Long-term effects of size and nutrition of the pregnant ewe on mammogenesis and lactation performance of offspring and growth of the grand offspring

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

Master of Animal Science

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Abstract

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Undernutrition of fetal sheep has resulted in conflicting reports on fetal mammary development. A cohort of such underfed offspring produced greater milk, lactose and crude protein yields at their first lactation, and their lambs grew faster to weaning, than offspring that ate *ad libitum*, but these effects were not repeated at their second lactation. This thesis reports continued studies of that cohort to examine potential fetal programming effects of maternal size and plane of nutrition during pregnancy on mammary gland development and subsequent lactational performance of the female offspring. Light (L) and heavy (H) twin-bearing dams (G0) were fed either *ad libitum* (A) or maintenance (M) nutritional regimens from day 21 until day 140 of pregnancy under pastoral grazing conditions. Fetal mammary glands from female offspring were collected at day 140 of gestation (H: n=16; L; n=19; A: n=17; M n=18) and were assessed by histological and imaging analysis, recording number and total area of ducts and the size, total area and total number of secretory cells. Milk yield and composition of ewe offspring (G1) were recorded weekly for the first six weeks of their third (n=52) and fourth (n=45) lactations. The birth weights and growth of the grand-offspring (G2) were also measured once weekly until the lambs were 42 days old.

Fetal offspring from *A*-dams had greater body weights (5.9 ± 0.1 kg *vs.* 5.2 ± 0.1 kg; $P<0.01$) and tended to have heavier mammary glands at day 140 (14.9 ± 0.9 g *vs*. 13.0 ± 0.7 g; $P<0.1$) compared to those from M-dams. There was a tendency for LA-fetuses to have a greater number of mammary ducts than all other treatment groups (LA: 5.8 ± 0.23 g *vs.* HA: 5.6 ± 0.23 g, HM: 5.4 ± 0.21 g, LM: 5.2 ± 0.21 g; $P<0.1$). An interaction between nutritional treatment and rank, single (S) or twin (T), was found ($P<0.05$) for mammary gland weight such that twin fetuses carried by M-dams had lighter mammary glands compared to all other nutrition by rank groups (TM: 10.66 ± 1.06^{a}; SM: 15.24 ± 0.99^{b}; TA: 14.87 ± 1.18^{b}; SA: 15.08 ± 1.13^{b}, g; $P<0.05$). Dam size had no significant effect on fetal mammary gland dimensions.

At the third lactation, there was an interaction ($P<0.01$) between dam size and nutrition such that LA-ewes had lower lactose percentages than HA-ewes and LM-ewes. Compared to H-ewes, L-ewes had higher milk fat percentages (6.3 vs. 6.8 ± 0.13% respectively; $P<0.05$) and yield (177.3 vs. 187.8 ± 3.8 g/day respectively; $P<0.05$) over the six-week trial period. There was a significant ($P<0.05$) effect of grand-dam size on grand-offspring weight during the third lactation, but not the fourth. During the third lactation, the lambs (G2) of H-ewes and A-ewes grew faster than G2 lambs from L-ewes and M-ewes, respectively (11.20 and 11.05 vs. 10.56 and 10.72 ± 0.17 kg respectively; $P<0.05$). At their fourth lactation, H-ewes had higher lactose percentage (5.39 vs. 5.32 ± 0.02%, $P<0.05$), lactose yield (132.45 vs. 125.11 ± 2.4 g/day, $P<0.01$), and higher crude protein yield (126.08 vs. 119.54 ± 2.24 g/day, $P<0.05$) than L-ewes. There was no effect of G0 nutrition on G1 milk yield, milk fat or lactose and crude protein overall percentages or yields during the third and fourth lactations.

In summary, poor dam nutrition increased fetal mammary gland development but effects reported in the first lactation of the offspring were not repeated in the second to fourth lactations. Grand-dam nutrition also has inconsistent intergenerational influence when comparing the offspring's first, second and third parity. In the first parity, a grand-dam maintenance diet accelerated grand-offspring growth, whereas it inhibited grand-offspring growth for the second and third parities. Development of strategies to overcome constraints imposed by size and nutrition has the potential to enhance lamb growth and production by offspring, thereby increasing the profitability of the lamb-production enterprise.

Acknowledgments

I dedicate this section to those of you who have supported and encouraged me these past couple of years.

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Introduction

In New Zealand, over 33 million lambs are born each year (MAF, 2007), generating 2.59 billion dollars from lamb exports (Beef + Lamb New Zealand Limited, 2011). Lamb growth is constrained by several factors including litter size, maternal size, nutrition and milk yield (Greenwood *et al.*, 1998; Luther *et al.*, 2007; Kenyon *et al.*, 2009). Therefore, development of strategies to overcome these constraints would enhance lamb growth and thereby increase the profitability of the lamb-production enterprise.

Lactation performance of the dam is a primary determinant of lamb survival and growth as milk is the only source of nutrition for offspring during early life (Abecia *et al.*, 2006; Munoz *et al.*, 2008). Ewe body size, body condition score, live weight, and plane of nutrition can influence colostrum production, milk quality, and milk quantity (Mellor & Murray, 1985; Banchero *et al.*, 2006) which in turn, can influence lamb birth weights, growth rates and thus survival rates (Annett & Carson, 2006; Ford *et al.*, 2007). A better understanding of the factors contributing to a ewe's potential lactation performance may lead to new strategies that enhance lamb survival and growth.

Sheep in New Zealand are maintained in a pasture-based system and they are pregnant during winter, when pasture growth is minimal and adequate nutrient intake may be difficult to maintain. Maternal nutrient intake has been shown to affect mammary gland development *in-utero,* as well as lamb birth weights and later life milk yield of the offspring's first and second lactations (Jenkinson, 2003; Corner, 2007; van der Linden *et al.*, 2009; Blair *et al.*, 2010). Maternal nutrition also has intergenerational effects, whereby it can influence the growth of at least the first two generations of grand-offspring (van der Linden *et al.*, 2009;

Blair *et al.*, 2010). The mechanisms underlying the effects of maternal size and nutrition on mammary gland development and subsequent lactation performance offspring have yet to be elucidated.

Understanding the mechanisms underlying the effect of dam size or nutritional programming of mammary development may contribute to understanding the factors that influence mammary gland development. In the future, this research could lead to the development of on-farm applications to improve lactational performance, thereby improving lamb growth.

1.1 Mammary Gland Development

1.1.1 Overview

The mammary gland is unlike other organs, because its development primarily occurs post-natally in cycles that re-occur with each pregnancy (Hennighausen & Robinson, 2001). Mammary gland development begins in fetal life, when the foundation of the mammary gland is formed. However, it is not until the animal is pregnant that the secretory cells differentiate into fully functional milk-producing cells (Akers, 2002). In sheep, there is very little growth and development during lactation and secretory cells degrade relatively slowly during involution (Akers, 2002). The mammary gland is composed of two general tissue types, known as parenchyma and stroma. Stroma is the component that is primarily composed of adipose tissue (fat pad) and surrounds the milk-synthesizing portion of the gland, providing supporting framework (Akers, 1990), whilst the parenchyma is composed of ducts and lobulo-alveolar components (Figure 1. 1) (Hovey *et al.*, 1999; Hovey *et al.*, 2002). Milk yield is

determined by the amount of fully differentiated mammary epithelial cells within the parenchyma (Knight & Peaker, 1982). Ducts branch into lobules and secretory cells, but the rate and depth at which ducts grow depends on the species. The lobules are comprised of alveoli, and each alveoli has secretory cells which go through cycles of apoptosis with each lactation (Chebel *et al.*, 2007), re-growing and differentiating during the next pregnancy (Hennighausen & Robinson, 2001). Myoepithelial cells contract due to stimulation from oxytocin. Myoepithelial cells lie along the ducts and alveoli to aid with milk let-down (Hennighausen & Robinson, 2001; Khokha & Werb, 2011). The mammary gland develops over three separate phases in mammals before the first lactation begins: fetal growth, pubertal growth, and growth during pregnancy (Akers, 2002). In the ewe there is no significant growth after pregnancy, therefore this review focuses on the fetal and pubertal stages. Mammary gland development in sheep and the way in which their development differs to other species will be a reviewed. Understanding mammary gland development, and the difference between sheep and other mammals, is a fundamental prerequisite to understanding the subsequent information of the effects from maternal nutrition and size on the mammary gland.

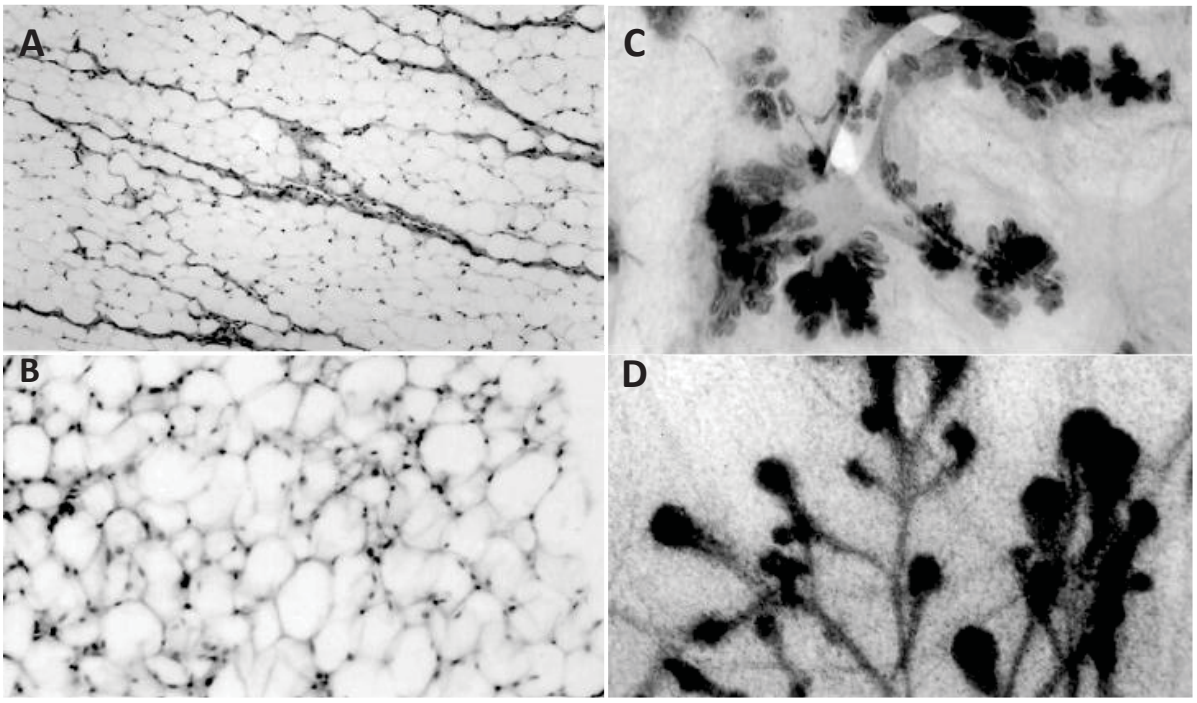

Figure 1. 1 A comparison of the cellular composition within the mammary fat pad of two species, at different stages of development (A) Mammary fat pad from post-natal ewe before puberty (B) mammary fat pad from post-natal mouse during puberty (C) Ducts from post-natal ewe before puberty (D) Parenchyma of post-natal mouse where ducts (terminal end buds) are proliferating into fat pad Source: Hovey *et al*. (1999).

1.1.2 Pre-natal Mammary Development

The pre-natal period is an important time forming the outline of the mammary gland from the ectoderm and mesenchyme. The mass of the mammary gland increases slowly, however, significant cellular development is occurring (Figure 1. 2) (Knight & Peaker, 1982). During the early fetal stage, animals undergo growth of the ectoderm, which separates the mammary gland into compartments (Hovey *et al.*, 2002) and forms mammary buds (Cowie, 1974). The ectoderm will eventually develop into the lobulo-alveolar, or secretory, part of the gland (Capuco & Akers, 1999). Mammary buds determine the exact position of the mammary glands (Knight & Peaker, 1982). Compacted ectodermal cells emerge on either side of the midline and are continuous from upper to lower limbs (Akers, 2002). A narrow edge of ectodermal

cells with a sheet of dense mesenchymal cells are known as the mammary line (Akers, 2002). The mammary line shortens as the ectodermal cells grow to form the mammary crest, which also shortens to form the inguinal region. Mammary development relies on signalling between the epithelium and the mesenchyme (Hennighausen & Robinson, 2001). Thus, the ectodermal cells continue inward growth, towards the mesenchymal layer and form fundamental components, such as the stroma and the circulatory system of the mammary gland (Akers, 2002). Primary spouts develop and they diverge into secondary spouts before puberty (Hovey *et al.*, 2002). The secondary sprouts in ruminants become lactiferous ducts from the formation of rows of epithelial cells that infiltrate the mesenchyme underneath (Akers, 2002). This row of epithelial cells will eventually form the parenchyma. Epithelial cells make up the active part of the mammary gland (Dublin, 1983). In contrast to the parenchyma, it is important to note that the stroma and the circulatory system are nearly completely developed at birth (Sejrsen & Purup, 1997). Therefore, the pre-natal period is an important time.

In sheep, the mammary gland grows five times faster than the body grows during mid-fetal development (days 44 to 70 of gestation), after which growth declines to 1.7 times the growth of the body (Knight & Peaker, 1982; Capuco & Akers, 1999; Hovey et al., 2002). Early and mid-fetal development of the mammary gland requires little energy intake, but there is still a high rate of cellular metabolic activity. The last third of the fetal development stage involves rapid tissue growth which requires a pregnant animal to use a greater amount of energy than a non-pregnant animal (Kenyon & Webby, 2007). This highlights the importance of the pre-natal stage of mammary development in sheep.

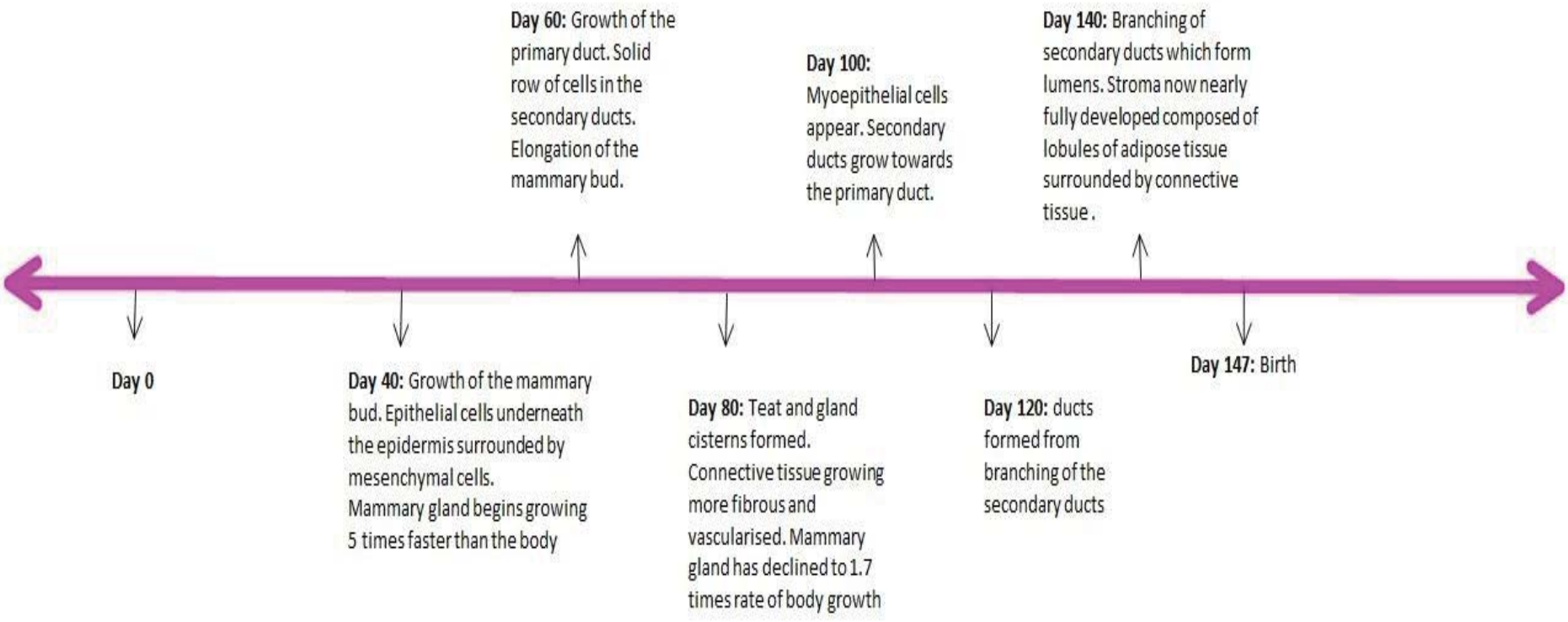

Figure 1. 2 Timeline of significant events of mammogenesis in fetal sheep. Adapted from Jenkinson (2003).

1.1.3 Post-natal Mammogenesis

In sheep, the post-natal period is primarily a time of parenchymal development. The mammary gland is comprised of stroma and parenchyma, and the interactions between these two tissue types determine functionality of the gland (Akers, 2002; Hovey *et al.*, 2002). Epithelial cells begin proliferating by growing into the stromal area (Akers, 2002) with lengthening ducts that eventually expand into the entire mammary fat pad (Ormerod & Rudland, 1984). The ducts divide, which expands the area of the parenchyma, and the spaces between are filled by parallel duct segments (Ormerod & Rudland, 1984). Expanding the parenchyma into the fat pad relies on the activity of the end buds, which are the structures at the end of growing ducts (Akers, 2002). The duct system develops into the fat pad, delaying the completion of proliferation until pregnancy (Knight & Peaker, 1982; Mitchell *et al.*, 2005). Thus, an interaction between the stroma and parenchyma is essential for the development of the mammary gland.

Stroma

Stroma is composed of the non-secretory parts of the mammary gland. It is widely accepted that adipose tissue is an endocrine organ that regulates metabolic hormones (Chemineau *et al.*, 1988; Hovey *et al.*, 1999), such as leptin, which can affect the development and composition of the mammary gland (Goldman, 2001). The composition and role of mammary adipose tissue varies between species. Rodent stroma is mostly composed of adipocytes, whilst ruminant stroma is composed of abundant, fibrous, connective tissue (Figure 1. 1) and human stroma has a distribution of both tissue types (Hovey *et al.*, 1999). The function of

mammary glands with differing quantities of stroma is not fully understood, however, it is proposed that a greater amount of connective tissue provides more functional support.

The mammary fat pad ultimately determines the amount of glandular epithelium that develops into a functional mammary gland (Sejrsen & Purup, 1997). The size of the mammary fat pad impacts the mammary gland growth rate, which then influences milk yield (Tucker, 1987). Excess adipose tissue in the mammary gland can hinder potential milk yield by taking up space and energy which would otherwise be used on secretory cell proliferation (Hennighausen & Robinson, 1998). This means that there is less ductal growth when more adipose is present in the gland. The presence of excess fat can limit epithelial cell proliferation, as indicated by decreased DNA accretion (Forsyth, 2007). It is important therefore, to have a large enough stromal area for the parenchyma to expand into, but not too much fat that it inhibits parenchymal growth.

Parenchyma

The parenchyma is the active portion of the mammary gland, made up of secretory epithelial cells, in alveolar clusters and ducts. The parenchyma is responsible for milk synthesis and secretion. The extent of the mammary gland development may be measured through the weight of the parenchyma and the parenchyma to stroma ratio. However, trying to differentiate stroma from parenchyma can often be difficult because the two tissue types develop in densely packed spaces. The volume of the parenchyma can affect milk yield because milk yield is a function of the quantity of mammary epithelial cells and their

functionality. Therefore, the parenchyma is the central component for mammary gland development (Akers, 2002).

Post-natal Growth Patterns

There is some controversy in the literature regarding mammogenesis in sheep. Allometric growth is a higher rate of growth of a specific area of the body, in comparison to the growth rate of the entire body (Akers, 2002). If there is a focus on growth in one area, then it allows the growing animal to prioritise energy towards the developing tissue, such as the mammary gland (Cowie, 1974). It should be noted that this prioritization can have positive or negative impacts for the development of the animal.

The end buds appear in the mammary gland when sheep are approximately 3 weeks old, (Gerlach & Aurich, 2000; Akers, 2002). Then, at around 16 weeks of age, sheep have been found to undergo rapid, allometric mammary gland development (Anderson, 1975). Lambs up to 16 weeks of age are also reported to have mammary glands mostly comprised of just adipose tissue (Anderson, 1975). However, others argue that starting from birth there is sizeable parenchymal tissue present, and through to 12 weeks of age, there is a 10-fold increase in parenchymal tissue alone (Ellis, 1995). In yet another study, the sheep mammary gland grew allometrically between 8 to 20 weeks of age (Chemineau *et al.*, 1988). At all other times, sheep have a slow but steady rate of mammary gland growth resulting in high levels of DNA in the mammary gland at the start of breeding (Anderson, 1975). DNA concentrations indicate total secretory epithelial cell numbers in the mammary gland, which is a good indicator of functionality (Akers, 2002).

1.1.4 Mammary Development during Pregnancy

Milk yield is dependent upon the function of secretory cells (also known as alveolar cells), which only complete their cycle of proliferation during pregnancy (Akers, 2000). Alveolar clusters emerge in mid-gestation, and late in gestation the alveoli proliferate rapidly, forming densely packed clusters (Akers, 2002). This is further evidence that the secretory capability of the gland is determined over many stages of development. The diameter of alveoli significantly influences the alveolar volume, which affects the storage and secretory capacity of the mammary gland (Akers, 2002), thus highlighting the importance of mammogenesis to maximize milk yield. An animal that develops over a longer period of time could potentially double the concentration of DNA in the mammary gland cells, in comparison to an animal that experiences a shorter period of development (Knight & Peaker, 1982).

Sheep do not begin to synthesize milkfat and protein until day 90 of gestation (Charismiadou *et al.*, 2000), which is later when compared to other species (Forsyth, 1986). In comparison to all other stages of mammary development, the growth during pregnancy is most substantial for sheep. In sheep, 78 % of mammary gland growth occurs during pregnancy (Hight & Sinclair, 1967).

1.1.5 Mammary gland development during lactation

During lactation, there is a change in the proportion of tissue that is occupied by epithelium, stroma and lumen, and the number of cells per alveoli. There may only be a small increase in cell number, but the parenchyma expands through the increasing volume of the alveolar cells as they store milk (Akers, 2002). In sheep, there is very minimal mammary gland growth

during lactation as it is completed by parturition (Anderson, 1975). In other species such, as cows and rodents, there is 10 to 50 % of growth, respectively, occurs during lactation (Forsyth, 1986). This suggests that mammary gland development during lactation is not a crucial feature in sheep, as it is in other species; therefore it is not reviewed extensively here.

1.1.6 Involution

Involution is a process in which the foundation of the mammary gland is kept intact, but some, or most of the secretory cells are discarded (Chebel *et al.*, 2007). Apoptosis is the process of cell death and the mechanism by which the mammary gland involutes (Capuco & Akers, 1999). Involution is an important process because it has been shown that allowing time for complete involution will maximize milk production in the next lactation. Cell death occurs during involution, which allows new cells to generate (Chebel *et al.*, 2007). Milk production is a function of the number of secretory cells and the secretory activity per cell. If an animal does not renew (involute) the secretory cells adequately then its production may decline as the alveolar cells degenerate (Akers, 2002). The ewe's mammary gland is capable of completing involution in as little as 30 days after lambs are weaned (Tatarczuch *et al.*, 1997; Chebel *et al.*, 2007) (Figure 1. 3). In contrast, involution in cows is drawn-out and involves a smaller amount of alveolar loss compared to other animals, which means that the cells do not regenerate in the same manner (Akers, 2002). Even after 42 days of no milk being extracted, some alveolar structure can be observed in cows (Capuco & Akers, 1999; Akers, 2002). Thus, dry cows have a larger percentage area that is occupied by epithelium and lumina, when compared to sheep (Nørgaard *et al.*, 2008), thus, involution in sheep is more extensive when compared to cows.

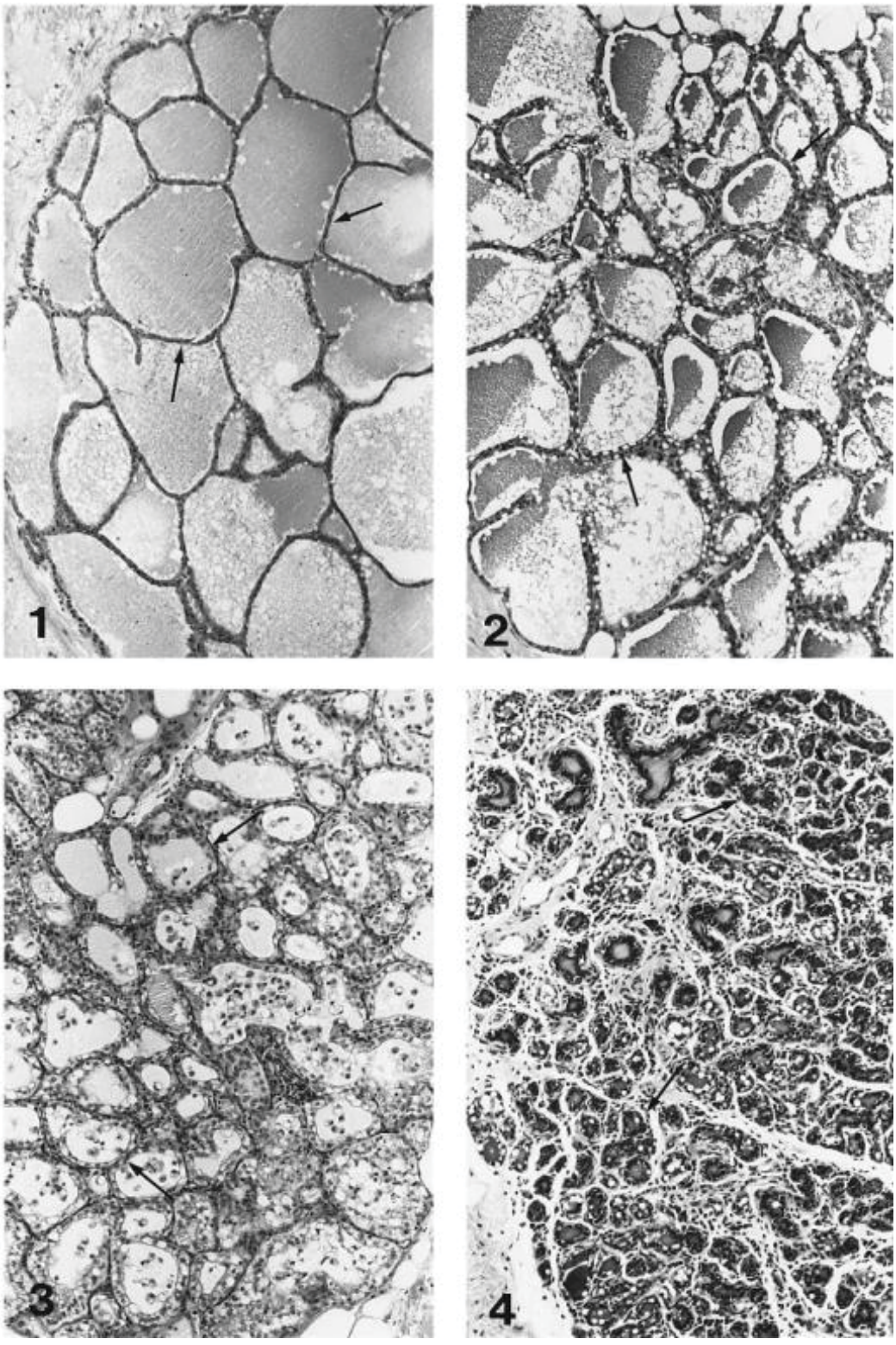

Figure 1. 3 Light scanned micrographs at x120 magnification showing the process of involution in sheep at (1) 2 days (2) 4 days (3) 7 days and (4) 30 days after weaning. Arrow indicates alveoli. Source: Tatarczuch *et al.* (1997).

1.1.7 Mammogenic Hormones

Hormone secretion begins while an animal is *in-utero* and sensitivity to the hormones slowly develops. The amount of hormone secretion and hormone sensitivity varies depending on the species, but for mammogenesis to occur, a combination of hormones is required (Ceriani, 1974). Mammary cells have receptors for growth hormones, glucocorticoids, prolactin, progesterone, estradiol, insulin, and thyroid hormone (Ollivier-Bousquet & Devinoy, 2005; Forsyth, 2007). Most of these hormones are permissive, but otherwise do not have an effect on mammary epithelial cell proliferation. The hormones of major importance will be briefly reviewed with a focus on endocrinology in sheep.

Mammogenic hormones are not universal: instead their roles are species-dependent. Estrogen, prolactin and growth hormone stimulate mammary growth in a cyclic pattern that is positively correlated with the ovarian cycle (Ceriani, 1974; Ollivier-Bousquet & Devinoy, 2005). Then during pregnancy and lactation, progesterone and prolactin combine to stimulate alveolar development (Hennighausen & Robinson, 2001). Studies in knock-out mice have shown that progesterone is essential for ductal development and lobulo-alveolar proliferation (Norman & Clark, 1998). Estrogen indirectly increases epithelial cell proliferation or stimulates elongation of the ducts by stimulating growth into the stroma (Hovey et al., 1999). The end buds appear in the mammary gland when sheep are approximately 3 weeks old, which is the time that a proliferative response to estrogen is also initially discernible (Gerlach & Aurich, 2000; Akers, 2002). Furthermore, ovariectomized sheep showed substantial parenchymal development after an injection of estrogen, suggesting that hormonal regulation of mammogenesis is different in sheep than in most other species that rely on progesterone to stimulate parenchymal growth (Ellis, 1998). Ovarian oestrogen production has been linked to

the pubertal allometric mammogenesis, and is essential for pre-pubertal mammogenesis in both cattle (Purup *et al.*, 1993) and sheep (Hovey et al., 1999).

Growth hormone (Webster *et al.*, 1991) and epidermal growth factor (EGF) stimulate ductal growth (Figure 1. 4) through the stroma (Hennighausen & Robinson, 2001). EGF is necessary for lobulo-alveloar development, but the presence of other substances such as progesterone, corticoids, growth hormone and prolactin for ductal growth and development varies according to the species (Webster *et al.*, 1991; Akers, 2002). However, insulin-like growth factor-1 (IGF-1) lowers the rate of cell turnover and enhances the production of mammary epithelial cells (Gerlach & Aurich, 2000). Insulin, on the other hand, stimulates DNA synthesis within the mammary gland (Forsyth, 1996). IGF-1 also stimulates DNA synthesis, but it is much more sensitive to binding the insulin receptor (Forsyth, 1996). The hormonal controls during involution remain to be discovered (Hennighausen & Robinson, 2001). More recently however, a leukemia inhibitory factor (LIF) has been identified as part of a key mechamism mediating mammary cell growth and apoptosis. LIF has been observed to work alongside prolactin, activating specific transcription factors which in turn regulate mammary cell death (Kritikou et al., 2003). These observations highlight the complexity of the hormone network needed to form a functional gland.

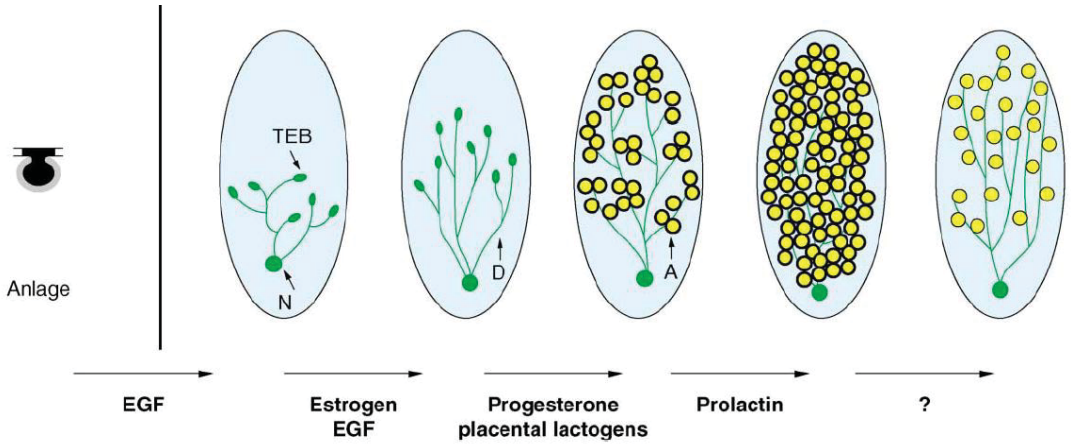

Figure 1. 4 The major endocrine control of mammogenesis from the embryonic stage, to involution. Epidermal growth factor (EGF) stimulates ductal growth. Then, EGF and estrogen control the ductal growth during puberty. Progesterone and placental lactogens stimulate the proliferation of the alveoli during pregnancy, and possibly combine with prolactin to proliferate during lactation, in some species. The hormonal controls during involution remain to be discovered. Source: Hennighausen & Robinson (2001)**.**

1.1.8 The Techniques Used to Measure Mammary Development

It is difficult to quantify mammary development in a reliable manner (Sejrsen & Purup, 1997), so the best option is to use more than one measurement technique (Akers, 2002). Slicing the mammary gland for histological analysis can allow for the estimation of area, thickness and volume of the parenchyma, and the surrounding fat pad. However, in ruminants it is difficult to differentiate epithelium and stroma, so basic quantifications of parenchyma area may be necessary (Akers, 2002). Parenchymal composition is important because the parenchyma weight does not always match the number of epithelial cells, and both wet and defatted dry weight can vary from 50 to 120 % (Nørgaard *et al.*, 2008). Therefore, weight can underestimate cellular development. Also, ruminants have a high proportion of non-secretory tissue (stroma) which means that measuring total size is not an accurate way of determining

the parenchymal size. The number of secretory cells in the gland can be an accurate method to determine mammary gland development, as secretory cells are the only cells that synthesize milk. The number of ducts can also be counted, with the proper histological technology and technique. Several studies measured stroma, epithelia and lumen alveoli area, by randomly choosing ten spots at 400 times magnification and differentiating cell types (Purup *et al.*, 1995; Nørgaard *et al.*, 2008). This is a more recent advance in imaging analysis, allowing measurement of quantity of cells, area, length (Tucker, 1987) and volume (Purup *et al.*, 1993). Milk production is directly related to the size of the mammary gland, which is directly related to the quantity of protein synthesis during lactation. Milk production is limited by the number of secretory cells (Ceriani, 1974). Using histological methods to quantify the development of the mammary glands is an important tool to understand factors influencing growth of the mammary glands.

Measuring mammary DNA concentrations may quantify the growth of the mammary gland, but the DNA concentration may not be directly equivalent to the growth of the mammary epithelial cells (Akers, 2002). Measuring the amount of DNA in tissue is accurate in mice, because their mammary glands are composed mainly of DNA from tissue which actively contributes to lactation. However, measuring DNA in cows is deceptive because the stroma has DNA that does not contribute to milk yield (Forsyth, 2007). Measuring the proliferation of cells by quantifying mitosis or DNA can indicate the rate of growth in any mammary gland, though it is typically only accurate during the later stages of pregnancy, when there are high rates of proliferation. It is possible to radioactively label the DNA in mammary epithelial cells, and then view the epithelial slides radiographically, to assess the amount of proliferation as a

measure of radiographic uptake (Hennighausen & Robinson, 2001). Measuring DNA is not practical in most cases because it involves invasive techniques.

1.2 Lactation

1.2.1 Mammary Function

The function of the mammary gland is to provide a nutrient-dense liquid for the offspring. Milk production occurs in a two-stage process known as lactogenesis and galactopoiesis. First, there is differentiation of epithelium during the onset of milk secretion, or lactogenesis. Galactopoiesis is the maintenance of milk secretion, due to the actions of prolactin and stimulation from oxytocin (Akers, 2002; Ollivier-Bousquet & Devinoy, 2005). Oxytocin has been found to affect the maintenance of milk secretion, poor milk let down is due to lack of oxytocin release (Bruckmaier & Blum, 1998; Lollovier & Marnet, 2005). Oxytocin may directly affect an animal's capacity for milk secretion, and indirectly affect it by stimulating prolactin secretion (Freeman *et al.*, 2000; Ollivier-Bousquet & Devinoy, 2005). Growth hormone has been shown to help maintain the maximum potential milk yield in some mammals (Freeman *et al.*, 2000). Milk is synthesized as molecules pass through and between cells with the assistance of hormonal stimulation. The number of mammary epithelial cells and their secretory activity influence the shape of the lactation curve (Capuco *et al.*, 2003). Reaching the maximum activity in each secretory cell can assist in reaching the maximum potential milk production, whereas simply increasing the number of epithelial cells may not have a great impact on the animal's milk production (Capuco *et al.*, 2003). Furthermore, with each

parturition there is an increasing potential milk yield (Takahashi, 1964), thus, the normal sheep lactation curve depends on the effect of parity (Figure 1. 5) (Takahashi, 1964). A standard lactation curve can be relied on for most production animals, because these animals have been bred to have a high milk yield (Akers, 2000). Studies show that selection pressures have created genetic differences in the animals and anatomical and physiological differences within the mammary glands (Sejrsen, 1994; Hovey *et al.*, 1999; Akers, 2000). Modifications of the mammary gland that may be influenced by genetics, include the number and size of mammary alveoli, weight of the mammary gland, parenchymal differentiation, RNA to DNA ratio, and possibly the ability to secrete α-lactalbumin (Keys *et al.*, 1989; Hovey *et al.*, 1999; Akers, 2000).

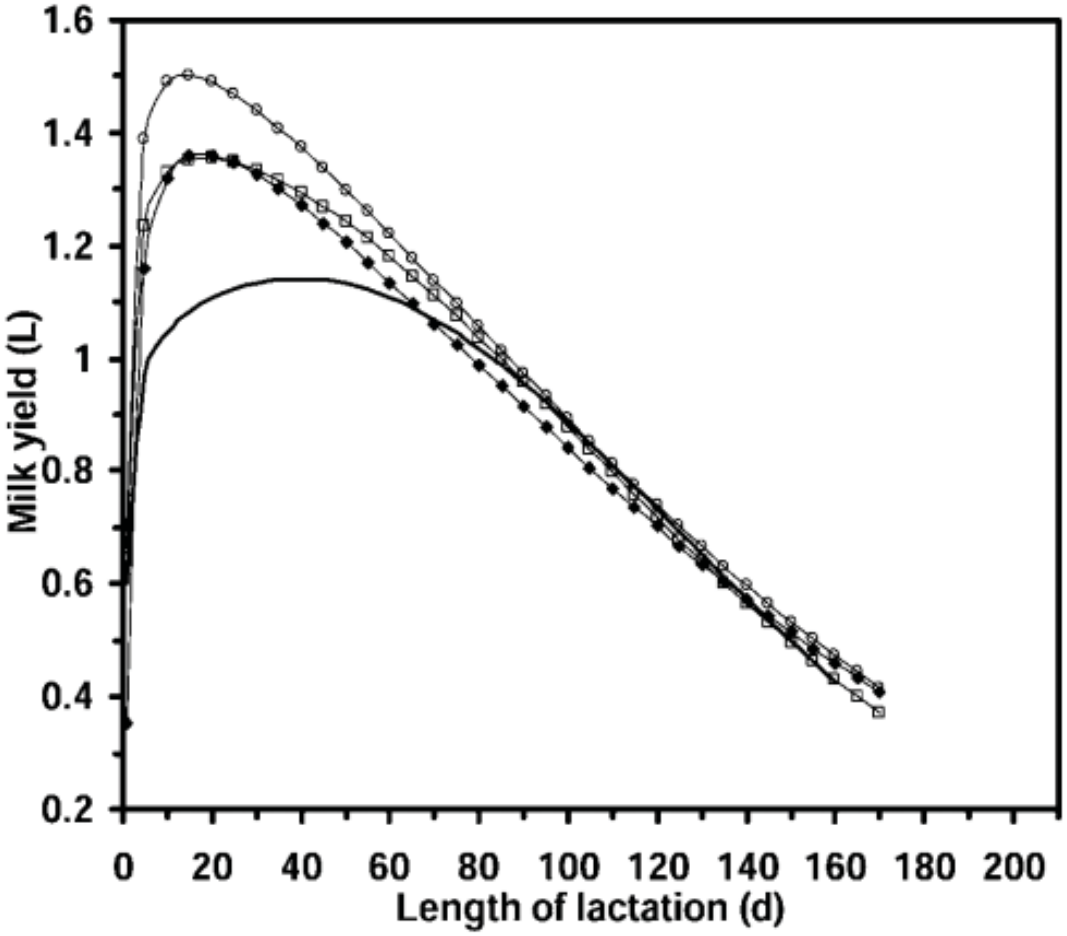

Figure 1. 5 Effect of parity on the shape of the lactation curve of laxta sheep.

1st parity sheep (_), 2nd parity sheep (□) 3rd, 4th, 5th or 6th parity sheep (○) and 7th or more parity sheep (♦). Source: Takahashi (1964).

1.2.2 Milk composition

The mean concentrations of the major milk components in twin-bearing ewes at day 42 of lactation were 6.4% fat, 5.3% protein and 4.6% lactose and there was no difference found between the four breeds of sheep (Snowder & Glimp, 1991). Milk composition varies according to the animal's physiological status, which alters their ability to transfer, produce and secrete the protein, lactose and fat components of milk (Figure 1.6) (Webster *et al.*,

1991). Carbohydrates, proteins and lipids are synthesized and secreted by secretory cells in the mammary gland, and these nutrients interact in a manner which can affect milk yield and synthesis of milk components (Akers, 2002).

An animal's milk yield is positively associated with the amount of lactose produced. Elevated lactose yields may be due to elevated mammary gland metabolic activity, or more secretory tissue (Nielsen *et al.*, 2007). Several studies found significantly higher milk yields of offspring from maintenance-fed ewes compared to ad-libitum fed ewes, who have also higher lactose percentages (Knight & Peaker, 1982; van der Linden *et al.*, 2009; Blair *et al.*, 2010). Lactose concentrations are difficult to alter, because they are regulated via an ion transport mechanism (Fedorcsák *et al.*, 2001).

Milk protein synthesis relies on the ion-dependent transport system to obtain the free amino-acids from the bloodstream. Higher milk protein concentrations may be a sign of immunoglobulins in the bloodstream (Akers, 2002), which may imply an underlying health problem. As milk yield increases during lactation, milk protein concentration decreases, in sheep and other animals (Ploumi *et al.*, 1998). Caseins assemble into micelles, which require additional nutrient components such as calcium in late gestation, thus, nutrition can directly affect milk yield and composition (Bauman *et al.*, 2006).

Milk fat is the most easily changeable milk component, and is often the first to change in response to factors such as ketosis, rumen fermentation, dietary fat intake or energy intake (Oravcova *et al.*, 2007). Therefore, there is a greater probability that milk fat concentrations would be more strongly influenced by external factors (Treacher & Caja, 2002). Fat intake is beneficial because lipolysis during pregnancy helps to support the energy requirements for lactogenesis (McNamara & Hillers, 1986). If an animal has experienced inhibited growth, then

the synthesis of milk components may be inhibited as well (O'Dowd *et al.*, 2008). In conclusion, many factors contribute to an animal's milk composition (Figure 1. 6) and maximizing individual milk components requires a healthy animal that is allowed time to fully develop, on a diet composed of essential nutrients and energy (Bencini & Pulina, 1997; Symonds, 2007).

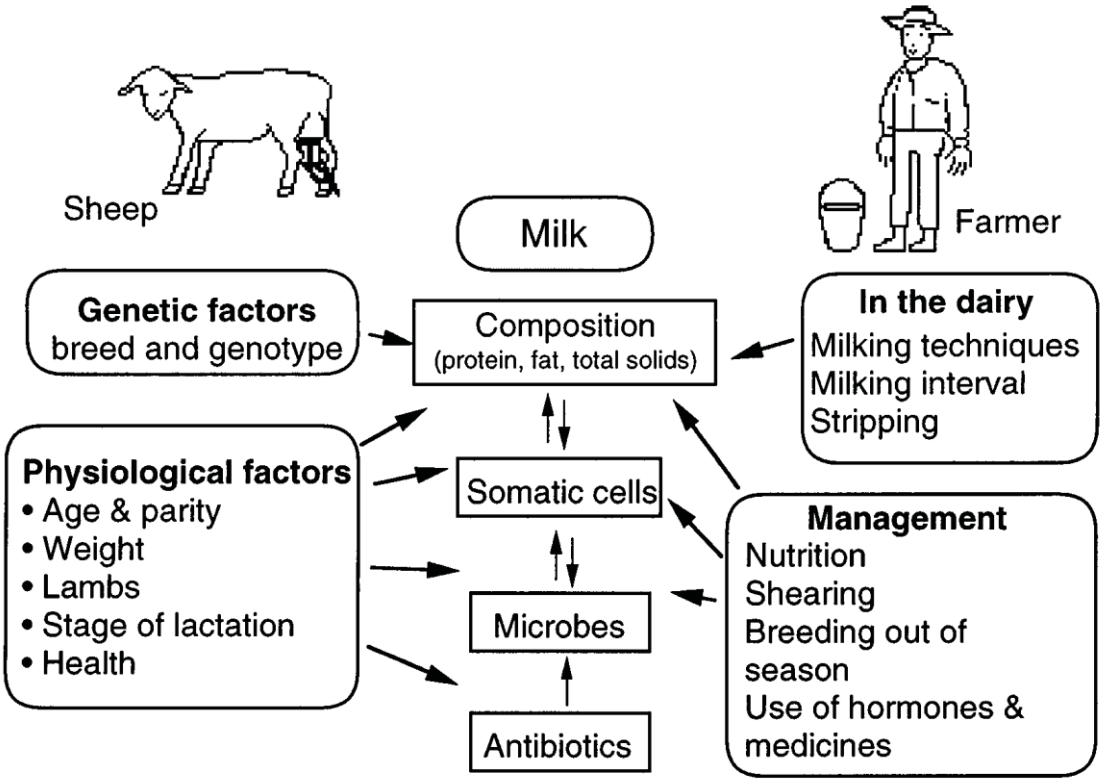

Figure 1. 6 Factors affecting milk composition: Input comes from the farmer, their milking techniques, milk interval, stripping, shearing, breeding, hormones and medical treatment also the animal influences milk composition through the breed, age, parity, size, litter size and health. Source: Bencini & Pulina (1997).

1.2.3 Lactogenic Hormones

Several endocrinological factors directly and indirectly affect milk secretion control at the tissue level (Brown *et al.*, 2005). The onset of milk secretion is stimulated by a decrease in progesterone concentrations, and an increase in prolactin and glucocorticoids; then milk secretions are maintained via oxytocin stimulation (Adams *et al.*, 1997). Prolactin regulates milk secretion by directly or indirectly initiating and then maintaining milk secretion in ruminants, humans and rodents; the exact mechanisms are unclear, but several studies in cattle have shown that exogenous prolactin increases milk secretion, perhaps by affecting α-lactalbumin concentrations (Akers, 1985). Prolactin's role was discovered with the use of an agonist that reduces prolactin secretions to only 20% of their normal concentrations; consequently, milk yields were reduced by half in the first 10 days, as were α-lactalbumin, lactose and fatty acid concentrations and the number of mammary epithelial cells (Clarke *et al.*, 2009) demonstrating the importance of prolactin for mammary alveoli differentiation and lactogenesis. Oxytocin binds to receptors on myoepithelial cells and mammary epithelial cells stimulating contraction (Lollivier *et al.*, 2001; Ollivier-Bousquet & Devinoy, 2005). Furthermore, it has been hypothesized that oxytocin affects secretory products by directly affecting the regulation of milk synthesis and secretion (Ollivier-Bousquet & Devinoy, 2005). The function of oxytocin is not completely understood, but it has been shown that oxytocin increases the permeability of tight junctions, allowing larger amounts of water and milk components into and out of cells (Nguyen & Neville, 1998). Oxytocin also stimulates pituitary prolactin secretion in ruminants (Freeman *et al.*, 2000), providing evidence that the endocrinology of mammogenesis and lactation is a complex matrix of hormones that are able to influence each other. In ewes, prolactin has been found to assist in the initiation of

lactogenesis, thereby prolactin is essential to maximize the ewe's potential milk production (Peterson, 1992).

1.2.4 Lamb Growth

In early neonatal life, lamb growth depends on efficient utilization of the ewe's milk (Smith & Clarke, 2010a, 2010b). For lambs up to four weeks of age, sheep milk typically meets all of their nutritional requirements, so there is a direct link between milk yield and lamb growth (Snowder & Glimp, 1991). After that, the link between lamb growth and milk consumption decreases steadily because lambs begin eating pasture (Smith & Clarke, 2010b). This steady decrease continues until lambs are 12 weeks to 16 weeks of age, and average daily gain and milk yield are no longer correlated (Smith & Clarke, 2010a). On average, pasture is not as nutrient-dense as ewe's milk and must be consumed in greater quantities to compensate for milk deficiencies. Thus, pasture must be consumed at a ratio of 5 to 1 relative to ewe milk (Brewer & Balen, 2010). Twin-reared lambs might provide an example of this nutrient discrepancy between milk and pasture because a twin lamb gets less milk than a single-reared lamb and their growth is impaired compared to a single-reared lamb (Figure 1. 7) (Morgan *et al.*, 2007; Norman, 2010). There is evidence that twin-born but single-reared lambs grow faster, but that they do not fully catch up to a single-born and single-reared lamb (McCoard *et al.*, 2010). Therefore, twin-reared lambs are lighter than single-reared lambs, but the mechanisms controlling the effects of rank are not known. The thrifty phenotype indicates that the fetal and early neonatal environment can prepare offspring for their future environment (Armitage *et al.*, 2004). Therefore, the single versus twin, and nutritional environment can affect the development of the offspring. Whilst the quantity and quality of milk produced is directly linked to the growth of the offspring it is evident that there are other

factors contributing to lamb growth. The numerous influences on lamb growth indicate that the relationship between lamb growth and ewe milk yield is variable.

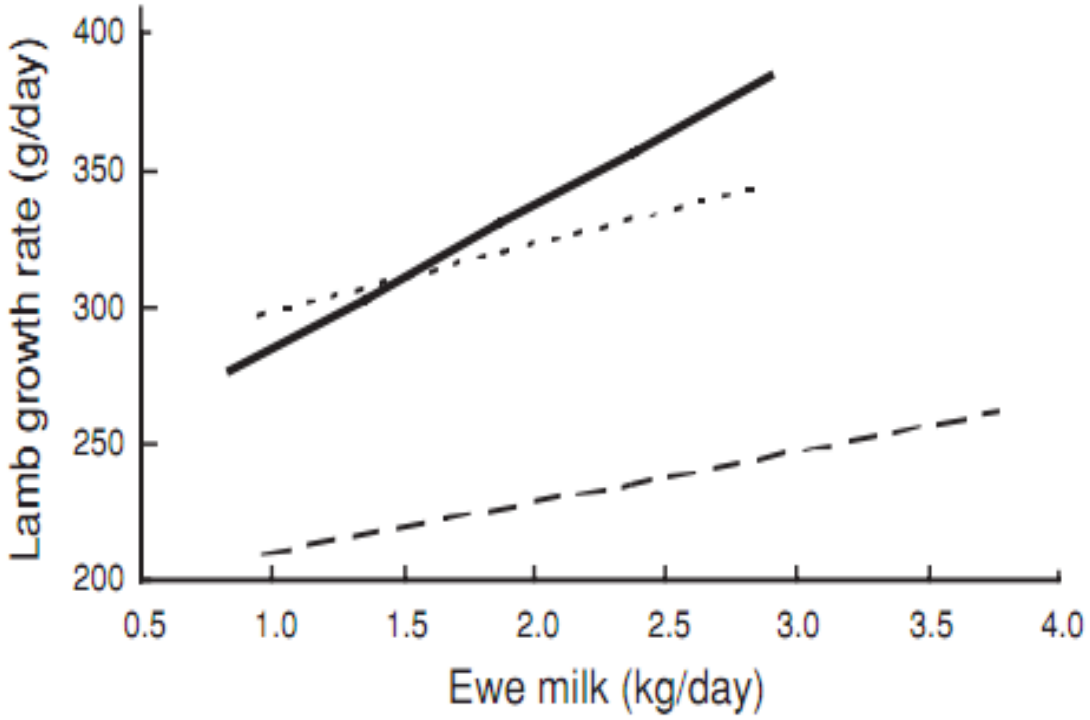

Figure 1. 7 The linear relationship between the predicted lamb growth rate and ewe milk yields in the first 4 weeks for: single born and single reared (solid line); twin born and single reared (short-dashed line); and twin born and twin reared(long-dashed line). Source: Morgan *et al.* (2007).

1.3 Factors that influence the development of the mammary gland

1.3.1 The correlation between hormones and effects of nutrition

Hormones play a key role in intrauterine fetal programming. Hormones can inhibit growth and development, especially when nutrient intake is diminished (Fowden & Forhead, 2004).

There are many types of hormones which affect development and production. There are reproductive hormones that can directly affect mammary gland development and milk synthesis, metabolic hormones which respond to nutrition, and mammary hormones that encourage specific tissue growth (Neville *et al.*, 2002).

Nutrient deficits can quickly lead to endocrine and developmental changes. High or low nutrient levels can affect leptin concentrations in fetuses, thereby affecting lipogenesis and insulin secretion (McMillen *et al.*, 2004). If sheep are under-fed, insulin levels may be decreased, and lipolysis may increase, contributing to poor body condition score in late gestation (Figure 1. 8) (Vernon *et al.*, 1981). There are long-term studies in rats finding negative effects due to *in-utero* undernutrition, such that they suffer from obesity, hyperleptinemia, hyperinsulinism and hypertension as adults (Vickers *et al.*, 2000).

In sheep, the function of the hypothalamic-pituitary-adrenal axis after birth has been found to change after only 10 days of under-nutrition *in-utero* (Vriend *et al.*, 1987). Thyroid hormones may play a role in fetal programming because they affect fetal tissue deposition and differentiation (Figure 1. 8) because they stimulate insulin-like growth-factor production and metabolic efficiency of oxygen in the tissues (Fowden & Forhead, 2004). Under-nutrition alters glucocorticoid concentration, changes the availability of other hormones and alters intracellular signalling pathways. After the peak of lactation, the decline in milk synthesis and secretion is dependent on the loss of secretory cells. Nutrition, estrogen and progesterone can affect the rate of secretory cell death (Svennersten-Sjaunja & Olsson, 2005). These alterations can reset endocrine pathways, including the hypothalamic-pituitary-adrenal axis. Ultimately, hormones come together in a circular mechanism causing, and acting as a consequence of the effects of nutrition (Fowden & Forhead, 2004).

The mammary gland goes through cyclical phases of remodelling, a time where the epithelial cells turnover in order to maintain normal function of the gland (Khokha & Werb, 2011). Mammary gland reprogramming requires a balancing of mitogens to stimulate epithelial cell differentiation, and protein inhibitors to stimulate apoptosis (Khokha & Werb, 2011). The morphology of the ovine mammary gland requires estrogen, identified as playing a key role (Gerlach & Aurich, 2000; Hennighausen & Robinson, 2001), along with gonadotropin-releasing hormone, luteinizing hormone, and follicle-stimulating hormone, all of which may also be required during the hormone-stimulated remodelling of the epithelium (Khokha & Werb, 2011). Understanding the hormones that are up- or down-regulated due to nutrition may lead to measures that prevent negative effects on growth and development via exogenous hormone manipulation.

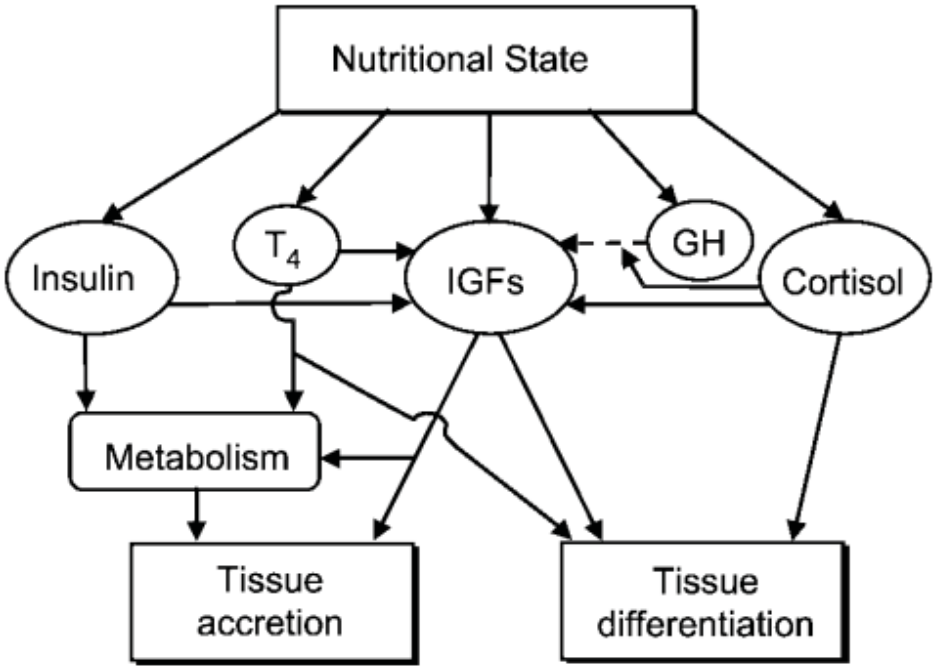

Figure 1. 8 A diagrammatic representation of the connection, and dependence, between nutritional state, hormones and metabolism on the body's growth and development. Source: Fowden & Forhead (2004).

1.3.2 The effect of nutrition on growth, mammary gland development and lactation performance of the offspring

Any significantly negative influence on the *in-utero* environment could have a significant long-term impact on the animal's lactation, because development during the fetal stage makes up the crucial building blocks for the mammary gland. The pubertal period is another critical time for mammogenesis and disturbances during this period could have long-lasting negative effects on the mammary gland (Sejrsen & Purup, 1997). Puberty attainment is influenced by environmental factors such as nutrition, temperature and day-length (Papachristoforou *et al.*, 2000) as well as non-environmental impacts such as breed, dam size, litter size and birth weight (Sejrsen, 1994; Godfrey *et al.*, 2007; van der Linden *et al.*, 2007; Minge *et al.*, 2008). In sheep and cows, it has been shown that a high plane-of-nutrition before puberty can decrease mammary growth rates and, thus, stunt mammogenesis (Johnsson *et al.*, 1985; Sejrsen & Purup, 1997). In cows, pigs and sheep, increased feed intake can increase growth rate and, thus, decrease the time taken to reach puberty (Dziuk & Bellows, 1983). This may negatively impact mammary gland development and subsequent lactation as decreasing the time to reach puberty may limit the time for crucial secretory cell replication and development (Ceriani, 1974). If an animal reaches puberty early, they have less time for pre-pubertal development; therefore, nutrition is important for endocrine function, growth, and development. In heifers, mammary growth is affected by changes in feeding levels before puberty, but not after puberty, highlighting the importance of the pre-pubertal period (Sejrsen *et al.*, 1982). Sheep exhibit significant mammary growth after puberty (Nørgaard *et al.*, 2008) therefore, direct comparisons to cows may not be accurate. These observations

emphasize the importance of nutrition during the early pre-pubertal period for the development and growth of the mammary gland.

There have been several studies examining the effects of maternal nutrition on subsequent mammary development and lactation of offspring, which implicate underlying effects of fetal programming (Mellor, 1983; Cleal *et al.*, 2007; Corner, 2007; Kenyon *et al.*, 2009; van der Linden *et al.*, 2009; Belkacemi *et al.*, 2010; Blair *et al.*, 2010). One such study found that fetuses from maintenance-fed dams had smaller mammary gland duct area at day 100 of gestation when compared to fetuses from dams that were fed 1.5 times maintenance (Jenkinson, 2003). A different study by van der Linden (2009) examined the effects of nutrition using 450 heavy (60.8 ± 0.18 kg) and 450 light (42.5 ± 0.17 kg) dams fed pasture either *ad libitum* (2304.0 ± 156.8 kg DM/ha) or maintenance (1330 ± 140.0 kg DM/ha) throughout pregnancy. Fetuses from maintenance-fed dams had heavier mammary glands than fetsuses from *ad libitum* fed dams, but there was no difference in duct area of the fetal mammary glands at day 100 of gestation (van der Linden, 2009). These studies (Jenkinson, 2003; van der Linden, 2009) may have differing results because they used different techniques to measure duct area. It is also possible that the quantity or quality of feed differed between the two studies due to differing maintenance restrictions, or climatic conditions caused different pasture growths. It was also found that the offspring from maintenance-fed and heavy dams had greater milk yields at day 7 and 28 compared to the offspring from *ad libitum*-fed or light dams during their first lactation (van der Linden et al., 2009). Lactose percentages were also greater in the offspring from both heavy and maintenance-fed dams when compared to offspring from light and *ad libitum*-fed dams in their first lactation. Despite the inconsistent results, these studies demonstrate that there are

effects of dam nutrition and dam size on fetal mammary gland development and subsequent offspring lactation performance.

Adequate nutrition during pregnancy is important for offspring growth and development. If daily nutrient requirements are not met, then the growth and development of the fetus will be compromised. Towards the end of pregnancy, daily nutritional requirements increase by 75% in comparison to a non-pregnant animal. Even small changes in intake can negatively impact the growth and development of the animal, for example, feeding pasture at 90% compared to 110% of maintenance energy requirements has been found to decrease adult mammary gland weights (Charismiadou *et al.*, 2000). Homeorhetic mechanisms indicate that nutrients are prioritized according to the organ or tissue's metabolic demand, which means that a pregnant animal will shift nutrients and energy from fat, muscle and bone, to the growing fetus (Redmer *et al.*, 2004; Nørgaard *et al.*, 2008). If an animal experiences a nutrient restriction and does not have adequate energy reserves stored as fat, then the fetus may be deprived of adequate energy and normal organ development may be inhibited (Barker *et al.*, 1993). This is further supported by findings that maternal under-nutrition can severely stunt development of the heart, pancreas, kidneys and thymus in fetal sheep, while the fetal body weight does not change (Harding & Johnston, 1995; Osgerby *et al.*, 2002). Vascular maturation and cell proliferation are also suppressed after nutrient deprivation (Redmer *et al.*, 2004). These observations indicate that maternal nutrition can compromise fetal development.

There is limited knowledge on the long-term effects of maternal nutrition on offspring mammary development and lactation. Total colostrum yield has been found to decrease by 50% when the ewe's nutrient intake was restricted during pregnancy. However by day five of

lactation, the effect of nutrition on milk yield was no longer observed. By day 30 the ewes under dietary restrictions had significantly higher milk yields compared to ewes fed *ad libitum* (Nørgaard *et al.*, 2008). The effects of nutrition on mammary gland development and offspring milk yield are variable and require further study.

Dam nutrition plays a key role in mammary development in the offspring, however, the findings to date have been variable and often contradictory, so further research is required to increase our knowledge regarding the effect of dam nutrition on mammary gland development. Furthermore, the effects of dam nutrition on fetal mammary gland development require offspring to be studied throughout adulthood to determine if subsequent effects on milk production are evident.

1.3.3 The effect of maternal size on offspring mammary gland development and production

Maternal size influences fetal and post-natal growth. In general, a larger body size enables animals to consume larger amounts of food, resulting in higher concentrations of precursors, glucose and amino acids, for milk synthesis (Revell *et al.*, 1998). Larger animals may have more body reserves, fat and muscle, and are thus capable of providing more energy for milk synthesis in comparison to animals with fewer body reserves (Revell et al., 1998). The weight or condition score of the dam may indirectly or directly relate to the size of the fat pad. The mammary parenchyma expands into the fat pad, implying that the size of the fat pad and, therefore, the weight or condition score of the dam is a significant factor contributing to the potential milk yield (Knight & Peaker, 1982; Mitchell *et al.*, 2005). Dam size has been found

to interact with fetal genotype to control the fetal growth rate and placental size (Steinlechner & Niklowitz, 1992). Fetal growth is restricted by the size of the placenta (Kelly, 1992), which is directly linked to the dam's body size (Hanson & Gluckman, 2008). Smaller animals have a smaller placenta, potentially limiting the quantities of nutrients available to support fetal growth in comparison to larger animals (Mellor, 1983). However, there is no clear evidence indicating that bigger animals have bigger mammary glands.

The weight of an animal does not always correlate exactly to their body fat composition, instead it could indicate a larger body frame. For example, heifers with similar body weights may not have the same capacity to produce milk due to differences in body composition, in particular, body fat reserves (Hondo *et al.*, 1995). Animals with less fat or a smaller body frame, compared to animals of the same weight that have more fat, may have less body reserves (Armstrong & Prescott, 1970). The amount of body reserves available involves an interaction between dam size and dam nutrition. An animal with less body reserves may have a decreased capacity to lactate due to a lack of available energy, when compared to an animal with available body reserves (Khan *et al.*, 2002). A lactating animal requires a much greater energy intake than a non-pregnant animal (Pryce *et al.*, 2001), thus an animal's milk yield is in part, a function of energy intake and energy reserves (Pryce *et al.*, 2001; van der Linden *et al.*, 2010).

Dam size may influence mammary gland development, milk production and growth of the offspring. Fetuses from heavy dams had greater mammary duct area, but no difference in the number of ducts at day 100 of gestation compared to those from light dams (Jenkinson, 2003). During the first lactation, the offspring from heavy dams were heavier, than the offspring from light-dams. However, during the second lactation, the offspring from heavy-

dams were no longer heavier-ewes than ewes from light dams. Therefore, either dam size was not a stably inherited trait, or there was less pasture in the second year, which had more of an impact on the previously heavier ewes. Furthermore, as adults, offspring from heavy dams had higher milk yields 14 days and 21 days post-partum, and greater total lactose yields (van der Linden *et al.,* 2009) in their first lactation. In the second lactation however, no differences in ewe live weight, milk yield or milk composition were apparent. Grand-offspring from heavy *ad libitum*-fed dams were heavier at birth than the grand-offspring from light-maintenance fed and light-*ad libitum* and heavy-maintenance fed dams (Blair *et al.*, 2010). These observations indicate an intergenerational effect whereby grand-dam size affects grand-offspring growth. The effects of dam size on milk yield and milk composition are not permanent, further indicating a variable or reversible mechanism (Blair *et al.*, 2010). Thus, there is a differential effect of dam nutrition on lamb growth and subsequent potential function of the offspring mammary glands.

The variable effects of dam size imply that dam size exerts its effects on offspring growth, development and lactational performance through epigenetic mechanisms. Programming of the offspring induces changes in the physiological makeup of the animal. Epigenetic mechanisms are a viable hypothesis, because they are reversible, or able to be 'silenced' in different environmental situations, potentially explaining the variability of findings across an animal's lifetime and subsequent generations (Wu *et al.*, 2004). Additionally, epigenetics may be one mechanism that mediates fetal programming events, but, more work needs to be done to establish the true underlying mechanisms. Furthermore, it is likely that there are multiple mechanisms in effect, as indicated by the variable effects on lamb growth and offspring milk production.

1.4 Fetal programming of mammary gland development and lactation performance

1.4.1 Proposed mechanisms for the effects of dam size and nutrition

There has been evidence put forth indicating that certain environmental events *in-utero* can program the fetus in such a way that alter development and affect the future production. Many terms have been coined and hypotheses and mechanisms proposed to describe and explain this phenomenon. Fetal programming mechanisms involve an exchange of non-genetic information that alters the development of the fetus in response to the *in-utero* environment (Barker *et al.*, 1993). Epigenetic mechanisms are patterns of gene expression that alter the expressed phenotypes in the offspring and may or may not be inherited through generations (Gicquel *et al.*, 2008). The thrifty phenotype hypothesis suggests that the fetus prepares for its future post-natal environment according to its exposure *in-utero.* The mismatch hypothesis works in conjunction with the thrifty phenotype hypothesis, whereby a fetus becomes physiologically prepared for the wrong post-natal environment due to *in-utero* experiences (Youngson & Whitelaw, 2008). Lastly, there are reprogramming mechanisms which have the potential to eliminate programming effects of nutrition via cell death and regeneration. The hypotheses mechanisms, and knowledge regarding their actions and effects is limited.

Fetal programming describes permanent effects, or adaptations, stemming from the *in-utero* environment (Youngson & Whitelaw, 2008). For example, it has been reported in humans, that there is an inverse relationship between birth weight and cardiovascular disease, (Barker *et al.*, 1993) suggesting that fetal programming changes an offspring's physiology, in a

permanent manner (Youngson & Whitelaw, 2008). Fetal programming resulting from altered dam nutrition may be important for mammary gland development. For example, a nutrient restricted *in-utero* environment coupled with a nutrient-rich post-natal environment can alter metabolism. It has been shown that fetal mammary gland development may be inhibited (Jenkinson, 2003; Nørgaard *et al.*, 2008; van der Linden *et al.*, 2009).

Epigenetic effects are phenotypes which may be inherited, through mitotically, meiotically or modified replicated genes (Wu *et al.*, 2004). There are many possible mechanisms underlying epigenetic effects, one of which is gene-expression modifications via post-translational changes, such as deoxyribonucleic acid methylation and histone variations (Wu *et al.*, 2004). These epigenetic mechanisms are, in principal, a transfer of specific genetic material to the fetus through mitotic or meiotic replication, and expression of specific phenotypes (Youngson & Whitelaw, 2008). Trans-generational epigenetic inheritance is the phrase used when chromosomes are altered and passed onto the next generations, (Youngson & Whitelaw, 2008). Most of the molecular studies focus on human or rodent models, but there is a gap in the literature relating to epigenetic modifications in production animals. However, there have been comparable physiological and metabolic changes in sheep and human studies, which imply the effects may be the same. For example, in sheep, studies have shown that fetal gluconeogenesis is up-regulated by maternal nutrient restriction (Gardner *et al.*, 2005; Limesand *et al.*, 2007). The effect of nutrition on gluconeogenesis may be caused by fetal programming. However, both sheep and human nutrition studies have identified DNA methylations, an epigenetic mechanism (Sinclair *et al.*, 2007).

It is possible that the fetus adapts to the environment it is subjected to, enhancing the probability of survival. This is referred to as the 'thrifty phenotype hypothesis'. These

adaptations to the *in-utero* environment may be reversible or irreversible responses that may be immediately beneficial or only beneficial if the offspring is subjected to this environmental situation again later in life (Figure 1. 9) (Gluckman *et al.*, 2007). Molecular research has been performed in humans to provide evidence that these adaptations exist, however, there is no direct evidence in sheep. This is a gap in research, which should be remedied with molecular research on the effects of maternal nutrition and size in sheep.

The thrifty phenotype can be detrimental if the animal is prepared for an environment different from the one it is born into; this is referred to as the mismatch hypothesis. For example, if the fetus is prepared for a nutrient-deprived environment, but is born into a nutrient-rich environment, there may be negative consequences because the offspring is prepared for the wrong environment. These negative consequences may entail a variety of risks, including metabolic and cardiovascular disorders (Hales & Barker, 2001; Gluckman *et al.*, 2007).

The effects on the fetus while *in-utero* are often thought to be permanent because they change the gene expression, or normal physiology in the fetus. However, another theory is that the effects from the environment *in-utero* do not remain permanent because certain tissues, such as the mammary gland, go through cyclical phases of remodelling, a time where the epithelial cells turnover in order to maintain adequate function (Khokha & Werb, 2011). During this period of remodelling, which occurs as animals age and at the conclusion of each lactation (Sternlicht, 2006; Khokha & Werb, 2011), many epithelial cells undergo cell-death and are renewed prior to the next lactation. Sheep experience more cell-turnover and involution of their mammary gland (Chebel *et al.*, 2007) than other animals, such as the dairy cow (Nørgaard *et al.*, 2008). Reprogramming can also occur in other tissues because the

epithelial cells in those tissues go through cycles of cell-turnover, however, this is a gap in the literature. Therefore, involution provides an opportunity for reprogramming of the mammary gland, which could remodel the mammary gland to the extent that the effects of nutrition are eliminated.

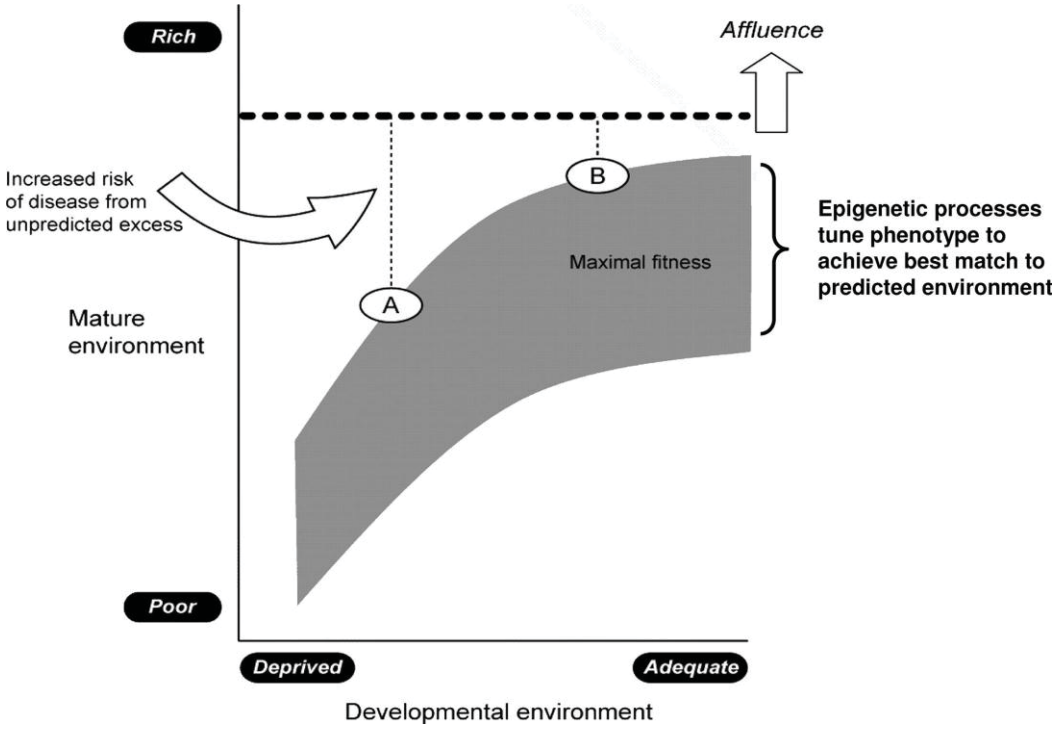

Figure 1. 9 A graph of the mismatch hypothesis, showing the relationship between the *in-utero* environment and the post-natal/adult environment. The epigenetic mechanisms modify genes *in-utero* to produce phenotypes that will best prepare the fetus for the predicted future environment. The gray area indicates the post-natal environment matching the *in-utero* environment, optimizing the potential survival and 'fitness' of the offspring. Outside the gray area implies that the offspring is prepared for the wrong environment and the resultant phenotype is detrimental to offspring growth and development. Source: Gluckman *et al.* (2007).

In summary, there are many possible mechanisms that could explain the effects stemming from the *in-utero* environment on offspring growth, development and production. There are fetal programming mechanisms, epigenetic modifications, the 'thrifty phenotype' hypothesis and the 'mismatch hypothesis', some of which may work in conjunction with one another.

Mammary gland reprogramming may explain why the effects are variable over time. Further research is required in order to understand these mechanisms and the full extent of their effects.

1.4.2 Fetal programming: The effect of maternal size and nutrition in the long term

Nutritional effects can continue affecting health, pregnancy and mammary gland performance after the nutrition of the animal has changed. There are limited number of studies on the effects of dam size and nutrition for longer than one year. The study performed by our group found that the effects vary by parity because the effects of dam level of nutrition and dam size were not repeated in the offspring's second lactation (Blair *et al.*, 2010). The grand-offspring from maintenance-fed dams had lower live-weights when compared to grand-offspring from *ad libitum* fed dams; however, there were no longer any significant differences in offspring milk, lactose or protein yields, when compared to the first lactation of the offspring (Blair *et al.*, 2010). Further studies are required in order to provide conclusive evidence of the long-term effects of the maternal plane of nutrition on subsequent lactations of the offspring.

1.5 Conclusion

As outlined in this review, there has been considerable research into the regulation of mammary development and function. The importance of fetal programming events has also

received attention and highlights the importance of dam size and nutrition on growth, development and production of offspring.

Milk yield is influenced by factors that stimulate the number of alveoli during lactation and the factors that control differentiation (Akers, 2002). Dam size and plane of nutrition during pregnancy can influence mammary gland development, milk yield and composition, and lamb growth. It has not been discovered how the plane of nutrition and dam size influence subsequent lactations of the offspring, and the growth of grand-offspring. It is hypothesized that the underlying mechanisms may be epigenetic or fetal programming, but further evidence remains to be gathered. The study on the effects of maternal size and plane of nutrition on the offspring's first and second lactations provides a useful experimental system to study fetal programming events (van der Linden *et al.,* 2009). In particular, the potential to influence the development of the fetal mammary gland and subsequent lactation performance of offspring. Understanding the mechanisms that underpin these effects is important because they may provide the tools for the eventual prevention of negative effects and manipulation of milk yield and composition.

The challenge for the future will be to identify the effects of maternal plane of nutrition and live weight during pregnancy on fetal mammary gland development and the long-term effects on subsequent lactations. The importance of dam size and nutrition for the production industry will be emphasized once the long-term effects are clarified.

The objectives of this research are to: (1) identify the effect of maternal size and plane of nutrition on fetal mammary gland development, and (2) evaluate the effect of maternal size and nutrition on lactational performance of offspring in their third and fourth lactations, and

on growth rates of the grand-offspring. The results from this study will generate new knowledge, beyond the results seen in the first and second lactations.

Chapter 2:

The effect of dam size and nutrition during pregnancy on fetal mammary gland development

Abstract

The objective of this study was to identify the effects of dam nutrition and size during pregnancy on fetal mammary gland development, through measures of mammary duct epithelial cell development. Light (L) and heavy (H) single-bearing and twin-bearing ewes (G0) were fed either *ad libitum* (A) or maintenance (M) nutritional regimens from day 21 until day 140 of pregnancy. At day 140 of pregnancy, fetal mammary glands were collected and preserved (H: n=16; L; n=19; A: n=17; M n=18). Fetal mammary gland development was analysed by histological and imaging analyses; total number of ducts, total area of ducts, total secretory cell area, estimated cell size, and total number of epithelial cells. Offspring from *ad libitum*-fed dams were heavier than offspring from maintenance-fed dams (5.87±0.15 kg *vs.* 5.19±0.14 kg; $P<0.01$). Fetuses from A-dams tended to have heavier mammary glands compared to those from M-dams (14.92 ± 0.93 g *vs*. 13.03 ± 0.77 g; ($P<0.1$). There was a tendency for LA-fetuses to have a greater number of ducts than those in all other treatment groups (LA:5.8±0.23 g *vs.* HA:5.6±0.23 g, HM:5.4±0.21 g, LM:5.2±0.21 g; $P<0.1$). Twin offspring (T) from M-dams had lighter mammary glands compared to any other group (TM: 10.66±1.06 g *vs.* SM: 15.24±0.99 g, SA: 15.08±1.13 g, TA: 14.87±1.18 g; $P<0.05$). No dam nutritional effects were found on total duct area, total lumen area, total secretory cell area, estimated cell size or total number of epithelial cells. Dam size had no effect on the parameters measured. These results highlight the importance of dam nutrition and may have important implications for future productivity.

Introduction

Critical aspects of mammary gland development occur during fetal life which can influence the glands subsequent functionality (Knight & Peaker, 1982). There is now data to indicate a differential growth effect of nutrition that depends on the time period of the nutritional insult on fetal mammary gland development, identifying a period of organogenesis of the mammary gland early in embryogenesis (Martín, 2011). Sub-maintenance levels of nutrition from days 21 to 50 of gestation resulted in lighter fetal mammary glands, compared to maintenance or *ad libitum* levels of nutrition (Martín, 2011). Moreover, this effect could not be overcome with a switch to *ad libitum* feeding from days 50 to 140 of gestation, highlighting the importance of nutrition during early pregnancy. Previous studies by our group have shown that altering the maternal environment through changes in the plane of nutrition affects fetal mammary gland development (Jenkinson, 2003; van der Linden *et al.*, 2009) and subsequent lactation performance of the offspring in their first lactation . At day 101 of fetal age the size of the mammary gland, as measured by total duct area, was one and a half times greater in fetuses whose dams had been exposed to a high level of feeding from days 19 to 101 relative to those whose dams remained at maintenance; the weights of the fetal mammary glands were not affected (Jenkinson, 2003; van der Linden *et al.*, 2009). These results differ from a follow-up study in which fetuses from maintenance-fed dams had heavier mammary gland than fetsuses from *ad libitum* (A) fed dams, but there was no difference in duct area (van der Linden *et al.*, 2009). Fetuses from heavy (H) dams had greater mammary duct area compared to fetuses from light (L) dams, but no difference in the number of ducts at day 100 (van der Linden *et al.*, 2009). Subsequently, offspring from H- or maintenance-fed (M) dams had greater milk yields than offspring from L- or A-dams in their first lactation.

Epithelial tissue later constitutes the active, secretory part of the mammary gland, including the lining of the mammary ducts (Knight & Sorensen, 2001; Hurley, 2002). It is estimated that the amount of epithelial tissue per gland increases approximately 24 times prior to birth (Jenkinson 2003). The fat pad interacts with ducts and promotes ductal morphogenesis which is where secretory cells proliferate, linking ductal development to future milk production of the animal (Robinson *et al.*, 1999; Knight & Sorensen, 2001). By the time an animal is born, their stroma has almost reached its full growth potential, however, pubertal growth is still important (Ormerod & Rudland, 1984; Walden *et al.*, 1998; Hovey *et al.*, 1999). Therefore, negative influences on mammogenesis during fetal development may impact the animal's subsequent lactation (Knight & Peaker, 1982). However, parenchymal morphogenesis and its role in milk production (Akers, 1990) is poorly understood.

Small and large breeds of sheep (Dickinson *et al.*, 1962; Gootwine *et al.*, 2007), cattle (Joubert & Hammond, 1958), pigs (Wilson *et al.*, 1998) and horses (Walton & Hammond, 1938; Allen *et al.*, 2002) have been used in crossbreeding and embryo transfer studies to show that fetal growth can be altered from the normal genetic potential by varying dam size. Our own studies with embryo transfer and crossbreeding in sheep have shown dam size can influence growth of the embryo (Sharma, 2010), birth weight and postnatal growth of the offspring (Jenkinson, 2003). A positive correlation is observed between dam size and offspring mammary gland weight and lamb (grand-offspring) live weight (Maria *et al.*, 1993; Nasholm & Danell, 1996; Kenyon *et al.*, 2004; Kenyon *et al.*, 2009; van der Linden *et al.*, 2009).

The objective of this study was to identify the effects of dam nutrition and size during pregnancy on fetal mammary gland development through measures of duct area and epithelial cell number on day 140 fetal mammary glands. It was hypothesized that offspring

born to light dams or dams fed maintenance during pregnancy would have enhanced fetal mammary gland development compared to offspring born to heavy dams or dams fed *ad libitum* during pregnancy.

2.1 Materials and methods

All procedures in this study were approved by the Massey University Animal Ethics Committee.

2.1.1 Animals and treatments

Mammary gland samples used in this study were derived from the larger production studies previously described (van der Linden *et al.*, 2009; Blair *et al.*, 2010). At day 21 of pregnancy (P21), heavy (H; 60.8 kg ± 0.2) and light (L; 42.5 kg ± 0.2) dams (G0) were randomly allocated to either *ad libitum* (A) or maintenance nutrition until P140. The aim of the M-nutritional regimen was to ensure that, throughout pregnancy, total dam liveweight gain was similar to that of the expected increase in conceptus mass (Rattray *et al.*, 1974). The aim of the A-nutritional regiment was to allow dams to eat to appetite throughout pregnancy, thus allowing the dam to maintain or increase live weight and body condition score in addition to growth of the conceptus.

2.1.2 Histology Samples

Mammary glands of female fetuses (HA: n=8; HM; n=10; LA: n=11; LM n=8) that were singles (S: n=18) or twins (T: n=19) were collected at day 140 of gestation. The left half of the

mammary gland from each fetus was preserved in Bouin's fixative for 20 hours, washed in two changes of 70% ethanol and stored in 70% ethanol before processing into paraffin (Leica Histoembedder, Leica Instruments GmbH, Nussloch, Germany).

Sections 5 µm and 2 µm thick were cut parallel to the long axis of the teat in the anterior-posterior plane for evaluation of total duct area and mammary epithelial cell number and size, respectively. Every 10^{th} section was evaluated under the microscope until the complete duct system was located. Sections were mounted individually onto pre-cleaned slides (Superfrost, Menzel-Glaser, Menzel GmbH & Co KG, Braunschweig) and oven-dried overnight at 60 degrees Celsius. Sections were automatically stained with haematoxylin and eosin (Leica Auto Stainer XL), and cover slips were mounted automatically using xylene-containing rapid-mounting medium (Entallan, Merck, kGaA, Darmstadt, Germany) and slides were stored at room temperature until analysed.

2.1.3 Morphological Measurements

Total duct area and the number of ducts were determined by capturing digital images (Zeiss Axiophot, Texas A&M Research, College Station, TX) and tracing the perimeter of each individual duct (Figure 2. 1) using a graphic tablet and pen (mousepen i608, Genius, KYE Systems Corp., Taipei) and a java-based image processing and analysis platform (Rasband, 1997). The same approach was used to measure the duct lumen area, which was subtracted from the total duct area, to enable the total area occupied by epithelial cells (TMEC; μm^2) to be determined for each gland. To confirm the accuracy of this measurement approach, an automated image analysis assay (Dragunow, 2008) was used to measure the lumen area in the same digital images using Metamorph image analysis package (Molecular Devices,

Sunnyvale, CA), as described previously (Blair *et al.*, 2010). The automated image analysis program which relies on a grey-scale could not be used to measure the total area of ducts occupied by mammary epithelial cells, as preferential staining of the epithelial cells to sufficiently distinguish the ducts from the stroma was not possible. Therefore, manual measurement was required. There was <5% variation between the manual measuring technique and the automated imaging analysis of measuring lumen area, confirming the accuracy of the manual measurement approach.

The 2-µm thick sections were used for an estimation of mammary epithelial cell size and number. Mammary epithelial cell number was estimated by randomly selecting four regions of interest per gland (Figure 2. 2, Figure 2. 3). Within each of these four areas, two separate regions of ducts containing approximately 50 epithelial cells each were individually measured with the manual measuring technique previously described, and the number of nuclei in each area was counted. The secretory cell area was divided by the number of cells in that area to estimate average epithelial cell size and epithelial cell number per unit area. This approach to estimate epithelial cell size was validated by individually measuring 20 cells randomly selected from the regions of interest used to generate the direct cell size from four differential animals. The variation between the indirect estimation of cell size and direct estimate of cell size was <10% confirming the validity of this approach.

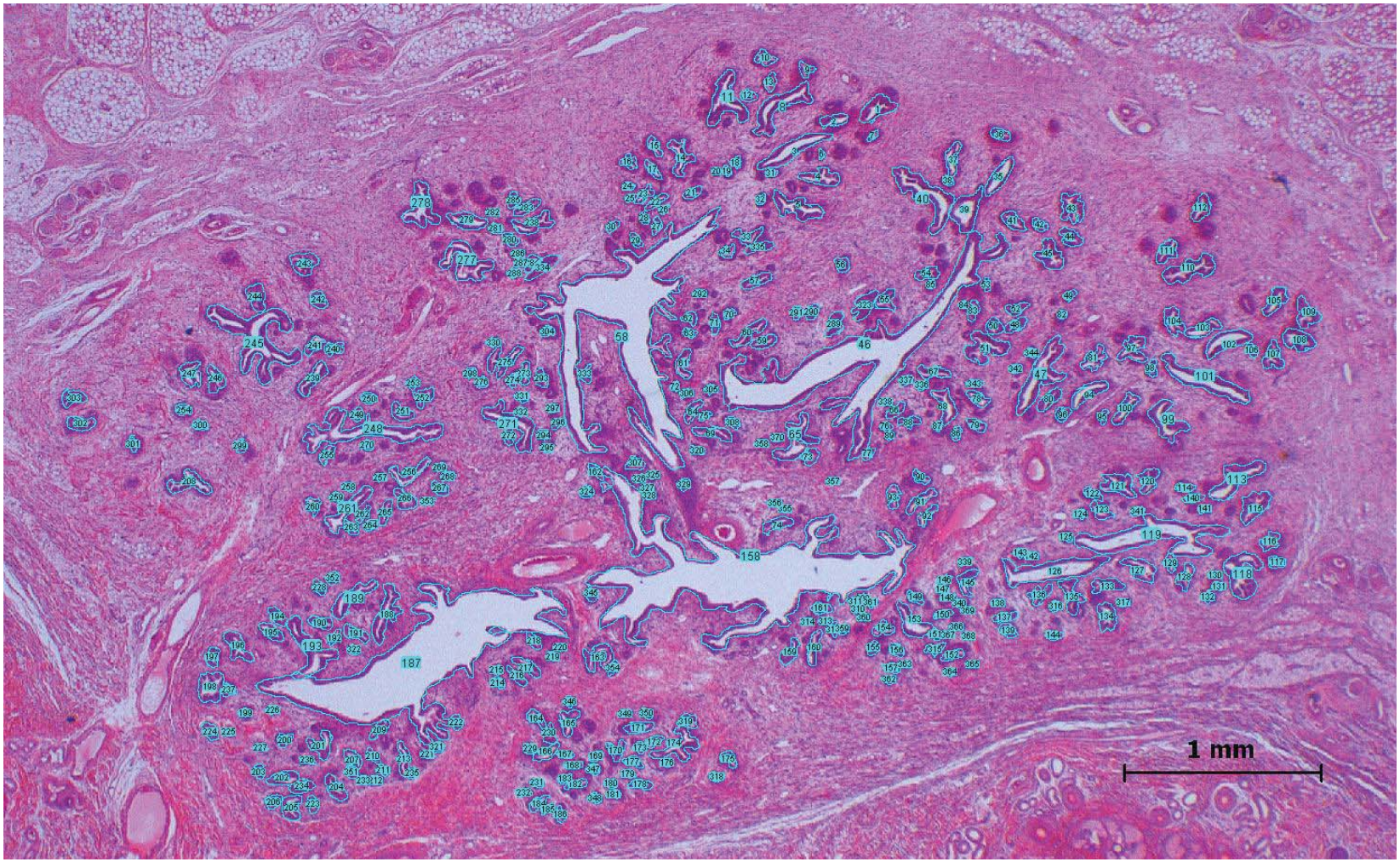

Figure 2. 1 Image of a fetal mammary gland at d140 displaying the method to count the number of ducts and measure their size

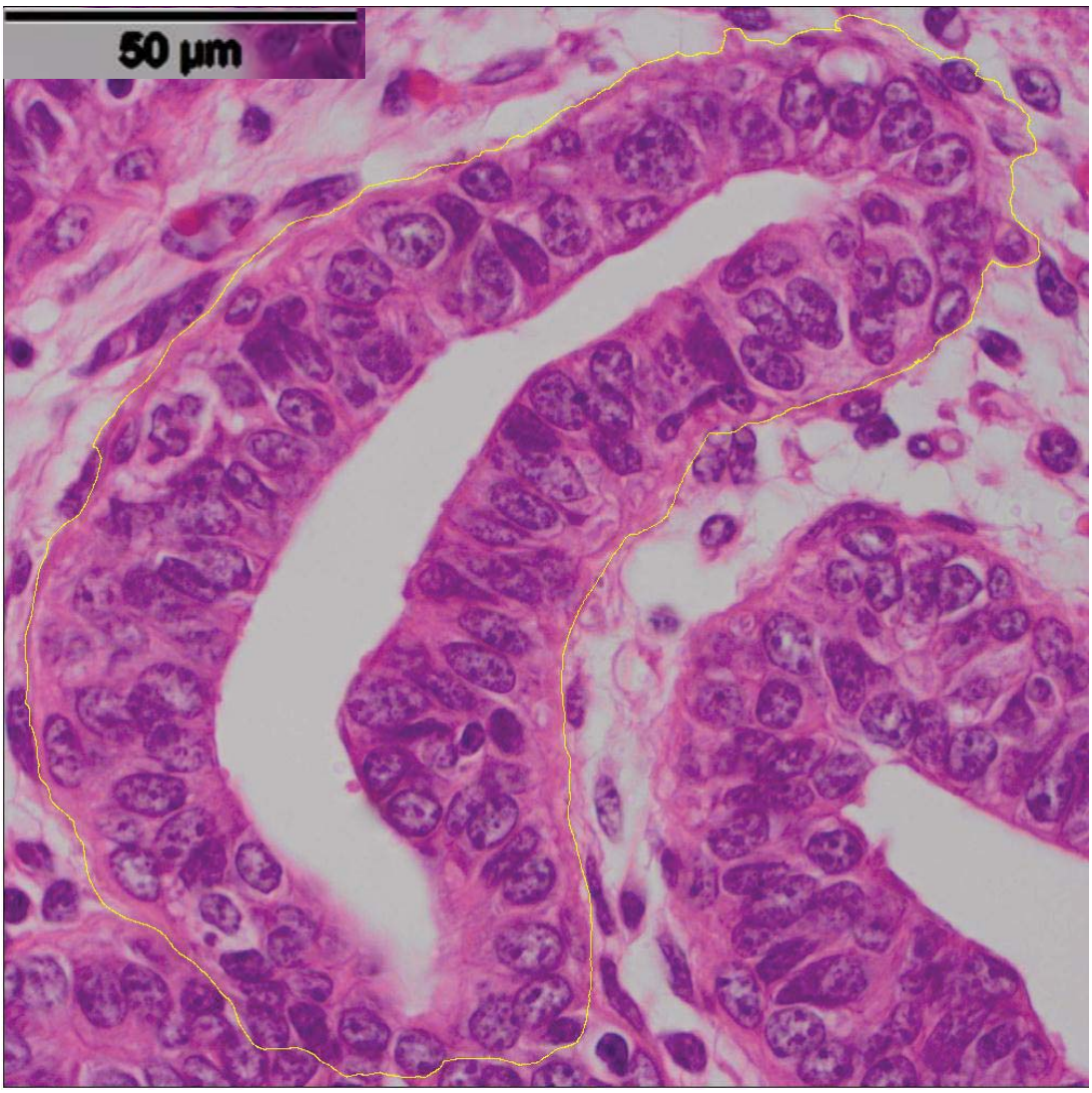

Figure 2. 2 Image of a duct from a fetal mammary gland at d140 displaying the method to count the epithelial cells. The total duct area was measured (outlined in yellow), then the area of the lumen was subtracted (white area within yellow perimeter). The number of cells within the yellow perimeter was counted.

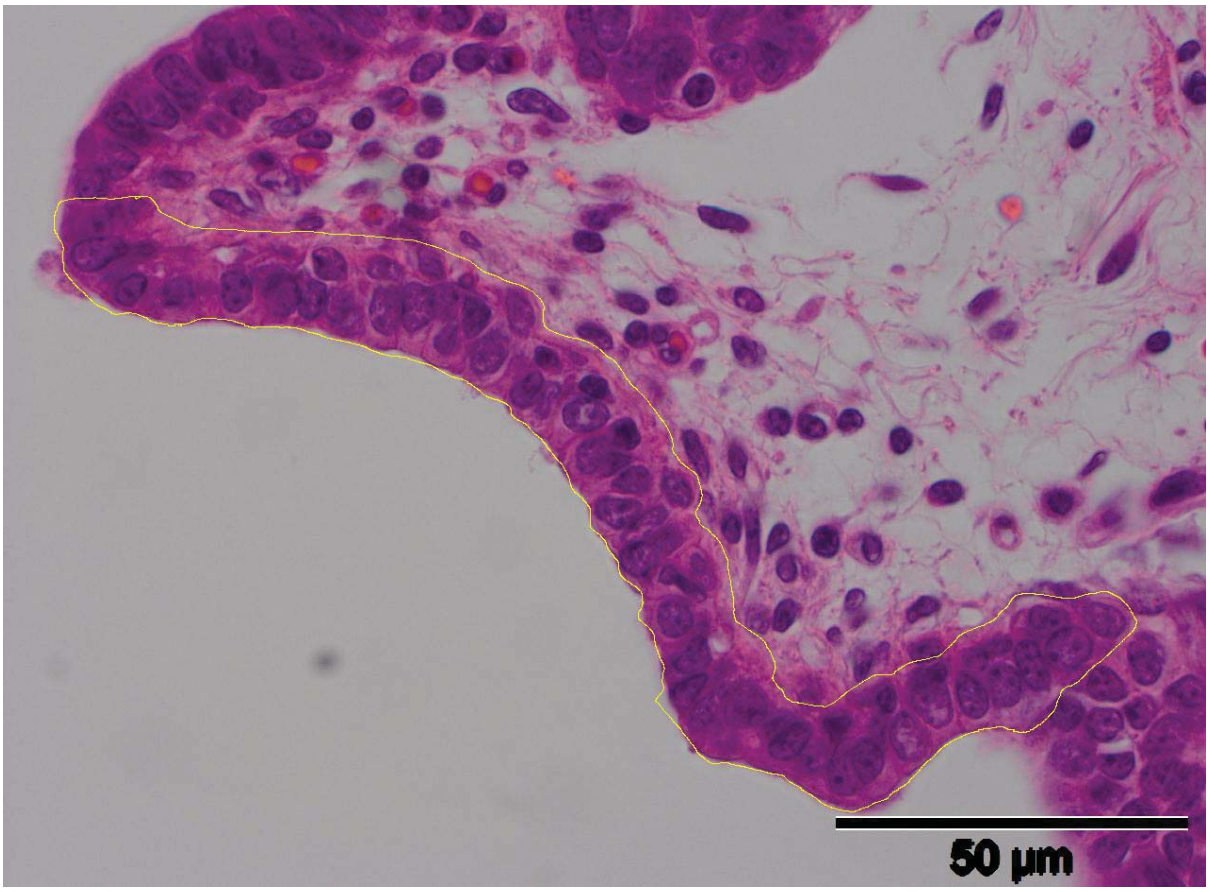

Figure 2. 3 Image of a duct from a fetal mammary gland at d140 displaying the method to count the epithelial cells when the lumen was larger than the field of view. The secretory cell area was measured, and the nuclei were counted within the measured area.

2.1.4 Statistical analysis

Data were analysed using the GLM procedure (Version 9.2, of the SAS System for windows Copyright © 2008, SAS Inst. Inc., Cary, NC) with a linear model that included the fixed effects of dam size (L or H) and dam nutrition (M or A) and the interaction of dam size by dam nutrition. Covariates (Birth rank – single versus twin, dam live weight, fetal weight, or fetal mammary gland weight) that were not significant (P>0.05) were removed from the model. Data are expressed as least square means ± SE.

2.2 Results

Fetuses from A- dams were heavier (P<0.01) than fetuses from M-dams (Table 3.1) at day 140 of pregnancy. Fetuses from A-dams tended (P<0.10) to have heavier mammary glands compared to M-fetuses. No dam nutritional effects were found on total duct area, total lumen area, total secretory cell area, estimated cell size or total epithelial cell number. Dam size had no effect on the parameters measured (Table 3.1).

An interaction between dam size and nutritional treatment was found, such that fetuses carried by LA-dams tended (P<0.10) to have a greater number of ducts than all other treatment groups (LA: 260.4±24.6 versus HA:106.1±28.6; HM:185.5±21.9; LM: 153.6±24.6).

Singleton fetuses had heavier body (P<0.01) and mammary gland (P<0.05) weights than twin fetuses (Table 2.1). Total duct and secretory cell areas were greater (P<0.05) in singleton fetuses compared to twin fetuses (P<0.05). Twin fetuses had a greater (P<0.05) number of ducts than singleton fetuses (Table 2.1). An interaction between nutritional treatment and

rank was found ($P<0.05$) for mammary gland weight, such that twin fetuses carried by M-dams had lighter mammary glands compared to all other nutrition by rank groups (TM: 10.66 ± 1.06^{a}; SM: 15.24 ± 0.99^{b}; TA: 14.87 ± 1.18^{b}; SA: 15.08 ± 1.13^{b}, g). The treatment group with a differing superscript has a significantly different mammary gland weight.

2.2.1 Effects of ewe size and nutrition on fetal mammary gland development

Table 2.1: Effects of dam size, light (L) and heavy (H), and nutritional treatment, maintenance (M) and *ad libitum* (A), from day 21-140 of pregnancy on: fetal weight, mammary gland weight, total duct area (TDA), total lumen area (TLA), secretory cell area (SCA), total number of ducts, estimated epithelial cell size (duct area/number of cells), and total epithelial cell number per gland at day 140 of gestation.

		Trait							
Treatment[1]	n	Fetal wt (kg)	Mammary gland wt (g)	TDA (um^2)	TLA (um^2)	SCA (um^2)	Total no. ducts	Cell size (um^2)	Total cell number
Rank									
Single	17	6.18±0.14[b]	15.16±0.75[b]	76888±7234[b]	26937±3387	49951±4412[b]	162±23[a]	70.3±1.5	1102±100
Twin	18	4.88±0.14[a]	12.77±0.79[a]	53846±6815[a]	23034±3191	30812±4157[a]	236±21[b]	70.2±1.6	843±103
Ewe size									
L	19	5.39±0.14	13.78±0.72	68324±6699	25208±3137	43116±4086	225±21	69.9±1.5	1066±99
H	16	5.67±0.15	14.14±0.88	62411±7486	24763±3505	37647±4565	174±24	70.5±1.6	879±104
Ewe nutrition									
M	18	5.19±0.14[a]	12.95±0.73[†]	64848±6834	22264±3200	42583±4168	183±22	70.8±1.5	996±99
A	17	5.87±0.15[b]	14.92±0.87[†]	65886±7337	27706±3435	38180±4475	216±23	69.6±1.6	949±104

[1] No interactions between dam size and dam nutrition were detected (P>0.10); therefore, only the main effects are reported.
† P < 0.10 [a b] P<0.05 indicate significance.

2.3 Discussion

The aim of this study was to determine the effects of dam size and plane of nutrition on fetal mammary gland development at day 140 of pregnancy. Previous studies by our group have shown that changes in the plane of nutrition affects fetal mammary gland development such that fetuses from *ad libitum* and heavy dams had greater mammary duct area compared to fetuses from light dams. Therefore, it was hypothesized that offspring born to light dams or dams fed maintenance during pregnancy would have enhanced fetal mammary gland development compared to offspring born to heavy dams or dams fed *ad libitum* during pregnancy. Fetal mammary gland development in this study is defined by the area of the ducts, the number of ducts, and the average secretory cell size.

2.3.1 Dam nutrition

At day 140 of gestation, there was a trend for mammary glands to be heavier in fetuses carried by dams fed *ad libitum* compared with their maintenance-fed counterparts. These results add to the observations by Martin (2011) who showed heavier mammary glands in fetuses carried by *ad-libitum* and maintenance-fed dams when compared to those carried by sub-maintenance-fed dams. Jenkinson (2003) observed no effects of maternal nutrition on fetal mammary gland weight at day 140 of gestation. In this study dam nutrition alone had no affects on the other mammary gland parameters: duct area, lumen area, secretory cell area, number of ducts or estimated cell size. There are also an absence of effects from dam

nutrition on fetal mammary gland development from the same cohort at day 100 (van der Linden *et al.*, 2009).

Larger fat pads may account for the A-fetuses having heavier mammary glands than M-fetuses. The mammary gland fat pad facilitates the action of growth hormone in the development of the mammary gland, implying that a larger fat pad inhibits growth (Walden *et al.*, 1998; Sternlicht *et al.*, 2006), which in turn could explain the subsequent greater milk yield by the M-ewes. However, no study has measured the ratio of parenchyma, versus fat pad and the effects of this ratio on subsequent milk production. Therefore, this theory will have to be evaluated in the future.

There was an interaction between dam size and dam nutrition on the total number of ducts in the fetal mammary glands. The LA-fetuses had the greatest total number of ducts compared to any other group (LM, HM or HA-fetuses). The results were similar to the study reported by Jenkinson (2003), in which the A-fetuses had a greater number of ducts than the M-fetuses. The numbers of ducts is one of the only histological measurements that were affected by fetal rank, dam size and nutrition, implying that the total number of ducts may be more easily influenced than other indicators of mammary gland development. However, in previous studies (van der Linden *et al.*, 2009), there were no interactions among fetal rank, dam size or dam nutrition observed; therefore a clear comparison cannot be made.

The only other interaction was between fetal rank and dam plane of nutrition. Twin fetuses from maintenance-fed dams had lighter mammary glands compared to all other nutrition-by-rank groups. The absence of any interactions of fetal rank and dam nutrition in the parenchymal parameters, such as duct area, suggests that there would be no subsequent effect on milk production, which is in agreement with the lack of interactions on the first milk

production of the siblings of these fetuses (van der Linden *et al.*, 2009). No other study has analysed the interaction between fetal rank and maternal nutrition on mammary gland development, and the subsequent effect on milk production.

Fetal rank had an effect on fetal weight and mammary gland development. Single fetuses weighed more, and had heavier mammary glands than twin fetuses ($P<0.05$). Furthermore, single fetuses had greater total duct area, and secretory cell area ($P<0.05$). However, twin fetuses tended to have a greater number of ducts than single fetuses. Because twin fetuses are lighter, with lighter mammary glands, less duct and secretory cell area, but greater total number of ducts, it implies that their ducts are significantly smaller at day 140 of gestation. It has been reported however, that twin fetuses have a higher growth rate earlier in gestation, and it slows later in gestation to the point that it is below that of single fetuses (Rattray *et al.*, 1974). This finding supports the possibility that twin fetuses have a greater number of ducts because they had a higher mammary gland growth rate early in gestation, but then the growth rate slowed, and there was less time for epithelial cell proliferation compared to single fetuses. At day 100 of gestation, fetuses from the same cohort had no difference in the number of ducts. No other study has reported an effect of rank on mammary gland development.

The current study was performed on day 140 fetuses, but contradicts findings from the day 100 fetuses, implying that any true differences in mammary gland weight would not appear until the end of gestation. The mammalian target of rapamycin (mTOR) is a kinase that is involved with several critical processes for cell growth and milk production (Moshel *et al.*, 2006). Another study on these same fetal mammary glands indicated that there is no effect of dam nutrition on the mTOR pathway in day 100 fetuses, but there is an effect by day 140 (Q.

Sciascia, Ag Research, Palmerston North, New Zealand, personal communication) which supports the theory that true effects appear towards the end of gestation. These molecular effects of dam nutrition inside animal tissues, such as mammary gland DNA, highlight an opportunity for future research.

2.3.2 Dam Size

No differences in fetal mammary gland weights, or parenchymal parameters were found between fetuses carried by heavy or light dams. However, fetuses from light dams on *ad libitum* nutrition levels tended to have a greater number of ducts compared with the other size by nutrition groups. No other study found any interactions between dam size and nutrition, on fetal mammary gland development. Smaller animals have a smaller placenta, indicating that there are lesser quantities of nutrients available to the fetus, and thus the fetus has less growth capabilities (Mellor, 1983). The size of the placenta, is directly linked to the dam's body size (Hanson & Gluckman, 2008). Since no dam size effects are observed in the mammary glands of day 140 fetuses, but there are dam size effects in the subsequent lactations of the offspring, it is likely that the physiological effects from dam size appear post-natally.

2.4 Conclusion

This study showed that dam nutrition during pregnancy affects fetal mammary gland weight. Moreover, twin fetuses were more susceptible to the effects of maternal nutrition than their singleton counterparts. Dam size had no effect on size and development of the fetal

mammary gland. This knowledge allows for the possibility of prevention of these effects in the future, and opening areas for future research. These findings are important because it is brought to attention that the combination of an animal's single or twin status with the dam's nutritional levels, can make a difference in the effect on mammary gland development. In the future, studies could focus on twins, as they are of greater economic value to the farmer compared to singletons. Therefore, future studies should analyse the impact of dam nutrition in twins, on mammary gland development during the fetal, post-natal and adult stages. These effects should be followed through, by researching the subsequent effects on long-term milk production. The mechanisms involved in the effects of nutrition and dam size require further investigations.

Chapter 3:

The effect of dam size and nutrition during pregnancy on the third and fourth lactations of the offspring, and growth of the grand-offspring in sheep

Abstract

Potential fetal-programming effects of maternal size and plane of nutrition during pregnancy on the lactation of the daughters were examined in sheep. Light (L) and heavy (H) twin-bearing dams (G0) were fed either *ad libitum* (A) or maintenance (M) nutritional regimens from d 21 until d 140 of pregnancy under pastoral grazing conditions. Milk production and composition of ewe offspring (G1) during their third (n=52) and fourth (n=45) lactation, and the birth weights and growth of the grand-offspring (G2) were measured. Time-series MANOVA was used within years to analyse milk and growth data. During their third lactation, there was a significant ($P<0.01$) interaction between dam size and nutrition such that LA-ewes had lower milk lactose percentages than HA- and LM-ewes. L-ewes had higher mean milk fat percentage (6.80 vs. 6.29 ± 0.13%; $P<0.05$) and yield (187.78 vs. 177.26 ± 3.80 g/day $P<0.05$) than H-ewes over the six-week lactation. The grand-offspring (G2) of H-dams and A-dams were heavier ($P<0.05$) than grand-offspring of L-dams and M-dams, respectively. After their fourth lambing, H-ewes had higher lactose percentage (5.39 vs. 5.32 ± 0.02%, $P<0.05$), lactose yields (132.45 vs. 125.11 ± 2.4 g/day, $P<0.01$), and higher crude protein yield (126.08 vs. 119.54 ± 2.24 g/day, $P<0.05$) than L-ewes. There was no effect of G0 size on the offspring's (G1) milk yield or crude protein percent and yield in the third lactation and no effect on milk yield, crude protein percent, or milk fat percent and yield in the fourth lactation. There were no effects of G0 nutrition on G1 milk yield, milk fat, lactose and crude protein percentages or yields during the third and fourth lactations. G0 size and nutrition had no effect on lamb growth to weaning by the fourth lambing of the G1. These results indicate that dam size can affect the composition of milk in the offspring, and the growth of the grand-offspring after

several lactations. Dam nutrition does not have consistent effects on the lactation over four of the offspring's parities, but grand-dam nutrition maintains an influence on the growth of the grand-offspring from the first through the third lambing.

Introduction

Dam size and nutrition during pregnancy influence mammary gland development, milk production of the offspring and growth of the grand-offspring (Jenkinson, 2003; Corner *et al.*, 2008; Kenyon *et al.*, 2009; van der Linden *et al.*, 2009; Blair *et al.*, 2010). Dam size, as measured by live weight, can affect the growth of the fetus via maternal constraint mechanisms. Maternal constraint mechanisms can limit nutrients and restrict growth through the placenta (Mellor, 1983) and the amount of body reserves that provides energy for growth and development of the offspring (Russel, 1984; Caldeira *et al.*, 2007). A restricted plane of nutrition can inhibit fetal mammary gland development, fetal weight, milk yield and composition of the offspring and growth of the grand-offspring (Wallace, 2000; Cafe *et al.*, 2006; Blair *et al.*, 2010). However, the mechanisms underpinning these effects remain to be elucidated.

In 2005, a study was initiated to investigate the trans-generational effect of maternal (G0) size and plane of nutrition on the growth and lactational performance of the offspring (G1) and grand-offspring (G2). Heavier dams positively influenced milk yield and lactose yield of the G1 offspring in their first lactation, and the birth and weaning weights of the grand-offspring (van der Linden *et al.*, 2009). In their second lactation, dam (G0) nutrition also affected the milk yield and milk composition of the G1 offspring and the growth rate of the G2 grand-offspring

(Blair *et al.*, 2010). This paper reports on the third and fourth lactations of the G1 offspring from the same cohort of ewes.

The first objective was to identify the effect of nutrition and size of the pregnant dam (G0) on milk yield and milk composition in the third and fourth lactations of the offspring (G1). The second objective was to identify the effect of G0 maternal size and plane of nutrition on the birth weight and growth of the grand-offspring (G2). Increased knowledge of the effects of the plane of nutrition during pregnancy on the performance of the offspring and grand-offspring could enable farmers to enhance their production.

3.1 Materials and Methods

The Massey University Keeble Sheep and Beef farm, 5 km south of Palmerston North, New Zealand was the site used for this study. All procedures in this study were approved by the Massey University Animal Ethics Committee.

3.1.1 Dams

The animals used in this study were generated as previously described (Kenyon *et al.*, 2009; van der Linden *et al.*, 2009; Blair *et al.*, 2010). Briefly, dams (G0) were selected by taking 450 of the heaviest (H; 60.8 kg ± SE 0.18) and 450 of the lightest (L; 42.5 kg ± SE 0.17) Romney ewes from a flock of 2,900. They were bred using AI and randomly allocated to an *ad libitum* (A) or a maintenance (M) diet from day 21 to day 140 of pregnancy in 2005 (HA, n=151; HM, n

= 153; LA, n = 155; LM, n = 153) under New Zealand pastoral grazing conditions. Maintenance-fed dams were fed to maintain their non-pregnant weight, so the fetus and placenta were the only source of weight gain. Pasture was the only source of nutrients. The average pre-grazing cover for M-fed dams was 1,330 ± 140 kg of DM/ha and the average post-grazing cover was 804 ± 133 kg of DM/ha, whereas the A-fed dam average pre-grazing cover was 2,304 ±157 kg of DM/ha and post grazing cover was 1,723 ±149 kg of DM/ha (Kenyon *et al.*, 2007).

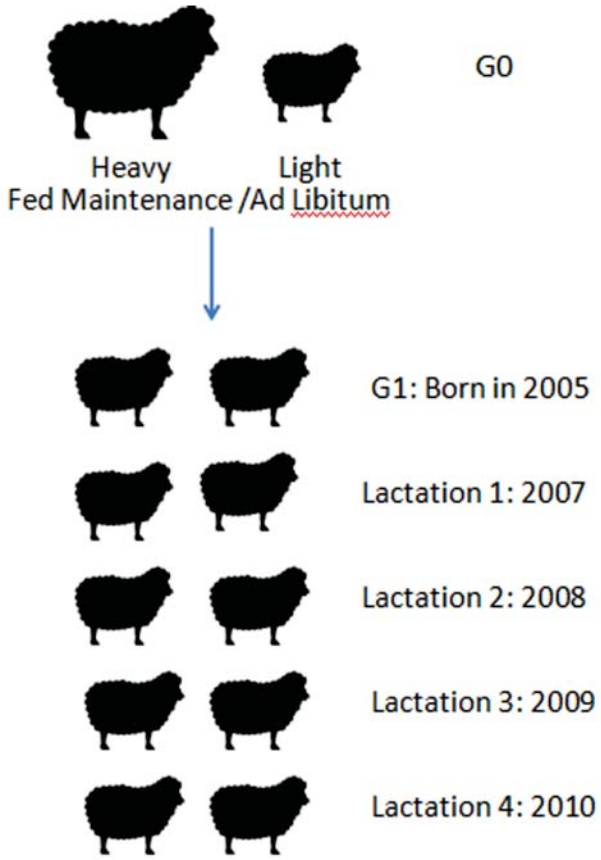

Figure 3. 1. A diagram of the experimental design. Dam (G0) size and nutritional treatments were implemented in 2005. After that, the offspring (G1) were kept together under normal New Zealand farming management.

3.1.2 Offspring in 2009 and 2010

Dams (G0) were H or L, fed either A or M, but their offspring (G1) were all grazed together throughout their lives at pasture under normal New Zealand farming conditions (Figure 3.1). The G1 offspring are referred to as H-ewes, L-ewes, M-ewes, A-ewes, indicating their dam's treatment group. Prior to mating with harnessed rams, the ewes were dosed with an anthelmintic drench capsule (Matrix Low Mineral, Ancare New Zealand Ltd., Auckland, New Zealand) and oestrus was synchronized using controlled internal drug releasers (CIDRs) that contained 0.3 grams progesterone (InterAg, Hamilton, NZ). Ultrasound pregnancy scanning at approximately day 70 of gestation was conducted to identify twin-bearing ewes, which were subsequently managed as a single group. Only twin-bearing ewes were used due to their economic importance for New Zealand agriculture.

In 2009 (third lactation), 54 twin-bearing ewes (HA n=14; HM n=15; LA n=11; LM n=14) and in 2010 (fourth lactation), 41 twin-bearing ewes (HA n=10; HM n=10; LA n=9; LM n=12) were milked once a week for six weeks, starting from an average of 7 ± 1 days post-partum using the oxytocin method (McCance, 1959; Peterson *et al.*, 1997). Lambs were separated from the ewes between morning and afternoon milking, weighed and offered by bottle 250 ml of milk collected from the dams during the morning milking. At each milking, ewes were given an intravenous injection of 1 IU of synthetic oxytocin (Oxytocin V, 10 IU/mL, Pheonix, Auckland, NZ) diluted in 0.9 mL physiological saline prior to the commencement of milking. Ewes were milked by machine followed by hand-stripping to ensure all milk was removed from the gland and the time was recorded when the udder was empty. The time of milking when the udder was empty was recorded. The ewes were milked in the afternoon and the time and total weight of milk for each ewe were recorded in order to calculate daily milk yield. Sub-samples

of milk from every ewe were taken in the afternoon and refrigerated at 4 degrees Celsius until analysis of composition (fat, protein and lactose) using a FT120-FTIR calibrated for sheep milk (Dairy NZ, Hamilton, New Zealand). Two reference checks for fat and crude protein were taken during lactation to account for changes in the matrix of the milk with stage of lactation (Dairy NZ, Hamilton, New Zealand). Before milking in the afternoons, udder dimensions were measured based on the technique described by Mellor and Murray (1985). Three dimensions (a, b, and c) were measured, and added together, by following the contours of the udder with measuring tape. Dimension a was the length of the posterior edge to the anterior edge along the midline. Dimension b was the distance between the left to right lateral margins, and Dimension c was the distance from the top margin to bottom, parallel to the midline (Figure 3.2).

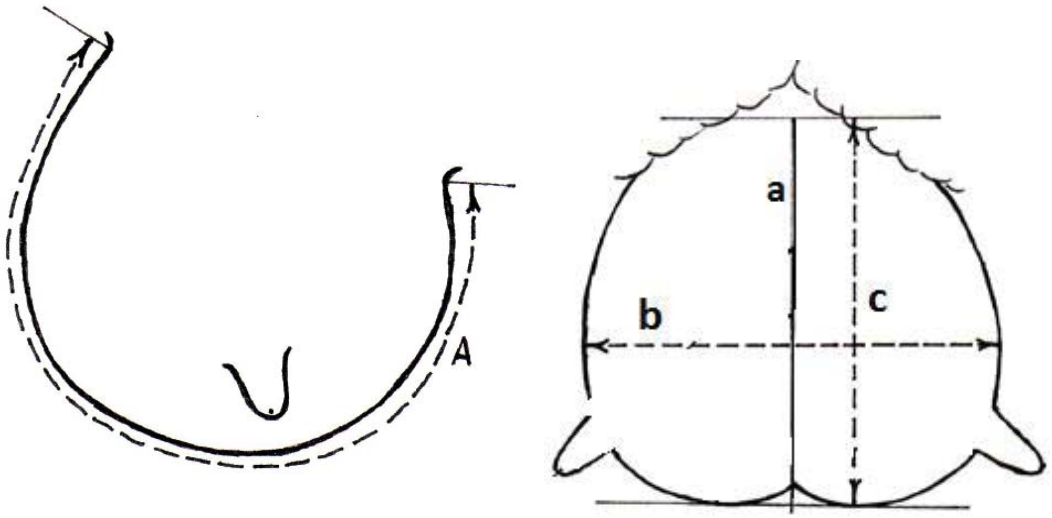

Figure 3.2. A diagram of the udder (lateral and posterior views) and the technique used to measure udder dimensions. Dimension a was the posterior edge to the anterior edge along the midline. Dimension b was the distance between the left to right lateral margins, and dimension c was the distance from the top margin to bottom, parallel to the midline.

3.1.3 Grand offspring in 2009 and 2010

Within 48 hours of birth, lambs born in 2009 (HA n=28; HM n=30; LA n=22; LM n=28) and 2010 (HA n=20; HM n=20; LA n=18; LM n=24) were ear-tagged, weighed, and their mothers were identified. On milking days, lambs were separated from the ewes in the morning. Lambs were weighed and bottle fed up to 250 ml of milk that was taken from the ewes that morning. Grand-lambs are identified according to the treatment groups of their grand-dams, and are referred to as H-lambs, L-lambs, M-lambs, A-lambs.

3.1.4 Calculations and Analysis of Data

Accumulated milk yields and milk composition data were analysed using two different methods. A third-degree orthogonal polynomial was one method, because it was used in the first two years of this study (van der Linden *et al.*, 2009; Blair *et al.*, 2010).

Method One: Third-degree orthogonal polynomial

Daily milk yield was calculated using the formula:

$$y_i = \alpha_0\varphi_{0i} + \alpha_1\varphi_{1i} + \alpha_2\varphi_{2i} + \alpha_3\varphi_{3i} + e_i$$

Where y_i is the record of milk yield or composition taken at day i, α_n is the n regression, and φ_{ni} is the rescaled value of day in milk i, calculated as

$$\varphi_{0i} = 1;\ \varphi_{1i} = x;\ \varphi_{2i} = \frac{(3x^2 - 1)}{2};$$

$$\varphi_{3i} = \frac{(5x^3 - 3x)}{2};\ x = \frac{2[i - (50 + 1)}{(50 - 1)}$$

Accumulated yields of milk, lactose, CP and fat were calculated over a 50-d lactation period for each ewe, using the estimates of the regression coefficients of the third-degree orthogonal polynomial.

Method Two: Direct Summation of Data

Daily milk yield was also calculated using the formula:

$$\frac{1440 \text{ minutes}}{\text{Interval between milkings (min)}} \times \text{milk yield at afternoon milking}$$

Daily yields of the individual milk components were calculated using the formula:

$$\frac{C_i}{100} \times MY_i$$

Such that *C* is the milk component in % on day *i*, and MY is the 24-hour milk yield as calculated above.

Adding the six daily yields for each ewe, resulting in one accumulated value for each milk component was the second method for calculating the accumulated yields. This allowed for a simpler analysis of milk yield and composition and was undertaken using the MIXED procedure with a linear model that included the fixed effects of dam size, dam nutrition and their interaction. The two methods to calculate the accumulated yields were compared, but both methods yielded results that were not statistically significant; therefore, the simpler method that used the raw data was chosen.

Within each year, analyses of ewe live weight, body condition score at breeding and near term (~140d gestation), the accumulated milk yields and milk component yields, udder

measurements, and lamb weight were undertaken using the MIXED procedure. Repeated-measures analysis of milk yield, composition and lamb weight were also undertaken using the MIXED procedure (SAS Inst. Inc., Cary, NC) with a linear model that included the fixed effects of dam size, dam nutrition and their interaction, and lamb sex (for lamb weight analysis only). Milk yield, G1 rank, ewe live weight and ewe body condition score at mating and lambing were included separately as covariates. Significant ($P<0.05$) covariates remained in the model. A simple linear regression analysis of milk yield on lamb live weight was also performed. Milk yield, ewe live weight and ewe body condition score at mating and lambing were included separately as covariates. Significant ($P<0.05$) covariates remained in the model. The effect of G1 birthrank for all the analyses was found to be non-significant ($P>0.10$) and data are not shown.

3.2 Results

In the present study there were no significant interactions between dam size and nutrition affecting milk parameters during the offspring's third and fourth lactation, the exception being lactose yield. Therefore, all milk parameters will be presented based on size and nutrition effects separately, except for lactose. Treatment effects that were not observed to have statistical significance ($P < 0.05$) may not be presented in a graph.

3.2.1 Effects of dam size and nutrition on lactational performance of offspring

Third-lactation (2009)

Offspring milk yield decreased significantly ($P<0.01$) from day 7 to day 42 in all groups, but there were no significant effects of dam nutrition or dam size on offspring's milk yield (Figure 3.3). There were also no effects of dam size or nutrition on accumulated yields (Table 3.1), crude protein percent (Figure 3.4) or yield (Table 3-1). However, L-ewes had significantly ($P<0.05$) higher milk fat percentage (Figure 3.6) and yield (187.8 vs. 177.3 ± 3.8 g/day $P<0.05$) than H-ewes. There was a significant ($P<0.01$) interaction between dam size and nutrition such that LA-ewes had lower lactose percentages than HA- and LM-ewes (Figure 3.5). There were no effects of dam size or nutrition on the udder dimensions (Figure 3.2) of the ewe mammary glands (M: 66.5 ± 1.0; A: 66.1 ± 0.8; L: 65.8 ± 0.9; H: 68.7 ± 0.9 cm) over the first six weeks of the third lactation.

Fourth-lactation (2010)

Milk yield (Figure 3.3) and milk fat percentages (Figure 3.6) and yields were not affected by dam size, but milk yield decreased significantly ($P<0.01$) from day 7 to day 42 in all groups. There were also no dam-size effects on accumulated yields (Table 3.1). H-ewes had significantly ($P<0.05$) higher mean daily crude protein yield (126.08±2.24 versus 119.54±2.12 g/day) and percent (Figure 3.6), and mean daily lactose yields (132.46 ±2.39 versus 124.11 ±2.27 g/day) and percent (Figure 3.7) than L-ewes. There were no effects of dam size or nutrition on the udder dimensions of the ewe mammary glands (M: 68.5 ± 1.0; A: 68.1 ± 0.8; L: 67.8 ± 0.9; H: 68.7 ± 0.9 cm) over the first six weeks of the fourth lactation. Over the six-week trial period, no significant effects of nutrition were found on milk yield, lactose, milk fat, or crude protein yield and percent.

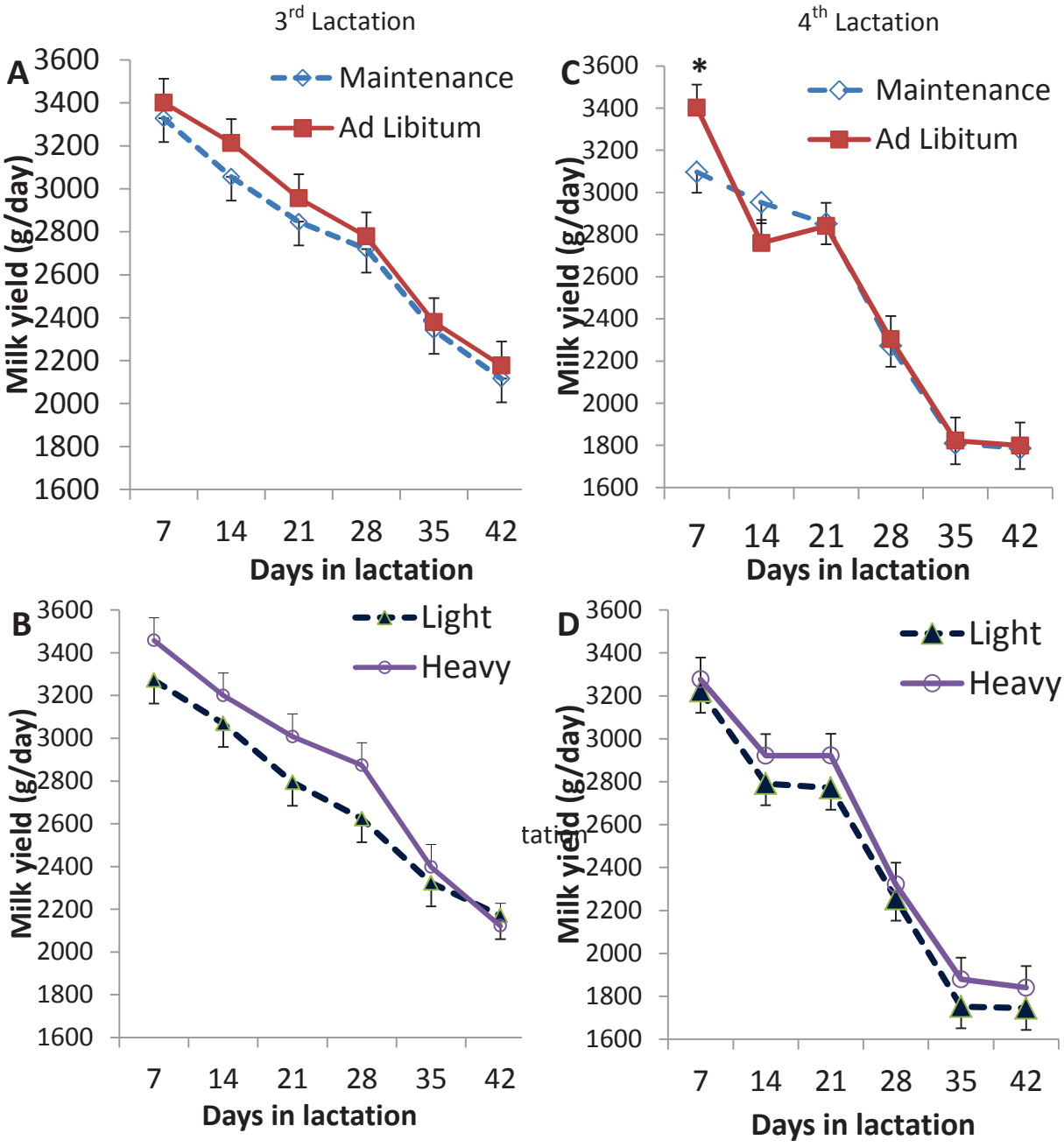

Figure 3. 3. Milk yield of offspring (G1) for the first 42 days in their third lactation and fourth lactation, G1 were born to dams (G0) fed *ad libitum* (A: n=25) (C: n=20) or maintenance (A: n=25) (C: n=25) from d21 to d140 of pregnancy and G1 born to heavy (B: n=29) (D: n= 21), or light (B: n=25) (D: n=24) dams. Data are presented as least square means (±SEM) * P<0.05 indicates significance obtained by univariate analysis. Repeated measures MANOVA showed no significant effects of maternal size or nutrition over the lactation period.

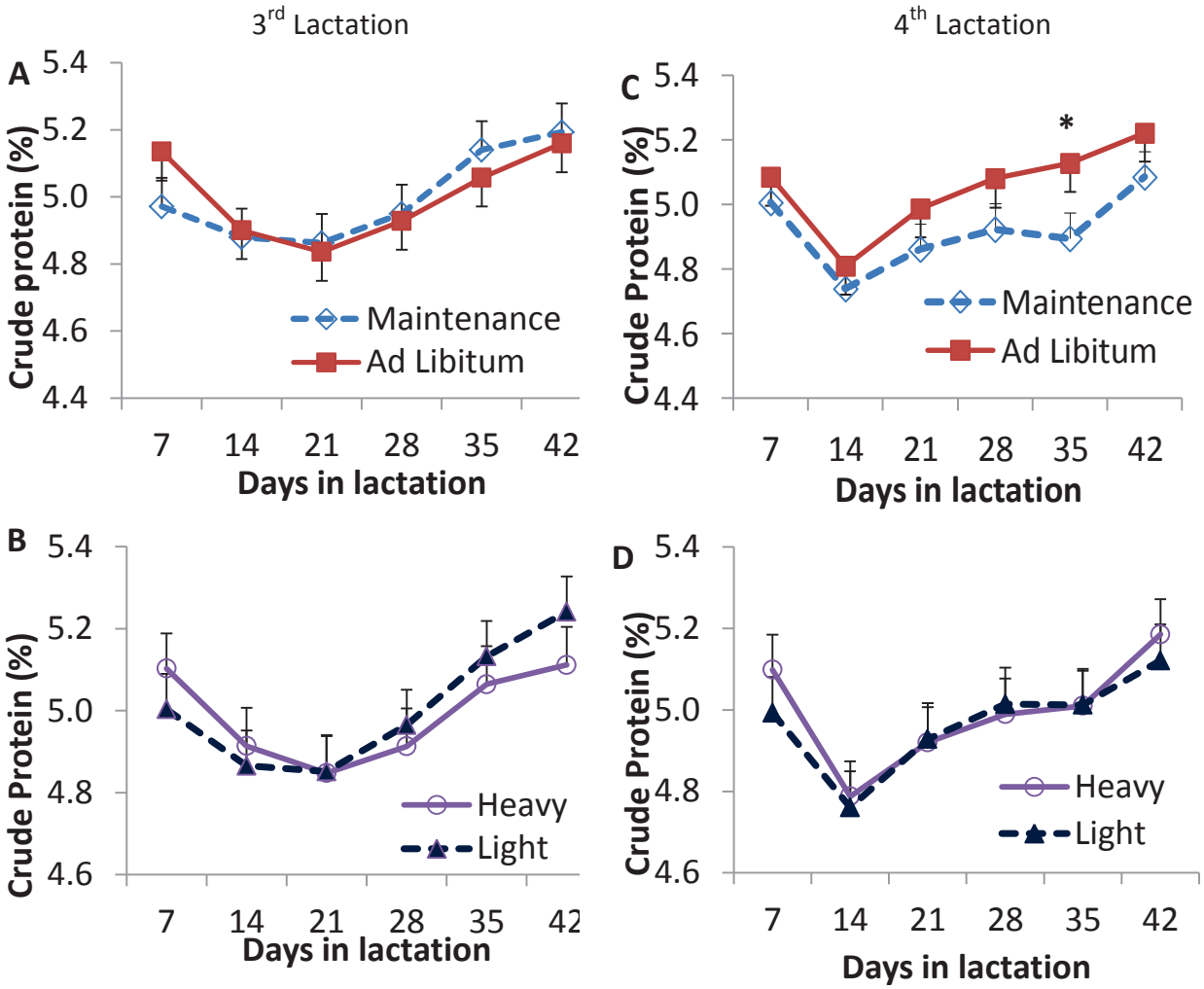

Figure 3.4. Crude protein of ewe offspring (G1), in the first 42 days, that were born to dams (G0) fed *ad libitum* in the third lactation (A: n=25), and fourth lactation (C: n=20) or maintenance (A: n=29) (C: n=25) from d21 to d140 of pregnancy, and ewes born to heavy (B: n=29) (D: n= 21), or light (B: n=25) (D: n=24) dams. There were no significant effects of maternal size or nutrition on crude protein percentages in the third or fourth lactations. Data are presented as least square means (± SEM). † $P < 0.10$ * $P<0.05$

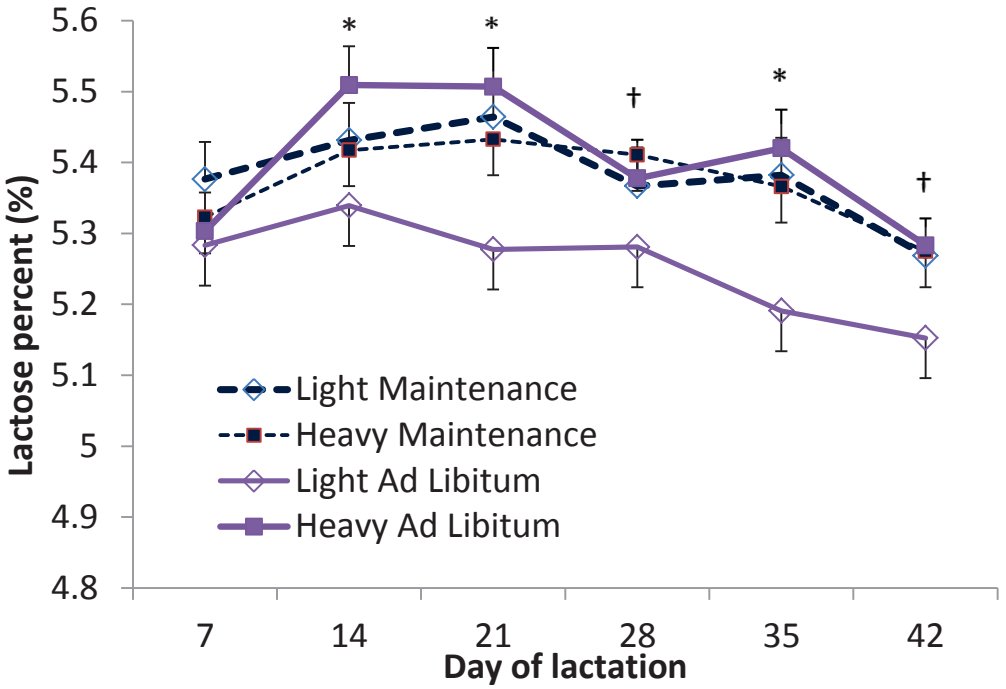

Figure 3.5. Milk lactose percentage of ewe offspring (G1) in the first 42 days of their third-lactation (A) born to heavy (H) or light (L) dams fed either maintenance (M) or *ad libitum* (A) during pregnancy. HA-ewes (n=14) had greater (P<0.05) lactose % than LA-ewes (n=11), and LM-ewes (n=14); had greater (P<0.05) lactose % than LA-ewes. HM-ewes (n=15) were not significantly different than any other group. Data are presented as least square means (± SEM). † P < 0.10 * P<0.05 indicate significance obtained by univariate analysis.

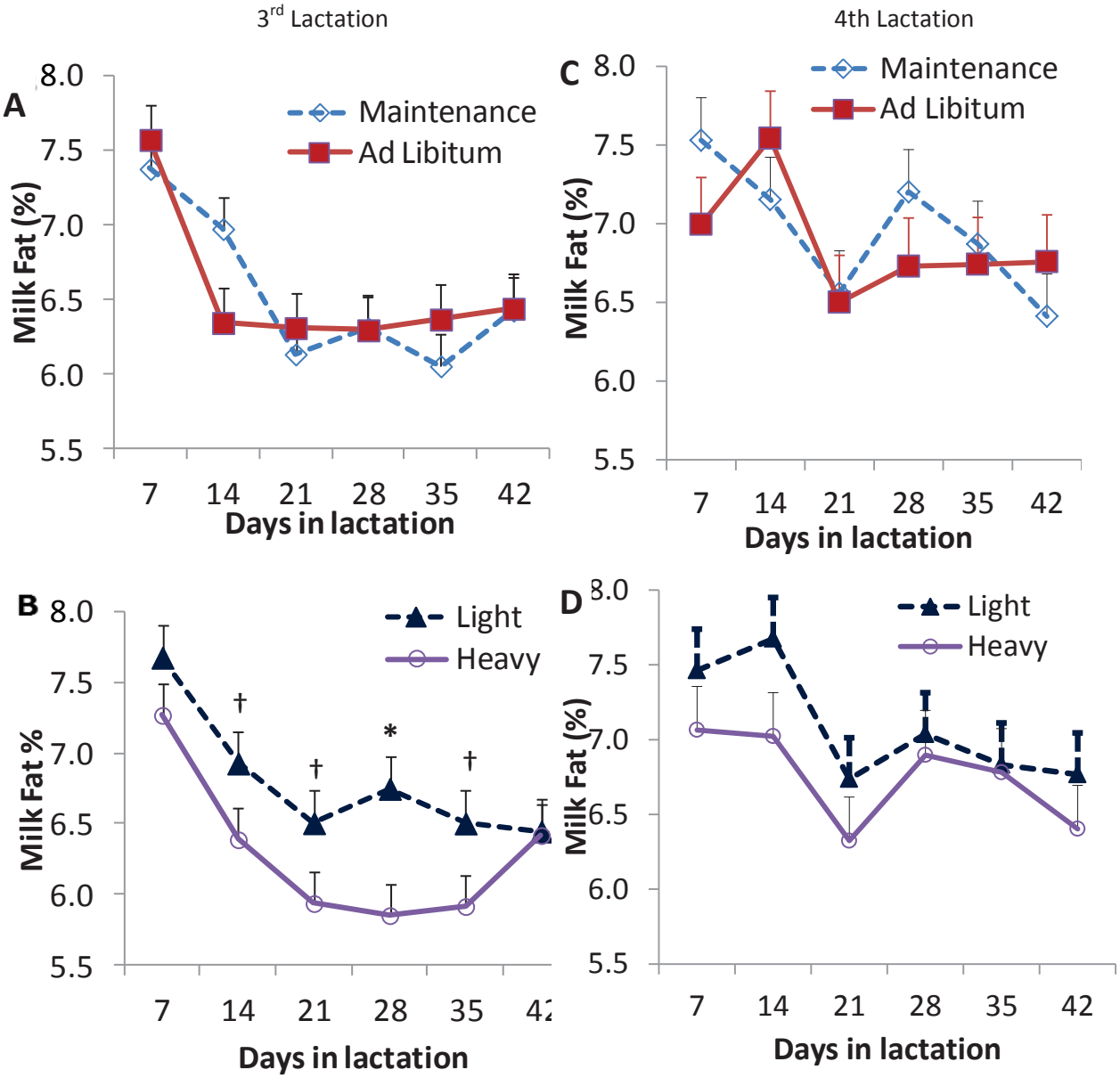

Figure 3.6. Milkfat percentage of ewe offspring (G1) in the first 42 days, that were born to dams (G0) fed *ad libitum* in their third lactation (A: n=25), and fourth lactation (C: n=20) or maintenance (n=29)(n=25) from d21 to d140 of pregnancy and ewes born to heavy (B: n=29) (D: n= 21), or light (n=25) (n=24) dams. In the third lactation there were no significant effects of maternal nutrition, but offspring from light dams had greater ($P < 0.05$) milkfat than offspring from heavy dams. In the fourth lactation, there were no significant effects of maternal size or nutrition. Data are presented as least square means (± SEM). † $P < 0.10$ * $P<0.05$ indicate significance obtained by univariate analysis

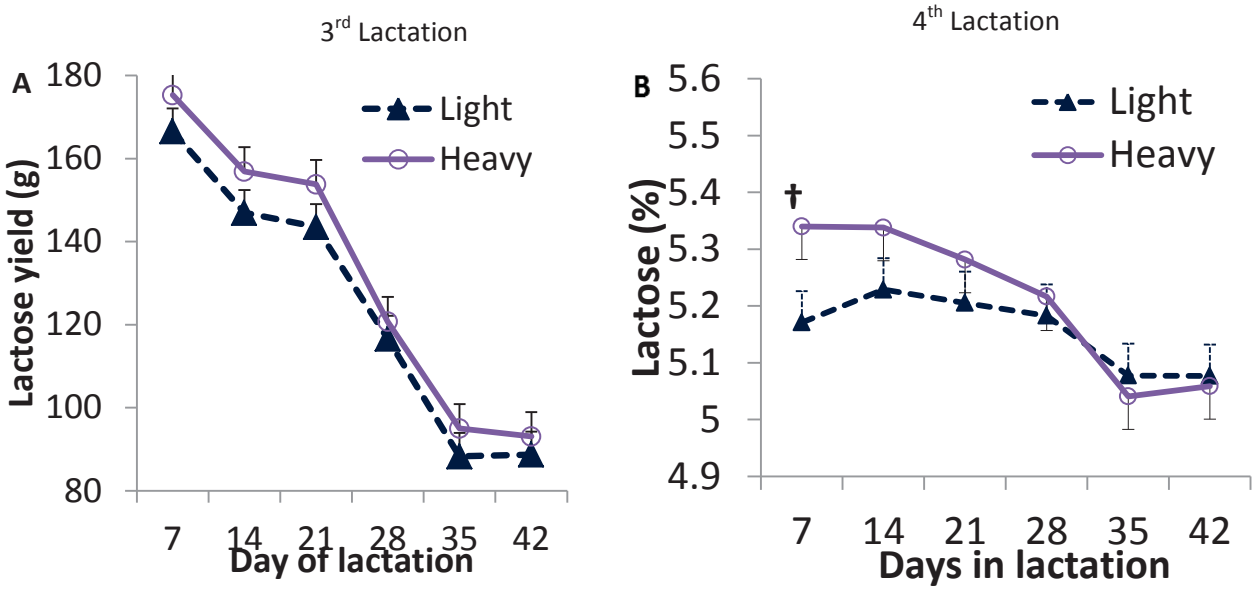

Figure 3.7. Lactose yield (A) and percentage (B) of ewe offspring (G1) in the first 42 days of their fourth-lactation (A), born to heavy (n= 21), or light (n=24) dams. The offspring from heavy dams produced greater (P<0.05) lactose yields than the offspring from light dams. Data are presented as least square means (±SEM). †P< 0.10 indicates significance obtained by univariate analysis.

Table 3.1: Accumulated milk yields and yields of milk components of the offspring, whose dams were heavy (H) or light (L) and fed *ad libitum* (A) or maintenance (M) during pregnancy. Data are presented in grams, as least square means (±SEM).

Accumulated Yield (g)	M	A	L	H	Nutrition Effect (P-value)	Size Effect (P-value)
Milk Yield 2009	15059 ±340	14543 ±399	14561 ±354	14999 ±389	0.55	0.54
Milk Yield 2010	14941 ±354	14593 ±393	14623 ±363	14911 ±384	0.51	0.59
Fat yield 2009	1023 ±32	984 ±33	1034 ±33	977 ±32	0.70	0.69
Fat yield 2010	1012 ±31	993 ±34	1022 ±31	982 ±33	0.68	0.39
Crude Protein yield 2009	735 ±24	699 ±25	733 ±25	690 ±24	0.97	0.84
Crude Protein yield 2010	740 ±19	709 ±21	723 ±19	726 ±20	0.28	0.88
Lactose yield 2009	760 ±22	734 ±24	750 ±23	766 ±24	0.82	0.40
Lactose yield 2010	772 ±21	756 ±23	757 ±21	770 ±22	0.61	0.66

3.2.2 Effects of ewe size and nutrition on lamb weights

Live weight of the grand-offspring (2009)

There was a significant ($P<0.05$) effect of grand-dam size on grand-offspring weight over the six-week trial period. H-lambs had greater mean live-weights than L-lambs over the six week trial period (11.02 vs. 10.52 ± 0.17 kg) (Figure 3.8). The weight of the H-lambs and L-lambs began to diverge at day 14 and became significantly different from day 21 to 35, then converged by day 42 (Figure 3.8B). Also, the A-lambs were heavier ($P<0.05$) over the six-week period compared with M-lambs. The effect of nutrition began by 28 ($P<0.1$) days of age and was significant ($P<0.05$) from age 35 to 42 days,(Figure 3.8). However, according to the univariate analysis, by day 42 there were no significant grand-dam size effects on lamb weights. The regression of lamb live weight on milk yield of their dams was not significant.

Live weight of the grand-offspring (2010)

There were no significant effects of grand-dam size or nutrition on lamb weights (Figure 3.8 C and D).

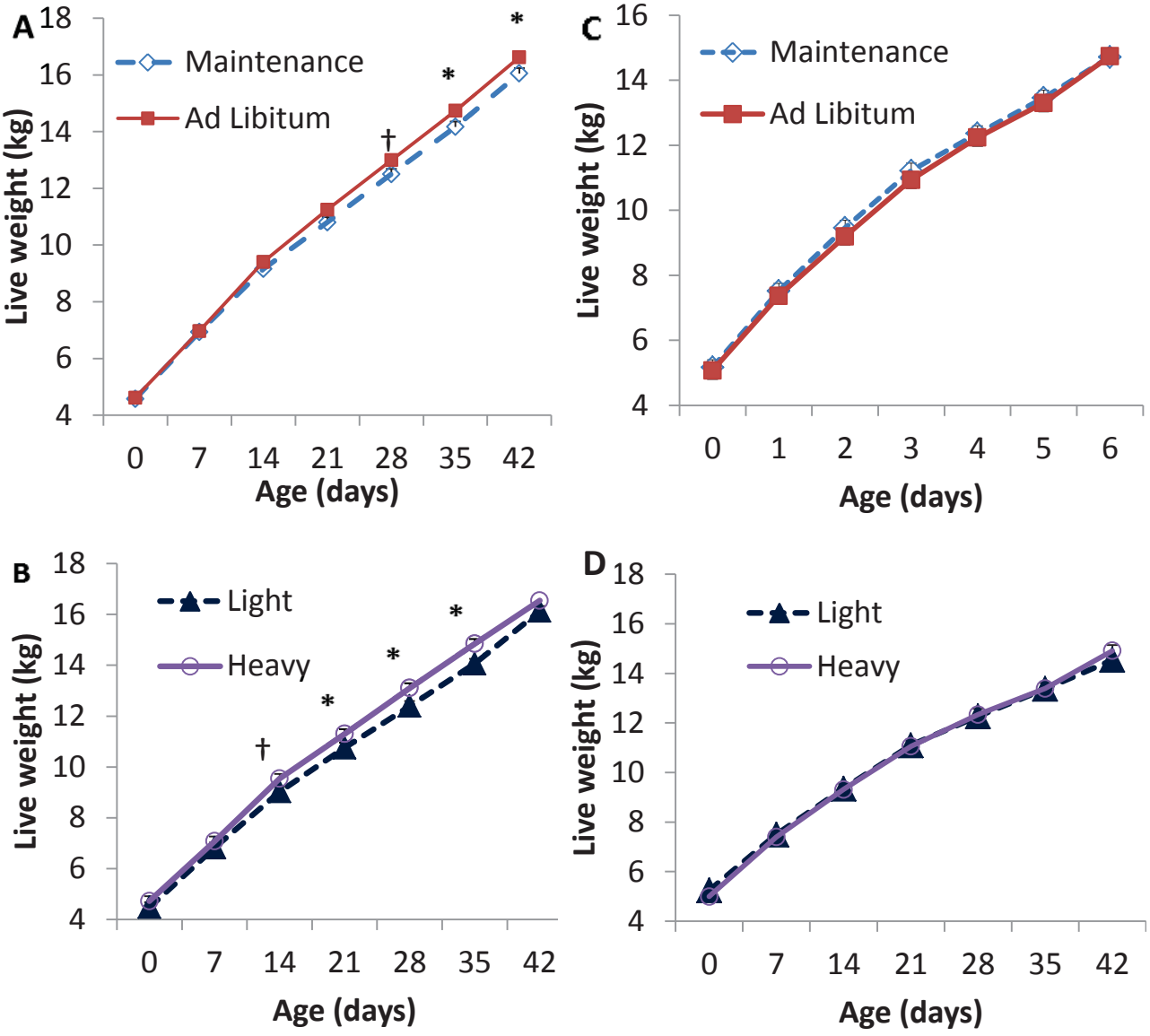

Figure 3.8. Lamb weight in 2009 (A,B) and 2010 (C,D) from birth weight until day 42 (A) for lambs (G2) whose grand-dams (G0) were fed *ad libitum* (n=40) or maintenance (n=50) from d21 to d140 of pregnancy, and (B) whose grand-dams were heavy (n=21), or light (n=24). Offspring from *ad libitum* or heavy dams produced lambs with greater (P<0.05) growth rates compared to offspring from maintenance-fed or light dams, respectively in 2009. There were no effects of grand-dam size or nutrition on lamb growth in 2010. Data are presented as least square means (±SEM). † P< 0.10 * P<0.05 indicates significance obtained by univariate analysis.

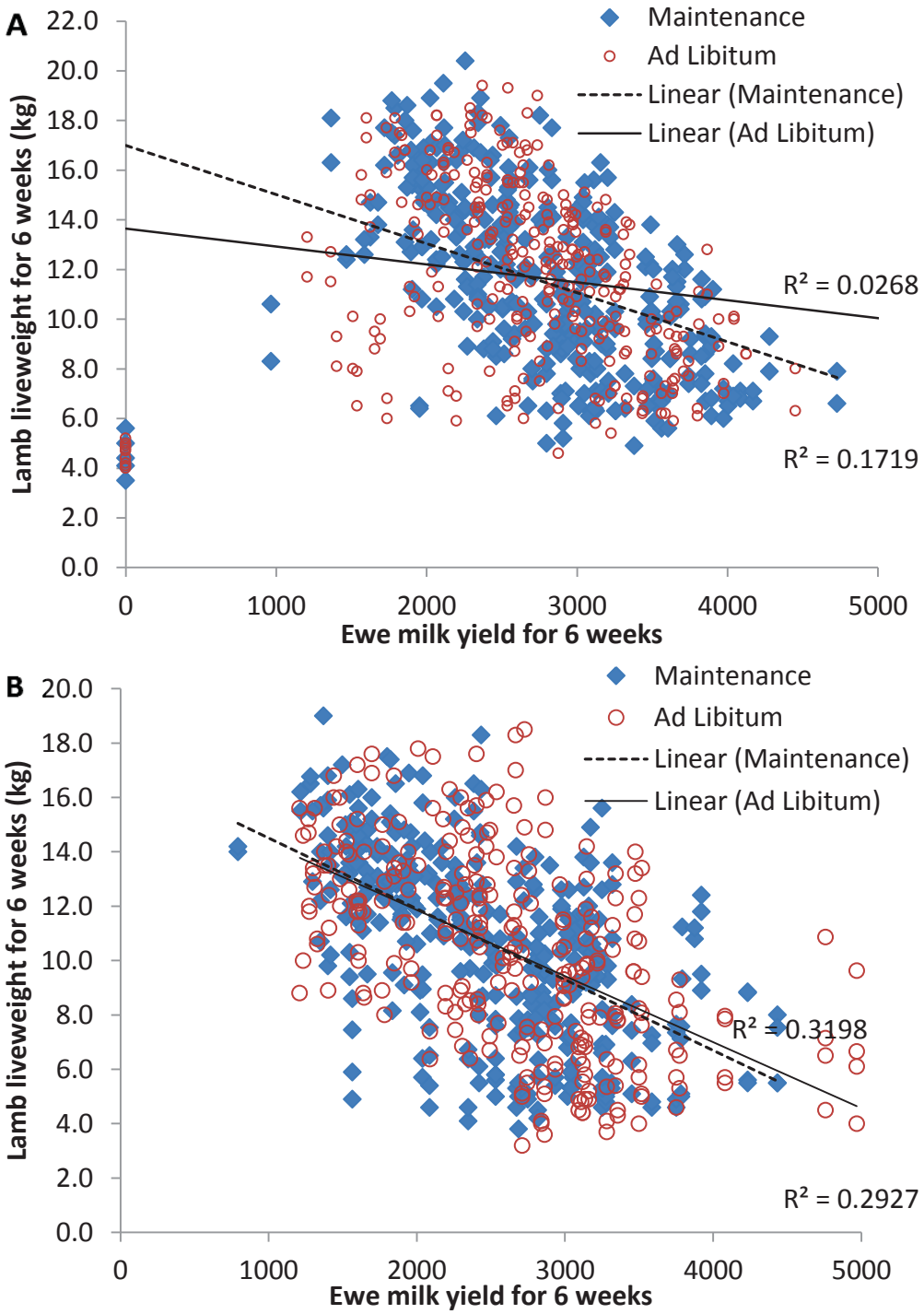

Figure 3.9. The linear relationship between lamb (G2) body weight and milk yield of the ewe (G1) during the third lactation (P>0.10) in 2009 (A) and fourth lactation (P>0.10) in 2010 (B). Grand-dams (G0) were fed *ad libitum* (n=40) or maintenance (n=50) from d21 to d140 of pregnancy.

3.3 Discussion

3.3.1 Overview

The objective of this study was to examine the effects of dam size and nutrition on the third and fourth lactation of the offspring, and growth of the grand-offspring. In the present study there were no significant interactions between dam size and nutrition, the exception being lactose yield. Therefore, all milk parameters will be discussed based on size and nutrition effects separately, except for lactose. The findings in the present study will then be compared with the findings in the first two lactations, which were previously reported (lactation one reported by van der Linden *et al.* (2009); lactation two reported by Blair *et al.* (2010)).

3.3.2 Effect of dam size

Dam size did not influence milk yield in the offspring's third or fourth lactations, which is consistent with the findings in the second lactation (Blair *et al.*, 2010). In contrast, in the first lactation (van der Linden *et al.*, 2009) the H-ewes produced more milk than the L-ewes. This was likely due to an effect of ewe live weight on lactation performance, as H- compared to L-ewes were heavier themselves in their first lactation (van der Linden *et al.*, 2009) while no difference in ewe live weight or milk yield was observed in the second (Blair *et al.*, 2010), third or fourth lactations (the present study). A similar effect of live weight on milk yield has been reported in cows, such that if any difference, larger cows were found to be less efficient at producing milk than smaller cows (Hansen, 2000). This is an important result from a farm

management perspective, showing that in the short-term, offspring from heavy dams produce more milk in their first lactation, but there may not be any long-term effects.

In the third lactation, the L-ewes had greater fat yields than the H-ewes, consistent with observations in both the first (van der Linden *et al.*, 2009) and second lactation (Blair *et al.*, 2010). In contrast, dam size did not influence the offspring's milk fat yield in the fourth lactation. It is known that milk fat is the most easily changeable milk trait, and can be changed by dietary fat intake, or ketosis (McNamara & Hillers, 1986). In this study, the ewes were managed as a single group and fed *ad libitum* during the first, second, third and fourth lactations. Their dietary intake was not measured after the first lactation, but ewe live weight at breeding and near term (day 140 of gestation) was included as a covariate in the statistical analysis performed in this study, and the covariate was not statistically significant, indicating that the effects on milk composition are not due to live weight gains or losses during that year. As ewes age, they produce milk with greater concentrations of fat (Bencini & Pulina, 1997); therefore, the effect seen in the third lactation may be the pivotal point between age and dam-size effect, after which the effect of age may become a more significant factor than size.

In the current study, there were no effects of dam size on crude protein yields in the third lactation, consistent with observations in the first lactation (van der Linden *et al.*, 2009). However, during the fourth lactation, H-ewes had greater crude protein yields than the L-ewes, consistent with observations in the second lactation (Blair *et al.,* 2010). The mechanisms behind these effects remain to be discovered.

In the third lactation, there was an interaction between dam size and dam nutrition such that HA-ewes had greater lactose yields than LA-ewes, but there was no such relationship in any

offspring from maintenance-fed dams. The absence of effects in M-ewes, indicates that this could be an effect of dam size that is only expressed when the dams were fed *ad libitum*. Lactose yields in the fourth lactation were affected by dam size, such that the H-ewes had higher yields than the L-ewes, consistent with the results in the first lactation (van der Linden *et al.*, 2009). However, in the second lactation, the effect of dam size was not evident in the lactose yield (Blair *et al.,* 2010). Lactose is not an easily changeable milk component because it is regulated by a strict ion transport mechanism (Peaker & Larson, 1978). Typically, differences in lactose yield should follow the differences in milk yield. However, in lactations two, three and four there were no differences in the milk yield, while there were differences found in lactose yields during the third and fourth lactations. Therefore, the progeny with higher lactose yields may have altered activity of the transport mechanism or have an altered membrane potential (Fedorcsák *et al.*, 2001). Since effects on lactose have been observed in three of the four lactations, there may be trans-generational effects of dam size on the physiological activity of the mammary gland. However, dam size effects on lactose yields of the offspring were not evident in the second year. Thus, either these are random effects on milk composition, or the potential for a programming effect and possible mechanisms involved requires further research.

There was an effect of dam size on milk yield in the first lactation, but this was not observed in the second, third or fourth lactations. The milk components, milk fat, crude protein, and lactose, were all affected by dam size in the current study, in either the third or fourth lactations. Milk fat yield was affected by dam size in the first, second and third lactations. Crude protein yield was affected in the second, third and fourth lactations. Lactose yield was affected in the first, third and fourth. The milk composition data overall, across all four

lactations, was extremely variable. In the first lactation, because the milk yield did not peak until day 21 (van der Linden *et al.*, 2009), the change in milk yield over time may have been less, when compared to the third and fourth lactations, in which peak yields were reached in week one. The difference between the first lactation curve, compared to the curves in the subsequent years, may be supporting evidence for the ability of the function of the mammary gland to change.

The variability of the effects of dam size over the parities may be explained by mammary gland reprogramming. Reprogramming of the mammary gland occurs with age and involution after lactation (Bissell & Inman, 2008; Boulanger & Smith, 2009; Khokha & Werb, 2011). After lambs are weaned, a larger portion of the secretory cells undergo apoptosis in the sheep mammary gland, when compared to most ruminants (Furth *et al.*, 1997). Reprogramming is a viable mechanism for the variable trans-generational effects of dam size due to the plasticity of the mammary gland, the ducts and the epithelium. Even though there is variability across the four lactations, these results indicate that dam size can affect the milk composition of the offspring if data from each individual milk component is taken into consideration across all four lactations. This evidence leads to the conclusion that the mammary gland may be partially reprogrammed.

3.3.3 Effect of dam nutrition

The results of the current study showed no effects of dam nutrition on the offspring's milk yield or crude protein yields, during the third or fourth lactation, consistent with the findings in the second lactation (Blair *et al.*, 2010). In the third lactation, there was an interaction

between dam size and dam nutrition such that HA-ewes had greater lactose yields than LA-ewes, but there was no such relationship in any offspring from maintenance-fed dams. These observations contrast with the first lactation in which M-ewes showed a trend towards greater milk yields, greater accumulated crude protein yields and greater accumulated lactose yields when compared with A-ewes (van der Linden *et al.*, 2009). The results of the current study also showed no effect of dam nutrition on the offspring's fat yields in the third or fourth lactations, which is consistent with findings in the first lactation. The second lactation was the only year in which there was an effect on milk fat; A-ewes produced greater accumulated fat yields than M-ewes (Blair *et al.,* 2010). The differences in the first lactation may not occur subsequently due to reprogramming of the mammary gland that occurs with age and involution (Furth *et al.*, 1997; Boulanger & Smith, 2009; Khokha & Werb, 2011).

Dam nutrition may affect the lactational performance of offspring in the first lactation, but the effects on milk yield and milk composition do not occur consistently in subsequent lactations. Epigenetic modifications or mammary gland reprogramming may be behind these effects of dam nutrition (McMillen & Robinson, 2005; Khokha & Werb, 2011). Molecular research is needed in order to identify the mechanism responsible for these effects.

3.3.4 Effect of grand-dam size and nutrition on growth of the grand-offspring

A-lambs and H-lambs were heavier than M-lambs and L-lambs, respectively in the third parity. This is similar to findings in the first parity, in which the H-lambs were heavier at weaning than the L-lambs and the M-lambs were heavier throughout the seven-week trial period than

A-lambs (van der Linden *et al.*, 2009). During the second parity, the birth weights of M-lambs were greater than those of A-lambs, but no grand-dam size or nutrition effects were observed in subsequent weeks (Blair *et al.,* 2010). The regression of lamb live weight on milk yield was non-significant in the third and fourth lactations (Figure 3.9) and the milk yield was unaffected by (G0) dam nutrition. This indicates that the effect of grand-dam size and nutrition on lamb weight may not be caused through the ewe's milk yield. In the current study, there were no significant effects of dam size or dam nutrition on grand-offspring growth during the G1 fourth parity. The effect of dam size and nutrition that is observed in parities one, two and three suggests that there is a fetal programming effect, but the mechanism is unknown.

The live weight measurements of the grand-offspring lead to the conclusion that dam size affects the growth and development of the grand-offspring. No molecular analysis has been performed in this trial. However, it is hypothesized that epigenetic mechanisms, independent of the milk yield of the dam, underlie the effects on growth of the grand-offspring.

3.3.5 The current evidence of fetal programming

Previous studies have found that poor nutrition during pregnancy can inhibit growth of the offspring (Cleal *et al.*, 2007; Corner *et al.*, 2008; Belkacemi *et al.*, 2010). There have been trans-generational studies showing that up to the third-generation there are reduced birth weights of the offspring, and increased disease risk through intergenerational transmission of genetic predispositions in rats and human (Drake & Walker, 2004; Drake *et al.*, 2005; Zambrano *et al.*, 2005). There have also been some observational studies performed in human populations (Lumey, 1998; Elias *et al.*, 2005; Kaati *et al.*, 2007), which indicate that the

greater the availability of food, the higher the risk that the growth of grand-offspring will be restricted through epigenetic mechanisms. These findings are similar to the effects observed in studies by our group, during the first (van der Linden *et al.*, 2009) and second lambing (Blair *et al.*, 2010), in which maternal nutrition positively affected birth weight. However, the effects of dam nutrition on birth weight did not continue on to the third and fourth lambing, and there was no data collected to indicate the effects on health of the offspring. Studies in other species have shown that epigenetic mechanisms may underlie the effects of nutrition with a genetic modification observed across generations when offspring are exposed to a certain *in-utero* nutritional environment (Kaati *et al.*, 2007). Furthermore, certain epigenetic effects can be reversed with a histone inhibitor to remove the added modification (Weaver *et al.*, 2004; Gicquel *et al.*, 2008). Thus, such epigenetic reversal could explain why some of the effects of nutrition are no longer seen in subsequent lactations. A study on human twins with identical epigenetic modifications showed that unspecified post-natal environmental factors can differentiate the expression of certain phenotypes (Blanc *et al.*, 2004). The variable effects of dam size and nutrition each year might also be explained by mammary gland reprogramming (Khokha & Werb, 2011). Fetal programming effects have previously been shown to be reversible and the information provided by Khokha and Werb (2011) support the hypothesis that the effects observed in this study could result from partial reprogramming of essential tissues.

3.3.6 Future research

This research sheds light on the long-term trans-generational and inter-generational effects, and possible underlying mechanisms of sub-optimal feeding and size of the dam during

pregnancy on the continuing lactational performance of the offspring and growth of the grand-offspring. Further research is needed in order to identify the epigenetic and programming mechanisms stimulated by dam size and nutrition which result in differences in the lactation of offspring and growth of grand-offspring.

3.4 Conclusion

This extends the results of previous studies that were done on this flock in their first and second lactations (van der Linden *et al.*, 2009; Blair *et al.*, 2010). These results further our understanding of long-term effects of dam size and nutrition on growth, development and production of the offspring. This study has shown that dam size during pregnancy can have long-term effects on the lactose yields of offspring and growth-rates of grand-offspring. This study has also shown that dam nutrition can have significant effects on grand-offspring growth, over three years after the dam's restricted plane of nutrition. Since the importance of dam size and nutrition relies on its relationship to later functionality and milk production, responses in gland development and subsequent lactation performance deserve further investigation.

Chapter 4: Discussion

A discussion of the effects of dam size and nutrition during pregnancy on fetal mammary gland development and lactational performance in the offspring

This thesis set out to identify the effects of dam size and dam nutrition on mammary gland development, milk yield and lamb growth. Fetal mammary glands are the foundation of the adult mammary gland and their development influences the ability to wean healthy offspring in the future. Dam size and nutrition can inhibit mammary gland development and the function of the mammary gland in the offspring (Jenkinson, 2003; Fowden *et al.*, 2006; van der Linden *et al.*, 2009). However, there is a limited amount of data available on the long-term effects of dam size and nutrition in sheep. Future research trials can use the information provided by this thesis to formulate ways that limit the impact of dam nutrition on mammary gland development and milk production of offspring.

In this study, fetal offspring at day 140 weighed more, and had heavier mammary glands when they were carried by *ad libitum*-fed dams compared to maintenance-fed dams. Further, dam nutrition, dam size and the rank of the offspring interacted in a way that together, they resulted in an increase in the total number of ducts in the fetal mammary glands in the fetuses from large *ad libitum*-fed dams. To my knowledge, no prior study has reported interactions of dam nutrition, size, or rank on fetal mammary gland measurements in sheep.

The nutrient intake of the dam during pregnancy is a significant contributor to the *in-utero* environment, and sub-optimal dam nutrition may result in lighter offspring (Cleal *et al.*, 2007; Corner, 2007; Belkacemi *et al.*, 2010). In this study, the growth rates of the 'grand'offspring, during the offspring's third parity, were positively affected when the 'grand'dams were allowed pasture *ad libitum*. Thus, the nutrition of the dam during one pregnancy can affect the growth rates of future generations. These findings contradict earlier studies, in which restricting a pregnant dam to a maintenance diet was beneficial to the fetal growth and development of the female offspring (Jenkinson, 2003; van der Linden *et al.*, 2009; Martín,

2011). The study on the same cohort of ewes found that maintenance-fed dams had greater milk yields and lamb growth at their first lactation and lambing (van der Linden *et al.*, 2009). The findings from the first lactation also contradict this study during the third lactation and lambing, in which there was no effect of nutrition on milk yields. In conclusion, this study has identified that there are no long-term effects of dam nutrition on the size of the adult mammary glands, or the quantity of milk produced by the offspring in the second, third and fourth lactations. However, feeding dams *ad libitum* can positively affect 'grand'offspring growth in subsequent parities.

Dam size can also influence the *in-utero* development of offspring. Dam size affects the fetus through the size of the placenta, limiting nutrient and growth capabilities (Mellor, 1983). Heavier animals may be capable of providing more energy for milk synthesis in comparison to lighter animals because they may have more energy reserves in fat and muscle (Revell *et al.*, 1998). There were no effects of dam size on the day 140 fetuses, but there are effects seen in the quality of milk and grand-offspring growth rates in the third and fourth lactations (the current study). It is likely that the physiological effects of dam size appear post-natally, given that no dam size effects were observed in the mammary glands of day 140 fetuses, but having a heavy dam positively affected milk yield and composition in the subsequent lactations. Further studies will need to be performed in order to identify the time which dam size effects are observed in the post-natal mammary glands. Greater dam weights also positively affected lactose yields in the long-term, as identified in the third and fourth lactation of the offspring. Lactose is a difficult component to change, because it is regulated by a strict ion-transport mechanism (Peaker & Larson, 1978). Typically, differences in lactose yield should follow the differences in milk yield. However, in lactations three and four there were no differences in

the milk yield, but there were differences in lactose yields. Therefore, the activity of the transport mechanism or the trans-cellular membrane potential may be different in the progeny with higher lactose yields (Fedorcsák *et al.*, 2001). Dam size does not have a consistent effect on fetal development or lifetime production, but heavier dams do require a greater energy intake (Morel & Kenyon, 2006). Ewes that do not produce high quantities of milk may produce offspring that are more efficient at nutrient utilization for growth, and adapt faster to eating pasture than ewes that do produce high quantities of milk (Geenty & Rattray, 1987). Larger animals have been found to be less efficient compared with their smaller counterparts (Gardner & Hogue, 1966; Hansen *et al.*, 1999). Based on this data, it may be beneficial to select light dams as it may make no difference to the overall lifetime production, and they consume less pasture.

There have been trans-generational studies in rats and humans showing that the offspring have reduced birth weights, and increased disease risk through the transmission of expressed phenotype through generations (Drake & Walker, 2004; Zambrano *et al.*, 2005). This current study found that a grand-dam that was heavy or *ad libitum*-fed was advantageous to the growth rates of the third set of grand-offspring when compared to light and maintenance-fed grand-dams. It is possible therefore, that the future germline (G2) are programmed when the offspring (G1) are *in-utero* (Youngson & Whitelaw, 2008). In the first lactation, there was a positive effect of dam size on ewe live weight and milk yield, but no effects on growth of the 'grand'offspring. The current study shows that the long-term productive performance of female offspring can be altered due to their *in-utero* experience. However, further research is required to investigate the effect of dam size on lifetime production.

Future studies should focus on identifying the time at which the effects of dam size and nutrition are evident in the mammary gland development of the offspring. This can be accomplished by analysing post-natal tissue development of offspring that were exposed to different *in-utero* environments. To negate the potential effects of unequal levels of available pasture between years, the pasture offered to offspring should be closely monitored throughout their lives and adjusted for accordingly. Altering the plane of nutrition has variable effects on mammary gland development, and further work is required to establish the role of nutrition on mammary gland development and function. Studies in sheep and cows found that a high plane of nutrition before puberty can decrease mammary growth rates, and greatly stunt post-natal mammogenesis (Johnsson *et al.*, 1985; Sejrsen & Purup, 1997). A different study showed that feeding sub-maintenance levels of nutrition during pregnancy can decrease mammary gland growth rates (Charismiadou *et al.*, 2000). Between these two studies, plus the current study, there may be an optimal level of nutrition that maximizes the potential mammary gland development. The contradicting findings support the conclusion that an animal can be programmed during early life, but the effects of dam nutrition require further research. The molecular changes underlying the effects of size and nutrition should be identified at the key life cycle stages.

This study was performed with normal pastoral farming practices and the nutritional treatments were not managed for individual sheep. The size of the dam was determined by live weight. Guidelines for choosing dams based on weight, and feeding levels during pregnancy to increase lactational performance and lamb weights may be created based on this research. Recommendations could then be easily translated and duplicated in a farm setting, having direct implications for farmers.

This thesis showed that *ad libitum* levels of dam nutrition can enhance fetal mammary gland development and future growth of the 'grand'offspring. Dam size can change quality of the milk and enhance lamb growth in the long-term. This thesis highlights the importance of research into the trans-generational effects of on offspring growth, development and production. It offers information regarding the positive effects of nutrition and recommendations for farmers to maximize the pasture that is offered to pregnant ewes. Maximizing milk production can be economically beneficial, as lamb sales are a primary income for sheep farmers. Thus, this research could change the practice of restricting feed intake at the beginning of pregnancy. Finally, it offers information for future investigations to continue improving our knowledge of animal production and the effects of dam nutrition and size.

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