_{D21-50}-$M_{D50-140}$ fetus (#5b) was excluded because the girth measurement was too small (26cm). For heart fat, one female L_{D21-50}-$H_{D50-140}$ fetus (#23b) had no data, and two fetuses (female M_{D21-50}-$M_{D50-140}$ #4a and male M_{D21-50}-$H_{D50-140}$ #10 b) were excluded since their values were too high compared to the rest (13.14 and 11.26 g

respectively). For the adrenal glands, the data of one male H_{D21-50}-$M_{D50-140}$ fetus (#56 b) was discarded because the gland was too small (0.14g), and two others (male and female M_{D21-50}-$M_{D50-140}$, #9b and #19b) because their values were too high (0.91 and 0.97g respectively). One female L_{D21-50}-$M_{D50-140}$ fetus (#53b) had an extremely large pineal gland so it was excluded from the analysis.

Log 10 was used to transform and normalise the data of empty caruncle numbers and placentome D weight, and square root was used for the data of total caruncle number and placentome B and C weight per fetus. One H_{D21-50}-$H_{D50-140}$ ewe (#6) was excluded from placentome A analysis, one H_{D21-50}-$H_{D50-140}$ (#29) from placentome B and one M_{D21-50}-$M_{D50-140}$ (#16) from placentome D analysis, due to incomplete fetal records. These three ewes were discarded for analysis on the total placentome and caruncle numbers. For the total placentome and caruncle numbers, three fetuses (male H_{D21-50}-$H_{D50-140}$ #6b, female H_{D21-50}-$H_{D50-140}$ #29b and male M_{D21-50}-$M_{D50-140}$ #16a) were excluded due to incomplete data. For the placentome A weight and total placentome weight per fetus, one male M_{D21-50}-$M_{D50-140}$ fetus (#16a) was discarded for the same reason.

CHAPTER 4 RESULTS

4.1 Pasture

Pasture results shown in Table 4-1 have previously been published by Kenyon *et al*. (2011). During D21 to D50, pre- and post-grazing masses of the H_{D21-50} treatment were greater than those of M_{D21-50} which in turn were greater than L_{D21-50}. Likewise, pre- and post-grazing masses of $H_{D50-140}$ were greater than $M_{D50-140}$ during D50 to D140. The pre-grazing herbage metabolisable energy (ME) values of L_{D21-50}, M_{D21-50} and H_{D21-50} did not differ during D21 to D50. Similarly, there was no difference in ME values between $M_{D50-140}$ and $H_{D50-140}$ during the period D50 to D140.

Table 4-1: Effect of nutritional treatments during D21-50 (Low (L) vs. Medium (M) vs. High (H)) and D50-140 (Medium (M) vs. High (H)) on pasture quality (ME MJ/kg DM) and pre- and post-grazing masses (kg DM/ha). Source: Kenyon *et al*. (2011).

Nutritional regimen	Pasture quality (MEMJ/kgDM)	Pasture mass (kgDM/ha)	
		Pre-grazing	Post-grazing
D 21-50			
L	12.35 ± 0.26	996^{a} ± 89	814^{a} ± 54
M	12.67 ± 0.26	1479^{b} ± 107	1112^{b} ± 59
H	12.14 ± 0.23	2331^{c} ± 82	1649^{c} ± 54
D 50-140			
M	12.67 ± 0.11	1450^{a} ± 84	1011^{a} ± 33
H	12.91 ± 0.11	1828^{b} ± 76	1301^{b} ± 37

Values are least-square means ± standard error of the mean. Different alphabetical superscripts within main effects and columns indicate significant differences (P<0.05).

4.2 Ewe

4.2.1 Ewe live weight

At D50, H_{D21-50} ewes were heavier (P<0.05) than both L_{D21-50} and M_{D21-50} ewes (Table 4-2). At D137, ewe D21-50 nutritional treatment had no effect (P>0.05) on ewe live weight but $H_{D50-140}$ ewes were heavier (P<0.05) than $M_{D50-140}$. There were no interactions (P>0.05) between nutritional treatments in D21-50 and D50-140 for ewe live weight.

4.2.2 Change in ewe live weight

Live weight changes differed (P<0.05) between D21-50 nutritional treatments (-0.128 ± 0.019, -0.014 ± 0.020 and 0.076 ± 0.020 g/d for L_{D21-50}, M_{D21-50} and H_{D21-50} respectively). During D50-140, $H_{D50-140}$ ewes gained more weight (P<0.05) than $M_{D50-140}$ ewes (0.258 ± 0.009 vs. 0.188 ± 0.009 g/d). There were no significant interactions (P>0.05) between nutritional treatments across both periods for ewe live weight gain (Table A- 1 in Appendix).

4.2.3 Ewe condition score

At D50, H_{D21-50} ewes had greater (P<0.05) condition score than M_{D21-50} ewes, and these ones had greater (P<0.05) body condition than L_{D21-50} ewes (Table 4-3). At Day 137, $H_{D50-140}$ ewes had greater condition scores (P<0.05) than the $M_{D50-140}$ ewes. There were no significant interactions (P>0.05) between nutritional treatments across both nutritional periods.

4.2.4 Ewe carcass weight, GR depth and abdominal fat weight

Ewe nutritional treatment during the period D21-50 had no effect (P>0.05) on carcass weight, but tended (P=0.06) to have an effect on the abdominal fat weight and GR depth (Table 4-4), such that H_{D21-50} ewes had more (P<0.05) abdominal fat than the L_{D21-50} ewes. No pairwise differences (P>0.05) were found for the GR depth during the period D21-50. $H_{D50-140}$ ewes had greater (P<0.05) carcass weight, abdominal fat weight and GR depths than the $M_{D50-140}$ ones. There were no interactions (P>0.05) between nutritional treatments across periods D21-50 by period D50-140 for ewe carcass weight, GR depth or abdominal fat weight measured.

4.2.5 Ewe organs weight, mammary gland weight and uterine and placental weight

Ewe nutritional treatments during either period D21-50 or D50-140 had no effects (P>0.05) on liver, total and left mammary gland, total placenta membranes or gravid uterus weights (Table A-2 in Appendix). There were also no significant interactions (P>0.05) for these ewe parameters.

Table 4-2: Effect of nutritional treatments during D21-50 (Low (L) vs. Medium (M) vs. High (H)) and D50-140 (Medium (M) vs. High (H)) on ewe live weight (kg) on Days -1, 21, 50 and 137.

Nutritional regimen		Live weight (kg)							
		n	Day -1		Day 21		Day 50		Day 137	
D21-50							**			
L		20	66.10	±1.42	66.13	±1.45	62.55[a]	±1.48	84.50	±1.76
M		19	67.26	±1.46	66.38	±1.49	65.98[a]	±1.52	86.03	±1.81
H		19	68.46	±1.46	69.04	±1.49	71.16[b]	±1.52	86.14	±1.86
D50-140									**	
M		28	67.73	±1.20	67.51	±1.23	66.82	±1.25	82.95[a]	±1.49
H		30	66.82	±1.16	66.85	±1.18	66.30	±1.20	88.17[b]	±1.47
D21-50xD50-140										
L	M	10	66.40	±2.00	66.20	±2.05	62.25[a.]	±2.09	80.95	±2.49
L	H	10	65.80	±2.00	66.05	±2.05	62.85[ab]	±2.09	88.05	±2.49
M	M	9	68.11	±2.11	67.06	±2.16	66.56[abc]	±2.20	83.61	±2.63
M	H	10	66.40	±2.00	65.70	±2.05	65.40[abc]	±2.09	88.45	±2.49
H	M	9	68.67	±2.11	69.28	±2.16	71.67[c.]	±2.20	84.28	±2.63
H	H	10	68.25	±2.00	68.80	±2.05	70.65[bc]	±2.09	88.00	±2.63

Values are least-square means ± standard error of the mean. Main effects P-values: *P<0.1; **P<0.05. Different alphabetical superscripts within main effects and columns indicate significant differences (P<0.05).

Table 4-3*:* Effect of nutritional treatments during D21-50 (Low (L) vs. Medium (M) vs. High (H)) and D50-140 (Medium (M) vs. High (H)) on ewe condition score on Days -1, 21, 50 and 137.

Nutritional regimen		Condition Score							
		n	Day -1		Day 21		Day 50		Day 137	
D21-50							**			
L		20	2.80	±0.12	2.80	±0.10	2.08[a]	±0.10	2.83	±0.13
M		19	2.95	±0.13	2.98	±0.11	2.60[b]	±0.11	2.91	±0.13
H		19	3.00	±0.13	2.84	±0.11	3.06[c]	±0.11	3.14	±0.14
D50-140							**		**	
M		28	2.92	±0.11	2.86	±0.09	2.73[b]	±0.09	2.70[a]	±0.11
H		30	2.92	±0.10	2.88	±0.08	2.48[a]	±0.08	3.22[b]	±0.11
D21-50xD50-140										
L	M	10	2.80	±0.18	2.85	±0.15	2.25[a]	±0.15	2.60[a]	±0.18
L	H	10	2.80	±0.18	2.75	±0.15	1.90[a]	±0.15	3.05[ab]	±0.18
M	M	9	2.94	±0.19	3.00	±0.15	2.72[b]	±0.15	2.67[ab]	±0.19
M	H	10	2.95	±0.18	2.95	±0.15	2.65[b]	±0.15	3.15[ab]	±0.18
H	M	9	3.00	±0.19	2.72	±0.15	3.22[b]	±0.15	2.83[ab]	±0.19
H	H	10	3.00	±0.18	2.95	±0.15	2.90[b]	±0.15	3.44[b]	±0.19

Values are least-square means ± standard error of the mean. Main effects P-values: *P<0.1; **P<0.05. Different alphabetical superscripts within main effects and columns indicate significant differences (P<0.05).

Table 4-4: Effect of nutritional treatments during D21-50 (Low (L) vs. Medium (M) vs. High (H)) and D50-140 (Medium (M) vs. High (H)) on ewe carcass weight (kg), abdominal fat weight (g) and GR depth (mm) on Day 140.

Feeding regimen	n	Carcass (kg)†			Abdominal fat (g)		GR (mm)	
D21-50					*		*	
L	20	1.48	±0.01	(30.38)	1474[a]	±130	22.22	±1.45
M	19	1.49	±0.01	(31.00)	1604[ab]	±141	22.36	±1.57
H	19	1.52	±0.01	(33.25)	1901[b]	±139	26.44	±1.54
D50-140			**		**		**	
M	28	1.47[a]	±0.01	(29.49)	1452 [a]	±112	20.51[a]	±1.24
H	30	1.52[b]	±0.01	(33.59)	1866[b]	±123	26.84[b]	±1.37
D21-50xD50-140								
L M	10	1.45[a]	±0.02	(28.44)	1295[a]	±187	19.73[a]	±2.07
L H	10	1.51[ab]	±0.02	(32.33)	1652[ab]	±179	24.71[ab]	±1.99
M M	9	1.46[ab]	±0.02	(29.11)	1396[a]	±182	18.60[a]	±2.02
M H	10	1.51[ab]	±0.02	(32.89)	1811[ab]	±205	26.13[ab]	±2.27
H M	9	1.48[ab]	±0.02	(30.93)	1666[ab]	±196	23.22[ab]	±2.17
H H	10	1.55[b]	±0.02	(35.57)	2135[b]	±198	29.67[b]	±2.20

Values are least-square means ± standard error of the mean. Main effects P-values: *P<0.1; **P<0.05. Different alphabetical superscripts within main effects and columns indicate significant differences (P<0.05).

† Log 10 of Carcass was used to analyse the data. Unadjusted mean values are presented in brackets.

4.3 Fetus

4.3.1 Fetal size measurements

Ewe D21-50 nutritional treatment had no effect (P>0.05) on fetal weight, crown-rump length (CRL), girth circumference, foreleg, hind-leg and femur length (Table 4-5). Further, ewe D50-140 treatment had no effect (P>0.05) on CRL and femur length. There was an interaction (P<0.05) between sex of the fetus and D50-140 nutritional treatment for fetal weight, girth circumference and hind-leg length, and tended (P=0.07) to be an interaction for fore-leg length (data not shown). Pairwise analysis found no differences (P>0.05) between groups for fetal weight, girth circumference and fore-leg length. However, the hind-legs of male fetuses were longer (P<0.05) than the hind-legs of female fetuses born to $H_{D50-140}$ ewes (36.79 ± 0.27 vs. 35.72 ± 0.23 cm respectively); no such relationship (P>0.05) was observed for those fetuses born to $M_{D50-140}$ ewes (36.18 ± 0.26 vs. 36.35 ± 0.27 cm for male and female fetuses). There was an interaction (P<0.05) between ewe nutritional treatment on D21-50 and D50-140 on *semitendinosus* weight, but pairwise analysis found no differences (P>0.05) between the groups.

Adjusted for fetal weight:

Ewe nutritional regimen in either period had no effect (P>0.05) on the fetal CRL, girth circumference, foreleg, hind-leg or femur lengths after adjustment for fetal weight (Table 4-6). There was a significant interaction (P<0.05) between nutritional treatments during D21-50 and D50-140 for the *semitendinosus* muscle adjusted weight, such that the muscle from fetuses born to H_{D21-50}-$H_{D50-140}$ ewes were lighter (P<0.05) compared to those from L_{D21-50}-$H_{D50-140}$ and M_{D21-50}-$M_{D50-140}$ dams, and the fetal muscle from L_{D21-50}-$M_{D50-140}$ dams were lighter (P<0.05) than L_{D21-50}-$H_{D50-140}$ and tended to be lighter (P=0.07) than M_{D21-50}-$M_{D50-140}$.

4.3.2 Fetal organ and system measurements

Main organs and glands

Neither D21-50 nor D50-140 nutritional periods had any effect (P>0.05) on the kidneys, spleen, lungs, heart or adrenal glands weights (Table 4-7 and Table 4-9). In addition, ewe D21-50 nutrition had no effect (P>0.05) on liver, thymus or thyroid weight. Dam D21-50 nutritional treatment tended (P=0.09) to have an effect on the pancreas weight but ewe D50-140

nutritional treatment did not (P>0.05). Fetuses from $H_{D50-140}$ dams had heavier (P<0.05) thyroids compared to the $M_{D50-140}$ ones. There was a significant interaction (P<0.05) between sex of the lamb and ewe D50-140 nutrition for fetal liver weight, as female fetuses from $H_{D50-140}$ dams had lighter (P<0.05) livers than the males in the same treatment (96.63 ± 2.94 vs. 109.60 ± 3.37 g respectively); no such effect was found in the $M_{D50-140}$ treatment (106.57 ± 3.34 vs. 106.46 ± 3.22 cm for female and male fetuses). Tendencies of interaction between fetal sex and D50-140 nutrition were also found for thymus weight (P=0.051, data not shown), and a three-way interaction between fetal sex, D21-50 and D50-140 periods for heart fat (P=0.054), but pairwise analysis did not show any significant differences between the groups (data not shown).

Adjusted for fetal weight:

Neither ewe nutritional regimens in either period had any effect (P>0.05) on the fetal liver, kidneys, spleen, lungs, heart, thymus, pancreas or adrenal glands weights after adjustment for fetal body weight (Table 4-8 and Table 4-10). Ewe D21-50 nutritional regimen had no effect (P>0.05) on the thyroid weight, whilst the fetuses from $H_{D50-140}$ dams had heavier (P<0.05) thyroids compare those born to $M_{D50-140}$ ewes. There was also a sex difference (P<0.05) on the adjusted thyroid weight, such that the females had heavier (P<0.05) thyroids than the males. There was a significant interaction between sex of the lamb, D21-50 and D50-140 nutrition for heart fat weight (P<0.05, data not shown) after adjustment for fetal weight, but no differences were found using the pairwise analysis.

Head and brain

Fetuses from L_{D21-50} ewes had wider (P<0.05) heads than the fetuses from H_{D21-50} ewes (Table 4-11). There was an interaction (P<0.05) between the fetal sex and ewe D21-50 nutritional level for the head length and they tended to interact (P=0.08) for brain weight, although there were no significant differences (P>0.05) between treatments under pairwise analysis (data not shown). Tendencies between sex and D50-140 nutrition were found for head length (P=0.06), but again no differences were found on the pairwise testing. There was a significant interaction (P<0.05) between ewe nutritional treatments during D21-50 and D50-140 for brain weight, such that the brains from the M_{D21-50}-$H_{D50-140}$ fetuses were heavier (P<0.05) than those from fetuses born to L_{D21-50}-$H_{D50-140}$ ewes. There was a tendency (P=0.08) of a three way interaction between the sex of the fetus and nutritional level across the two periods for the head width, but no differences were found between groups (data not shown). Neither sex of

the fetus, D21-50 or D50-140 nutrition had any effect (P>0.05) on the weight of the pineal gland.

Adjusted for fetal weight:

Fetuses from L_{D21-50} ewes had wider heads (P<0.05) than the fetuses from M_{D21-50} or H_{D21-50} treatments after adjustment for fetal weight (Table 4-12). Ewe D50-140 nutritional treatment had no effect (P>0.05) on adjusted head length. There was an interaction (P<0.05) between the sex of the fetus and ewe D21-50 nutritional level for the adjusted head length and these parameters tended (P=0.07) to interact for the adjusted brain weight, but no differences were found in the pairwise analysis (data not shown). The interaction between D21-50 and D50-140 nutritional levels was significant (P<0.05) for the adjusted brain weight, with a tendency of fetuses from M_{D21-50}-$H_{D50-140}$ ewes to have heavier (P=0.06) brains than the L_{D21-50}-$H_{D50-140}$ fetuses. Ewe D21-50 and D50-140 nutritional treatments tended (P=0.09) to have an effect on the adjusted head width, such that fetuses from L_{D21-50}-$M_{D50-140}$ ewes had wider (P<0.05) heads than fetuses from M_{D21-50}-$H_{D50-140}$ and H_{D21-50}-$M_{D50-140}$ ewes. Sex of the fetus, D21-50 and D50-140 nutrition tended (P=0.06) to have an effect on the adjusted head width, such that female fetuses from L_{D21-50}-$M_{D50-140}$ ewes had wider (P<0.05) heads than male fetuses from M_{D21-50}-$H_{D50-140}$ ewes and females from H_{D21-50}-$M_{D50-140}$ ewes (data not shown). Fetal sex, D21-50 or D50-140 nutritional treatments had no effect (P>0.05) on the weight of the pineal gland after adjustment for fetal weight.

Reproductive system

Ewe nutrition during either period did not affect (P>0.05) the weight of the testes in the male fetuses (Table 4-13). For the female fetuses, D21-50 nutrition had no effect (P>0.05) on the ovaries weights, but there was a tendency (P=0.08) of the fetuses from $H_{D50-140}$ ewes to have heavier (P=0.08) ovaries than the fetuses from $M_{D50-140}$ ewes. Female fetuses from L_{D21-50} dams during D21-50 had lighter (P<0.05) mammary glands than the M_{D21-50} and H_{D21-50} ones. There were no differences (P>0.05) on the mammary gland weight for nutritional levels during D50-140.

Adjusted for fetal weight:

There was no nutritional effect in either period (P>0.05) for the testes weight after adjustment for fetal weight (Table 4-14). Ewe D21-50 nutrition had no effect (P>0.05) on the ovaries weight; D50-140 nutrition tended (P=0.07) to affect ovary weight after correction for fetal

body weight, but no differences (P>0.05) were found on pairwise analysis. The adjusted weight of the mammary gland of the fetuses from L_{D21-50} were lighter (P<0.05) than the M_{D21-50} and H_{D21-50} fetuses. Ewe nutrition during D50-140 did not affect (P>0.05) the weight of the mammary gland after adjustment for fetal weight.

Weight ratios

Sex of fetus, D21-50 or D50-140 nutritional treatments had no effect (P>0.05) on the liver:fetus or fetus:placentome weight ratios (Table 4-15). Nutrition during D21-50 had also no effect (P>0.05) on brain:fetus or liver:brain weight ratios. There was an interaction (P<0.05) between the sex of the fetus and ewe D50-140 nutritional level for the brain:fetus ratio (data not shown), but no differences were found on the pairwise analysis. Sex and ewe D50-140 nutrition tended (P=0.052) to interact for the liver:brain ratio, such that the females in $M_{D50-140}$ treatment tended to have greater ratio compared to females in $H_{D50-140}$ (2.053 ± 0.062 vs. 1.843 ± 0.054 respectively, P=0.06); this difference was not found in the male fetuses.

Table 4-5: Effect of the fetal sex (female vs. male) and nutritional treatments during D21-50 (Low (L) vs. Medium (M) vs. High (H)) and D50-140 (Medium (M) vs. High (H)) on the fetal size measurements (body weight, *semitendinosus* muscle weight, crown-rump length (CRL), girth length, fore and hind-leg lengths and femur length) on Day 140.

	n	Weight (g)		Length (cm)				
		Fetus	Semitendinosus	CRL ^	Girth ^	Fore-leg ^	Hind-leg ^	Femur ^
Sex							*	
female	61	5453 ±72	7.80 ±0.16	54.02 ±0.48	35.99 ±0.23	30.75 ±0.18	36.03 ±0.18	10.69 ±0.13
male	55	5577 ±77	7.75 ±0.17	53.55 ±0.52	36.35 ±0.25	30.94 ±0.19	36.48 ±0.19	10.59 ±0.14
D21-50								
L	40	5410 ±88	7.63 ±0.19	53.58 ±0.59	35.99 ±0.29	30.55 ±0.22	35.98 ±0.22	10.68 ±0.16
M	38	5578 ±90	8.08 ±0.20	53.70 ±0.61	36.14 ±0.29	31.10 ±0.23	36.46 ±0.22	10.72 ±0.17
H	38	5556 ±90	7.61 ±0.20	54.06 ±0.61	36.37 ±0.29	30.87 ±0.23	36.33 ±0.22	10.53 ±0.17
D50-140								
M	56	5510 ±74	7.74 ±0.16	53.82 ±0.50	36.04 ±0.24	31.01 ±0.19	36.26 ±0.18	10.72 ±0.14
H	60	5520 ±72	7.80 ±0.16	53.74 ±0.49	36.29 ±0.23	30.67 ±0.18	36.25 ±0.18	10.56 ±0.13
D21-50xD50-140			**					
L M	20	5357 ±128	7.08 ±0.28	54.13 ±0.87	35.76 ±0.43	30.44 ±0.32	35.82 ±0.31	10.71 ±0.24
L H	20	5464 ±128	8.18 ±0.27	53.03 ±0.85	36.23 ±0.42	30.67 ±0.32	36.14 ±0.31	10.65 ±0.23
M M	18	5514 ±128	8.08 ±0.28	53.49 ±0.87	36.18 ±0.42	31.43 ±0.32	36.50 ±0.31	10.86 ±0.24
M H	20	5642 ±127	8.07 ±0.28	53.91 ±0.86	36.09 ±0.42	30.77 ±0.32	36.41 ±0.31	10.58 ±0.24
H M	18	5658 ±137	8.06 ±0.30	53.83 ±0.93	36.18 ±0.45	31.16 ±0.35	36.46 ±0.34	10.59 ±0.25
H H	20	5453 ±132	7.17 ±0.29	54.29 ±0.89	36.57 ±0.43	30.58 ±0.33	36.21 ±0.32	10.47 ±0.24

Values are least-square means ± standard error of the mean. Main effects P-values: *P<0.1; **P<0.05. Different alphabetical superscripts within main effects and columns indicate significant differences (P<0.05).

^ Raw data non-normally distributed.

Table 4-6: Effect of the fetal sex (female vs. male) and nutritional treatments during D21-50 (Low (L) vs. Medium (M) vs. High (H)) and D50-140 (Medium (M) vs. High (H)) on the fetal size measurements (*semitendinosus* muscle weight, crown-rump length (CRL), girth length, fore and hind-leg lengths and femur length) on Day 140, adjusted for fetal body weight.

	n	Weight (g)		Length (cm)									
		Semitendinosus		CRL ^		Girth ^		Fore-leg ^		Hind-leg ^		Femur ^	
Sex													
female	61	7.89	±0.11	54.24	±0.42	36.12	±0.15	30.82	±0.15	36.10	±0.14	10.75	±0.11
male	55	7.65	±0.12	53.30	±0.45	36.18	±0.17	30.83	±0.16	36.37	±0.15	10.52	±0.12
D21-50													
L	40	7.80	±0.14	53.99	±0.51	36.23	±0.19	30.68	±0.19	36.11	±0.17	10.79	±0.14
M	38	7.97	±0.14	53.44	±0.52	35.96	±0.19	31.00	±0.19	36.34	±0.18	10.65	±0.14
H	38	7.54	±0.14	53.88	±0.52	36.25	±0.19	30.80	±0.19	36.25	±0.17	10.48	±0.14
D50-140													
M	56	7.74	±0.12	53.82	±0.43	36.03	±0.16	31.00	±0.16	36.25	±0.14	10.72	±0.12
H	60	7.80	±0.11	53.73	±0.42	36.26	±0.15	30.65	±0.15	36.22	±0.14	10.56	±0.11
D21-50xD50-140		**											
L M	20	7.27^{ab}	±0.20	54.59	±0.75	36.14	±0.28	30.66	±0.27	36.07	±0.25	10.83	±0.21
L H	20	8.33^{c}	±0.20	53.39	±0.73	36.32	±0.27	30.70	±0.26	36.15	±0.25	10.74	±0.20
M M	18	8.09^{bc}	±0.20	53.51	±0.74	36.16	±0.27	31.41	±0.27	36.47	±0.25	10.87	±0.20
M H	20	7.84^{abc}	±0.20	53.37	±0.74	35.76	±0.27	30.58	±0.27	36.21	±0.25	10.43	±0.20
H M	18	7.86^{abc}	±0.22	53.34	±0.80	35.80	±0.29	30.93	±0.29	36.21	±0.27	10.46	±0.22
H H	20	7.22^{a}	±0.21	54.42	±0.77	36.71	±0.28	30.66	±0.28	36.29	±0.26	10.50	±0.21

Values are least-square means ± standard error of the mean. Main effects P-values: *P<0.1; **P<0.05. Different alphabetical superscripts within main effects and columns indicate significant differences (P<0.05).

^ Raw data non-normally distributed.

Table 4-7: Effect of the fetal sex (female vs. male) and nutritional treatments during D21-50 (Low (L) vs. Medium (M) vs. High (H)) and D50-140 (Medium (M) vs. High (H)) on the fetal organs (liver, kidneys, spleen, lungs, heart and heart fat) on Day 140.

	n	Weight (g)													
		Liver		Left Kidney		Right Kidney		Spleen		Lungs		Heart		Heart fat	
Sex		*													
female	61	101.60	±2.20	12.39	±0.26	12.22	±0.26	6.25	±0.19	155.50	±4.25	37.52	±0.77	5.17	±0.15
male	55	108.03	±2.36	12.97	±0.28	12.70	±0.28	6.28	±0.20	153.00	±4.56	37.98	±0.83	5.37	±0.16
D21-50														*	
L	40	102.03	±2.71	12.38	±0.32	12.15	±0.32	6.19	±0.23	152.70	±5.21	36.80	±0.95	4.93	±0.19
M	38	105.75	±2.77	12.93	±0.33	12.74	±0.33	6.14	±0.24	155.00	±5.36	38.68	±0.98	5.52	±0.19
H	38	106.66	±2.76	12.74	±0.33	12.50	±0.33	6.46	±0.24	155.00	±5.33	37.77	±0.97	5.35	±0.18
D50-140															
M	56	106.51	±2.29	12.75	±0.27	12.52	±0.27	6.32	±0.20	158.20	±4.42	37.98	±0.81	5.19	±0.15
H	60	103.11	±2.21	12.62	±0.26	12.41	±0.26	6.21	±0.19	150.30	±4.26	37.51	±0.78	5.35	±0.15
D21-50xD50-140															
L M	20	103.84	±3.95	12.45	±0.47	12.18	±0.47	6.43	±0.34	163.20	±7.63	37.40	±1.39	4.70	±0.26
L H	20	100.21	±3.95	12.31	±0.46	12.12	±0.46	5.95	±0.33	142.20	±7.46	36.19	±1.36	5.16	±0.29
M M	18	108.01	±3.94	13.01	±0.47	12.71	±0.47	6.23	±0.34	153.40	±7.61	38.42	±1.39	5.47	±0.27
M H	20	103.50	±3.92	12.86	±0.46	12.78	±0.46	6.06	±0.34	156.50	±7.57	38.94	±1.38	5.57	±0.26
H M	18	107.69	±4.22	12.79	±0.50	12.67	±0.50	6.29	±0.37	157.90	±8.16	38.13	±1.49	5.39	±0.28
H H	20	105.63	±4.06	12.69	±0.48	12.33	±0.48	6.62	±0.35	152.10	±7.85	37.41	±1.43	5.31	±0.27

Values are least-square means ± standard error of the mean. Main effects P-values: *P<0.1; **P<0.05. Different alphabetical superscripts within main effects and columns indicate significant differences (P<0.05).

Table 4-8: Effect of the fetal sex (female vs. male) and nutritional treatments during D21-50 (Low (L) vs. Medium (M) vs. High (H)) and D50-140 (Medium (M) vs. High (H)) on the fetal organs weight (liver, kidneys, spleen, lungs, heart and heart fat) on Day 140, adjusted for fetal body weight.

	n	Weight (g)													
		Liver		Left Kidney		Right Kidney		Spleen		Lungs		Heart		Heart fat	
Sex															
female	61	102.60	±1.65	12.54	±0.20	12.38	±0.20	6.35	±0.16	156.90	±3.94	38.03	±0.52	5.20	±0.14
male	55	106.50	±1.77	12.81	±0.21	12.54	±0.21	6.17	±0.17	151.40	±4.24	37.43	±0.56	5.32	±0.15
D21-50															
L	40	103.90	±2.03	12.66	±0.25	12.43	±0.24	6.37	±0.19	155.40	±4.86	37.73	±0.64	5.32	±0.17
M	38	104.20	±2.08	12.76	±0.25	12.56	±0.25	6.03	±0.20	153.30	±4.97	38.09	±0.66	4.99	±0.18
H	38	105.60	±2.06	12.62	±0.25	12.38	±0.24	6.38	±0.20	153.80	±4.93	37.37	±0.65	5.49	±0.18
D50-140															
M	56	106.40	±1.71	12.75	±0.21	12.52	±0.20	6.32	±0.16	158.20	±4.09	37.98	±0.54	5.33	±0.14
H	60	102.70	±1.65	12.61	±0.20	12.40	±0.20	6.20	±0.16	150.20	±3.94	37.48	±0.52	5.19	±0.14
D21-50xD50-140															
L M	20	107.00	±2.96	12.76	±0.36	12.49	±0.35	6.63	±0.28	166.20	±7.09	38.44	±0.94	5.35	±0.25
L H	20	100.70	±2.90	12.55	±0.35	12.37	±0.34	6.11	±0.27	144.60	±6.93	37.02	±0.92	5.29	±0.26
M M	18	107.70	±2.94	13.02	±0.36	12.72	±0.35	6.24	±0.28	153.50	±7.04	38.46	±0.93	5.15	±0.27
M H	20	100.70	±2.95	12.49	±0.36	12.41	±0.35	5.82	±0.28	153.00	±7.05	37.71	±0.93	4.82	±0.25
H M	18	104.40	±3.17	12.47	±0.38	12.34	±0.38	6.08	±0.30	154.70	±7.58	37.04	±1.00	5.50	±0.25
H H	20	106.70	±3.04	12.78	±0.37	12.42	±0.36	6.68	±0.29	152.90	±7.26	37.70	±0.96	5.47	±0.25

Values are least-square means ± standard error of the mean. Main effects P-values: *P<0.1; **P<0.05. Different alphabetical superscripts within main effects and columns indicate significant differences (P<0.05).

Table 4-9: Effect of the fetal sex (female vs. male) and nutritional treatments during D21-50 (Low (L) vs. Medium (M) vs. High (H)) and D50-140 (Medium (M) vs. High (H)) on the fetal glands (thymus, thyroid, pancreas and adrenals) on Day 140.

	n	Weight (g)									
		Thymus †			Thyroid †			Pancreas		Adrenal ^	
Sex											
female	61	1.36	±0.02	(24.00)	0.14	±0.01	(1.45)	4.67	±0.14	0.561	±0.012
male	55	1.35	±0.02	(23.65)	0.11	±0.01	(1.32)	4.74	±0.15	0.558	±0.013
D21-50								*			
L	40	1.34	±0.02	(23.02)	0.10	±0.02	(1.29)	4.39	±0.17	0.546	±0.014
M	38	1.35	±0.02	(23.27)	0.14	±0.02	(1.43)	4.87	±0.18	0.555	±0.015
H	38	1.37	±0.02	(25.17)	0.14	±0.02	(1.44)	4.86	±0.18	0.577	±0.015
D50-140						**					
M	56	1.36	±0.02	(24.22)	0.10[a]	±0.01	(1.31)	4.65	±0.15	0.557	±0.013
H	60	1.35	±0.02	(23.43)	0.15[b]	±0.01	(1.46)	4.75	±0.14	0.561	±0.012
D21-50xD50-140											
L M	20	1.35	±0.03	(23.79)	0.09	±0.02	(1.29)	4.4	±0.26	0.528	±0.021
L H	20	1.32	±0.03	(22.25)	0.10	±0.02	(1.29)	4.37	±0.25	0.564	±0.021
M M	18	1.32	±0.03	(21.79)	0.10	±0.02	(1.33)	4.55	±0.26	0.553	±0.022
M H	20	1.38	±0.03	(24.76)	0.17	±0.02	(1.53)	5.19	±0.25	0.556	±0.021
H M	18	1.40	±0.03	(27.07)	0.10	±0.03	(1.31)	5.02	±0.27	0.591	±0.024
H H	20	1.35	±0.03	(23.27)	0.19	±0.03	(1.57)	4.70	±0.26	0.564	±0.022

Values are least-square means ± standard error of the mean. Main effects P-values: *P<0.1; **P<0.05. Different alphabetical superscripts within main effects and columns indicate significant differences (P<0.05).

† Log 10 of Thymus and Thyroid was used to analyse the data. Unadjusted mean values are presented in brackets.

^ Raw data non-normally distributed.

Table 4-10: Effect of the fetal sex (female vs. male) and nutritional treatments during D21-50 (Low (L) vs. Medium (M) vs. High (H)) and D50-140 (Medium (M) vs. High (H)) on the fetal glands weight (thymus, thyroid, pancreas and adrenals) on Day 140, adjusted for fetal body weight.

	n	Weight (g)										
		Thymus †			Thyroid †			Pancreas		Adrenal ^		
Sex						**						
female	61	1.36	±0.01	(24.42)	0.14[b]	±0.01	(1.46)	4.73	±0.13	0.564	±0.011	
male	55	1.34	±0.01	(22.97)	0.10[a]	±0.01	(1.31)	4.68	±0.14	0.554	±0.012	
D21-50												
L	40	1.35	±0.02	(23.80)	0.10	±0.02	(1.32)	4.49	±0.16	0.551	±0.014	
M	38	1.34	±0.02	(22.59)	0.13	±0.02	(1.41)	4.81	±0.16	0.551	±0.015	
H	38	1.36	±0.02	(24.69)	0.14	±0.02	(1.43)	4.81	±0.16	0.576	±0.014	
D50-140						**						
M	56	1.36	±0.01	(24.15)	0.10[a]	±0.01	(1.31)	4.65	±0.13	0.558	±0.012	
H	60	1.35	±0.01	(23.24)	0.15[b]	±0.01	(1.46)	4.75	±0.13	0.561	±0.011	
D21-50xD50-140												
L M	20	1.38	±0.02	(25.18)	0.10	±0.02	(1.32)	4.51	±0.23	0.534	±0.020	
L H	20	1.33	±0.02	(22.42)	0.11	±0.02	(1.32)	4.46	±0.23	0.569	±0.020	
M M	18	1.32	±0.02	(21.64)	0.10	±0.02	(1.33)	4.55	±0.23	0.552	±0.021	
M H	20	1.36	±0.02	(23.54)	0.16	±0.02	(1.49)	5.06	±0.23	0.549	±0.020	
H M	18	1.37	±0.03	(25.61)	0.09	±0.02	(1.28)	4.90	±0.25	0.587	±0.023	
H H	20	1.36	±0.02	(23.77)	0.19	±0.02	(1.58)	4.73	±0.24	0.565	±0.021	

Values are least-square means ± standard error of the mean. Main effects P-values: *P<0.1; **P<0.05. Different alphabetical superscripts within main effects and columns indicate significant differences (P<0.05).

† Log 10 of Thymus and Thyroid was used to analyse the data. Unadjusted mean values are presented in brackets.

^ Raw data non-normally distributed.

Table 4-11: Effect of the fetal sex (female vs. male) and nutritional treatments during D21-50 (Low (L) vs. Medium (M) vs. High (H)) and D50-140 (Medium (M) vs. High (H)) on the fetal head measurements and the weight of the brain and pineal gland at Day 140.

	n	Head length (cm)^		Head width (cm)^		Weight (g)					
						Brain		n (Pineal)	Pineal †		
Sex											
female	61	17.51	±0.10	10.72	±0.11	52.51	±0.43	60	-1.95	±0.02	(0.0123)
male	55	17.63	±0.11	10.92	±0.12	53.55	±0.47	55	-1.95	±0.02	(0.0117)
D21-50				**							
L	40	17.62	±0.13	11.12[b]	±0.14	52.63	±0.55	39	-1.99	±0.02	(0.0108)
M	38	17.71	±0.13	10.72[ab]	±0.14	53.77	±0.55	38	-1.94	±0.02	(0.0126)
H	38	17.38	±0.13	10.63[a]	±0.13	52.69	±0.54	38	-1.92	±0.02	(0.0126)
D50-140											
M	56	17.61	±0.11	10.90	±0.11	52.84	±0.45	55	-1.94	±0.02	(0.0124)
H	60	17.53	±0.10	10.74	±0.11	53.23	±0.44	60	-1.96	±0.02	(0.0115)
D21-50xD50-140						**					
L M	20	17.74	±0.18	11.21	±0.19	53.50[ab]	±0.77	19	-1.98	±0.04	(0.0115)
L H	20	17.50	±0.19	11.04	±0.21	51.76[a]	±0.80	20	-2.01	±0.03	(0.0101)
M M	18	17.74	±0.18	10.96	±0.19	52.30[ab]	±0.77	18	-1.92	±0.03	(0.0133)
M H	20	17.68	±0.18	10.49	±0.19	55.25[b]	±0.77	20	-1.96	±0.03	(0.0120)
H M	18	17.36	±0.19	10.54	±0.21	52.71[ab]	±0.83	18	-1.92	±0.04	(0.0126)
H H	20	17.40	±0.19	10.71	±0.20	52.67[ab]	±0.80	20	-1.93	±0.04	(0.0126)

Values are least-square means ± standard error of the mean. Main effects P-values: *P<0.1; **P<0.05. Different alphabetical superscripts within main effects and columns indicate significant differences (P<0.05).

† Log10 of Pineal was used to analyse the data. Unadjusted mean values are presented in brackets. ^ Raw data non-normally distributed.

Table 4-12: Effect of the fetal sex (female vs. male) and nutritional treatments during D21-50 (Low (L) vs. Medium (M) vs. High (H)) and D50-140 (Medium (M) vs. High (H)) on the fetal head measurements and the weight of the brain and pineal gland on Day 140, adjusted for fetal body weight.

	n	Head length (cm)^		Head width (cm)^		Weight (g)					
						Brain		n (Pineal)		Pineal †	
Sex											
female	61	17.54	±0.09	10.76	±0.10	52.65	±0.41	60	-1.95	±0.02	(0.0124)
male	55	17.57	±0.10	10.87	±0.11	53.40	±0.44	55	-1.96	±0.02	(0.0116)
D21-50		*		**							
L	40	17.67	±0.11	11.18[b]	±0.12	52.88	±0.52	39	-1.98	±0.02	(0.0110)
M	38	17.65	±0.11	10.67[a]	±0.12	53.61	±0.51	38	-1.94	±0.02	(0.0125)
H	38	17.34	±0.11	10.59[a]	±0.12	52.58	±0.51	38	-1.93	±0.02	(0.0125)
D50-140											
M	56	17.60	±0.09	10.90	±0.10	52.83	±0.42	55	-1.94	±0.02	(0.0125)
H	60	17.51	±0.09	10.73	±0.10	53.22	±0.41	60	-1.96	±0.02	(0.0115)
D21-50xD50-140				*		**					
L M	20	17.85	±0.16	11.31[b]	±0.17	53.78	±0.73	19	-1.97	±0.04	(0.0117)
L H	20	17.49	±0.16	11.05[ab]	±0.19	51.98	±0.75	20	-2.00	±0.03	(0.0102)
M M	18	17.72	±0.16	10.94[ab]	±0.17	52.31	±0.72	18	-1.92	±0.03	(0.0133)
M H	20	17.58	±0.16	10.39[a]	±0.17	54.92	±0.72	20	-1.97	±0.03	(0.0117)
H M	18	17.24	±0.17	10.45[a]	±0.19	52.41	±0.78	18	-1.93	±0.04	(0.0124)
H H	20	17.44	±0.16	10.74[ab]	±0.18	52.76	±0.75	20	-1.93	±0.04	(0.0126)

Values are least-square means ± standard error of the mean. Main effects P-values: *P<0.1; **P<0.05. Different alphabetical superscripts within main effects and columns indicate significant differences (P<0.05).

† Log10 of Pineal was used to analyse the data. Unadjusted mean values are presented in brackets.

^ Raw data non-normally distributed.

Table 4-13: Effect of nutritional treatments during D21-50 (Low (L) vs. Medium (M) vs. High (H)) and D50-140 (Medium (M) vs. High (H)) on the weight data of the fetal reproductive organs (testes, ovaries and mammary gland) on Day 140.

	Male fetuses			Female fetuses					
	n	Testes (g)		n	Ovaries (g) †			Mammary gland (g)	
D21-50								**	
L	14	5.39	±0.30	26	-1.17	±0.03	(0.071)	13.13[a]	±0.55
M	20	5.86	±0.25	18	-1.19	±0.04	(0.069)	16.74[b]	±0.66
H	21	5.43	±0.27	17	-1.12	±0.04	(0.086)	15.40[b]	±0.65
D50-140						*			
M	29	5.40	±0.21	27	-1.20	±0.03	(0.068)	14.93	±0.56
H	26	5.71	±0.23	34	-1.12	±0.03	(0.083)	15.25	±0.49
D21-50xD50-140									
L M	9	5.13	±0.39	11	-1.18	±0.05	(0.072)	12.84[a]	±0.90
L H	5	5.64	±0.50	15	-1.17	±0.04	(0.070)	13.43[a]	±0.71
M M	10	5.54	±0.34	8	-1.20	±0.06	(0.062)	17.51[b]	±0.99
M H	10	6.18	±0.37	10	-1.17	±0.05	(0.077)	15.98[ab]	±0.91
H M	10	5.53	±0.38	8	-1.22	±0.06	(0.069)	14.43[ab]	±1.13
H H	11	5.32	±0.38	9	-1.02	±0.06	(0.103)	16.36[ab]	±1.00

Values are least-square means ± standard error of the mean. Main effects P-values: *P<0.1; **P<0.05. Different alphabetical superscripts within main effects and columns indicate significant differences (P<0.05).

† Log 10 of Ovaries was used to analyse the data. Unadjusted mean values are presented in brackets.

Table 4-14: Effect of nutritional treatments during D21-50 (Low (L) vs. Medium (M) vs. High (H)) and D50-140 (Medium (M) vs. High (H)) on the fetal reproductive organs weights (testes, ovaries and mammary gland) on Day 140, adjusted for fetal body weight.

	Male fetuses			Female fetuses					
	n	Testes (g)		n	Ovaries (g) †			Mammary gland (g)	
D21-50								**	
L	14	5.51	±0.26	26	-1.17	±0.03	(0.071)	13.26[a]	±0.49
M	20	5.82	±0.22	18	-1.19	±0.04	(0.068)	16.30[b]	±0.60
H	21	5.33	±0.23	17	-1.12	±0.04	(0.086)	15.20[b]	±0.58
D50-140						*			
M	29	5.51	±0.19	27	-1.20	±0.03	(0.067)	14.41	±0.52
H	26	5.59	±0.20	34	-1.12	±0.03	(0.084)	15.44	±0.43
D21-50xD50-140									
L M	9	5.39	±0.34	11	-1.18	±0.05	(0.072)	12.70[a]	±0.80
L H	5	5.63	±0.44	15	-1.16	±0.04	(0.071)	13.83[ab]	±0.64
M M	10	5.70	±0.30	8	-1.21	±0.06	(0.060)	16.80[b]	±0.90
M H	10	5.94	±0.33	10	-1.17	±0.05	(0.077)	15.81[ab]	±0.80
H M	10	5.44	±0.33	8	-1.22	±0.06	(0.068)	13.73[ab]	±1.02
H H	11	5.22	±0.33	9	-1.02	±0.06	(0.103)	16.68[b]	±0.89

Values are least-square means ± standard error of the mean. Main effects P-values: *P<0.1; **P<0.05. Different alphabetical superscripts within main effects and columns indicate significant differences (P<0.05).

† Log 10 of Ovaries was used to analyse the data. Unadjusted mean values are presented in brackets.

Table 4-15: Effect of the fetal sex (female vs. male) and nutritional treatments during D21-50 (Low (L) vs. Medium (M) vs. High (H)) and D50-140 (Medium (M) vs. High (H)) on the weight ratios between Brain and Fetus, Liver and Fetus, Liver and Brain, and Fetus and Placentomes on Day 140.

	n	Relative Weight								
		Brain : Fetus		Liver : Fetus		Liver : Brain^		n	Fetus : Placentome	
Sex										
female	61	0.0097	±0.0001	0.0186	±0.0003	1.948	±0.041	60	13.080	±0.351
male	55	0.0097	±0.0001	0.0193	±0.0003	2.011	±0.044	51	13.520	±0.389
D21-50										
L	40	0.0099	±0.0002	0.0188	±0.0004	1.941	±0.053	39	13.780	±0.466
M	38	0.0097	±0.0002	0.0190	±0.0004	1.983	±0.051	35	12.930	±0.460
H	38	0.0096	±0.0002	0.0190	±0.0004	2.015	±0.051	37	13.180	±0.432
D50-140										
M	56	0.0097	±0.0001	0.0193	±0.0003	2.026	±0.042	54	12.960	±0.366
H	60	0.0098	±0.0001	0.0186	±0.0003	1.933	±0.042	57	13.640	±0.374
D21-50xD50-140										
L M	20	0.0100	±0.0002	0.0194	±0.0005	1.965	±0.073	20	13.300	±0.617
L H	20	0.0097	±0.0003	0.0182	±0.0006	1.917	±0.081	19	14.270	±0.737
M M	18	0.0095	±0.0002	0.0197	±0.0005	2.083	±0.073	16	12.370	±0.657
M H	20	0.0099	±0.0002	0.0184	±0.0005	1.883	±0.072	19	13.490	±0.623
H M	18	0.0095	±0.0002	0.0189	±0.0006	2.030	±0.078	18	13.210	±0.662
H H	20	0.0097	±0.0002	0.0192	±0.0006	2.000	±0.075	19	13.150	±0.652

Values are least-square means ± standard error of the mean. Main effects P-values: *P<0.1; **P<0.05. Different alphabetical superscripts within main effects and columns indicate significant differences (P<0.05).

^ Data non-normally distributed.

4.4 Placenta

4.4.1 Per ewe

Neither the ewe nutritional regimen in D21-50 or D50-140 had any effect (P>0.05) on the number of placentome type A, B, C or D (Table A-3 in Appendix). Type A placentome was always the most numerous. There was an interaction (P<0.05) between ewe nutritional treatments on D21-50 and D50-140 on the total caruncle number, but no differences (P>0.05) were found on the pairwise analysis.

4.4.2 Per fetus

The total placentome number per fetus was not affected (P>0.05) by nutrition during either D21-50 or D50-140 (Table 4-16). Interaction of dam nutritional levels during D21-50 and D50-140 had a significant effect (P<0.05) on the total caruncle number, and it tended (P=0.09) to affect the empty caruncle number. However, no differences (P>0.05) were found on pairwise analysis.

Ewe D21-50 or D50-140 nutrition had no effect (P>0.05) on placentome A, C and D weights (Table 4-17). Placentome B from H_{D21-50} ewes were heavier (P<0.05) than the L_{D21-50} ones, but ewe D50-140 nutrition had no effect (P>0.05) on the placentome B weight. Ewe nutrition during D21-50 had no effect (P>0.05) on the total placentome weight, but ewe D50-140 nutritional regimen tended (P=0.08) to affect, although no differences (P>0.05) were found on pairwise testing.

Adjusted for fetal weight:

No effect (P>0.05) was found either for sex, D21-50 or D50-140 nutrition on the weights of placentomes A, C and D after adjustment for fetal body weight (Table A-4 in Appendix). Fetal sex and dam D50-140 nutrition did not affect (P>0.05) the adjusted placentome B weight, but there was a tendency (P=0.06) for treatment during D21-50 period. Sex of fetus and dam D21-50 nutrition had no effect (P>0.05) on the adjusted total placentome weight. There was a tendency (P=0.08) for D50-140 nutrition to affect the adjusted placentome weight, but no differences (P>0.05) were found following pairwise analysis.

Table 4-16: Effect of the fetal sex (female vs. male) and nutritional treatments during D21-50 (Low (L) vs. Medium (M) vs. High (H)) and D50-140 (Medium (M) vs. High (H)) on total caruncle number, placentome number and empty caruncle number, per fetus, on Day 140.

	n	Total number								
		Caruncle #			Placentome			Empty Caruncle †		
Sex										
female	61	7.96	±0.09	(63.85)	54.65	±1.27		0.85	±0.04	(9.19)
male	55	8.12	±0.10	(66.39)	55.32	±1.36		0.95	±0.05	(10.80)
D21-50										
L	40	8.11	±0.11	(66.29)	55.50	±1.54		0.96	±0.06	(10.73)
M	38	7.97	±0.11	(64.07)	55.69	±1.60		0.83	±0.05	(8.27)
H	38	8.04	±0.12	(65.01)	53.77	±1.64		0.90	±0.05	(10.97)
D50-140									*	
M	56	7.97	±0.09	(64.00)	55.44	±1.31		0.84	±0.05	(8.51)
H	60	8.11	±0.09	(66.25)	54.53	±1.28		0.95	±0.04	(11.47)
D21-50xD50-140			**						*	
L M	20	8.03	±0.16	(64.79)	55.63	±2.26		0.94	±0.09	(9.17)
L H	20	8.19	±0.16	(67.78)	55.37	±2.21		0.98	±0.08	(12.29)
M M	18	8.17	±0.17	(67.70)	57.82	±2.31		0.85	±0.08	(9.54)
M H	20	7.76	±0.16	(60.44)	53.55	±2.23		0.82	±0.08	(7.00)
H M	18	7.70	±0.17	(59.51)	52.86	±2.42		0.74	±0.08	(6.81)
H H	20	8.37	±0.18	(70.52)	54.67	±2.47		1.06	±0.08	(15.13)

Values are least-square means ± standard error of the mean. Main effects P-values: *P<0.1; **P<0.05. Different alphabetical superscripts within main effects and columns indicate significant differences (P<0.05).

#Total Caruncle number is the sum of Placentome and Empty Caruncle. Square root of Total Caruncle number was used to analyse the data. Unadjusted mean values are presented in brackets.

† Log 10 of Empty Caruncle number was used to analyse the data. Unadjusted mean values are presented in brackets.

Table 4-17: Effect of the fetal sex (female vs. male) and nutritional treatments during D21-50 (Low (L) vs. Medium (M) vs. High (H)) and D50-140 (Medium (M) vs. High (H)) on the weight of the placentomes (total and grouped in A, B, C and D type) per fetus, on Day 140.

	n	Placentome weight (g)											
		A		B#			C#			D†			Total	
Sex														
female	61	259.5	±17.08	7.99	±0.44	(73.63)	7.08	±0.51	(63.85)	1.43	±0.09	(53.64)	428.00	±13.60
male	55	244.1	±18.57	7.79	±0.47	(70.51)	7.12	±0.54	(62.30)	1.57	±0.10	(73.64)	416.80	±14.64
D21-50					**									
L	40	237.1	±21.35	6.78[a]	±0.53	(55.67)	7.24	±0.65	(64.66)	1.58	±0.11	(88.40)	405.60	±16.67
M	38	264.1	±21.83	8.25[ab]	±0.54	(78.18)	6.82	±0.63	(63.12)	1.40	±0.11	(45.88)	430.20	±17.36
H	38	254.2	±21.44	8.63[b]	±0.56	(82.35)	7.25	±0.64	(61.45)	1.52	±0.10	(56.63)	431.50	±17.06
D50-140														*
M	56	260.1	±18.11	8.30	±0.45	(79.87)	7.33	±0.52	(66.11)	1.57	±0.09	(63.77)	440.20	±14.23
H	60	243.5	±17.14	7.48	±0.44	(64.26)	6.88	±0.54	(60.04)	1.43	±0.09	(63.51)	404.70	±13.64
D21-50xD50-140														
L M	20	269.5	±31.87	6.91	±0.77	(56.97)	7.23	±0.95	(65.68)	1.65	±0.16	(77.48)	424.50	±24.44
L H	20	204.7	±30.03	6.65	±0.77	(54.38)	7.26	±0.95	(63.64)	1.52	±0.15	(99.32)	386.80	±23.90
M M	18	262.5	±31.46	8.59	±0.76	(88.34)	6.88	±0.88	(66.73)	1.59	±0.15	(75.26)	455.90	±25.04
M H	20	265.7	±30.47	7.92	±0.76	(68.01)	6.75	±0.92	(59.51)	1.21	±0.16	(16.50)	404.40	±24.22
H M	18	248.1	±32.81	9.38	±0.85	(94.30)	7.88	±0.94	(65.93)	1.49	±0.15	(38.56)	440.10	±26.11
H H	20	260.2	±31.61	7.89	±0.83	(70.40)	6.63	±0.99	(56.97)	1.55	±0.16	(74.71)	422.80	±25.15

Values are least-square means ± standard error of the mean. Main effects P-values: *P<0.1; **P<0.05. Different alphabetical superscripts within main effects and columns indicate significant differences (P<0.05).

Square root of Placentome B and C weight was used to analyse the data. Unadjusted mean values are presented in brackets.

† Log 10 of Placentome D weight was used to analyse the data. Unadjusted mean values are presented in brackets.

CHAPTER 5 DISCUSSION

The aim of this study was to investigate the effects of early (day 21 to 50) and mid- to late-(day 50 to 140) pregnancy nutrition on the anatomical development of twin sheep fetuses, with particular focus on the fetal mammary gland. The pre- and post-grazing herbage masses, live weight and condition score changes during both early and mid- to late-pregnancy indicate that the ewe nutritional regimens were successful in achieving their targets. These observations match the findings in the larger study from which the ewes were sourced (Kenyon *et al.*, 2011).

Despite the changes in the physiological status of the ewes, there were few differences in the placenta. The total weight of membranes did not vary between treatments, possibly because the nutrient restriction was during early gestation only, when placental development is not at its peak. In mid- to late- pregnancy, the energy requirements were either met or exceeded by the treatments. Furthermore, the nutritional treatments did not affect placentome number, level of eversion, weight of type A, C and D placentomes or fetus:placentome weight ratio at D140, an indicator of placental efficiency. These findings are consistent with previous studies (McCrabb *et al.*, 1991; McCrabb *et al.*, 1992; Clarke *et al.*, 1998; Heasman *et al.*, 1998; Vonnahme *et al.*, 2003; Munoz *et al.*, 2008; Symonds *et al.*, 2010). Consequently, in the absence of significant placental differences, the lack of an effect on fetal weight and size was not unexpected.

Indeed, nutritional treatments in both early and mid- to late-pregnancy failed to affect fetal weight or general size measurements (crown-rump length, girth circumference, femur or fore-leg length) at day 140 of gestation. Interestingly, birth weight and crown-rump length of twin lambs born to $M_{D50-140}$ ewes were greater compared to the twin lambs born to ewes fed $H_{D50-140}$ in the larger cohort of ewes kept alive (Kenyon *et al.*, 2011). It was likely that the ewes fed *ad libitum* in mid- to late-gestation were unable to convert their additional nutrition towards enhancing total lamb birth or weaning weight (Kenyon *et al.*, 2011). The lack of an effect of ewe live weight loss in early pregnancy indicates that any potential negative effects of under-nutrition in early pregnancy can be compensated by maintenance or above at later stages (Munoz *et al.*, 2008). This concurs with the idea that under-nutrition in early gestation is less likely to negatively affect birth weight (Gardner *et al.*, 2007; Kenyon, 2008) and some potential effects on the fetal growth appear to be transitory with no differences detectable at birth (Robinson *et al.*, 1999b).

Significant interaction between nutritional treatments during D21-50 and D50-140 was found for the *semitendinosus* muscle weight. Muscle growth at the cellular level, is a combined increase in the number of myofibres (hyperplasia) and in their diameter and length (hypertrophy) (Swatland & Cassens, 1973; Bonnet *et al.*, 2010). Muscle growth occurs mainly through hyperplasia during fetal development (Bonnet *et al.*, 2010) and it is highly responsive to maternal dietary treatments (Reed *et al.*, 2007; Firth *et al.*, 2008). It has been argued that if dietary restriction is removed prior to the end of the major proliferation of muscle fibres (day 80, Swatland & Cassens (1973)), some compensatory growth can occur (Brameld & Daniel, 2008; Munoz *et al.*, 2008) and that an effect on the growth potential of lambs is unlikely (Annett & Carson, 2006). Thus, heavier weights of the *semitendinosus* muscles from L_{D21-50}-$H_{D50-140}$ fetuses compared to L_{D21-50}-$M_{D50-140}$ (after adjustment for fetal weight) indicate that, after a restriction in early pregnancy, muscle growth can be improved if ewes are fed well in late pregnancy. However, late pregnancy nutrition had no effect on muscle development when there was no restriction in early pregnancy (i.e. M_{D21-50} or H_{D21-50}).

Maternal nutrition in early gestation affected the mammary gland size in twin sheep fetuses. Fetuses from undernourished dams in early pregnancy had lighter mammary glands compared to the M_{D21-50} and H_{D21-50} fetuses, and these differences remained after adjustment for fetal weight. This new information helps clarify previous findings (Jenkinson, 2003; van der Linden *et al.*, 2009; Blair *et al.*, 2010) by partitioning the period of nutritional manipulation to more precise stages during gestation. Jenkinson (2003) fed ewes either a maintenance or *ad libitum* diet throughout gestation. In that study, the weights of the fetal mammary glands were not affected at day 103 or 137 of gestation (Blair *et al.*, 2010). In contrast, van der Linden and colleagues (2009) also fed ewes a maintenance or *ad libitum* diet, and found that the mammary glands of fetuses from ewes fed maintenance were heavier at day 100 (van der Linden *et al.*, 2009) and tended to be lighter at day 140 (Blair *et al.*, In Press) of gestation than those from the ewes fed *ad libitum*. In this study, the weight of the fetal mammary gland was affected by maternal nutrition only during early gestation and this could not be overcome with improved nutrition in mid- to late-gestation. Thus, the current results indicate that the critical window of early mammary gland development is somewhere between days 21 to 50 of gestation. Later critical windows, in terms of long-term milk production, have been described around birth and puberty for different species (Johnsson & Hart, 1985; Peclaris *et al.*, 1997; Sejrsen *et al.*, 2000; Knight & Sorensen, 2001; Jenkinson, 2003; Sorensen *et al.*, 2006; Hovey & Aimo, 2010; Villeneuve *et al.*, 2010), but this is the first study to show an important period in early fetal life in sheep.

It is anticipated that the lower weight of fetal mammary gland in the restricted fetuses during early gestation, will correlate with a reduced milk production later in life. This is likely because interactions between the mammary epithelium and the fat pad that occur during embryogenesis, lead to the morphogenesis and development of the mammary ducts, and this is the place where secretory cells proliferate in the adult animal during late pregnancy and lactation (Robinson *et al.*, 1999a; Knight & Sorensen, 2001; Cowin & Wysolmerski, 2010). In the study of Jenkinson (2003), fetuses from ewes fed a maintenance diet had a smaller duct area, less ducts and smaller secretion cell area than those in the *ad libitum* group (Blair *et al.*, 2010). In contrast, van der Linden *et al.* (2009) found no effect on total duct area or total number of ducts at day 100 of gestation. Paradoxically, the offspring from the maintenance group produced more milk and milk of altered composition at 18 months of age, which resulted in heavier and faster growing lambs until weaning (van der Linden *et al.*, 2009). In view of the works of Jenkinson (2003) and van der Linden (2009), there is a need to examine the response of gland development on their subsequent lactation.

Maternal nutrition affected other organs and glands, including thyroids, liver, brain and ovaries. Thyroid glands were lighter in fetuses from $M_{D50\text{-}140}$ dams compared to the $H_{D50\text{-}140}$ ones. This difference remained after adjustment for fetal weight. In addition, females had heavier thyroids than males. It is known that thyroid hormone concentrations in plasma are nutritionally sensitive, and that the thyroid status in fetal animals is largely determined by the nutrient supply to the fetus (Symonds, 1995; Rae *et al.*, 2002b). Hence, the weight of the thyroid gland could be correlated with its functionality. Thyroid hormones are necessary for promoting maturation of many tissues and organs over the final stages of fetal development (Symonds, 1995), and they may play an important role mediating the effects of maternal under-nutrition on fetal reproductive development (Rae *et al.*, 2002b). These reports are consistent with the current findings of lighter ovaries in the female fetuses from $M_{D50\text{-}140}$ ewes compared to those from $H_{D50\text{-}140}$ ewes, and a lower reproductive performance in these offspring might be expected (Rae *et al.*, 2002a).

Male and female fetuses were affected by maternal nutrition in a different way. Male fetuses from $H_{D50\text{-}140}$ ewes had longer hind-legs and heavier livers than the females in the same treatment, but no such effect was found in the $M_{D50\text{-}140}$ treatment. In contrast, in the cohort of ewes that gave birth, lambs from *ad libitum* fed ewes in mid- to late-gestation had shorter hind-legs than the maintenance group, with no differences due to sex of the lamb (Kenyon *et al.*, 2011). These differences in the results could be partially explained by the higher nutritional pressure and fetal growth in the last few days of gestation (Rattray *et al.*, 1974; Kenyon &

Webby, 2007; Symonds *et al.*, 2007) and because males could have taken better advantage of the extra maternal nutrition from mid- to late-gestation as they mature later or grow faster (Pedersen, 1980). Nevertheless, the results might suggest that female fetuses are more affected by maternal under-nutrition in early fetal life than males, with potentially negative effects on their adult reproductive performance, although for the majority of organs measured, there was not an interaction between nutrition and sex of the fetus.

CHAPTER 6 CONCLUSION

The small number of differences in placental and fetal traits, with evident changes on ewe live weight and condition score, suggests that the feto-placental unit was able to adapt to the different nutritional environments explored in this study. However, some tissues were differentially affected and female fetuses seemed to be more affected by maternal under-nutrition in early fetal life than males, with potential negative effects on their adult reproductive performance.

It is clear from this study that nutrition in early gestation affected the fetal mammary gland weight, independently of fetal weight or size. The results indicated that the critical window of early mammary gland development is between days 21 to 50 of gestation. Since the importance of the mammary gland weight relies on its relationship to later functionality and milk production, and in view of the previous work done in sheep (Jenkinson, 2003; van der Linden *et al.*, 2009), responses in gland development and subsequent lactational performance deserve further investigation.

The mammary glands of the fetuses in this study and a cohort of siblings from these animals have been kept. Mammary gland tissue will be dissected for analysis of duct area, duct number and fat pad, and thus the relationship between mammary weight and development will be established. The live animals of the larger cohort will be monitored for their lifetime performance, including milk production at first and second lactations, again assisting in relating the mammary weight and development with its functionality.

The current work has the potential to change current farming practices, allowing farmers to affect the productive performance of future generations. If the lighter fetal mammary glands are related to an impaired milk production in terms of volume and/or composition, this will affect lamb survival and growth to weaning. The consequences for sheep farmers can be of great economic importance, as their main income is the sale of those lambs at or after weaning. Thus the general practice of restricting feed intake at the beginning of pregnancy would change. However, the critical window proposed from this work (day 21 to 50 of gestation) is still a relatively long period. Future research should break down this window into two or three stages, and give recommendation for feeding levels to farmers. Further research should also be carried out in other animal species, particularly in cattle, as this could affect the worldwide dairy industry. Likewise, if the nutrition of the pregnant woman can affect the

mammary gland development of her daughter and thus, the nutrition of her grandchildren, it is timely for a review of some fundamentals of human nutrition and health.

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APPENDIX

Materials and Methods

Recording sheets for the measurements and tissue collection for ewe and fetal euthanasia on day 140:

Recording sheet for maternal organs Date _____

	Ewe tag	Live weight kg	Carcass kg	GR mm	Skirt fat (around rumen) kg	Gravid uterus kg
1						
2						
3						
4						
5						

Recording sheet for placenta (combined twin placenta) Date_____

	Ewe tag	Total weight of placenta*	Total placentome weight	Number of placentomes	Number of empty caruncles	Placentome type**			
						A	B	C	D
1									
2									
3									
4									
5									

*Total weight of placenta = membranes plus placentomes

**Placentome type = number of placentomes in each category

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Recording sheet for fetal measurements

Fetus A / B

Ewe tag _____ Date _____

	Parameter	Measure/weight	Units
1	Sex of fetus (M or F)		
	Size		
2	Weight of fetus		g/kg
3	Crown-rump length		cm
4	Heart girth		cm
5	Fore-limb length (left)		cm
6	Hind-limb length (left)		cm
	Organs		
7	Brain		g
8	Liver		g
9	Kidneys (x2)		g
10	Total kidney fat		g
11	Pancreas		g
12	Spleen		g
13	Heart		g
14	Heart fat		g
15	Lungs (x2)		g
16	Thymus		g
17	Thyroid (x2)		g
18	Adrenals (x2)		g
19	Semitendinosus muscle (left)		g
20	Testes (x2)		g
21	Ovaries (x2)		g

Results

Non-nutritional effects: sire and slaughter day

There were sire effects (P<0.05) for the following fetal measurements: fetal, *semitendinosus* muscle and pancreas weights; for the adjusted (for fetal weight) right kidney weight, spleen weight and head width; and for the unadjusted and adjusted (for fetal weight) hind-leg length, liver, heart, thymus, thyroid, pineal gland, brain and ovaries weights.

The slaughter day had an effect (P<0.05) on the fetal: weight, girth circumference, hind-leg and head lengths, spleen, thymus, pancreas and mammary weights, as well as unadjusted and adjusted (for fetal weight) *semitendinosus* muscle weight, femur length, head width, liver, heart, kidneys (x2) and thyroid weights.

Other nutritional effects

The following tables present results that have been described in the Results chapter.

Table A- 1: Effect of nutritional treatments during D21-50 (Low (L) vs. Medium (M) vs. High (H)) and D50-140 (Medium (M) vs. High (H)) on ewe live weight change (g/d).

Feeding regimen	n	Weight change (g/d)					
		Day 21-50		Day 51-140		Day 21-140	
D21-50		**		**			
L	20	-0.128[a]	±0.019	0.255[b]	±0.010	0.161	±0.008
M	19	-0.014[b]	±0.020	0.233[b]	±0.011	0.172	±0.009
H	19†	0.076[c]	±0.020	0.180[a]	±0.011	0.154	±0.009
D50-140				**		**	
M	28			0.188[a]	±0.009	0.135[a]	±0.007
H	30			0.258[b]	±0.009	0.190[b]	±0.007
D21-50xD50-140							
L M	10			0.217[b]	±0.015	0.129[a]	±0.012
L H	10			0.293[c]	±0.015	0.193[bc]	±0.012
M M	9			0.198[a]	±0.015	0.145[ab]	±0.012
M H	10			0.268[bc]	±0.015	0.200[c]	±0.012
H M	9			0.147[a]	±0.015	0.132[a]	±0.012
H H	9			0.214[b]	±0.015	0.176[abc]	±0.012

Values are least-square means ± standard error of the mean. Main effects P-values: *P<0.1; **P<0.05. Different alphabetical superscripts within main effects and columns indicate significant differences (P<0.05).

Table A-2: Effect of nutritional treatments during D21-50 (Low (L) vs. Medium (M) vs. High (H)) and D50-140 (Medium (M) vs. High (H)) on ewe liver weight (g), mammary gland weight (as total and left mammary gland only in g), total placenta membranes weight (g) and gravid uterus weight (g), at Day 140.

Feeding regimen	n	Liver (g) †			Mammary Gland (g) †			Left Mammary Gland (g) †			Total Membranes (g)		Gravid Uterus (g) †		
D21-50											*				
L	20†	3.03	±0.01	(1085)	3.06	±0.03	(1164)	2.75	±0.03	(573)	2572	±61	4.20	±0.01	(16079)
M	19	3.02	±0.01	(1057)	3.04	±0.03	(1122)	2.74	±0.03	(556)	2744	±66	4.23	±0.01	(17167)
H	19	3.04	±0.01	(1095)	3.09	±0.03	(1281)	2.78	±0.03	(627)	2700	±65	4.23	±0.01	(16871)
D50-140															
M	28†	3.02	±0.01	(1063)	3.06	±0.02	(1171)	2.75	±0.02	(578)	2618	±52	4.22	±0.01	(16603)
H	30	3.04	±0.01	(1094)	3.07	±0.03	(1207)	2.76	±0.03	(593)	2726	±58	4.22	±0.01	(16809)
D21-50xD50-140															
L M	10†	3.03	±0.02	(1077)	3.05	±0.04	(1145)	2.74	±0.04	(565)	2604	±87	4.20	±0.02	(15849)
L H	10	3.04	±0.02	(1093)	3.06	±0.04	(1184)	2.75	±0.04	(581)	2539	±84	4.21	±0.01	(16309)
M M	9	3.01	±0.02	(1042)	3.02	±0.04	(1089)	2.71	±0.04	(527)	2652	±85	4.22	±0.01	(16649)
M H	10	3.03	±0.02	(1071)	3.06	±0.05	(1156)	2.77	±0.05	(585)	2835	±96	4.25	±0.02	(17684)
H M	9	3.03	±0.02	(1071)	3.10	±0.04	(1281)	2.79	±0.04	(642)	2599	±91	4.24	±0.01	(17309)
H H	10	3.05	±0.02	(1120)	3.08	±0.04	(1282)	2.76	±0.04	(612)	2802	±93	4.22	±0.02	(16433)

Values are least-square means ± standard error of the mean. Main effects P-values: *P<0.1; **P<0.05. Different alphabetical superscripts within main effects and columns indicate significant differences (P<0.05).

† Log 10 of Liver, Mammary (total and left) and Gravid Uterus was used to analyse the data. Unadjusted mean values are presented in brackets.

Table A-3: Effect of nutritional treatments during D21-50 (Low (L) vs. Medium (M) vs. High (H)) and D50-140 (Medium (M) vs. High (H)) on the ewe placentome numbers (total and in A, B, C and D type) and caruncle numbers at Day 140.

Feeding regimen	n	Placentome number										Total Caruncle number†		
		A		B		C†		D†		Total				
D21-50														
L	20	60.73	±6.65	14.22	±1.97	1.03	±0.11 (15.27)	0.82	±0.15 (20.13)	110.10	±3.24	2.12	±0.01	(133.20)
M	19	65.07	±7.24	17.04	±2.13	1.06	±0.11 (14.84)	0.74	±0.17 (14.25)	110.40	±3.72	2.11	±0.01	(129.50)
H	19	60.57	±7.20	17.42	±2.23	1.04	±0.11 (14.35)	0.91	±0.16 (14.00)	105.90	±3.72	2.11	±0.01	(131.10)
D50-140														
M	28	63.87	±5.73	17.43	±1.70	1.08	±0.09 (14.89)	0.83	±0.13 (15.16)	110.60	±2.87	2.11	±0.01	(129.00)
H	30	60.37	±6.34	15.02	±1.90	1.01	±0.10 (14.75)	0.82	±0.14 (17.10)	107.00	±3.16	2.12	±0.01	(133.50)
D21-50xD50-140													**	
L M	10	63.41	±9.52	14.42	±2.84	1.04	±0.16 (14.59)	0.87	±0.22 (18.36)	110.40	±4.66	2.11	±0.02	(130.50)
L H	10	58.05	±9.15	14.02	±2.72	1.02	±0.15 (15.95)	0.78	±0.20 (21.91)	109.90	±4.47	2.13	±0.02	(135.90)
M M	9	65.82	±9.29	19.04	±2.75	1.10	±0.14 (15.63)	0.80	±0.22 (15.76)	115.30	±4.90	2.13	±0.02	(135.90)
M H	10	64.32	±10.49	15.04	±3.10	1.03	±0.16 (14.05)	0.68	±0.24 (12.75)	105.40	±5.12	2.09	±0.02	(123.10)
H M	9	62.37	±10.07	18.83	±2.97	1.10	±0.15 (14.46)	0.82	±0.21 (11.36)	105.90	±4.91	2.08	±0.02	(120.60)
H H	10	58.76	±10.71	16.01	±3.22	0.98	±0.16 (14.25)	1.00	±0.23 (16.64)	105.90	±5.62	2.15	±0.02	(141.50)

Values are least-square means ± standard error of the mean. Main effects P-values: *P<0.1; **P<0.05. Different alphabetical superscripts within main effects and columns indicate significant differences (P<0.05).

† Log 10 of Placentome C, D and total caruncle number was used to analyse the data. Unadjusted mean values are presented in brackets.

Table A-4: Effect of the fetal sex (female vs. male) and nutritional treatments during D21-50 (Low (L) vs. Medium (M) vs. High (H)) and D50-140 (Medium (M) vs. High (H)) on the adjusted weight of the placentomes (total and grouped in A, B, C and D type) at Day 140, per fetus.

	n	Placentome weight (g)											
		A		B#			C#			D†			Total	
Sex														
female	61	258.70	±17.25	8.03	±0.44	(74.18)	7.13	±0.51	(64.68)	1.44	±0.09	(54.74)	431.00	±13.41
male	55	245.00	±18.74	7.75	±0.47	(69.99)	7.09	±0.54	(61.72)	1.57	±0.10	(72.98)	413.90	±14.43
D21-50					*									
L	40	235.90	±21.60	6.85	±0.54	(56.65)	7.33	±0.65	(66.12)	1.60	±0.11	(90.37)	410.90	±16.54
M	38	265.00	±22.00	8.20	±0.54	(77.47)	6.78	±0.63	(62.54)	1.40	±0.11	(46.69)	426.50	±17.11
H	38	254.60	±21.55	8.62	±0.56	(82.13)	7.22	±0.64	(60.93)	1.50	±0.10	(54.52)	429.90	±16.75
D50-140														*
M	56	260.30	±18.19	8.30	±0.45	(79.88)	7.37	±0.52	(66.87)	1.58	±0.09	(64.82)	439.90	±13.96
H	60	243.40	±17.22	7.49	±0.44	(64.29)	6.85	±0.54	(59.52)	1.42	±0.09	(62.90)	405.00	±13.39
D21-50xD50-140														
L M	20	268.40	±32.10	7.00	±0.77	(58.09)	7.44	±0.96	(69.33)	1.67	±0.16	(80.70)	430.60	±24.14
L H	20	203.40	±30.27	6.71	±0.77	(55.22)	7.21	±0.95	(62.91)	1.52	±0.15	(100.04)	391.20	±23.54
M M	18	262.80	±31.60	8.59	±0.76	(88.39)	6.91	±0.88	(67.26)	1.60	±0.15	(77.04)	454.50	±24.58
M H	20	267.10	±30.75	7.81	±0.77	(66.55)	6.65	±0.92	(57.81)	1.21	±0.16	(16.34)	398.60	±23.91
H M	18	249.60	±33.09	9.30	±0.85	(93.15)	7.77	±0.94	(64.01)	1.47	±0.15	(36.73)	434.80	±25.73
H H	20	259.60	±31.76	7.94	±0.83	(71.11)	6.68	±0.99	(57.85)	1.53	±0.16	(72.32)	425.10	±24.70

Values are least-square means ± standard error of the mean. Main effects P-values: *P<0.1; **P<0.05. Different alphabetical superscripts within main effects and columns indicate significant differences (P<0.05).

†Log 10 of Placentome D weight was used to analyse the data. Unadjusted mean values are presented in brackets.

Square root of Placentome B and C weight was used to analyse the data. Unadjusted mean values are presented in brackets.