Maternal nutritional programming in the sheep: Effects on post-natal growth, mammogenesis and lactation in adult-ewe offspring

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Abstract

Developmental programming is the concept that environmental factors, particularly during foetal life, can alter development, metabolism and physiology of an organism and this can have consequences later in life. There is growing interest in developmental programming in livestock species, particularly effects of maternal pregnancy nutrition, which is easy to manipulate. Recent research, using a sheep model, has shown that milk production in ewe offspring may be susceptible to maternal nutritional programming, such that over nutrition (*ad libitum*) of the pregnant dam, compared with maintenance nutrition, may impair their first lactation performance and result in the weaning of lighter lambs. The present study however revealed that maternal nutritional programming effects on lactation performance in ewe offspring did not persist over their productive lifetime.

In a new study, the critical programming period was narrowed down to early gestation, coinciding with early mammogenesis in the foetus. In this study only twin-born ewes were examined due to their economic significance in commercial sheep production and due to their increased susceptibility to nutritional insult *in-utero*. It was revealed that, in addition to over nutrition (*ad libitum*), under-nutrition (sub-maintenance) of the dam during early pregnancy also impaired first lactation performance of twin-born ewe offspring when compared with maintenance.

Transcriptomics analysis using RNA-seq identified that nutritional programming affects late pregnancy mammogenesis, rather than secretory cell function during lactation, in ewe offspring during their first parity. Ewes born to *ad libitum*-fed dams, in particular, appeared to have impaired regulation of cell cycle while ewes born to sub-maintenance-fed dams had reduced expression of genes associated with the extracellular matrix, both of which may influence cell proliferation. As a consequence, both ewes born to *ad libitum* and sub-maintenance-fed dams may have had fewer mammary secretory cells, resulting in reduced lactation performance.

The findings of this thesis indicate that differences in first-lactation performance of ewe offspring, as a result of maternal nutritional programming, may be mediated by impaired proliferation of secretory epithelial cells. These findings contribute to our knowledge of the mechanisms of developmental programming of the mammary gland and presents a platform for future investigations which may ultimately lead to the ability to manage and manipulate lactation performance.

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Table of contents

List of figures

List of tables

List of abbreviations

Ad = *ad libitum*
AdAd = *ad libitum* dam nutrition during early and mid-to-late pregnancy
AdHv = *ad libitum*-fed, heavy dam
AdLt = *ad libitum*-fed, light dam
AdM = *ad libitum* dam nutrition during early pregnancy and maintenance dam nutrition during mid-to-late pregnancy
ADP = adenosine diphosphate
Ad_{P21-50} = *ad libitum* dam nutrition during early pregnancy
$Ad_{P50-140}$ = *ad libitum* dam nutrition during mid-to-late pregnancy
AI = artificial insemination
ATP = adenosine triphosphate
BCS = body condition score
BF = back fat
BHB = beta hydroxybutyrate
BR = birth rank
cDNA = copy DNA
CoA = co-enzyme A
CP = crude protein
CPY = crude protein yield
Cq = quantitation cycle (in RT-qPCR
CY = casein yield
dsDNA = double-stranded DNA
E = ewe
E = oestrogen
EEF = eukaryotic translation elongation factor
EIF = eukaryotic translation initiation factor
EMA = eye-muscle area
ER = oestrogen receptor
ERα = oestrogen receptor type alpha
ERβ = oestrogen receptor type beta
ETC = electron transport chain
FAS = fatty acid synthase
FEC = faecal egg count
FGF = fibroblast growth factor
FL = fore-leg
FY = fat yield
G0 = generation zero (dams fed differentially during pregnancy)
G1 = generation one (offspring born to G0 dams)
G2 = generation two (lambs born to G1 ewes, grand-offspring of G0 grand-dams)
gDNA = genomic DNA
GH = growth hormone
GHR = growth hormone receptor
GnRH = gonadotropin releasing hormone
HGF = hepatocyte growth factor

HL = hind-leg
HPA axis = hypothalamic-pituitary-adrenal axis
HTS = high-throughput sequencing
Hv = heavy
IDH = isocitrate dehydrogenase
IGF1 = insulin-like growth factor 1
IGF1-R = insulin-like growth factor 1 receptor
IGF2 = insulin-like growth factor 2
IGFBP3 = insulin-like growth factor binding protein 3
IGFBP6 = insulin-like growth factor binding protein 6
IHC = immune-histochemistry
ISH = in situ hybridization
JAK = Janus kinase
KGF = keratinocyte growth factor
L = lactation
LP = late pregnancy
LR = leptin receptor
Lt = light
LW = live weight
LWG = live weight gain (growth rate)
LY = lactose yield
M = maintenance
MAd = maintenance dam nutrition during early pregnancy and *ad libitum* dam nutrition during mid-to-late pregnancy
MAPK = mitogen-activated protein kinase
MD = muscle depth
MEC = mammary epithelial cells
MHv = maintenance-fed, heavy dam
MLt = maintenance-fed, light dam
MM = maintenance dam nutrition during early and mid-to-late pregnancy
$M_{P21\text{-}50}$ = maintenance dam nutrition during early pregnancy
$M_{P50\text{-}140}$ = maintenance dam nutrition during mid-to-late pregnancy
mRNA = messenger RNA
mTOR = mammalian target of rapomycin
NADH = nicotinamide adenine dinucleotide
NADPH = nicotinamide adenine dinucleotide phosphate
ncRNA = non-coding RNA
NE = net energy
NEY = net energy yield
P = day of pregnancy, e.g., P0 = day zero of pregnancy.
P = progesterone
P21-50 = day 21 to 50 of pregnancy (early pregnancy)
P50-140 = day 50 to 140 of pregnancy (mid-to-late pregnancy)
p53 = putative tumour protein 53
PCR = polymerase chain reaction
PL = placental lactogen

PPAR = peroxisome proliferator-activated receptors
PPP = pentose phosphate pathway
PR = progesterone receptor
Prl = prolactin
PrlR = prolactin receptor
R = ram
RER = rough endoplasmic reticulum
rRNA = ribosomal RNA
RT-PCR = reverse transcriptase PCR
RT-qPCR = quantitative (or real-time) RT-PCR
S = single (lamb)
SER – smooth endoplasmic reticulum
SmAd = sub-maintenance dam nutrition during early pregnancy and *ad libitum* dam nutrition during mid-to-late pregnancy
SmM = sub-maintenance dam nutrition during early pregnancy and maintenance dam nutrition during mid-to-late pregnancy
$Sm_{P21\text{-}50}$ = sub-maintenance dam nutrition during early pregnancy
SREBP = sterol regulatory element binding protein
STAT = signal transducing activator of transcription
T = twin (lamb)
TAG = triacyl glycerol
TCA = citric acid cycle
TEB = terminal end buds
TGF = transforming growth factor
TGFβ = transforming growth factor beta
TP = true protein
TPY = true protein yield
TS = twin born, single reared (lamb)
VFA = volatile fatty acid
VLDL = very low density lipoprotein

1 Maternal nutrition during pregnancy and foetal programming of growth, mammary gland development and lactation potential in the sheep: a review of literature

1.1 Introduction

During foetal development important physiological and metabolic parameters can be reset by environmental influences, including dam nutrition during pregnancy (Barker, 2004a; Gluckman & Hanson, 2004). These perturbations in early life may have consequences in adult life, this concept is termed developmental programming. Effects of nutritional programming during foetal life may be mediated through tissue remodelling, abnormal hormone exposure such as excessive glucocorticoids, or epigenetic mechanisms such as DNA methylation, histone modification and non coding RNAs (ncRNA), which may alter gene expression in the offspring (Waterland & Garza, 1999; Duncan *et al*., 2014). In human epidemiological studies and animal models, both maternal under- and over nutrition during pregnancy has been linked to predisposing offspring to obesity, metabolic syndrome (e.g., impaired glucose tolerance and insulin resistance) and cardio-vascular disease (Hales & Barker, 2001; Barker, 2004a & b; Gluckman & Hanson, 2004; Wu *et al*., 2004; Langley-Evans, 2006; Li *et al*., 2011).

In livestock, maternal nutritional programming has been reported to affect production traits in offspring including growth, body composition and carcass traits, fibre production, reproduction and milk production (Wu *et al.*, 2004; Kenyon, 2008; Ashworth *et al*., 2009; Kenyon & Blair, 2014). In many pastoral livestock systems animals are dependent on available pasture for nutrition, however, there may often be periods of feed deficit throughout the year. In New Zealand pastoral sheep production systems, pregnancy of the ewe occurs during winter when pasture supply can be limited, and nutrition may not be optimal (Kenyon & Webby, 2007). Thus there is potential for consequences to the long-term productive performance of offspring. To overcome this, farmers may restrict pasture allocation during early pregnancy in order to ensure adequate feed for later in pregnancy when energy demands for foetal growth are highest (Kenyon & Webby, 2007). However, early pregnancy is a critical period for development of foetal organs and tissues, and perturbations during this time may impair foetal development and production (e.g., growth, reproduction and health) performance of offspring in adult life (Robinson *et al*., 1999).

Of particular interest, and economic significance, are the effects on reproduction and lambing performance in ewe offspring that are retained for breeding. There are many studies that have reported impaired ovarian development and reduced ovulation in ewes subjected to dam over- and under-nutrition during gestation (Rhind, 2004; Kenyon & Blair, 2014), which may affect the number of lambs weaned and have consequences for farm profits. Effects on offspring lactation performance are also important as milk is the sole source of nutrients for the new-born lamb and has a role in immunity and growth. Therefore, the objective of this chapter was to review the current literature

on the effects of maternal nutritional programming on ewe offspring, specifically on lactation performance, and identify key areas for future research.

To date, only one study by van der Linden *et al*. (2009) has examined the effects of maternal nutritional programming on lactation performance of ewe offspring, and reported *ad libitum* pregnancy nutrition of the dam impaired milk production of ewe offspring in their first lactation. Van der Linden *et al.* (2009), however, did not examine lifetime effects. The period of dam nutritional treatment in that study was relatively long, from day 21 to 140 of gestation, and thus did not allow for identification of potentially critical smaller windows during pregnancy in which maternal nutrition may be acting to influence foetal mammary gland development. Further research is warranted to identify the critical period by investigating different levels of nutrition at varying periods of pregnancy. Finally, the mechanisms behind observed differences in offspring lactational performance are unknown. Milk production is highly correlated to the number of differentiated epithelial cells in the mammary gland and their secretory activity (Capuco *et al*., 2001, Boutinaud *et al*., 2004, Wall & McFadden, 2012). Thus, altered expression of genes responsible for proliferation, differentiation and metabolic activity of mammary epithelial cells (MECs) may underlie production differences in ewe offspring as a result of maternal nutritional programming. At a higher level, hormones and/or epigenetic mechanisms may mediate changes in gene expression in the mammary gland. Recent advances in genome-wide transcriptome research may provide a means to investigate differences in patterns of gene expression and related biological functions and molecular signalling pathways.

1.2 Developmental programming

1.2.1 Introduction to developmental programming

Developmental plasticity is the term used to describe the process by which the foetus interprets the environment *in utero*, and makes developmental adjustments to generate phenotypes which may improve immediate survival or enhance performance later in life (Hales & Barker, 1992; Barker, 1997; Hales & Barker, 2001; Gluckman & Hanson, 2004). If there is a mismatch between the predicted and actual environment, the adaptive changes may be detrimental (Gluckman *et al*., 2005). In some cases, changes are obvious and immediate to increase foetal survival (McMillen & Robinson, 2005). For example, in a situation of suboptimal nutrient availability the foetus may alter nutrient partitioning to more critical organs such as the brain at the expense of other organs, which may impair their development and subsequently their functionality later in life (Barker, 1995). In other cases foetal programming effects may be more subtle, for example, altering regulation of gene expression in such a way that no obvious effects are observed until adult life (McMillen & Robinson,

2005). There are a number of factors which can contribute to programming of the foetus, many of which are maternally mediated including weight, age, nutritional status and endocrine status of the dam as well as placental composition (Fowden *et al*., 2005; Reynolds *et al*., 2010). Maternal nutrition, in particular, is a major factor which can alter growth and gene expression in the foetus and may result in developmental adaptations that have long-term effects on body composition, physiological function and metabolic health of the offspring (Wu *et al*., 2004; Symonds *et al*., 2006).

1.2.2 Maternal nutrition and developmental programming

The placenta mediates the transfer of substrates from the mother to the foetus, ensuring that the foetus is adequately nourished, and buffers the effects of variation in the maternal diet. However, the placenta may not be able to adjust for severe deviation from adequate maternal nutrition (i.e., under- and over nutrition) and the foetus, and placenta itself, may suffer impaired development and function (Godfrey, 2002; Wu *et al*., 2004; Reynolds *et al*., 2010). Tissue remodelling as a result of maternal nutritional programming may change the number of cells or cell types present in a tissue which may affect function (McMillen & Robinson, 2005). For example, in rats, a maternal diet low in protein has been shown to reduce the number and size of islets in the pancreas of offspring leading to altered insulin secretion and glucose homeostasis (Snoeck *et al* 1990; Dahri *et al* 1995). In sheep, an obesogenic diet has been shown to accelerate development of pancreatic β-cells (Ford *et al*., 2009).

Maternal nutrition may also influence maternal circulating concentrations of hormones resulting in inappropriate hormonal exposure of the developing foetus. Glucocorticoid exposure has been proposed as a mediator of developmental programming effects (Seckl, 2001; Seckl & Holmes, 2007; Fowden & Forehead, 2009). Glucocorticoids are steroid hormones that are able to diffuse through the placenta, and have been shown to accelerate development of foetal organs (Bian *et al*., 1992; Ward, 1994). There is evidence from animal studies of maternal under-nutrition to suggest that excess glucocorticoid exposure may be a mechanism of programming hypertension and renal defects in offspring (In the rat: Langley-Evans *et al*., 1996; Langley-Evans, 1997, and in the sheep: Dodic *et al*., 1998).

Epigenetic modifications are also proposed as a mechanism of developmental programming. Substrates from the maternal diet may be utilised in biochemical processes involved in epigenetic programming of the foetal genome (Wu *et al.,* 2006; Dolinoy *et al*., 2007; Sinclair *et al.,* 2007). Epigenetic regulation of gene promoters is established during development and is responsible for regulating patterns of gene expression and silencing in adults. Perturbations to this process, from modulation in maternal pregnancy nutrition, may lead to persistent alterations in offspring

phenotype (James *et al*., 2010). Alteration of the epigenetic state of the foetal genome involves stable and possibly heritable modifications to gene expression, which do not involve changes in the nucleotide sequence (Goldberg *et al*., 2007). Modification of the expression of genes may result in permanent changes to morphology, physiology, metabolism and growth of the offspring (Jammes *et al*., 2010), and if present in the germline, may be passed on to subsequent generations (Painter *et al*., 2008). Epigenetic regulation of gene expression can include DNA-methylation, ncRNAs and modifications of histone proteins, which can lead to altered chromatin state (summarised in Figure 1.1, Duncan *et al*., 2014).

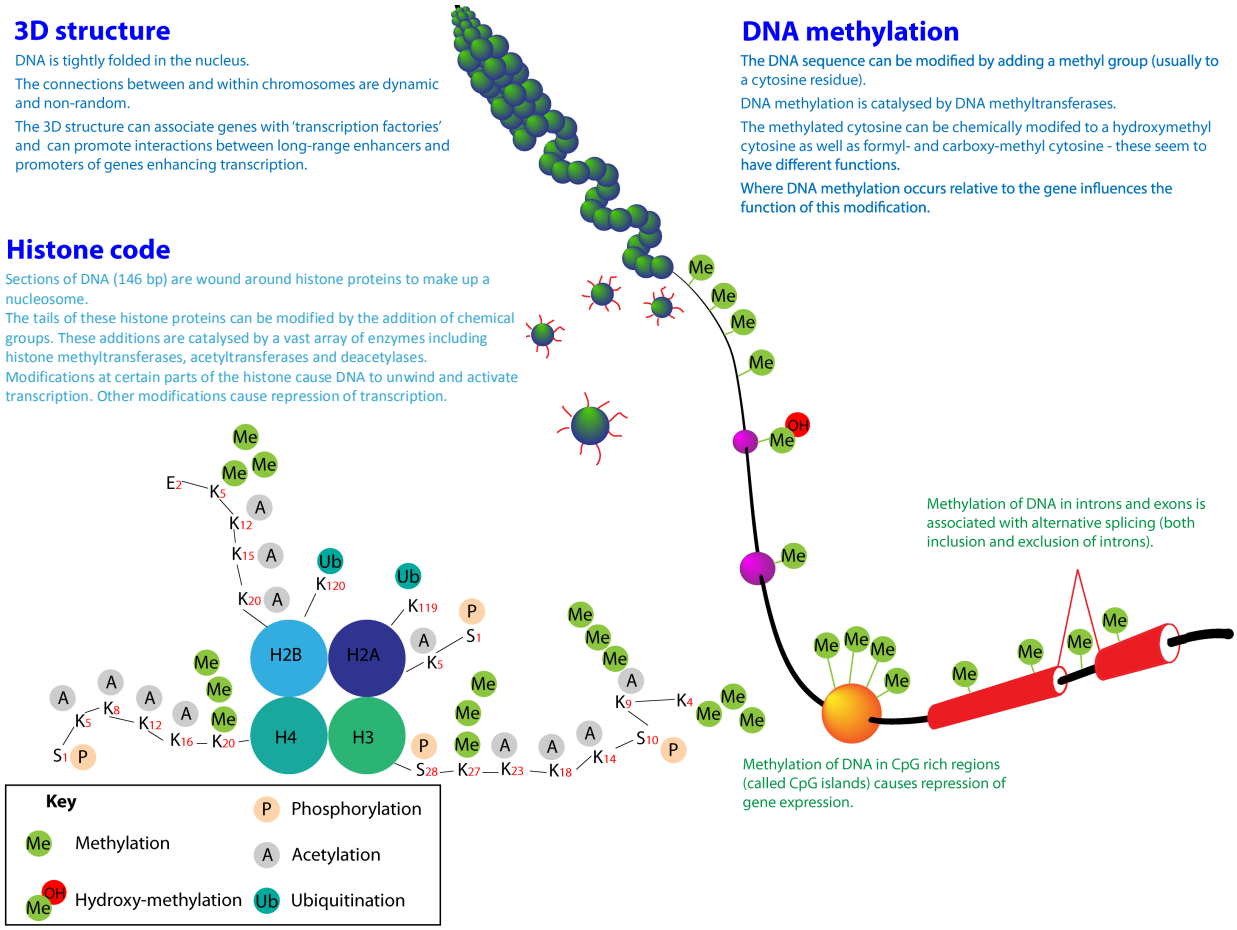

Figure 1.1 Summary of epigenetic modifications and their potential effects on gene expression (Duncan *et al.,* 2014)

Both under- and over nutrition of the mother during pregnancy have been shown to be detrimental for offspring. Human epidemiological studies have linked maternal under-nutrition during pregnancy to increased risk of hypertension, cardio-vascular disease and metabolic disorders including obesity, insulin resistance and type-2 diabetes mellitus in offspring (Hales & Barker, 2001; Roseboom *et al*., 2001). In livestock species, under-nutrition has been shown to affect development of foetal organs and tissues (Robinson *et al*., 1999), and alter production traits in adult offspring,

including growth, carcass composition, reproductive performance, immune function, production of wool, and production of milk (Bell, 2006; Wu *et al*., 2006; Kenyon, 2008; Ashworth *et al*., 2009).

In humans, maternal over nutrition or obesity during pregnancy may result in increased risk of metabolic disease in offspring (Heerwagen *et al*., 2010). This has also been shown in rodent models of over-nourished pregnant dams (particularly high-fat diets), which increase the incidence of obesity, altered glucose metabolism, risk of high blood pressure, cardio-vascular disease and metabolic syndrome (e.g., glucose intolerance and insulin resistance) in offspring (Armitage *et al*., 2005; Li *et al.*, 2011). In production animals, maternal over-feeding during pregnancy has been shown to impair growth of the developing foetus (Wu *et al*., 2006). However in the sheep, evidence of negative effects of maternal over nutrition on offspring have only been demonstrated when the dam is adolescent and there is competition for nutrients between the developing foetus and the growing mother (Wallace *et al*., 1996; Wallace *et al*., 2001). In this model, excess nutrients result in increased partitioning to maternal tissues and can result in intra-uterine growth restriction of the foetus and low birth weight lambs (Wallace *et al*., 1998). This response, however, has not been observed when the dam is mature (Wallace *et al*., 2005). In the study of van der Linden *et al. (*2010a), ewe offspring born to over-nourished dams produced less glucose in response to an epinephrine challenge, in comparison to ewe offspring born to maintenance-fed dams. Van der Linden *et al. (*2010a) suggested that gluconeogenic/glycolytic capacity may be impaired in the ewe offspring of over-nourished dams compared with those born to maintenance-fed dams, raising the possibility that they may be at a disadvantage under physiological stressful situations (e.g., pregnancy and lactation). This finding does suggest that over nutrition in mature ewes can affect the offspring and may have consequences for their productive performance. Further research is required to investigate the effects of overnutrition.

Developmental programming effects are highly dependent on the timing of nutritional insult and reflect the specific organs and systems that are most rapidly developing (Robinson *et al*., 1999). For example, in rodent models and human epidemiological studies, maternal nutrition during early pregnancy has been linked with increased risk of cardiovascular disease in offspring. Whereas maternal nutrition during mid-pregnancy is linked to renal dysfunction and obesity in offspring, and maternal nutrition during late pregnancy may alter metabolic function in adult offspring which has been linked to increased risk of diabetes.

1.2.2.1 Overview of pregnancy stages in the sheep

In the sheep, gestation is approximately 147 days in duration. The period of early to mid-pregnancy is critical for embryogenesis, early organogenesis and tissue hyperplasia, and placental

development (Heasman *et al*., 1998; Robinson *et al*., 1999). Following elongation of the trophectoderm and its spread throughout the uterus from day 15 to 25 of gestation, growth of the placenta commences (Ehrhardt & Bell, 1995). Energy requirements for foetal growth are low during early pregnancy, however, the metabolic activity and specific growth rate of the foetus is high (Harding & Johnston, 1995; Robinson & Symonds, 1995; Redmer *et al*., 2004). In the sheep, the critical period for placental development is thought to be approximately between days 40 to 80 of gestation (Ehrhardt & Bell, 1995). The placenta is vital for substrate transfer between the mother and foetus and impaired placental growth can restrict foetal development and size of the neonate (Reynolds *et al*., 2010). In humans, deviations from normal placental-to-foetal weight ratio has been linked to cardio-vascular disease (Barker *et al*., 1990). In the sheep, suboptimal dam nutrition during early to mid-pregnancy has been demonstrated to influence the development of the foetal kidneys, liver, ovary, gastro-intestinal tract and muscle, which may have important effects on offspring metabolism and productive traits in adult life (Robinson *et al*., 1999; Bell, 2006).

Late pregnancy is traditionally considered the most important period for managing maternal nutrition. During late pregnancy there is rapid increase in foetal size and associated energy demands, making the foetus particularly susceptible to perturbations in maternal nutrition (Mellor & Matheson, 1979; Mellor, 1983; Mellor, 1987). There is a high correlation between nutrition of the dam during late pregnancy and both foetal and birth weight and size (Mellor, 1983; Gardner *et al*., 2007). In an animal-production context, this may be important for neonatal survival and optimal post-natal growth trajectory (Dalton *et al*., 1980; Alexander, 2009).

1.3 Relevance to animal production

Nutrition during pregnancy of livestock is important not only for successful conception and pregnancy (Robinson *et al*., 2002), but also with regards to developmental programming of production traits in offspring. Inadequate nutrition during pregnancy, often due to seasonal fluctuations in the quality and quantity of forage feeds, is a common problem worldwide. Animal production systems which rely on pasture and that have little or no nutritional supplementation, and in which multiple-bearing pregnancies are common, may be particularly vulnerable to nutritional constraint during pregnancy (Kenyon & Webby, 2007).

1.3.1 Developmental programming in pastoral sheep production

The majority of sheep breeds have a seasonal reproductive cycle and in many countries, including New Zealand, pregnancy of the ewe coincides with winter, a period with low pasture growth rate. One practice, common in New Zealand sheep-production systems, is to utilise rotational grazing to restrict feed to ewes during early gestation, thus conserving pasture to allow

for greater levels of nutrition during late gestation and lactation (Kenyon & Webby, 2007). Through either a shortage in pasture availability or restrictive feeding management practices, pregnant ewes may be exposed to suboptimal nutrition. Inadequate levels of maternal nutrition during pregnancy may result in reduced lamb birth weight and size, but also may result in long-term changes in physiology and metabolism which may affect production traits including growth, immunity and reproduction (Kenyon & Blair, 2014).

Effects of maternal nutrition on the postnatal live weight (LW) and growth rates (LWG) of offspring have been extensively studied, although results vary considerably (Mellor, 1983; Robinson *et al*., 1999; Harding, 2001; Brameld & Daniel, 2008; Kenyon, 2008; Ashworth *et al*., 2009; Brameld *et al*., 2010). Nutrition of the dam during mid-to-late gestation, rather than early gestation, appear to be critical for influencing offspring birth weight and postnatal growth (Gardner *et al*., 2007; Kenyon, 2008; Ashworth *et al*., 2009). Early pregnancy, however, is a critical time for developing organs and tissues, including muscle fibre and adipose tissue. There is evidence that maternal nutrient restriction during early pregnancy can affect the number of muscles fibres formed during foetal life, which may lead to reduced muscling in adult offspring (Robinson *et al*., 1999; Brameld & Daniel, 2008). In contrast, under-nutrition, particularly during early pregnancy, is associated with increased adipose tissue in offspring. There is also evidence from human epidemiological and experimental animal studies that maternal nutrition may program immune competence in offspring, altering susceptibility to infection (Shanks & Lightman, 2001; Cronje, 2003). The study of Rooke *et al*. (2010) reported that 3-month old lambs born to ewes which were nutrient restricted during early to mid-pregnancy had increased faecal egg counts (FEC). In contrast the study of (Paganoni, 2005) found no difference in the FECs of offspring born to ewes which were differentially fed from mid-pregnancy through to early lactation. Thus, it would appear that there may be a critical window during early pregnancy whereby maternal nutritional insults may program specific cell populations and/or organs of the immune system, which may impart lasting effects on offspring susceptibility to parasites. More research is required to develop clear conclusions about nutritional programming of immunity.

Maternal nutrition, during several stages of gestation, may alter development of the foetal reproductive system, with potential effects on adult reproductive function (Rhind *et al*., 2001; Rhind, 2004; Kenyon, 2008; Ashworth *et al*., 2009; Dupont *et al.*, 2012). Dam under-nutrition during early to mid-pregnancy has been shown to reduce follicular development and ovarian mass in foetuses (Borwick *et al*., 1997; Rae *et al*., 2001), increase pituitary sensitivity to gonadotropin releasing

hormones (GnRH) and the number of follicles in the ovary in 10 month old ewe offspring (Kotsampasi *et al*., 2009), and reduce ovulation rates in 20 month old offspring (Rae *et al*., 2002).

1.3.2 Effects of maternal gestational nutrition on lactational performance of offspring

Lactational performance of the ewe is an important contributing factor for production efficiency. Milk is the sole source of nutrients for new-born lambs and greatly influences survival and growth (Stephenson *et al*., 1981; Mellor 1983; Jordan & Mayer, 1989; Degen & Benjamin, 2005). The production of milk is tightly governed by hormones and is well regulated by local autocrine/paracrine mechanisms (Tucker, 2000; Hennighausen & Robinson, 2001; Hovey *et al*., 2002; Neville *et al*., 2002). Ultimately, the number of secretory cells and their metabolic activity determines the lactation potential of the ewe, and therefore factors which influence this are likely to have implications for milk production (Capuco *et al*., 2001; Boutinaud *et al*., 2004; Wall & McFadden, 2012).

Mammary gland development begins early in foetal life when the rudiments of the mammary gland are formed prior to birth (Forsyth *et al*., 1999; Hovey *et al*., 2002). The mammary gland then undergoes further development at critical stages during postnatal life, with the majority of development in the ewe occurring during gestation (Anderson, 1975; Knight & Peaker, 1982). Perturbations to normal development in foetal life, such as suboptimal maternal gestational nutrition, may alter subsequent development at later stages, and ultimately affect function during lactation (Capuco & Akers, 2010; Singh *et al*., 2010a). In a study reported by Blair *et al*. (2010), foetuses from ewes which were *ad libitum*-fed from day 19 of gestation, had increased area of mammary ducts and numbers of insulin-like growth factor 1 receptors (IGF1-R) at days 103 and 137 of foetal life, although no difference in total mass of the mammary gland was observed. Increased ductal area may provide a platform for increased proliferation of mammary secretory cells during pregnancy-related mammogenesis in adult life. Insulin-like growth factor 1 (IGF-1) is thought to be involved in mammary gland development, largely through its role in mediating the proliferative effects of growth hormone (GH) and oestrogen through stromal-epithelial cell interactions (Forsyth *et al*., 1999; Hovey *et al*., 2002; Akers & Capuco, 2006). Thus, a higher number of IGF-1R may stimulate higher levels of cell proliferation in the mammary gland. In the study of Blair *et al. (*2010), no offspring were retained to investigate lactation performance in adult offspring. In contrast, the study of van der Linden *et al. (*2009) reported reduced mass of mammary glands of day (d) 100 twin-foetuses from ewes that were offered *ad libitum* feeding from day 21 of pregnancy, compared with those from ewes offered maintenance feeding. In the latter study, there was no difference in duct area, and van der Linden *et al. (*2009) speculated that the lighter mass might have been due to a

smaller fat pad. In a cohort of adult offspring, van der Linden *et al. (*2009) reported that ewes born to *ad libitum*-fed dams produced less milk in their first lactation, with lower lactose and crude protein (CP) yields, compared with ewes born to maintenance-fed dams. Blair *et al. (*2010) investigated the second lactation performance and found no differences in milk yields of ewe offspring, although ewes born to dams that were *ad libitum*-fed had higher milk fat and milk net energy yields. In a more recent study by Martin *et al. (*2012) there were no differences between mammary gland weights of day 140 foetuses from dams that were offered maintenance or *ad libitum* feed from day 21 of pregnancy. However, foetuses exposed to an additional dam nutritional treatment of sub-maintenance feeding from day 21 to 50 of gestation, had lighter mammary glands than foetuses from maintenance- and *ad libitum*-fed dams.

The combined findings of these studies, while inconsistent, demonstrate that the foetal mammary gland is susceptible to the level of maternal nutrition, particularly during early pregnancy. Furthermore, responses of the foetal mammary gland to maternal gestational nutrition appear to translate into differences in milk production in adult ewe offspring during their first lactation (van der Linden *et al*., 2009). Further investigation of effects on milk production in ewe offspring over their productive lifetime is warranted. In addition, understanding the developmental programming mechanisms during critical periods of gestation may allow for manipulation of lactation performance in ewe offspring.

1.4 Mammary gland development and function

1.4.1 Evolution of the mammary gland and importance of lactation

Lactation and development of the mammary gland is an important part of the mammalian reproductive strategy, enabling successful rearing of offspring and survival to reproductive age (Capuco & Akers, 2009). Through production of milk, both nutrients and passive-immunity are transferred to the young. Differences between mammalian species exist in the structure (number and location) and morphology of mammary glands, hormonal regulation, metabolic capabilities and adaptations, and in the composition of their milk (Capuco & Akers, 2009). The production of milk by domestic ruminants has long been exploited by humans with cow, goat and sheep milk all being used for the manufacture of dairy products. Many studies have focused on milk production by cattle due to the economic significance of the dairy sector to the economy. However, milk production is arguably as important in pastoral livestock systems such as the New Zealand pastoral sheep production system, for the production of meat and/or fibre (Dalton *et al*., 1980). In pastoral sheep production, milk is the sole source of nutrients for the new-born lamb and may affect survival, growth to weaning and future productive outputs (Stephenson *et al*., 1981; Mellor 1983; Jordan &

Mayer, 1989; Degen & Benjamin, 2005). Thus improved understanding of the factors influencing mammary development and function in the sheep may be of use for productive gains.

1.4.2 Mammary gland structure and morphology

The mammary gland is comprised of two main tissue components, the parenchyma and the stroma (Nickerson & Akers, 2011). The parenchyma refers to the functional secretory and ductal tissue. The secretory tissue is made up of structures referred to as alveoli, that are comprised of epithelial cells organised in a single layer lining a hollow lumen. The MECs are responsible for the synthesis of milk, therefore, the number and secretory activity of these cells directly influences lactation performance (Capuco *et al*., 2001; Boutinaud *et al*., 2004; Wall & McFadden, 2012). The epithelial cells are surrounded by myoepithelial cells, which contract upon stimulation by oxytocin, and cause stored milk to be ejected into the lumen (Nickerson & Akers, 2011). Alveoli are also surrounded by capillaries which are responsible for the transport and exchange of nutrients, waste and hormones between blood and epithelial cells. Groups of alveoli cluster together in lobules and drain into a common duct, this is referred to as a lobulo-alveolar unit. The mammary stroma (or fat pad) contains supportive tissue, including connective tissue, fibroblasts, adipose tissue, nerve tissue, blood vessels and lymph vessels (Hurley & Loor, 2011; Nickerson & Akers, 2011). The fat pad provides structural support to the mammary gland and allows access to vasculature, lymphatics, lipids and local growth factors which stimulate development (Hovey *et al*., 1999; Hurley & Loor, 2011).

1.4.3 Overview of mammary developmental events

The mammary gland is unique in that it can undergo repeated cycles of growth, functional differentiation and regression in alignment with reproductive state. Mammary gland development begins during foetal life when the rudiments of the stroma and parenchyma are formed and the foundations of the mammary gland are established (Jenkinson, 2003; Capuco & Akers, 2010). The majority of mammary development, however, occurs postnatally with the most extensive development during pregnancy (Anderson, 1975). Just prior to parturition, functional differentiation of epithelial cells is initiated (termed lactogenesis stage 1, Akers & Capuco, 2002). This stage is characterised by cytological changes (e.g., an increase in the size of the cytoplasm and presence of vacuoles and lipid droplets) and increasing expression of genes encoding milk proteins to prepare for lactation (Tucker, 1981; Akers & Capuco, 2002; Brisken & Rajaram, 2006). Lactogenesis stage 2, or secretory activation, is initiated by specific hormonal cues at parturition, in particular a decrease in progesterone, and suckling stimulus (Akers & Capuco, 2002; Neville *et al*., 2002). The secretory activation stage is characterised by the closure of tight junctions between alveolar epithelial cells,

and build up of pressure within cells triggers the release of milk secretion into the alveolar lumen (Akers & Capuco, 2002; Mather, 2011). Milk yield increases until peak lactation through increased number, size and activity of secretory cells (Capuco & Ellis, 2013). Decrease in milk yield after peak lactation is thought to be related to a decrease in the number of secretory cells (Capuco & Ellis, 2013). As a result of either gradual weaning, or intervention causing the cessation of milk removal, the mammary gland undergoes a program of apoptosis, and extensive tissue remodelling occurs during involution (Knight, 1982; Hurley & Loor, 2011).

The growth, developmental and functional processes of the mammary gland are tightly regulated by hormones of the endocrine system. External and local factors, including the uptake of nutrients by the gland, the surrounding stromal tissue, physiological state, photoperiod, and milk removal (Figure 1.4) can influence mammary development and function (Capuco & Akers, 2010). On a molecular level multiple signalling pathways, involving many genes, underlie the regulation and modulation of these effects (Hennighausen & Robinson, 2001). Aberrant signalling, however, may lead to impaired development and may affect lactation performance.

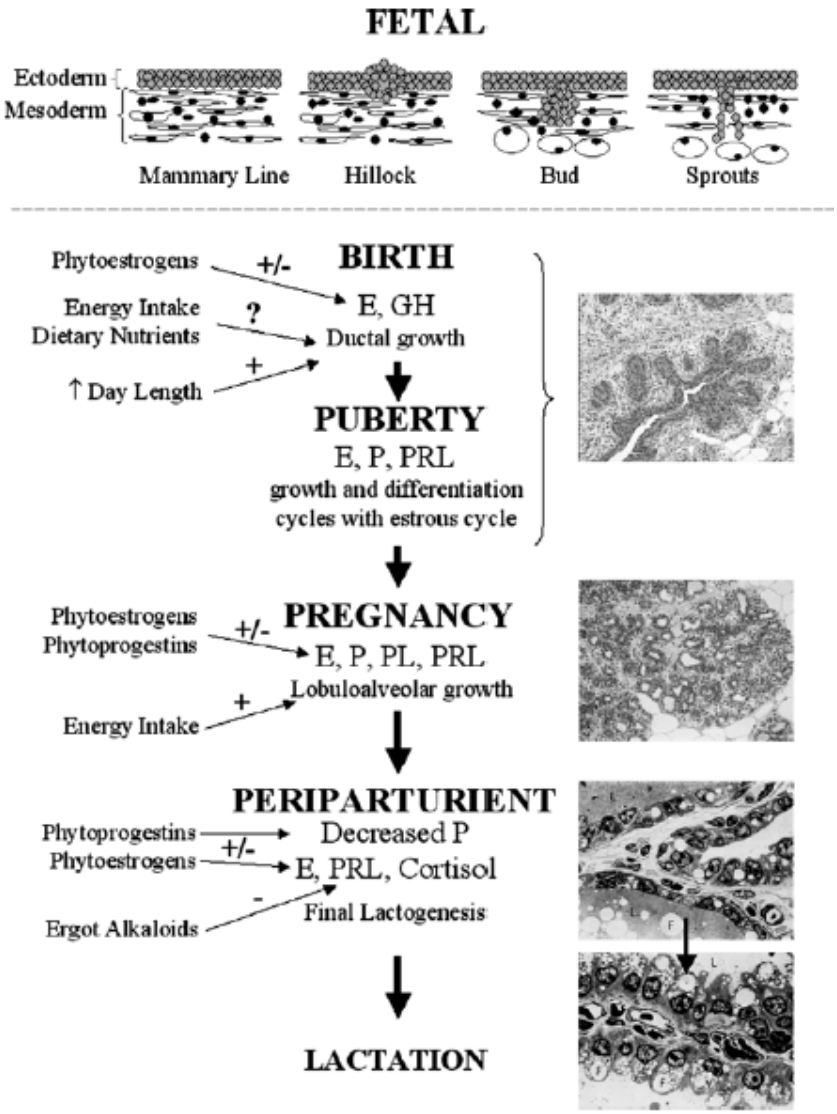

Figure 1.2 Foetal and postnatal mammary gland development depicted with the primary hormones and management factors which influence growth and differentiation (Capuco & Akers, 2010). E = oestrogen, GH = growth hormone, P = progesterone and PRL = prolactin.

1.4.4 Mammary development during foetal life

Organogenesis of the ovine mammary gland begins around day 20 of foetal life (Jenkinson, 2003). Prior to birth the foundations and precursor tissue that supports postnatal development is formed (Knight and Peaker, 1982). Initial stages involve a thickening of the ectoderm in which the mammary line and crest forms. This determines the positioning of the glands. The ectodermal cells sink inwards into the dermis along the mammary line and form the mammary buds. This determines the number and precise location of the individual glands and teats. Formation of the primary duct occurs and, through a process called canalisation, ultimately gives rise to the teat and gland cisterns. Teat formation occurs through proliferation of mesenchymal tissue under and around the mammary bud. Secondary ducts begin to branch off the primary duct during foetal life, towards the end of the

first trimester, which determines the basic structure and numbers in the mammary duct system (Jenkinson, 2003; Capuco & Akers, 2010; Hurley & Loor, 2011; Rowson *et al*., 2012). By birth the key structures, including the teat, teat cistern, gland cistern and primitive ductal system are present. In addition, ancillary structures, such as blood vessels, connective tissue, lymphatic tissue, nerves and myoepithelial cells, which make up the mammary stroma also begin to form (Jenkinson, 2003; Capuco & Akers, 2010). Adipose tissue also arises from the mammary mesenchyme to form the mammary fat pad. The fat pad is an important structure to support future proliferation and differentiation of the mammary epithelium through providing space, support and local controls for ductal elongation and eventually lobulo-alveolar development (Hovey *et al*., 1999; Hurley & Loor, 2011).

While mammary development occurs throughout foetal life to term and during post-natal life, much of the initial critical development which sets up future development has occurred within the first trimester for sheep (Jenkinson, 2003). Therefore it is important to consider how pre-natal influences, including nutrition of the dam during specific developmental periods, may be critical for future development of the mammary gland and lactational performance.

The mammary gland is considered to be non-functional at birth and foetal mammogenesis is thought to occur independently of hormones, instead relying on signalling of local factors between the epithelium and mesenchyme (Hennighausen & Robinson, 2001; Knight & Sorensen, 2001; Hennighausen & Robinson, 2005; Akers & Capuco, 2010). The predominant signalling pathways include the Wnt, Notch and Hedgehog pathways (Malhotra *et al*., 2011). There is, however, evidence that the foetal mammary gland may be capable of responding to the endocrine environment. In a rodent model, foetal mammary fragments taken at day 17 of gestation, and maintained in culture, were stimulated by hormones to produce ducts and mammary secretion (Ceriani, 1970a & b). Another example of hormone responsiveness in the early mammary gland is the phenomenon in humans known as ‘witches milk’. In these infants peri-parturient changes in hormones are thought to be responsible for abnormal precocious development and fluid secretion observed (Capuco & Akers, 2010). Indeed, several studies have demonstrated the presence of hormone receptors in the foetal mammary gland. Growth hormone receptor (GHR) protein and mRNA has been located in the bovine foetal mammary gland, in the epithelium, mesenchyme, endothelium and epidermis, using reverse transcriptase-PCR (RT-PCR), in-situ hybridisation (ISH) and immuno-histochemistry (IHC) techniques (Knabel *et al*., 1998). Insulin-like growth factors 1 (IGF-1) and 2 (IGF-2) and the receptor, IGF1-R, messenger RNA (mRNA) have also been identified in the stromal cells adjacent to the epithelium in the ovine foetal mammary gland, by ISH, at 10 to 20 weeks of foetal age (Forsyth *et al*.,

1999). Receptors of oestrogen (ER) and prolactin (PrlR) have been identified in foetal mammary glands of rodents, there are no findings reported for ruminants (Hovey *et al*., 2002). Collectively, these findings indicate that while hormone receptors may not be required in the foetal and early-neonatal mammary gland they are present. It is, therefore, possible that one mechanism by which maternal nutrition during pregnancy may impact foetal mammary gland development is through altered endocrine environment. Indeed, adverse hormonal exposures *in utero*, including those resulting from an altered maternal diet, have been proposed as mechanisms behind tumours in adult mammary tissue. Hilakivi-clarke & De Assis (2006) proposed that in humans, breast-cancer risk might be increased in daughters from *in utero* exposure to maternal high fat diets. It was suggested that a maternal diet high in fat might lead to an increased production of maternal oestrogen, a hormone that stimulates proliferation of terminal end buds (TEBs) in the mammary gland, which may increase breast-cancer targets in daughters. This theory was supported by evidence from rodent models (Hilakivi-Clarke *et al*., 1997).

1.4.5 Peri-pubertal mammary development

In the sheep, the mammary gland undergoes a period of allometric growth just prior to puberty (Anderson, 1975; Rowson *et al*., 2012). During pre-pubertal development, the ductal system, of which the basic structures were formed during foetal life, undergoes extensive proliferation and branching, invading the mammary fat pad (Capuco & Akers, 2010; Rowson *et al*., 2012). The ducts of the mammary gland support later proliferation of MECs that synthesise and secrete milk during lactation. Impairment of ductal outgrowth may limit the secretory capacity of the mammary gland and have consequences for lactation performance (Capuco & Akers, 2010).

1.4.6 Mammary development during pregnancy

The majority of post-natal mammary gland development occurs during pregnancy (Rattray *et al*., 1974; Anderson, 1975; Knight & Peaker, 1982) when there is continued elongation of the mammary ducts and marked expansion of the lobulo-alveolar network. The most extensive epithelial proliferation occurs during late pregnancy, and just prior to parturition functional differentiation of the mammary epithelium occurs in order to prepare the gland for milk production during lactation (Akers & Capuco, 2002; Hurley & Loor, 2011). This mammogenic period is particularly critical for lactation performance as it is the MECs of the alveoli developing during this stage that are responsible for synthesis and secretion of milk (Akers & Capuco, 2002; Wall & McFadden, 2012).

1.4.7 Mammary development and function during lactation

In the sheep, there is very little mammary gland growth post-partum. Anderson, (1975) reported that in sheep, based on DNA content, 98% of mammary gland growth from the beginning of pregnancy occurs during pregnancy while only 2% occurs in early-lactation.

1.4.7.1 Lactogenesis

Lactogenesis is the term given to the process of functional differentiation of MECs in order to produce milk during lactation (Akers & Capuco, 2002). During lactogenesis biochemical and structural changes occur in MECs to prepare them for the onset of milk synthesis and secretion. Lactogenesis is broken into two stages; lactogenesis stage 1 and lactogenesis stage 2. Stage 1 occurs in late pregnancy prior to parturition, and is characterised by an increase in synthesis of milk proteins and cytological changes (e.g., increase of cell cytoplasm in relation to the nucleus and increased presence of milk fat globules and vacuoles, Akers & Capuco, 2002). Lactogenesis stage 2, which initiates secretion of milk, is triggered by hormones at parturition and by external stimuli such as a suckling lamb (Akers & Capuco, 2002; Hovey *et al*., 2002). Lactogenesis stage 2 is characterised by structural changes including increased cell size, increased size and development of organelles such as the mitochondria, rough endoplasmic reticulum (RER) and Golgi apparatus, and polarisation of cells (Akers & Capuco, 2002). In fully differentiated cells the baso-lateral region is the site of uptake of metabolic precursors and protein and lipid synthesis. The basal membrane has receptors for transporters to facilitate the intake of nutrients into cells for milk biosynthesis. The apical membrane is populated with Golgi apparatus and is the site at which post-translation modification of proteins, protein packaging and lactose synthesis occurs (Stelwagen, 2011a & b). Vesicles containing protein and lactose bind to the apical membrane and are subsequently exocytosed into the alveolar lumen. Lactogenesis stage 2 also promotes increased metabolic activity, closure of tight junctions and increased translation of milk proteins to support milk production (Nickerson and Akers, 1984).

1.4.7.2 Milk protein synthesis

Milk protein provides an essential source of amino acids, antibodies and minerals to the neonate and is a commercially valuable component of the milk (Ng-Kwai-Hang, 2011; Stelwagen, 2011a). Proteins are synthesised in MECs from amino acids which can be transported from the blood or synthesised *de novo* (Bionaz & Loor, 2011). The casein and whey proteins make up the majority of milk proteins In addition IgA, lactoferrin, transferrin and serum albumin are also found in milk (Ng-Kwai-Hang, 2011; Stelwagen, 2011a). Casein proteins are phosphoproteins, of which there are four different types: αs1-casein, αs2-casein, β-casein and κ-casein. Whey proteins synthesised in the

sheep mammary gland include beta-lactoglobulin and alpha-lactalbumin (Ng-Kwai-Hang, 2011; Ramos & Juarez, 2011). Alpha-lactalbumin is important for lactose synthesis, as part of the lactose synthase enzyme complex, and for milk secretion (Brew, 2011). The biological function of beta-lactoglobulin is unknown, although it has been suggested to be some sort of transporter or merely a convenient nutritional protein (Creamer *et al*., 2011). There are two main variants of beta-lactoglobulin (A and B).

Synthesis of milk proteins can be regulated at the transcriptional level and also at the translational and post-translational levels (Chevalier, 2011; Stelwagen, 2011a). Lactogenic hormones regulate transcription of milk proteins. On a molecular level, this may be mediated by changes in chromatin conformation, nuclear receptors, transcription factors or transcriptional repressors, facilitating or inhibiting the transcription of genes (Rijnkels *et al*., 2010; Singh *et al*., 2010a). Milk protein gene expression is typically used as a marker of differentiated MECs. Expression of the casein genes and whey protein genes increases from late pregnancy, dramatically increasing at parturition (Naylor *et al*., 2005). Prolactin (Prl), growth hormone (GH) and glucocorticoids are known to promote expression of milk protein genes. Both Prl and GH can activate the Janus-kinase and signal transducer and activator of transcription (JAK-STAT) signalling cascade. Mitogen-activated protein kinase (MAPK) signalling and the mammalian target of rapomycin (mTOR) are also thought to be involved in transcription of milk protein genes (Naylor *et al*., 2005; Bionaz & Loor, 2011). Following translation, milk proteins are folded and post-translational modifications occur in the endoplasmic reticulum, after which they are packaged for transport out of the cell in the Golgi apparatus (Mather, 2011; Stelwagen, 2011a).

1.4.7.3 Lactose synthesis

Lactose is the main carbohydrate and osmotic component of milk. Lactose is a disaccharide synthesised from glucose and galactose (which is derived from glucose) within the Golgi apparatus (Stelwagen, 2011b). Glucose is the sole precursor required for synthesis of lactose. The mammary gland is unable to generate glucose due to the lack of glucose-6-phosphatase; therefore, glucose must be supplied to the mammary gland from the circulation (Finucane *et al*., 2008; Stelwagen, 2011b). Synthesis of lactose is catalysed by the lactose synthase enzyme complex, which is comprised of beta-1,4-galactosyl-transferase and the whey protein alpha-lactalbumin (Stelwagen, 2011b). Synthesised lactose is then packaged into secretory vesicles, along with milk proteins, which are pinched off from the Golgi. These secretory vesicles, assisted by the microtubule network, move through the cytosol to the apical membrane where they fuse and release their contents into the milk in the alveolar lumen (Mather, 2011). The osmotic gradient between the vesicles and cytosol, due

largely to the lactose content, pulls water into them increasing the volume of milk (Stelwagen, 2011b).

1.4.7.4 Lipid synthesis

Milk fat provides an essential source of energy for the neonate. The sheep has an average of 15% lipid content in their milk, which is higher than the cow which has ~10% (Ramos & Juarez, 2011). Milk fat is predominantly triacylglycerols (TAG), but also includes a small amount of diacylglycerides, monoacylglycerides, cholesterol, phospholipids (includes sphingolipids) and free fatty acids (Bauman *et al*., 2011). Synthesis of TAGs occurs in the smooth endoplasmic reticulum (SER) from glycerol and fatty acids. In ruminants, milk fat TAGs are largely from short to medium chain (4 to 18 carbon length) fatty acids. These short to medium chain fatty acids are synthesised *de novo* while longer chain fatty acids are supplied from circulation (Bionaz & Loor, 2008; Harvatine *et al*., 2009; Bauman *et al*., 2011). *De novo* synthesis of fatty acids in the mammary gland increases at parturition and increases to peak milk yield, then declines as milk yield declines. Expression of lipogenic genes also increases at parturition under the regulation of endocrine hormones, in particular decreased progesterone and increased prolactin (Naylor *et al*., 2005; Rudolph *et al*., 2007; Bionaz & Loor, 2008; Finucane *et al.*, 2008; Bionaz *et al*., 2012). In non-ruminant species, glucose is used as a precursor for fatty acid synthesis. In ruminants, however, a glucose sparing mechanism (Randle's effect, Randle, 1998) enables the use of acetate, and beta-hydroxybutyrate (BHB) to a small extent, as a precursor for fatty acid synthesis (Bauman *et al*., 2011).

1.4.7.5 Galactopoiesis

The increase in milk production at the onset of lactation is largely due to increased numbers of secretory cells as indicated by increased mammary DNA, while an increase in the metabolic activity of these cells then becomes an important factor for milk yield until peak lactation (Knight & Peaker, 1982; Knight, 2000; Akers & Capuco, 2002; Capuco & Ellis, 2013). The balance between cell proliferation and apoptosis, while at low levels during lactation, is an important factor of maintaining the secretory cell population. As lactation progresses this balance favours apoptosis, demonstrating that apoptotic cell death may be responsible for the decline in lactation (Knight & Peaker, 1982).

Galactopoiesis refers to the maintenance of established lactation. Increased galactopoiesis means increased lactation persistency which is of benefit in animal production systems. Lactation persistency depends upon maintenance of the population of secretory cells, therefore, factors which promote cell proliferation and survival and inhibit apoptosis may improve milk production in animals (Figure 1.3, Capuco & Ellis, 2013). Factors such as hormones, systemic and locally produced growth factors and signalling molecules, changes in milking frequency, antioxidants or exposure to reactive

oxygen species/oxidative stress, disease such as mastitis, blood flow and nutrient partitioning and other stressors may affect lactation persistency (Capuco & Akers, 2011; Collier *et al*., 2011; Wall & McFadden, 2012).

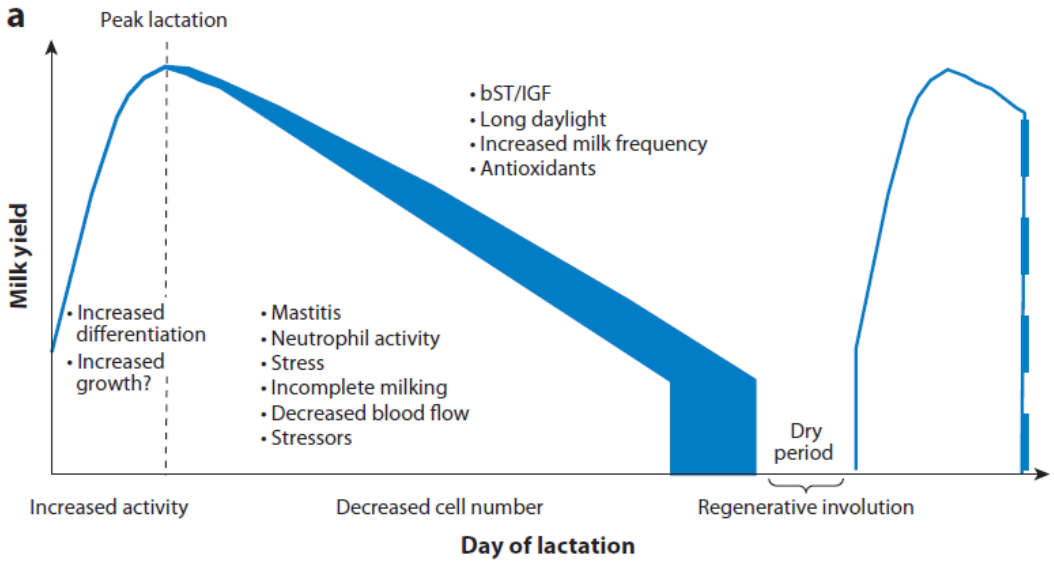

Figure 1.3 Factors contributing to changes in milk yield during a bovine lactation. Prior to peak lactation, milk yield increases owing to increased secretory activity per cell. After peak lactation, milk yield declines primarily because of a decline in epithelial cell number owing to apoptotic cell death. Factors that decrease loss of cells decrease the decline in milk yield (i.e., increase persistency). Factors shown above the lactation curve increase milk yield and persistency, and those below decrease milk yield and persistency (Capuco & Ellis, 2013).

1.4.8 Mammary regression during involution

After the cessation of milk removal the mammary gland undergoes involution where the alveolar units and secretory tissue completely regresses (Akers & Capuco, 1999; Rowson *et al*., 2012), and is not reformed until the next lactation (Figure 1.4, Capuco & Ellis, 2013). During involution cells undergo apoptosis and are removed by phagocytosis. This process is predominantly regulated by local factors, although systemic hormones are also involved (Hurley & Loor, 2011). During the dry period and subsequent pregnancy the mammary gland undergoes extensive remodelling. It is believed that the dry period between lactations is essential for regenerating of the mammary gland, in particular for replacing the progenitor cells, which have a limited life-span (Akers & Capuco, 1999; Capuco & Ellis, 2013). It is these progenitor cells that are responsible for expanding and maintaining subsequent populations of secretory cells and a decline in the replacement of the progenitor cell population may impair the redevelopment and productive capacity of the mammary gland in subsequent lactations (Capuco & Ellis, 2013). When there is insufficient remodelling there is increased numbers of senescent cells, increased apoptosis of cells, overall reduction in cell replacement, and ultimately reduced secretory activity.

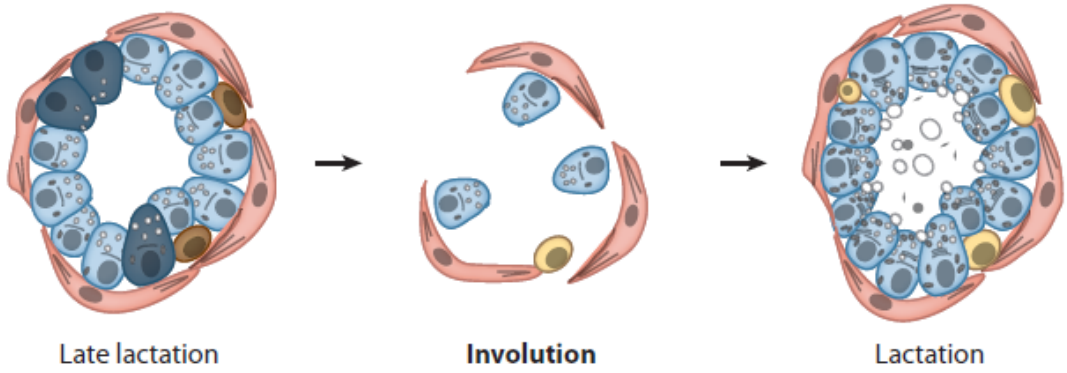

Figure 1.4 Bovine mammary alveoli during the transition from the cessation of milk removal to the following lactation. Alveolar structure is maintained during regenerative involution, and senescent (darkened) epithelial cells (blue) and progenitor cells (yellow) are replaced. Mammary involution in a non-pregnant animal results in the destruction of the alveolus, which is not restored until the next pregnancy. Myoepithelial cells are depicted as pink cells (Capuco & Ellis, 2013).

1.5 Hormonal regulation of mammary development and function

Post-natal mammary development and function is initiated and regulated by hormones of the reproductive system (e.g., oestrogen, progesterone, prolactin and placental lactogen), in addition to metabolic hormones (e.g., insulin, growth hormone and glucocorticoids). These hormones act during specific stages of mammary development (Figure 1.5, from Neville *et al*., 2002) which will be explained in more detail, for the key hormones, in the following sections.

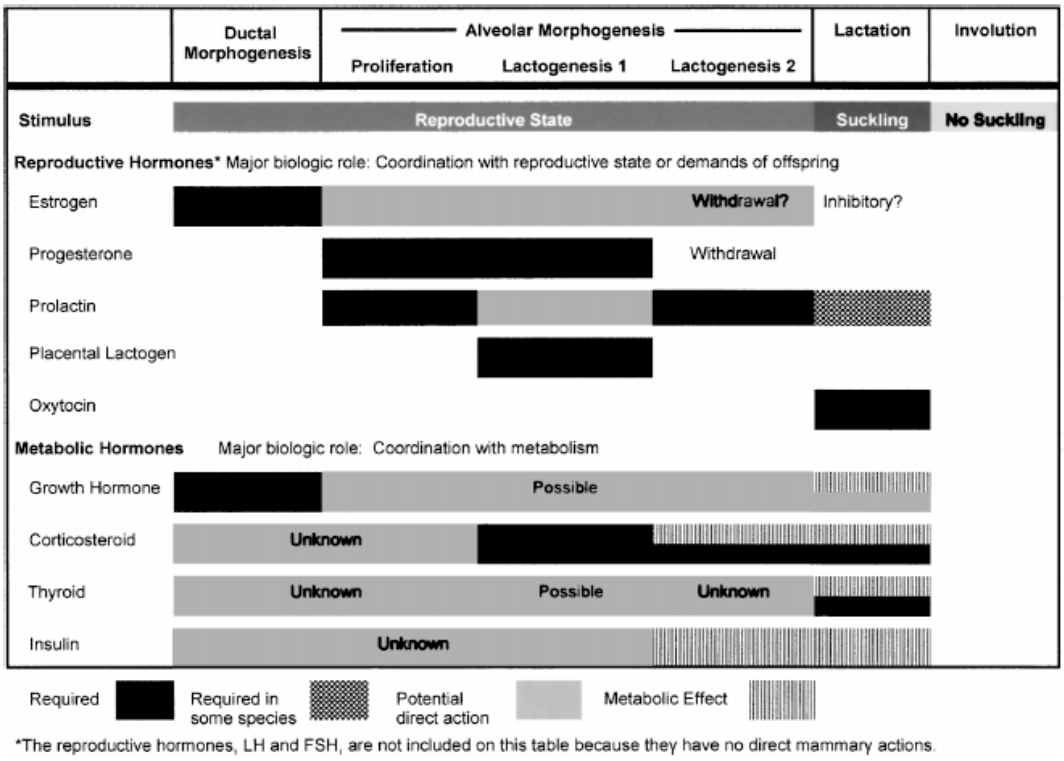

Figure 1.5 Summary of hormones and the stages in which they are involved in mammary gland development (Neville *et al*., 2002)

1.5.1 Oestrogen

Oestrogen is required for elongation of mammary ducts during puberty (Capuco & Akers, 2010) and early pregnancy and alveolar expansion during mid-to-late pregnancy (Hovey *et al*., 2002). The effects of oestrogen are mediated through receptors, of which there are two major forms: oestrogen receptor subtype alpha (ERα) and oestrogen receptor subtype beta (ERβ), which act as transcription factors upon steroid binding (Hennighausen & Robinson, 2005). It is believed that oestrogen exerts effects through a paracrine manner, potentially through inducing stromal production of growth factors (e.g., IGF-1, Surmacz, 2000; and hepatocyte growth factor (HGF), Gjorevski & Nelson, 2011). As previously mentioned, aberrant oestrogen signalling has been implicated in increased mammary tumour susceptibility in offspring of mothers fed pregnancy diets high in fat (Hilakivi-Clarke *et al*., 2001; Hilakivi-Clarke & Assis, 2006). This finding suggests that oestrogen may be a mechanism by which nutrition of the dam can influence development of the mammary gland in female offspring. The research of Hilakivi-Clarke *et al. (*2001) was conducted using rodent models and were directed for medical outcomes, therefore, the effects on milk production were not evaluated. It remains to be elucidated whether altered oestrogen activity/exposure may play a role in the sheep in mediating effects of over nutrition of the dam during pregnancy on mammary gland development and lactation performance of offspring.

1.5.2 Progesterone

Progesterone is important for alveolar development in the mammary gland during pregnancy (Neville *et al*., 2002; Hennighausen & Robinson, 2005). Activity of progesterone in the mammary gland is mediated through the two isoforms of the progesterone receptor [PR, isoforms A (PR-A) and B (PR-B)] (Hennighausen & Robinson, 2001; Conneely *et al*., 2003). The PR-B isoform, in particular, is thought to mediate the proliferative effects of progesterone in MECs (Mulac-Jericevic *et al.*, 2000). Progesterone also has inhibitory effects on lactation. Prior to parturition progesterone prevents commencement of lactogenesis stage 2 (milk secretion) through inhibition of alpha lactalbumin synthesis (Goodman *et al*., 1983; Akers, 1985; Tucker, 2000; Akers & Capuco, 2002). Alpha-lactalbumin is a key milk protein involved in lactose synthesis and regulation of milk yield. Thus, the regression of the corpus luteum and related decrease in progesterone is important for the onset of copious milk production at parturition.

1.5.3 Prolactin

Prolactin (Prl) and placental lactogen (PL), as their names suggest, are important lactogenic hormones required for mammary development and function (Horseman, 1999; Knight, 2001; Oakes *et al*., 2008). Placental lactogens are produced by the placenta and have roles in preparing the

mammary gland for lactation as well as supporting foetal growth and development (Forsyth, 1986). In the sheep, the mid-pregnancy increase in placental lactogen coincides with an increase in lobulo-alveolar development in the mammary gland (Akers, 1985). Prolactin is important for both lactogenesis stage 1 and stage 2. Binding of Prl to prolactin receptor (PrlR) stimulates structural and functional differentiation of the mammary epithelium, promoting proliferation of MECs during alveolar morphogenesis, and closure of tight junctions, increased glucose uptake and increased expression of lipogenic genes and milk protein genes during lactogenesis (Akers *et al.,*1981a & b; Goodman *et al.,*1983; Akers, 2006). The action of Prl is supported by other hormones, including GH, glucocorticoids and placental lactogens, which work synergistically to enhance the effects of Prl in the mammary gland (Horseman, 1999; Reichardt *et al*., 2001; Akers, 2006; Wall & McFadden, 2012).

1.5.4 Glucocorticoid

As previously mentioned, glucocorticoids work in synergy with Prl to stimulate development and functional differentiation in the mammary gland (Reichardt *et al.,* 2001; Akers, 2006). Glucocorticoid action in the mammary gland is mediated through the glucocorticoid receptor which regulates expression of milk protein genes (Reichardt *et al.,* 2001). A study by Reichardt *et al. (*2001), using a model with genetically-modified mice, suggested that the glucocorticoid receptor may use DNA-binding dependent mechanisms to regulate genes involved in MECs proliferation, while protein-protein interaction with transcription factors regulates differentiation and expression of milk protein genes.

1.5.5 Growth hormone

The role of GH in the mammary gland has been extensively studied (reviews by Plaut *et al*., 1993; Sjersen *et al*., 1999; Kelly *et al*., 2002; Akers, 2006; Kleinberg & Ruan 2008). GH is required for ductal elongation in the mammary gland during puberty, has been shown to stimulate mammary growth during pregnancy, and has galactopoietic effects during lactation (Akers, 2006). The action of GH in the mammary gland is thought to be mediated by IGF-1 (Hovey *et al.*, 1999; Akers, 2006; Kleinberg & Ruan 2008). Purup *et al. (*1993) demonstrated that exogenously administered GH increased circulating levels of IGF-1, which then act to stimulate mammary growth. GH receptors (GHR) have been localised to the mammary fat pad. These receptors induce local production of IGF-1 upon hormone-binding, and subsequently promotes growth and proliferation of MECs (Forsyth *et al*., 1999; Akers, 2000; Plath-Gabler *et al*., 2001; Hovey *et al*., 2002).

1.5.6 Leptin

Leptin is a hormone which is produced by adipose tissue and is largely associated with appetite regulation. Expression of leptin and leptin receptors (LR) have been demonstrated in the

mammary gland and, therefore, it may be involved in local regulation of mammary development and function (Laud *et al*., 1999; Chilliard *et al*., 2001; Bonnet *et al*., 2002). In ruminants leptin is thought to be a mediator of the negative effects of over nutrition on the mammary gland and is associated with decreased proliferation of MECs (Silva *et al*., 2002). Heifers which were reared on a high plane of nutrition prior to puberty were found to have increased plasma leptin levels, and reduced epithelial cell proliferation (Block *et al*., 2003). More recently, in mammary HC11 cell lines (Motta *et al*., 2007) and in cultured caprine mammary explants (Li *et al*., 2010), leptin has been shown to influence Janus-Kinase (JAK) signalling pathways, working in synergy with prolactin to increase expression of milk protein genes and MEC differentiation. However, the actions of leptin on the mammary gland is not fully understood and it is unknown if it has a role in mediating effects of over nutrition during foetal life on mammary gland development and function of adult offspring.

1.5.7 Growth factors

In addition to IGFs, which play a role in mediating effects of oestrogen and GH, a number of other locally produced growth factors are thought to be involved in post-natal mammogenesis (Hovey *et al*., 1999; Akers, 2006). Members of the epidermal growth factor (EGF) family have been shown to be key regulators of mammary growth in rodent models, particularly through stromal-epithelial cell interactions (Plaut, 1993; Forsyth, 1996; Akers, 2006). The EGF receptor (EGFR) plays a vital role in ductal elongation, at least in mice, where knock-out models have found that ductal growth does not occur in its absence (Wiesen *et al*., 1999). The EGF receptor has been detected in the ruminant mammary gland suggesting it may function in local regulation of mammary development (Akers, 2006). Other members of the EGF family, including transforming growth factor (TGF) which has TGFα and TGFβ isoforms, also appear to affect ductal growth and branching. TGFs appear to act as inhibitors of ductal branching and epithelial cell differentiation (Plaut, 1993). The TGFs also have a role in mammary gland regression during involution. Other growth factors including, hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), fibroblastic growth factor (FGF), and keratinocyte growth factor (KGF) may also be involved in mammogenesis (Hovey *et al*., 1999; Capuco & Akers, 2010).

1.6 Investigating gene expression differences in the mammary gland

As previously discussed, differential maternal nutrition can affect foetal mammary gland and adult offspring lactational performance. These effects may be, in part, mediated by differences in gene expression in the mammary gland. Recent advances in high-throughput sequencing, such as the advent of RNA-seq, provide tools to be able to investigate gene expression differences at the genome-wide scale (Mortazavi *et al*., 2008; Wang *et al*., 2009; McCabe *et al*., 2012; Wickramasinghe *et al*., 2012; Cameron *et al*., 2013b). RNA-seq is a recently developed approach to transcript profiling that uses deep sequencing technologies. RNA-seq provides a precise measurement of the levels of transcripts and, therefore, may be used to enable greater insight into the transcriptomic regulation of mammary development and molecular events underlying developmental programming. In other species (rodent, bovine and caprine), functional genomics research has highlighted the complex and coordinated networks of genes and molecular pathways involved in regulation of mammary development and lactation (Naylor *et al*., 2005; Ollier *et al*., 2007; Finucane *et al*., 2008; Casey *et al*., 2011; Bionaz *et al*., 2012). These studies have greatly advanced knowledge of the molecular processes and gene networks involved in mammary development, lactogenesis (Naylor *et al*., 2005; Finucane *et al*., 2008; Bionaz *et al*., 2012), milk fat synthesis (Bionaz & Loor, 2008), milk protein synthesis (Bionaz & Loor, 2011) and metabolism. Coordinated, large-scale, differences in gene expression are required to govern mammary development and function.

Transcriptomics technologies have also been proven useful for examining effects of specific treatments (e.g., nutrition, Ollier *et al*., 2007) or management practices (e.g., milking frequency, Littlejohn *et al*., 2010) on mammary growth and milk production. The findings of these studies will help pave the way for development of interventions and management strategies to improve lactation performance in production animals. To date, there are no studies that use RNA-seq to profile gene expression changes in the ovine mammary gland, and there are no studies that examine the effects of nutritional programming during foetal life on whole-scale gene expression in the mammary gland of adult offspring.

1.7 Summary and research objectives

In summary, maternal nutrition can affect the foetal mammary gland and subsequent milk production during the first lactation. It is unknown if this difference offers a benefit over their productive lifetime. Based on the research of Martin *et al.* (2012), the critical programming period is hypothesised to be early gestation, although differences on offspring lactational performance are yet to be investigated. Furthermore, the molecular mechanisms are yet to be elucidated. Genes involved in milk production are widespread and variable emphasising the enormity of defining mechanisms behind programming of lactational performance.

Based on the previous research it is hypothesized that:

1) Early pregnancy dam nutrition rather than mid-to-late pregnancy nutrition will affect offspring mammary gland development and lactation performance.

2) Over- and under-feeding of the dam will result in decreased lactation performance of the ewe progeny.

3) That gene expression, during late pregnancy mammogenesis and/or lactation, in the mammary gland of ewe offspring may be affected by maternal nutrition.

Therefore the aims of this thesis were to examine:

a) The lifetime effects of maternal nutrition, and dam weight, on offspring lactational performance (Chapter 2).

b) To determine the critical period for 'programming' of offspring puberty attainment, parasite susceptibility, body composition, LW and growth performance until breeding. (Chapter 4).

c) To determine the critical period for 'programming' of the mammary gland *in utero* which results in lactational differences (Chapter 5).

d) To examine the effects of granddam nutrition on grand-offspring growth performance (Chapter 6).

e) To determine the mechanisms behind maternal nutritional programming of offspring mammary gland function using transcriptomics (Chapter 7). This includes validation of transcriptome results with quantitative PCR (RT-qPCR) using stably expressed reference genes to normalise data. A house-keeping gene study was therefore conducted to identify appropriate reference genes (Chapter 8).

2 Effect of dam weight and pregnancy nutrition on average lactational performance of ewe offspring over five years

2.1 Abstract

The foetal mammary gland is sensitive to maternal weight and nutrition during gestation. This, in-turn, can affect offspring milk production. A previous study showed that ewes born to dams offered maintenance nutrition during pregnancy (day 21 to 140 of gestation) produced greater milk, lactose and crude protein yields in their first lactation when compared with ewes born to dams offered *ad libitum* nutrition. In addition, ewes born to heavier dams (ave. dam LW: 60.8 kg ± 0.18) produced greater milk and lactose yields when compared to ewes born to lighter dams (ave. dam LW: 42.5kg ± 0.17). While the first lactation performance is altered, it is unknown if dam weight or gestational nutrition has lasting effects on the milk production of ewe offspring. The objective of this study was to analyse and compare the lifetime lactation performance of the previously mentioned ewes, born to heavy or light dams that were offered maintenance or *ad libitum* pregnancy nutrition. Ewes were milked once per week, for the first six weeks of their lactation, for five years. Using daily milk yield and composition data, accumulated yields were calculated over a 42-d period for each year for milk, milk fat, crude protein, true protein, casein and lactose using a Legendre orthogonal polynomial model. Over the five-year period it was found that ewes born to heavy dams produced greater accumulated milk (P = 0.02), lactose (P = 0.01), crude protein (P = 0.02), true protein (P = 0.02) and casein (P = 0.03) yields than offspring born to light dams, while accumulated milk fat yields did not differ (P = 0.77). After adjusting for ewe live weight prior to lactation (day 140 of gestation), the differences in milk (P = 0.05) and lactose (P= 0.02) yields remained, and the differences in crude protein (P = 0.07), true protein (P = 0.09) and casein (P = 0.09) yields tended to remain. In addition, milk fat (P = 0.07) yields also tended to be greater in ewes born to heavy dams. Dam nutrition during pregnancy did not affect (P < 0.05) offspring milk yields or composition. These results indicate that maternal gestational nutrition appears to only affect the first-lactation performance of offspring, while dam weight can have lifelong effects on milk production of ewe offspring.

2.2 Introduction

Maternal influences (e.g., diet and weight) during early developmental stages, particularly during foetal life, can lead to physiological and metabolic changes which may have consequences later in life, often referred to as foetal programming (Barker, 1997; Hales & Barker, 1992; Hales & Barker, 2001; Gluckman & Hanson, 2004). With regard to livestock species, management of the intrauterine environment could be of importance for the productive performance of offspring (Bell, 2006, Wu *et al*., 2006; Kenyon, 2008; Ashworth *et al*., 2009). Production traits, including growth and reproductive performance, have been demonstrated to be affected by factors including birth rank, and nutrition, weight, body condition and age of the dam (Kenyon & Blair, 2014). However, there are few studies that investigate the implications of an adverse intrauterine environment on the performance of offspring over their entire productive lifetime. Knowledge of this would be useful, particularly with regards to selecting replacement animals for breeding.

Milk production of the ewe is an important contributing factor to her overall breeding efficiency. Milk is the sole source of nutrients for the lamb during the first three to four weeks of life (Degen & Benjamin, 2005), and is correlated with lamb survival and growth to weaning (Mellor, 1983). Previous research has demonstrated that the foetal mammary gland may be susceptible to programming effects from maternal pregnancy nutrition. Blair *et al. (*2010) reported that above-maintenance (1.5 × maintenance), compared with maintenance nutrition of the dam during gestation (days 19 to 137 of pregnancy), increased the ductal area in mammary glands, measured in late gestation foetuses, with no effects on the mammary gland mass. In contrast, van der Linden *et al. (*2009) reported that late gestation foetuses from dams offered *ad libitum* nutrition during gestation (days 21 to 140 of pregnancy) had lighter mammary glands compared to foetuses from dams offered maintenance nutrition, with no difference in duct area. In addition to maternal pregnancy nutrition, weight of the dam may also affect development of the foetal mammary gland. In the study of van der Linden *et al. (*2009) foetuses from heavy dams had reduced mammary gland duct area when compared with foetuses from light dams. Additionally, van der Linden *et al. (*2009) retained a cohort of ewe offspring for milking. They demonstrated that ewes born to dams offered *ad libitum* nutrition produced less milk with lower lactose and crude protein (CP) yields at their first lactation, when compared to ewes born to dams offered maintenance nutrition. In the same study, ewes born to light dams also produced less milk than ewes born to heavy dams. More importantly, the lower milk production of ewes born to dams offered *ad libitum* nutrition and heavy dams led to them weaning lighter lambs (van der Linden *et al*., 2009). In their second lactation, however, there were no differences in milk production based on dam live weight or pregnancy nutrition (Blair *et al*., 2010). This finding might suggest that the mammary gland may be able to 'reset' during

involutionary remodelling and redevelopment in subsequent lactation cycles, which may result in no long-term production effects.

The ewe offspring from the first parity study of van der Linden *et al. (*2009) and the second parity study of Blair *et al. (*2010) were retained by our research group. Milk production and lambing data were collected for the third, fourth, and fifth parities. The objective of this study was to utilise the data collected over the five parities, to evaluate the effects of dam weight and nutrition on the lactational and lambing performance of the ewe offspring.

2.3 Materials and methods

This study was conducted at the Massey Keeble Sheep and Beef farm, 5 km south of Palmerston North, New Zealand (Longitude: 175°36'36"E, latitude: 40°21'00"S, winter solstice 21st June). The study and all handling procedures were approved by the Massey University Animal Ethics Committee, Palmerston North, New Zealand.

2.3.1 Background of dam treatments

Briefly, as previously described by Kenyon et al. (2009), in 2005 Romney ewes that were either the heaviest (Hv: *n* = 450, LW = 60.8 ± 0.18 kg, and body condition score (BCS; scale 1 – 5) = 3.02 ± 0.03) or the lightest (Lt: *n* = 450, LW = 42.5 ± 0.17 kg, and BCS = 1.97 ± 0.03) were selected from a commercial flock of 2,900 and bred using artificial insemination (AI) and semen from Suffolk rams. From day 21 of pregnancy (P21), ewes (*n* = 612) were randomly allocated to one of two nutritional regimens until day 140 of pregnancy (P140): maintenance (M), or *ad libitum* (Ad), under pastoral grazing conditions. The average pre- and post-grazing pasture covers during P21 to P140 were: 1330 kg DM/ha ± 156.8 and 804.0 kg DM/ha ± 133.4 for the maintenance treatment. The average pre- and post-grazing covers for the *ad libitum* regimen were 2304.0 kg DM/ha ± 156.8 and 1723.3 kg DM/ha ± 149.7, respectively. The study, therefore, utilized a two-by-two factorial design including: heavy-*ad libitum* (HvAd), heavy-maintenance (HvM), light-*ad libitum* (LtAd) and light-maintenance (LtM), all of which contained both singleton- and twin-bearing dams.

From day 141 of gestation, all ewes were merged for lambing and managed under *ad libitum* nutrition conditions. For the remainder of the study the ewe offspring, born in 2005, were managed under New Zealand commercial grazing conditions.

2.3.2 Present study

To evaluate the effects of dam weight and nutrition on the ewe-progeny lifetime lactational performance, cohorts of twin-bearing adult ewe offspring, born to the previously mentioned Lt or Hv

dams offered either M or Ad nutrition were milked for five consecutive years; from 2007 to 2011, and the LW of their lambs, from birth until weaning, was recorded (see Table 2.1 for details).

2.3.3 Ewe live weight and body condition

In the cohorts of twin-bearing ewe offspring retained for milking in 2007, 2008, 2009, 2010 and 2011, LW and BCS were measured at breeding (P0), late pregnancy and weaning of their lambs (refer to Table 2.1 for number of animals for each year).

2.3.4 Lactation performance

In 2007, 2008, 2009, 2010 and 2011, breeding of the ewe-progeny was synchronised. A sub-sample of ewes that were identified as twin-bearing at pregnancy diagnosis, using trans-abdominal ultrasound, were retained for milking. Ewes were milked using the oxytocin method (McCance, 1959) once-per-week for the first six weeks of their lactations, starting at an average day 7 postpartum (L7). Ewes were milked in the morning and, after an interval of approximately 5 h, again in the afternoon. The time of each milking and weight (kg) of milk obtained was recorded. The lambs were separated from the ewes between morning and afternoon milking to prevent suckling and were bottle-fed as required. Daily MY was calculated using the formula:

$$\frac{24\text{ h}}{\text{time between milkings}} \times \text{MY at 2nd milking}$$

Milk obtained by machine and hand-stripping was mixed and sub-sampled for analysis of milk composition yields [fat yield (FY), crude protein yield (CPY), true protein yield (TPY), casein yield (CY) and lactose yield (LY)] by Fourier transform infrared spectroscopy (Milkoscan FT120, FOSS, Hillerød, Denmark) as described by van der Linden *et al*. (2009). The 2007 and 2008 milking data has been reported separately by van der Linden *et al*. (2009) and Blair *et al*. (2010), respectively.

2.3.5 Lamb live weights

In 2007, 2008, 2009, 2010 and 2011, within 24 h after birth, lambs were identified to their dam and weighed. Twin-born lambs, whose dams were retained for milking, were weighed weekly during the 6-wk milking period, and at weaning (Table 2.1). The present study only utilises the LW of the twin lambs born to ewes retained for milking. Data of lamb LW during the first 6 weeks of each lactation only included twin-born and twin-reared lambs. However, not all ewes successfully weaned both lambs, therefore, the data at weaning included both twin-born and reared lambs as well as twin-born and single-reared lambs.

2.3.6 Statistical analysis

2.3.6.1 Animal numbers.

All data were analysed with ewe as the experimental unit. To minimize variation from differing pregnancy and rearing rank, only data from ewes with complete twin-sets for the duration of the milking period were included. For this reason individual ewes may not be represented in each year of the study.

Table 2.1 Number of animals milked during each of the five experimental years (2007 until 2011), for each of the dam nutrition (maintenance; M, or *ad libitum*; Ad) × live weight (light; Lt, or heavy; Hv) treatments: MLt = maintenance-fed, light dam; MHv = maintenance-fed, heavy dam; AdLt = *ad libitum*-fed, light dam; and AdHv = *ad libitum*-fed, heavy dam.

	Year				
	2007	2008	2009	2010	2011
Ewe treatment					
MLt	12 (24)[1]	11 (22)	14 (28)	14 (28)	8 (16)
MHv	21 (42)	18 (36)	15 (30)	11 (22)	14 (28)
AdLt	15 (30)	10 (20)	12 (24)	10 (20)	9 (18)
AdHv	24 (48)	13 (26)	13 (26)	10 (20)	10 (20)
Total	72 (144)	52 (104)	54 (108)	45 (90)	41 (82)
Activity					
Breeding[2]	P02	P0	P0	P0	P0
Late pregnancy[2]	P140	P141	P142	P139	P134
Lambing[3]	L13	L1	L1	L1	L1
Weaning[3]	L78	L94	L97	L107	L101

[1](*n*) = number of lambs

[2]P = day of pregnancy

[3]L = day of lactation

2.3.6.2 *Calculation of lactation yields.*

Using daily milk yield (MY) data calculated from the once-per-week milkings in 2007, 2008, 2009, 2010 and 2011, accumulated yields were calculated for each lactation. This was done by fitting a third-degree orthogonal polynomial model to milk yield and composition data for each ewe milked each year:

$$yi = \alpha_0 \, \emptyset_{0i} + \alpha_1 \, \emptyset_{1i} + \alpha_2 \, \emptyset_{2i} + \alpha_3 \, \emptyset_{3i} + e_i$$

Where y_i is the record of milk or composition taken at day *I*, α_n is the *n* regression and $\emptyset_{ni}$ is rescaled value of days in milk *i* calculated as:

$$\emptyset_{oi} = 1; \; \emptyset_{1i} = x; \; \emptyset_{2i} = \frac{(3x^2 - 1)}{2}; \emptyset_{3\mathrm{i}} = \frac{(5x^3 - 3x)}{2}; \text{ where } x = \frac{2\big(i - (50+1)\big)}{(50-1)},$$

where e_i is the error term associated with each milk record.

Accumulated MY and composition (FY, CPY, TPY, CY and LY) yields were calculated over L1 to L42 for each ewe using estimates calculated from the regression coefficients of the third-degree orthogonal polynomial model.

Milk net energy (NE, MJ/L) was calculated for each ewe for each lactation using the following equation, based on cattle, from Holmes *et al.* (2002):

$NE = 0.376 \times F\% + 0.209 \times CP\% + 0.976$ (MJ/L),

where $F\%$ is milk fat percentage and $CP\%$ is milk crude protein percentage.

Accumulated milk net energy yield (NEY) was then calculated by multiplying the NE by the accumulated milk yield (kg) calculated from the Legendre orthogonal polynomial model.

Differences in the shape of the lactation curve were analysed by comparing the differences in MY at the start (day 1, MY_1), and end (day 42, MY_{42}) of the milking trial period. Differences in peak milk yield (MY_P) and day of peak milk yield (DMY_P), obtained from the Legendre orthogonal polynomial estimates, were also analysed. Lactation persistency for each parity was calculated by two measures: the first measure (Lactation persistency 1) was determined by the rate of decline of milk yield from DMY_P to day 42 of lactation, calculated by the following equation:

$$MY\ decline = \frac{MY_P - MY_{42}}{42 - dMY_p}$$

Where MY_P is milk yield at day of peak of lactation, MY_{42} is milk yield at day 42 of lactation, and dMY_p is day of peak of lactation. This persistency measure is useful for investigating differences in the decline of milk yield after peak lactation. Differences in decline of milk yield may indicate variation in rates of apoptosis and/or metabolic activity of milk secretory cells among the treatments.

The second measure (lactation persistency 2) was determined by dividing the lactation measurement period into three equal periods (A: L1 to L14, B: L15 to L28, and C: L29 to L42). The area under the curve (AUC) for each period was calculated to determine the correlation between AUC_C and AUC_A (AUC_c/AUC_A). The more correlated AUC_C and AUC_A (i.e. Closer to 1.0); the more persistent the lactation was considered. This persistency measure considers the early lactation period as well as the decline in milk yield after peak lactation and is more representative of changes in the lactation curve than persistency measure 1.

2.3.6.3 Analysis of lactation yields

Effects on lifetime MY, LY, FY, CPY, TPY, CY, NEY, MY_1, MY_{42}, MY_P, day of MY_P, lactation persistency (measure one and two), and lamb LW at, and growth, during birth and weaning were analysed. While each treatment was represented in each year, the individual animals varied; therefore, a repeat measure analysis was not used. Rather, a PROC MIXED in SAS 9.2 (SAS) was used with fixed effects of dam weight, dam nutrition, ewe birth rank and parity. In addition the two-way interaction of dam weight × dam nutrition and the three-way interaction of dam weight × dam nutrition × parity were included in models. Interactions which were not significant were not removed from the model in order to test the study design. Analyses of MY and composition yields of ewes were run both with and without ewe LW in late pregnancy fitted as a covariate. Analyses of lamb LW at birth and at weaning were carried out using the combined LW of both lambs (in a twin pair), with ewe fitted as a random effect. All lambs in the analyses were twin-born, however, not all ewes in the study successfully reared twins. Therefore, the number of lambs weaned per ewe was

fitted as a covariate for the analysis of lamb LW at weaning. Data of ewes which did not successfully wean any lambs were removed from the analysis. In addition, the analysis of lamb LW at weaning was run both with and without lamb LW at birth as a covariate.

2.4 Results

2.4.1 Average ewe live weight and condition score over five parities

Dam nutrition had a tendency to affect the average ewe LW at breeding over five years, such that ewe offspring born to Ad-dams tended (P = 0.07) to be heavier (by 2.3 ± 1.27kg) at breeding than ewe offspring born to M-dams (Table 2.2). The average LW of ewe offspring, over five years, during late pregnancy and weaning of their lambs was unaffected (P > 0.05) by dam nutrition during pregnancy.

Dam weight did not affect (P > 0.05) the average LW, over the five years, of ewe offspring at breeding and during late pregnancy. Ewes born to Hv-dams tended (P = 0.08) to be heavier, themselves, at weaning of their lambs, when compared to ewes born to Lt-dams (Table 2.2).

The average BCS, over five years, of ewe offspring at breeding, late pregnancy and weaning of their lambs was unaffected (P > 0.05) by dam weight and dam nutrition during pregnancy.

The average ewe LW and BCS, at breeding, late pregnancy and weaning of their lambs, varied over the five lactations (P <0.0001). Ewe LW at mating was the lowest prior to the first lactation. In addition, ewes had the lowest BCS at mating, breeding and weaning of their lambs in their first lactation. Ewe LW during late pregnancy, however, was the lowest during the second lactation, and LW at weaning of their lambs was lowest during the third lactation. Ewes had the heaviest average LW at breeding, late pregnancy and weaning of their lambs in their fifth lactation.

Table 2.2 Least-squares means ± SEM of average LW (kg) and BCS, over five parities, at breeding, late pregnancy and weaning of their lambs, of ewes born to either heavy (Hv) or light (Lt) dams that were fed either maintenance (M) or *ad libitum* (Ad) nutrition during pregnancy days 21 to 140 (P21-140).

	Live weight (kg)			Body condition score		
Treatments	Breeding	Late pregnancy	Weaning of lambs	Breeding	Late pregnancy	Weaning of lambs
Dam nutrition						
M	68.1 ± 0.84	76.6 ± 0.87	64.3 ± 0.72	3.0 ± 0.06	2.3 ± 0.05	2.2 ± 0.04
Ad	70.3 ± 0.90	77.9 ± 0.92	65.4 ± 0.77	2.9 ± 0.06	2.3 ± 0.05	2.1 ± 0.04
P value	0.07	0.35	0.31	0.23	0.30	0.30
Dam weight						
Lt	68.6 ± 0.89	76.6 ± 0.89	64.1 ± 0.77	3.0 ± 0.06	2.3 ± 0.05	2.2 ± 0.04
Hv	69.8 ± 0.78	78.3 ± 0.86	66.3 ± 0.73	3.0 ± 0.05	2.3 ± 0.04	2.1 ± 0.04
P value	0.30	0.21	0.08	0.51	0.40	0.40
Parity (year)						
1 (2007)	61.6 ± 0.89^{a}	75.9 ± 0.83^{b}	62.9 ± 0.67^{b}	2.5 ± 0.07^{a}	2.1 ± 0.05^{a}	1.4 ± 0.04^{a}
2 (2008)	67.8 ± 1.02^{b}	64.8 ± 0.94^{a}	64.1 ± 0.75^{bc}	3.2 ± 0.08^{c}	2.2 ± 0.10^{ab}	2.7 ± 0.05^{c}
3 (2009)	74.6 ± 1.00^{d}	78.8 ± 0.91^{c}	59.6 ± 0.72^{a}	3.7 ± 0.07^{d}	2.4 ± 0.05^{b}	2.3 ± 0.05^{b}
4 (2010)	70.0 ± 1.10^{bc}	80.2 ± 1.01^{c}	66.2 ± 0.79^{c}	2.9 ± 0.08^{bc}	2.4 ± 0.06^{b}	2.2 ± 0.06^{b}
5 (2011)	71.9 ± 1.13^{cd}	86.7 ± 1.02^{d}	71.5 ± 0.80^{d}	2.7 ± 0.08^{ab}	2.3 ± 0.06^{b}	2.2 ± 0.06^{b}
P value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

$^{a, b, c, d}$Differing superscripts within columns within treatments, indicate least-squares means that are significantly different ($P < 0.05$).

2.4.2 Milk and composition yields

Nutrition of the dam during pregnancy influenced the performance of ewe offspring during their first lactation such that ewes born to Ad-dams had reduced MY (P = 0.03) and LY (P = 0.01) compared with ewes born to M-dams (Tables 2.3 for MY and 2.4 for LY). There were, however, no differences (P > 0.05), attributed to dam nutrition, on ewe-offspring lactational performance during any other parity (second, third, fourth and fifth lactations). Ultimately, dam nutrition during pregnancy did not (P > 0.05) affect the average lactational performance of ewe offspring over five years, as evidenced by no differences in average MY, FY, CPY, TPY, CY, LY and NEY (Table 2.5).

Ewes born to Hv-dams tended to produce greater MY (P = 0.07, Table 2.3) and produced greater LY (P = 0.03, Table 2.4) during their first lactation, compared with ewes born to Lt-dams. Dam weight did not (P > 0.05) affect ewe-offspring MY and LY produced in their second, third, fourth or fifth lactations. However, on average over the five years, ewes born to Hv-dams produced greater MY (P = 0.04), CPY (P = 0.04), LY (P = 0.01), and tended to produce greater TPY (P = 0.06) and CY (P = 0.06), compared with ewes born to Lt-dams. There were no differences (P > 0.05) in average milk FY and NEY produced by ewe offspring.

2.4.3 Lactation curve and persistency

Dam weight and dam nutrition during pregnancy had no effect on the five-year average $MY_{1,}$ MY_{42}, MY_{P}, DMY_{P} (Table 2.6). Lactation persistency, as measured by rate of decline in milk yield from peak to day 42 (Persistency 1) and correlation between late and early lactation (persistency 2) also did not differ.

Table 2.3 Least-square means ± SEM of yearly average accumulated milk yield (MY, kg), for five consecutive lactations, of twin-bearing ewes born to heavy (Hv) or light (Lt) dams that were fed either maintenance (M) or *ad libitum* (Ad) nutrition during pregnancy days 21 to 140 (P21-140).

	Lactation (year)				
	One (2007)	Two (2008)	Three (2009)	Four (2010)	Five (2011)
Dam nutrition					
M	115.1 ± 2.46[b]	99.9 ± 2.24	116.9 ± 3.71	105.0 ± 3.57	132.4 ± 3.80
Ad	107.7 ± 2.24[a]	103.3 ± 2.52	125.1 ± 4.07	110.7 ± 4.07	137.0 ± 4.06
P value	0.03	0.36	0.19	0.32	0.45
Dam weight					
Lt	108.4 ± 2.60	101.0 ± 2.41	117.7 ± 3.45	106.4 ± 3.48	131.0 ± 3.90
Hv	114.4 ± 2.01	102.2 ± 2.01	124.3 ± 3.33	109.2 ± 3.75	138.5 ± 3.31
P value	0.07	0.69	0.17	0.59	0.15

[a, b]Differing superscripts within columns, within treatments, indicate least-square means that are significantly different ($P < 0.05$).

Table 2.4 Least-square means ± SEM of yearly average accumulated lactose yield (LY, kg), for five consecutive lactations, of twin-bearing ewes born to heavy (Hv) or light (Lt) dams that were fed either maintenance (M) or *ad libitum* (Ad) nutrition during pregnancy days 21 to 140 (P21-140).

	Lactation (year)				
	One (2007)	Two (2008)	Three (2009)	Four (2010)	Five (2011)
Dam nutrition					
M	6.1 ± 0.13[b]	5.2 ± 0.12	6.3 ± 0.21	5.4 ± 0.22	7.0 ± 0.21
Ad	5.6 ± 0.17[a]	5.4 ± 0.14	6.7 ± 0.22	5.7 ± 0.25	7.2 ± 0.22
P value	0.01	0.26	0.22	0.45	0.60
Dam weight					
Lt	5.7 ± 0.14[a]	5.3 ± 0.13	6.3 ± 0.19	5.4 ± 0.21	6.9 ± 0.21
Hv	6.0 ± 0.11[b]	5.3 ± 0.11	6.7 ± 0.18	5.7 ± 0.23	7.3 ± 0.18
P value	0.03	0.75	0.13	0.39	0.16

[a, b]Differing superscripts within columns, within treatments, indicate least-square means that are significantly different ($P < 0.05$).

Table 2.5 Least-squares means ± SEM of average lactation yields (kg), over five lactations, for: milk (MY), fat (FY), crude protein (CP), true protein (TP), casein (CY) and lactose (LY), and average milk net energy yields (NEY, MJ) of ewes born to either heavy (Hv) or light (Lt) dams that were fed either maintenance (M) or *ad libitum* (Ad) nutrition during pregnancy days 21 to 140 (P21-140).

	Yield (kg)						
Treatments	MY	FY	CPY	TPY	CY	LY	NEY (MJ)
Dam nutrition							
M	114.6 ± 1.54	8.2 ± 0.15	5.7 ± 0.08	5.2 ± 0.07	4.5 ± 0.06	6.0 ± 0.08	539.2 ± 7.81
Ad	115.0 ± 1.63	8.3 ± 0.16	5.6 ± 0.08	5.2 ± 0.08	4.5 ± 0.06	6.0 ± 0.09	540.0 ± 8.28
P value	0.87	0.87	0.64	0.63	0.66	0.95	0.94
Dam weight							
Lt	112.6 ± 1.60^{a}	8.2 ± 0.16	5.5 ± 0.08^{a}	5.1 ± 0.07	4.4 ± 0.06	5.9 ± 0.09^{a}	535.3 ± 8.12
Hv	117.0 ± 1.43^{b}	8.2 ± 0.14	5.7 ±0.07^{b}	5.3 ± 0.07	4.6 ± 0.06	6.2 ± 0.08^{b}	543.9 ± 724
P value	0.04	0.98	0.04	0.06	0.06	0.01	0.43
Parity (year)							
1 (2007)	110.7 ± 1.76^{b}	9.0 ± 0.17^{b}	5.1 ± 0.09^{a}	4.6 ± 0.08^{a}	4.0 ± 0.07^{a}	5.8 ± 0.10^{b}	551.7 ± 8.98^{b}
2 (2008)	101.7 ± 2.04^{a}	7.6 ± 0.19^{a}	5.0 ± 0.10^{a}	4.6 ± 0.10^{a}	4.0 ± 0.08^{a}	5.3 ± 0.12^{a}	489.5 ± 10.41^{a}
3 (2009)	119.8 ± 1.98^{c}	8.1 ± 0.19^{a}	6.0 ± 0.10^{b}	5.6 ± 0.09^{b}	4.9 ± 0.08^{b}	6.4 ± 0.11^{c}	545.32 ± 10.14^{b}
4 (2010)	107.7 ± 2.22^{ab}	7.5 ± 0.21^{a}	5.3 ± 0.11^{a}	4.9 ± 0.11^{a}	4.2 ± 0.09^{a}	5.6 ± 0.13^{ab}	498.7 ± 11.33^{a}
5 (2011)	134.0 ± 2.27^{d}	9.0 ± 0.21^{b}	6.8 ± 0.12^{c}	6.3 ± 0.11^{c}	5.3 ± 0.10^{c}	7.2 ± 0.13^{d}	612.7 ± 11.61^{c}
P value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

$^{a, b, c, d}$Differing superscripts within columns within treatments, indicate least-squares means that are significantly different ($P < 0.05$).

Table 2.6 Least-squares means ± SEM of average milk yields (kg): at one day after the mid-point of lambing (MY1), at peak production (MYP), and at day 42 post-partum (MY42); and of average day of peak milk yield (DMYP); and lactation persistency, 1.) as measured by decline in milk yield from peak to day 42 post-partum (persistency 1) and, 2.) as measured by dividing the lactation measurement period into three equal periods (A: day 1 to 14, B: day 15 to 28, and C: day 29 to 42 of lactation); the area under the curve (AUC) for each period was calculated to determine the correlation between AUC_C and AUC_A (AUC_C/AUC_A). The more correlated AUC_C and AUC_A (i.e. Closer to 1.0) the more persistent the lactation was considered as measured by the (persistency 2). Measured were made over five lactations, of ewes born to either heavy (Hv) or light (Lt) dams that were fed either maintenance (M) or *ad libitum* (Ad) nutrition during pregnancy days 21 to 140 (P21-140).

					Persistency (g/day)	
Treatments	MY_1 (kg)	MY_P (kg)	DMY_P (days)	MY_{42} (kg)	Persistency 1	Persistency 2
Dam nutrition						
M	3.0 ± 0.13	3.6 ± 0.09	9.1 ± 0.76	2.2 ± 0.04	43.8 ± 2.29	0.83 ± 0.035
Ad	3.1 ± 0.14	3.7 ± 0.10	9.4 ± 0.80	2.2 ± 0.04	44.1 ± 2.41	0.74 ± 0.037
P value	0.45	0.61	0.81	0.96	0.93	0.10
Dam weight						
Lt	3.0 ± 0.14	3.6 ± 0.09	9.7 ± 0.77	2.2 ± 0.04	44.3 ± 2.35	0.82 ± 0.036
Hv	3.1 ± 0.12	3.7 ± 0.09	8.8 ± 0.69	2.2 ± 0.04	43.6 ± 2.10	0.75 ± 0.032
P value	0.56	0.67	0.38	0.66	0.83	0.13
Parity (year)						
1 (2007)	2.3 ± 0.17^{a}	3.1 ± 0.12^{a}	14.1 ± 0.98^{b}	2.2 ± 0.05^{b}	30.8 ± 2.85^{a}	0.98 ± 0.041^{b}
2 (2008)	2.7 ± 0.20^{ab}	3.3 ± 0.14^{ab}	8.9 ± 1.14^{a}	2.0 ± 0.05^{c}	39.1 ± 3.31^{a}	0.89 ± 0.047^{b}
3 (2009)	3.5 ± 0.20^{c}	4.1 ± 0.13^{c}	6.9 ± 1.12^{a}	2.1 ± 0.05^{c}	53.8 ± 3.24^{b}	0.65 ± 0.046^{a}
4 (2010)	3.1 ± 0.22^{bc}	3.7 ± 0.15^{bc}	7.4 ± 1.25^{a}	1.7 ± 0.06^{a}	57.6 ± 3.63^{b}	0.52 ± 0.052^{a}
5 (2011)	3.6 ± 0.23^{c}	4.2 ± 0.15^{c}	8.7 ± 1.30^{a}	2.8 ± 0.06^{d}	38.2 ± 3.82^{a}	0.88 ± 0.053^{b}
P value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

$^{a, b, c, d}$Differing superscripts within columns within treatments, indicate least-squares means that are significantly different ($P < 0.05$).

2.4.4 Lamb live weights and growth to weaning

There were no ($P > 0.05$) 3-way (dam parity × granddam nutrition × granddam weight) or 2-way interactions (granddam nutrition × granddam weight) on LW of grand-offspring at birth or on their growth from birth to weaning (data not shown). There was, however, a tendency for a 3-way interaction of dam parity × granddam nutrition × granddam weight on grand-offspring LW at weaning, such that during the first parity, grand-offspring of AdLt-granddams were lighter than grand-offspring from all other treatments (AdLt = 44.5 ± 1.45 vs. AdHv = 50.4 ± 1.16, MLt = 52.5 ± 1.61 and MHv = 50.6 ± 1.22 kg). This difference was not observed in later parities. In addition, the interaction was no longer present when the model was adjusted for lamb LW at birth (data not shown).

There were no effects of granddam nutrition, granddam weight or dam birth rank on the LW of grand-offspring at birth and at weaning, or on their growth rates from birth until weaning (Table 2.7).

Table 2.7 Least-squares means ± SEM, recorded for five years (2007 to 2011), of average combined LW (kg) at birth and weaning, and growth from birth to weaning, of lambs born to ewes that were born to either heavy (Hv) or light (Lt) dams that were fed either maintenance (M) or *ad libitum* (Ad) nutrition during pregnancy days 21 to 140 (P21-140).

Treatments	Combined lamb LW at birth (kg)	Combined lamb LW at weaning (kg)	Combined lamb LWG birth to weaning (g/day)
Granddam nutrition			
M	9.7 ± 0.12	55.6 ± 0.51	509.1 ± 5.18
Ad	9.6 ± 0.13	55.2 ± 0.54	506.9 ± 5.48
P value	0.29	0.59	0.76
Granddam weight			
Lt	9.6 ± 0.13	54.9 ± 0.55	504.2 ± 5.66
Hv	9.7 ± 0.11	55.9 ± 0.50	511.7 ± 5.07
P value	0.63	0.19	0.32
Parity (year)			
1 (2007)	9.0 ± 0.14^{a}	49.6 ± 0.67^{a}	529.8 ± 6.20^{bc}
2 (2008)	9.2 ± 0.16^{a}	57.9 ± 0.79^{bc}	519.4 ± 7.27^{b}
3 (2009)	9.2 ± 0.16^{a}	55.2 ± 0.77^{b}	472.9 ± 7.03^{a}
4 (2010)	10.2 ± 0.17^{b}	59.66 ± 0.86^{c}	464.0 ± 7.86^{a}
5 (2011)	10.7 ± 0.18^{b}	54.8 ± 0.90^{c}	553.9 ± 8.23^{c}
P value	<0.0001	<0.0001	<0.0001

2.5 Discussion

Previous research has demonstrated that weight and pregnancy nutrition of the dam can affect first-parity lactational performance of offspring (van der Linden *et al*., 2009) while the second parity lactation appears to be unaffected (Blair *et al.,* 2010). The long-term effects on offspring lactation performance were unknown. The ewe offspring of the previously mentioned studies were retained and subsamples of twin-bearing ewes were milked each year for their third, fourth and fifth parities. Using milk production data from the five parities, the present study examined the effects of dam weight and pregnancy nutrition on the average lactational performance of offspring and the LW and growth of grand-offspring from birth to their weaning. In the present study there were no three-way interactions of parity × dam nutrition × dam weight, or two-way interactions of dam weight × dam nutrition on any of the ewe LW, BCS and milk production parameters measured in the offspring over the five lactations. The results, therefore, are discussed in relation to the main effects of dam nutrition and dam weight only.

2.5.1 Dam nutrition

Despite ewes born to Ad-dams having reduced milk yields in their first parity lactation, compared with ewes born to M-dams (van der Linden *et al.,* 2009), there appears to be no effect of dam pregnancy nutrition on offspring lactation performance over five parities. It appears that the programming effects in the first lactation are lost in subsequent parities thus explaining why there are no differences in lifetime lactational performance. It is possible that programmed differences may only be present in the mammary cells that undergo apoptosis during involution, and thus the effect is lost upon regression of the mammary gland after weaning (Akers & Capuco, 1999; Capuco & Ellis, 2013). In subsequent pregnancies the ewe offspring were maintained on the same nutrition and, therefore, may have undergone the same extent of mammary re-development. Furthermore, the adult ewe offspring were maintained on *ad libitum* nutrition which is unlikely to cause physiological stress. If the mismatch hypothesis is correct offspring born to M-fed dams may be programmed to be more thrifty under more constrained nutrition (Gluckman & Hanson, 2004); it may be worthwhile, in future studies, to test the productive performance of offspring in different post-natal nutritional environments.

Consistent with the lack of differences in average milk production over the five parities, there were no differences in the total birth weight, total weaning weight and total growth rates of lambs born to the subset of ewes that were milked. This finding is consistent with the study of Asmad, (2013) in which no differences were found on lifetime reproductive performance of ewe offspring (which included ewes in this analysis as well as those which were not milked). In this study

there were no differences in the number or weight of lambs weaned by ewe offspring, over their productive lifetime (Asmad, 2013). In terms of lamb production, collectively these findings indicate that there is no long-term advantage to be gained on future offspring performance through manipulating nutrition of the dam during pregnancy.

From a milk production perspective, however, it is interesting that the first lactation performance of offspring can be influenced by dam pregnancy nutrition. Understanding the mechanisms of this programming effect may help to explain the transient nature and may, therefore, be useful in developing manipulations or interventions to achieve long-term improvements of lactational performance. This should be a focus of future studies.

2.5.2 Dam weight

The study of van der Linden *et al. (*2009) demonstrated that offspring born to Hv-dams produced more milk in their first lactation with greater lactose yields compared with ewes born to Lt-dams. In the present study, there were no significant differences in milk yield each year, from 2008 to 2011, between ewes born to Lt- and heavy-dams. However, numerically ewes born to Hv-dams produced more milk and this is reflected in the average yearly production over five parities, such that of ewes born to Hv-dams had greater MY, LY, CPY, TPY and CY, compared with ewes born to Lt-dams. These findings demonstrate a long-term effect on offspring lactational performance as a result of dam weight. It is possible that ewes born to Lt-dams may have suffered from intrauterine growth restriction (IUGR): e.g., lighter dams may have less body reserves and reduced capacity to supply nutrients to the conceptus. In situations of IUGR, the foetus makes adaptation for immediate survival, such as partitioning nutrients to critical organs at the expense of less crucial tissues (Barker, 1995; McMillen & Robinson, 2005) such as the mammary gland. As a result, foetal mammary gland development may have been limited leading to impaired lactational performance in adult life. Indeed, consistent with this hypothesis, the study of van der Linden *et al. (*2009) reported smaller mammary duct area in foetuses of Lt-dams compared with foetuses from Hv-dams. These findings may be of relevance in dairy production enterprises as farmers may be able to select heavier dams for breeding in order to permanently improve milk production of the subsequent generation. Furthermore, studies using dairy cows and goats may be of interest in order to investigate if these results are consistent across species.

Despite ewes born to Hv-dams having higher milk production than ewes born to Lt-dams, there were no differences in the average LW of grand-offspring at weaning, indicating that the extra milk produced did not result in greater lamb growth. In the whole population, Asmad (2013) also reported no differences in the weights of lambs weaned. This finding raises the possibility that the

measure of milk production of the ewe does not reflect the intake of milk consumed by the lamb (van der Linden *et al.,* 2010c). Additionally, while milk is an important factor for lamb survival and growth, it is not the only influencing factor and solid feed intake (pasture), to some extent, will also contribute to lamb growth rates (Degen & Benjamin, 2005). Thus, while dam weight appears to have a lasting effect on the lactational performance of the ewe offspring, there appears to be no benefit for lamb production. Therefore, in lamb production enterprises, it may be more efficient to breed lighter dams, compared with heavier dams (Morel & Kenyon, 2006), as there appears to be no detrimental effects on offspring reproductive performance (Asmad, 2013).

2.6 Conclusions

Dam weight had lasting effects on offspring lactation performance, such that ewes born to Hv-dams produced more milk than ewes born to Lt-dams. This finding, suggests that ewes born to Lt-dams may have suffered from IUGR, impairing their mammary gland development and lifetime lactational performance. However, despite producing higher milk yields ewes born to Hv-dams did wean heavier lambs over their productive lifetime. Therefore, there may be no real lamb production advantage to be gained from having heavier dams. The findings may be of interest in farming enterprises where milk is harvested.

Nutrition of the dam, while influencing first lactation performance, did not have lifetime effects. This may be attributed to the nature of the mammary gland to regress and re-develop in subsequent lactations, thereby losing the programmed effects from the first lactation. Thus, manipulating dam nutrition to enhance offspring lactational performance does not appear to be a feasible option. Rather, future studies should focus on determining the mechanisms involved in programming first lactation performance to assist with development of interventions to achieve more long-term production advantages. In terms of nutritional management, there appears to be no benefit to offering *ad libitum* nutrition over maintenance nutrition to pregnant dams.

3 Background information on the experimental design and experimental animals

3.1 Introduction

The study reported by van der Linden *et al. (*2009) revealed that maintenance nutrition, compared with *ad libitum* nutrition, of the dam during gestation (P21-140) improved the first-lactation performance of the ewe offspring. However, this advantage did not persist into their second lactation (Blair *et al*., 2010), or throughout their productive lifetime (as measured over five lactations, reported in Chapter 2). These results have stimulated interest in identifying the critical period and underlying mechanisms whereby the foetal mammary gland is 'programmed' by maternal nutrition.

An experiment was designed by Kenyon *et al. (*2011a), in which dam nutritional treatments were split into the periods of early and mid-to-late pregnancy with the aim of investigating comparative and interacting effects on the offspring. The nutrition treatments during early pregnancy were applied from P21-50 to include the early stages of foetal mammogenesis (Forsyth *et al*., 1999; Hovey *et al*., 2002). It was hypothesised that perturbations during this period would have more of an influence on the foetal mammary gland and subsequent development and functionality later in life, compared with dam nutrition during later stages. Nutrition treatments during mid-to-late pregnancy were applied from P50-140, during which time there are increased energy demands for placental and foetal growth. Adverse influences in this period may affect offspring birth weight, post-natal growth trajectory and body composition (Robinson *et al*., 1999, Kenyon, 2008, Ashworth *et al*., 2009). This chapter provides a full description (adapted from Kenyon *et al*., 2011a) of dam nutritional treatment, to which the offspring studied in the remaining chapters of this thesis were subjected during their gestational development. Later chapters therefore provide only a brief summary of the dam-nutrition treatments applied.

3.2 Dam nutritional treatments

This study was conducted at Massey University Keeble Sheep and Beef farm, 5 km south of Palmerston North, New Zealand (Longitude 175˚36'36"E, latitude 40˚21'00"S, winter solstice 21st June). The study and all animal interventions were approved by the Massey University Animal Ethics Committee, Palmerston North, New Zealand.

Mixed-aged Romney ewes (generation 0, G0, dams; *n* = 1,169; average body condition score (BCS) = 2.96 ± 0.02; scale 0-5 (Jefferies 1969) and live weight (LW) = 66.3 ± 0.18 kg) from a commercial flock were synchronised using vaginally inserted Controlled Internal-Drug-Release devices (CIDR, 0.3 g progesterone; Pharmacia & UpJohn, Auckland, New Zealand) for 14 days (from 14 until 0 days before mating, P-14 until P0 of gestation) prior to artificial insemination (AI). The AI

was carried out surgically via intra-uterine laparoscopy using fresh semen which was randomly allocated from one of five Romney rams. Half of the ewes underwent AI on P0 (14th April 2009) while the remaining ewes underwent the same procedure the next day (day 1 of pregnancy, P1). Both cohorts of ewes were then merged and managed as one flock under commercial grazing conditions, with a minimum post-grazing pasture mass of 1,200 kg DM/ha. From day five of pregnancy until day 21 (P21), 12 crayon-harnessed entire Romney rams were introduced to the ewes to identify ewes that had returned to service (i.e., non-pregnant ewes). Any ewes displaying crayon-marks on their rump, indicating that they were still cycling, were removed for the remainder of the study.

From P21, the remaining ewes (*n* = 879) were randomly allocated to one of three pastoral nutritional treatments until day 50 (P50) of pregnancy (P21-50; sub-maintenance = $Sm_{P21–50}$, maintenance = $M_{P21–50}$, or *ad libitum* = $Ad_{P21–50}$; Figure 3.1 and Table 3.1). The aim of the $Sm_{P21–50}$ treatment was to achieve a loss of 100 g/d in total ewe LW (change achieved: –0.15 ± 0.02 kg/d). This was achieved through restricting access to pasture with pre- and post-grazing masses of 996 ± 89.3 and 814 ± 54.2 kg DM/ha, respectively (Table 3.2). The aim of the $M_{P21–50}$ treatment was to ensure no change in total ewe LW (change achieved: –0.02 ± 0.02 kg/d). This was achieved with pre- and post-grazing masses of 1,479 ± 107.7 and 1,112 ± 59.4 kg, DM/ha respectively (Table 3.2). The aim of the $Ad_{P21–50}$ treatment was to provide unrestricted access to pasture with a gain in total ewe LW of 100 g/d (LW change achieved: 0.15 ± 0.02 kg/d). This was achieved through providing ewes access to pasture of no less than 1,200 kg DM/ha (pre- and post-grazing pasture masses: 2,331 ± 82.0 and 1,649 ± 54.2 kg DM/ha, respectively, Table 3.2). Ewe LW and BCS, and pasture masses and quality, during P21-50 are reported in Tables 3.1 and 3.2, respectively.

At day 48 of pregnancy, all ewes underwent pregnancy scanning via trans abdominal ultrasonography. Ewes which were non-pregnant (*n* = 33), single- (*n* = 228), triplet- (*n* = 110) or quadruplet-bearing (*n* = 1) were removed from the trial. An additional 10 ewes were removed from the trial due to incomplete data.

At P50, the remaining ewes (*n* = 497) were randomly re-allocated to one of two pastoral nutritional treatments until day 140 (P140) of pregnancy (P50-140; pregnancy maintenance = $M_{P50-140}$ vs. *ad libitum* = $Ad_{P50-140}$; Figure 3.1). The aim of the $M_{P50-140}$ treatment was to achieve total ewe LW change to match change in conceptus mass (change achieved: 0.19 ± 0.01 kg/d). Pre- and post-grazing pasture masses were 1,450 ± 83.9 and 1,011 ± 32.8 kg DM/ha, respectively (Table 3.2). The $Ad_{P50-140}$ treatment offered unrestricted access to pasture (pre- and post-forage grazing masses: 1,828 ± 76.0 and 1,301 ± 37.8 kg DM/ha, respectively) and increased total ewe LW by 0.26 ± 0.01 kg/d. Therefore, the study had a 3 × 2 factorial arrangement, resulting in six dam nutritional

treatments (Figure 3.1). Ewe LW and BCS, and pasture masses and quality, during P50-140 are reported in Tables 3.1 and 3.2, respectively.

From P140, ewes from all nutrition treatments were merged and set stocked for lambing at a rate of 12.1 ewes/ha with an average pasture mass of 1558 ± 72 kg DM/ha. From lambing, all G0 dams and their offspring (generation 1, G1, who were foetuses at the time of G0 nutritional treatment) were managed under commercial grazing conditions with an *ad libitum* allocation of pasture (minimum pasture mass: 1,200 kg DM/ha). Kenyon *et al. (*2011) reported on the effects of the dam gestational nutrition treatments on the performance of the G0 ewes and their G1 lambs from birth until weaning. In addition Martin *et al. (*2012) euthanized a subset of G0 ewes (*n* = 60; 10 ewes per treatment) at day 141 of gestation in order to investigate the effects of dam nutrition on the anatomical development of twin-foetuses during late gestation.

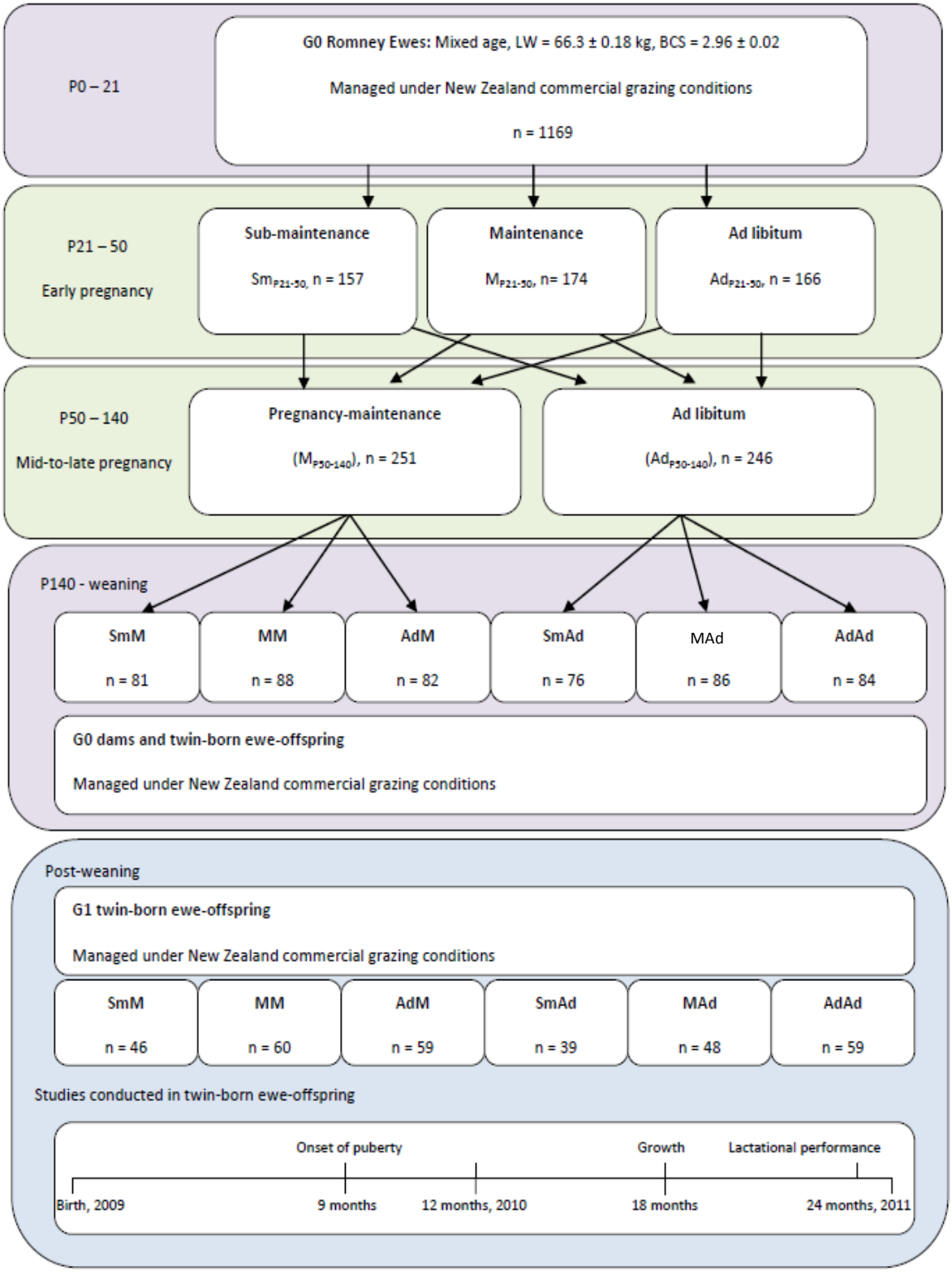

Figure 3.1 Experimental design of G0 dam nutritional treatments and overview of studies conducted in G1 twin-born ewe offspring.

Table 3.1 Least squares means ± S.E.M of live weight (kg) and body condition scores (scale 1 to 5) at days 21 (P21), 50 (P50) and 137 (P137) of pregnancy of ewes fed either sub-maintenance (Sm), maintenance (M), or *ad libitum* (Ad) nutrition during early pregnancy (P21-50) and either maintenance (M) or *ad libitum* (Ad) nutrition during mid-to-late pregnancy (P50-140). Adapted from Kenyon *et al*., 2011.

		Live weight			Body condition score		
Dam nutritional treatments	n	P21	P50	P137	P21	P50	P137
P21-50							
Sm	117	66.0 ± 0.58[a]	62.2 ± 0.56[a]	84.3 ± 0.68	3.0 ± 0.04	2.2 ± 0.04[a]	3.1 ± 0.05[a]
M	131	65.6 ± 0.55[b]	65.1 ± 0.53[b]	85.5 ± 0.67	2.9 ± 0.04	2.7 ± 0.04[b]	3.2 ± 0.05[ab]
Ad	134	66.2 ± 0.55[a]	69.5 ± 0.53[c]	86.4 ± 0.67	2.9 ± 0.04	3.2 ± 0.04[c]	3.3 ± 0.05[b]
P50-140							
M	194			82.6 ± 0.53[a]			2.9 ± 0.04[a]
Ad	188			88.2 ± 0.53[b]			3.5 ± 0.04[b]
P21-50×P50-140							
SmM	60			81.0 ± 0.95[a]			2.8 ± 0.08[a]
SmAd	57			87.7 ± 0.97[b]			3.4 ± 0.08[b]
MM	67			83.3 ± 0.90[a]			2.9 ± 0.07[a]
MAd	64			87.7 ± 0.92[b]			3.4 ± 0.07[b]
AdM	67			83.6 ± 0.92[a]			3.0 ± 0.07[a]
AdAd	67			89.1 ± 0.90[b]			3.6 ± 0.07[b]

[a, b] Differing superscripts within a column, within treatment groups, indicate least squares means that are significantly different ($P < 0.05$).

Table 3.2 Least squares means ± S.E.M of metabolisable energy (ME, MJ/ kg DM) and pre- and post-grazing pasture masses (kg DM/ha) for each dam nutritional treatment during early (day 21 to 50 of pregnancy, P21-50) and mid-to-late pregnancy (day 50 to 140 of pregnancy, P50-140). Adapted from Kenyon *et al*., 2011.

	Pasture energy and masses		
Dam nutritional treatments	ME (MJ/Kg DM)	Pre-grazing (kg DM/ha)	Post-grazing (kg DM/ha)
P21-50			
Sm	12.35 ± 0.26	996 ± 89.3[a]	814 ± 54.2[a]
M	12.67 ± 0.26	1479 ± 107.7[b]	1112 ± 59.4[b]
Ad	12.14 ± 0.23	2331 ± 82.0[c]	1649 ± 54.2[c]
P50-140			
M	12.67 ± 0.11	1450 ± 83.9[a]	1011 ± 32.8[a]
Ad	12.91 ± 0.11	1828 ± 76.0[b]	1301 ± 37.8[b]

[a, b] Differing superscripts within a column, within treatment groups, indicate least squares means that are significantly different ($P < 0.05$).

4 Effects of dam nutrition during early and mid-to-late pregnancy on postnatal growth performance and puberty attainment of twin-born ewe offspring

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4.1 Abstract

Pregnant ewes were allocated to either sub-maintenance (Sm_{P21-50}: -0.15 ± 0.02 kg/d live weight change), maintenance (M_{P21-50}: -0.02 ± 0.02 kg/d live weight change) or *ad libitum* (Ad_{P21-50}: 0.15 ± 0.02 kg/d live weight gain) nutrition from day 21 to 50 (P21-50) of pregnancy. At day 50 to 150 of pregnancy (P50-140), ewes were then re-allocated to either pregnancy maintenance nutrition ($M_{P50-140}$: designed to match change in conceptus mass; 0.19 ± 0.01kg/d live weight change) or *ad libitum* ($Ad_{P50-140}$: 0.26 ± 0.01 kg/d live weight gain) nutrition. The aim of this study was to investigate the effects of, and interactions between, ewe nutrition during the two periods of pregnancy on production traits measured in the twin-born ewe offspring including: live weight and live weight gain from birth until 18 months of age, body condition and composition, faecal egg counts and onset of puberty. Ewes born to dams fed maintenance throughout gestation (MM) were heavier on average, from birth until 18 months of age, (P = 0.03) compared with ewes born to dams fed sub-maintenance during P21-50 and maintenance during P50-140 (SmM). There was an interaction (P = 0.05) of time by dam nutrition during P50-140, such that female-offspring born to $M_{P50-140}$-dams were heavier (P = 0.002) than ewes born to $Ad_{P50-140}$-dams at birth but not at any other time. There was a transient effect that ewes born to Ad_{P21-50}-dams, compared with ewes born to M_{P21-50}-dams, had lower (P = 0.01) faecal egg counts at eight months, but not at nine months of age. A lower (P = 0.02) proportion of ewe offspring born to $Ad_{P50-140}$-dams reached puberty by nine months of age when compared with those born to $M_{P50-140}$-dams, however, after adjustment for live weight this difference was no longer present (P > 0.05). Body condition scores, back fat and eye muscle area of ewe offspring were unaffected by dam nutritional treatments. In summary, different levels of dam nutrition during both early and mid-to-late pregnancy resulted in only minor productive differences between ewe offspring, suggesting farmers can manipulate ewe feeding during pregnancy with minimal economic consequences being incurred in the offspring.

4.2 Introduction

The conflict between the optimal nutrient requirements for animal production and the actual feed that is available can be a challenge for many livestock systems. This is particularly the case for pasture-based systems, which are susceptible to seasonal- and climatic-induced fluctuations in the forage available (Valentine and Kemp, 2007). Most sheep are seasonal breeders such that their pregnancy typically coincides with winter when pasture availability can be limited. One practice, common in New Zealand sheep production systems, is to limit feed offered to ewes during early gestation, thereby conserving feed and enabling greater levels of nutrition during late-gestation (Kenyon and Webby, 2007). Therefore, through restrictive-feed management, pregnant

ewes may be exposed to nutritional constraints. This problem is further exacerbated for multiple-bearing ewes which have additional nutritional requirements (Rattray *et al*., 1974, Gootwine *et al*., 2007, Nicol & Brookes, 2007). Poor nutrition during pregnancy can impact on lamb birth weights and survival but may also have effects on the offspring which persist into later life (Metges & Hammon, 2005, Wu *et al*., 2006, Ashworth *et al*., 2009, Brameld *et al*., 2010, Greenwood *et al*., 2010). Understanding the consequences of both specific timing and level of nutrition during pregnancy may be valuable for developing feed-management strategies to optimise the productive performance of future generations.

Nutrition during different stages of pregnancy may affect postnatal phenotypes in the offspring. During early to mid-pregnancy, coinciding with the development of many foetal tissues and organs, dam nutrient restriction has been associated with increased adiposity, reduced muscle fibres (Robinson *et al*., 1999; Brameld & Daniel, 2008), impaired immunity (Shanks & Lightman, 2001; Cronje, 2003; Rooke *et al*., 2010) and decreased ovulation rates in offspring (Rae *et al*., 2002). During mid-to-late pregnancy, when energy demands for foetal growth are highest, suboptimal nutrition of the dam has been reported to reduce lamb birth weights (Borwick *et al*., 2003; Bielli *et al*., 2002; Gardner *et al*., 2007; Tygeson *et al*., 2007), increase adiposity (Gardner *et al*., 2005) and reduce growth rates of the offspring (Schinckel & Short, 1961; Tygesen *et al*., 2008)

It is apparent from the current literature that maternal nutrition can affect the foetus and may have consequences for productive performance in adult life. However, few studies have considered more than one period of nutritional insult or how they interact to affect production traits in sheep (Kenyon, 2008). There is a need to study the effects of variable maternal nutrition on the performance of the offspring as an adult, in particular for replacement breeding ewes in which long-term effects will impact on production (Brameld *et al*., 2010). This study was therefore designed to investigate the effects, and potential interactions between, maternal nutrition during early (P21-50) and mid-to-late (P50-140) pregnancy on the growth performance, body composition, puberty attainment and parasite susceptibility of twin-born ewe offspring, from birth to approximately 18 months of age.

4.3 Materials and methods

This study was conducted at Massey University Keeble Sheep and Beef farm, 5km south of Palmerston North, New Zealand (Longitude 175˚36’36”E, latitude 40˚21’00”S, winter solstice 21st June). The study and all animal handling procedures were approved by the Massey University Animal Ethics Committee, Palmerston North, New Zealand.

4.3.1 Experimental animals

The present study utilised the twin-born ewe offspring born to dams fed differentially during pregnancy. The dam nutritional treatments and management have been described in detail in Chapter 3. Briefly, ewes were fed either a sub-maintenance (Sm_{P21-50}), maintenance (M_{P21-50}) or *ad libitum* (Ad_{P21-50}) pasture allowance during early gestation (P21-50) and then reallocated to either a maintenance ($M_{P50-140}$) or *ad libitum* ($Ad_{P50-140}$) pasture allowance during mid-to-late gestation (P50-140). Therefore, the offspring generated, were from one of six dam nutritional treatment groups: SmM, SmAd, MM, MAd, AdM, and AdAd.

4.3.2 Experimental procedures and measurements

4.3.2.1 Live weight and growth

Live weight of the twin-born ewe offspring (*n* = 370) was recorded monthly from birth (average date of birth: 8 September 2009, one day postpartum, D1) to approximately 18 months (average 552 days after the mid-point of lambing) of age. Live weight gains (LWG) from birth to weaning (LWG_{b-w}, where weaning was approximately 90 days after the mid-point of lambing), weaning to six months of age (LWG_{w-6mo}), six to nine months (LWG_{6-9mo}), nine to 12 months (LWG_{9-12mo}), 12 to 15 months ($LWG_{12-15mo}$), and 15 to 18 months of age ($LWG_{15-18mo}$) were calculated.

4.3.2.2 Puberty attainment

Eight crayon-harnessed vasectomised rams were joined with ewe lambs (*n* = 370) at D261 (May 27th 2010) for a 17-d period. The presence of crayon marks on the rump of the ewe-lambs was taken as indicative of oestrus activity and was used to assess puberty attainment.

4.3.2.3 Faecal egg counts

Faecal samples, of approximately 5 to 10 g, were collected rectally from ewe-lambs at eight months (D244, *n* = 235) and nine months of age (D277, *n* = 228), and processed for faecal egg counts (FECs) on the same day. The FECs allowed the determination of infection by strongyloides and nematodirus. The number of strongylate and nematode eggs per gram faeces (epg) was determined using a modified McMaster technique as described by Stafford *et al*. (1994).

4.3.2.4 Ewe body condition, back-fat and eye-muscle area

Body condition score (BCS) was measured at approximately 13 and 14 months of age (D392 and D435, respectively). Body condition score is a subjective measurement, which involves the palpation of the spinous (vertical) and transverse (horizontal) process of the lumbar vertebrae in order to estimate the relative amount of subcutaneous fat in an individual animal (Jefferies, 1961;

Russel *et al*., 1969). Body condition score is on a scale ranked from one to five, including half scores, with one representing emaciated and five representing obese (Jefferies, 1961).

Ultrasonic measurements were taken of back-fat (BF) thickness and *Longissimus dorsi* (LD) muscle area (eye-muscle area, EMA) in the ewe offspring, at approximately 14 months of age (BF taken at D434 and EMA taken at D441), as an indicator of lean tissue characteristics. The ewes were ultrasound-scanned (Aloka SSD-500 ultrasound scanner, Aloka Co. Ltd., Tokyo, Japan) on their left-side, lateral of the spine in the area of the 3rd and 4th lumbar vertebrae, using a linear probe of 5MHz and mineral oil as a conductive medium. The EMA was calculated from measurements of muscle depth, taken on the long axis of the cross section of the LD, and muscle width, the largest perpendicular measurement of the section, using the following formula (Beef and Lamb NZ, 2010):

$$EMA = (muscle\ depth \times muscle\ width) \times 0.0077$$

4.3.3 Statistical analyses

Data were analysed using PROC MIXED in SAS 9.2 (SAS Institute, Cary NC, USA) unless otherwise stated. Post-hoc testing of differences between means were calculated using Tukey's HSD (honestly significantly different) test.

A repeated measures analysis was used to analyse ewe LW with fixed effects of time, dam nutritional treatment during P21-50 and P50-140 and their two- and three-way interactions. The three-way interaction of time by dam nutritional treatment in P21-50 by dam nutritional treatment in P50-140 (t×P21-50×P50-140) and the two-way interactions of time by dam nutritional treatments (t×P21-50 and t×P50-140) and dam nutritional treatment in P21-50 by P50-140 (P21-50×P50-140) remained in the model, regardless of significance, to allow for testing of the study design. Date of birth was fitted as a covariate. The analysis for LW was not adjusted for birth weight as it was a component of the maternal nutrition treatment effects.

A univariate analysis was used for LWG and FEC. Fixed effects of sire, dam nutritional treatments during P21-50, P50-140 and P21-50×P50-140 were fitted along with date of birth as a covariate. Non-significant two-way interactions between dam-nutrition treatment periods were not removed, to allow for testing of the study design. The FEC data were normalised using a square root transformation prior to analysis. Back-transformed means were obtained by squaring.

The total proportion of twin-born female offspring that were crayon-marked by harnessed vasectomised rams (indicating puberty attainment) during days 261 to 278 of age was analysed using the GENMOD procedure in SAS 9.2 (SAS Institute, Cary NC, USA). Fixed effects included sire, dam nutritional treatment during P21-50, P50-140, and P21-50×P50-140, both with and without live

weight at puberty as a covariate. In addition, date of birth was fitted as a covariate. Data were logit transformed prior to analysis and back-transformed using inverse logit to obtain the mean proportions (± 95% confidence intervals).

4.4 Results

4.4.1 Ewe-offspring live weight and live weight gain

There was no three-way interaction ($P > 0.05$) between time and dam nutritional treatments (t×P21-50×P50-140) on ewe-offspring LW (Figure 4.1). There was also no interaction ($P > 0.05$) effect of dam nutrition during both periods (P21-50×P50-140) on ewe-offspring LW (data not shown).

There was an interaction ($P = 0.05$) between time and dam nutrition during P50-140 (t×P50-140) on ewe-offspring LW. There was an initial difference, such that ewe lambs born to $Ad_{P50-140}$-dams were lighter ($P = 0.002$) at birth when compared to ewe lambs born to $M_{P50-140}$-dams, however this difference did not persist (Table 4.1). There was no interaction ($P > 0.05$) between time and dam nutritional treatment during P21-50 (data not shown).

There were no effects (Table 4.1), or interactions (data not shown), ($P > 0.05$) of dam nutrition during P21-50 and P50-140 on ewe-lamb LWG from birth until weaning. There were no P21-50×P50-140 interactions ($P >0.05$) on ewe-offspring LWG from six until nine months, nine until 12 months and 15 until 18 months of age (data not shown). There were, however, differences ($P = 0.01$) in LWG of ewe offspring between weaning and six months of age (SmM = 63.8 ± 4.25; SmAd = 75.0 ± 4.84; MM = 74.2 ± 3.71; MAd = 64.1 ± 4.09; AdM = 63.0 ± 3.68; AdAd = 72.7 ± 3.75 g/day), however, post-hoc comparison of means using Tukey's test did not show any differences.

During the period between six to nine months of age, ewe lambs born to $Ad_{P50-140}$-dams had lower ($P = 0.04$) LWG when compared with those born to $M_{P50-140}$-dams (Table 4.1). However, this impaired growth was reversed ($P = 0.04$) for LWG from 15 to 18 months of age, with no differences ($P > 0.05$) for LWG during 12 to 15 months of age. From nine to 12 months of age, there was a tendency for ewe lambs born to Ad_{P21-50}-dams to have lower ($P = 0.08$) LWG when compared with ewe lambs born to M_{P21-50}-dams, with those born to Sm_{P21-50}-dams not differing ($P > 0.05$) from either treatment. There were no effects ($P > 0.05$) of dam nutrition during P21-50 on ewe-offspring LWG during any other period (Table 4.1).

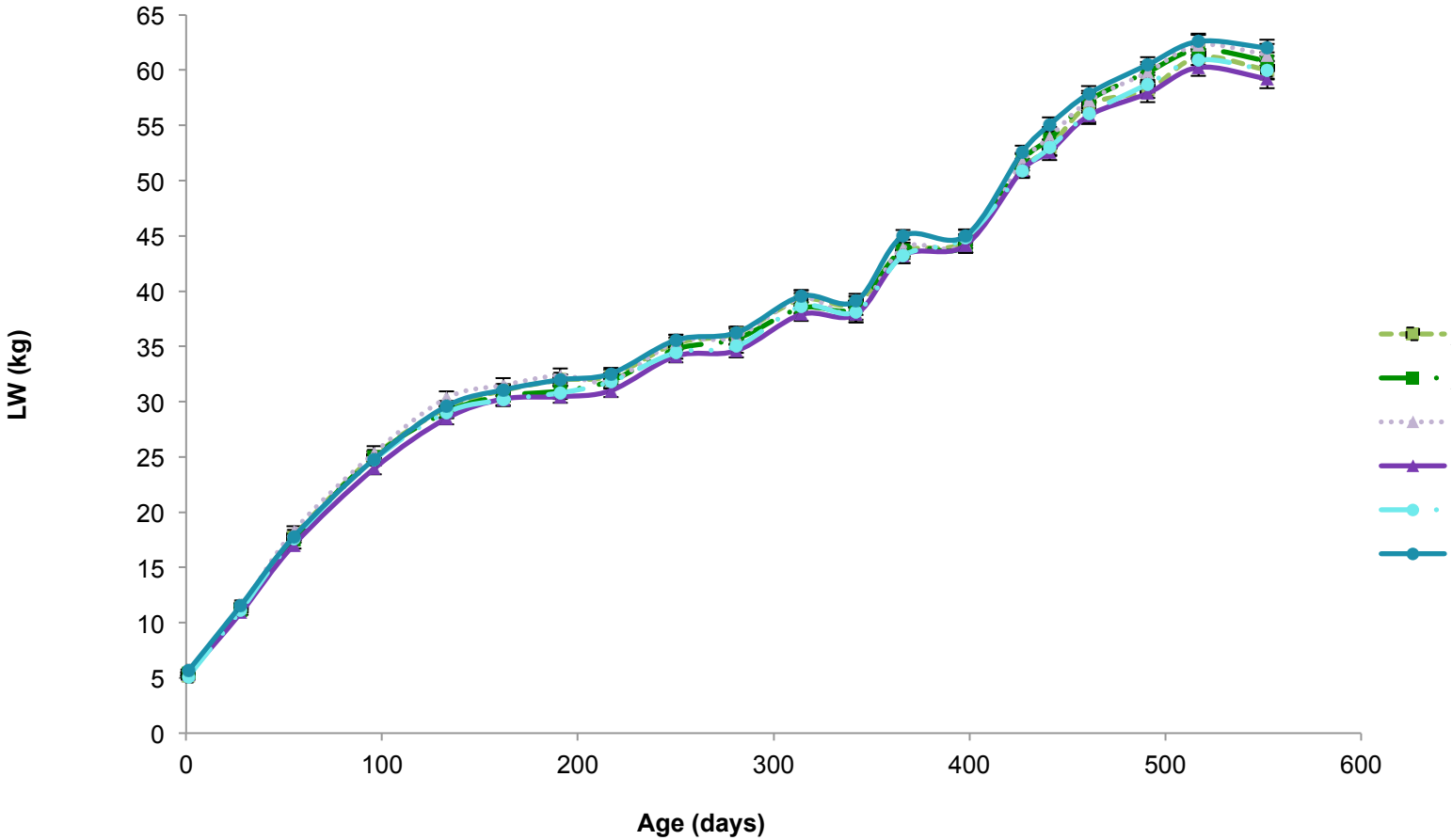

Figure 4.1 Least square means ± SEM of live weight (LW, kg), during the first 18 months of age (D1 to D552), of twin-born ewe offspring born to ewes fed either sub-maintenance (Sm), maintenance (M) or *ad libitum* (Ad) nutrition during early pregnancy (P21-50) and reallocated to either pregnancy maintenance (M) or *ad libitum* (Ad) nutrition during mid-to-late pregnancy (P50-140): P21-50×P50-140: SmM; *n* = 33, SmAd; *n* = 26, MM; *n* = 49, MAd; *n* = 34, AdM; *n* =48, and AdAd; *n* = 42.

Table 4.1 Least squares means ± pooled SEM of live weight (kg) at birth (day 1 postpartum, D1), weaning (D96), and six (D191), nine (D281), 12 (D366), 15 (D461), and 18 (D552) months of age; and live weight gain (g/day) of twin-born ewe offspring born to dams fed either sub-maintenance (Sm), maintenance (M) or *ad libitum* (Ad) nutrition during early pregnancy (P21-50) and either pregnancy maintenance (M) or *ad libitum* (Ad) nutrition during mid-to-late pregnancy (P50-140).

		Dam nutrition P21-50				Dam nutrition P50-140		
	n	Sm	M	Ad	SEM	M	Ad	SEM
Live weight (kg)								
Birth	266	5.3	5.4	5.3	0.07	5.5[b]	5.2[a]	0.06
Weaning	266	24.6	24.8	25.0	0.35	24.6	25.0	0.29
6 months	266	31.4	31.4	31.5	0.39	31.1	31.7	0.32
9 months	266	35.2	35.6	35.9	0.42	35.5	35.7	0.34
12 months	266	43.6	44.1	43.6	0.46	44.0	43.5	0.38
15 months	265	56.6	57.0	57.0	0.53	57.0	56.7	0.43
18 months	232	60.3	61.0	60.4	0.59	60.7	60.5	0.48
Live weight gain (g/day)								
Birth to weaning	306	219.2	214.3	223.0	3.52	217.2	220.4	2.90
Weaning to 6 months	265	69.4	69.1	67.9	2.84	67.0	70.6	2.35
6 months to 9 months	263	41.0	43.5	47.0	2.45	46.9[b]	40.8[a]	2.02
9 months to 12 months	264	97.5	101.7	91.9	3.28	99.9	94.2	2.71
12 months to 15 months	264	126.8	126.3	125.8	4.69	128.6	123.9	3.88
15 months to 18 months	259	66.0	66.9	67.7	3.15	62.9[a]	70.8[b]	2.60

[a, b] Differing superscripts within rows, within treatment groups, indicate least squares means that are significantly different ($P < 0.05$).

4.4.2 Ewe-offspring body condition score, back fat and eye-muscle area

There were no ($P > 0.05$) interactions between, or effects of, dam nutrition during P21-50 and P50-140 on ewe-offspring BCS at 13 (D392) and 14 (D435) months of age, or on BF and EMA (Table 4.2).

4.4.3 Puberty attainment

There was no P21-50×P50-140 interaction ($P > 0.05$) on the occurrence of puberty, as measured by tupping of ewe offspring (Table 4.3). Puberty attainment was unaffected ($P > 0.05$) by dam nutrition during P21-50 (Table 4.3). A lower proportion of ewe offspring born to $Ad_{P50-140}$-dams tended ($P = 0.08$) to reach puberty by mid-May, when compared with offspring born to $M_{P50-140}$-dams (Table 4.3). When adjusted for LW, measured at the time of testing (D261), this difference was slightly reduced ($P = 0.10$; -0.58 ± 0.226 (36.0%) vs. -0.08 ± 0.193 (47.9%), logit transformed means ± SE (back-transformed percentages) for ewe offspring born to $Ad_{P50-140}$-dams tending to be lower than those born to $M_{P50-140}$-dams, respectively.

4.4.4 Faecal egg counts

There was no P21-50×P50-140 interaction ($P > 0.05$) on offspring FEC's at approximately eight (D244) and nine (D277) months of age (Table 4.4). At D244, ewe lambs born to Ad_{P21-50}-dams had less ($P = 0.01$) strongyloides eggs than those born to M_{P21-50}-dams, while FECs of ewe lambs born to Sm_{P21-50}-dams did not differ (Table 4.4). In contrast, ewe lambs born to $Ad_{P50-140}$-dams tended ($P = 0.08$) to have more strongyloides eggs at D244 when compared to those born to $M_{P50-140}$-dams (Table 4.4). The effects of dam nutrition, during P21-50 and P50-140, on ewe offspring FECs were no longer apparent ($P > 0.05$) in the second sampling at D277, for both strongyloides or nematodirus eggs.

Table 4.2 Least squares means ± SEM of body condition scores (1-5 scale) on day (D) 392 and 435 of age, back fat (mm) on D434 of age, and eye muscle area (cm^3) on D441 of age of twin-born ewe offspring born to dams fed either sub-maintenance (Sm), maintenance (M) or *ad libitum* (Ad) nutrition during early pregnancy (P21-50) and either pregnancy maintenance (M) or *ad libitum* (Ad) nutrition during mid-to-late pregnancy (P50-140).

	Body condition score				Back fat (mm)		Eye muscle area (cm^3)	
Dam nutrition	*n*	D392	*n*	D435	*n*	D434	*n*	D441
P21-50								
Sm	68	2.8 ± 0.04	70	2.8 ± 0.04	70	4.6 ± 0.14	69	12.9 ± 0.17
M	93	2.9 ± 0.03	95	2.9 ± 0.03	94	4.9 ± 0.12	93	12.9 ± 0.15
Ad	97	2.9 ± 0.03	99	2.8 ± 0.03	99	4.7 ± 0.12	98	12.8 ± 0.14
P50-140								
M	141	2.9 ± 0.03	144	2.8 ± 0.03	142	4.8 ± 0.10	140	12.8 ± 0.12
Ad	117	2.8 ± 0.03	120	2.9 ± 0.03	121	4.7 ± 0.11	120	13.0 ± 0.13
P21-50 × P50-140								
SmM	39	2.8 ± 0.05	41	2.8 ± 0.05	41	4.5 ± 0.18	40	12.6 ± 0.23
SmAd	29	2.8 ± 0.06	29	2.8 ± 0.06	29	4.6 ± 0.22	29	13.1 ± 0.26
MM	52	2.9 ± 0.05	52	2.9 ± 0.04	51	4.9 ± 0.16	50	13.0 ± 0.20
MAd	41	2.9 ± 0.05	43	2.9 ± 0.05	43	5.0 ± 0.18	43	12.8 ± 0.22
AdM	50	2.9 ± 0.05	51	2.8 ± 0.04	50	4.9 ± 0.16	50	12.7 ± 0.20
AdAd	47	2.9 ± 0.05	48	2.9 ± 0.05	49	4.6 ± 0.17	48	13.0 ± 0.21

Table 4.3 Number (*n*), and least squares means of logit transformed proportions (± SE), and back-transformed percentages (± 95% confidence interval) of ewes to attain puberty (measured by display of oestrus) during days 261 to 278 of age. Ewes were twin-born offspring born of dams fed either sub-maintenance (Sm), maintenance (M) or *ad libitum* (Ad) nutrition during early pregnancy (P21-50) and either pregnancy maintenance (M) or *ad libitum* (Ad) nutrition during mid-to-late pregnancy (P50-140).

		Displayed oestrus		
Dam Nutrition	*n*	Number (*n*)	Proportion (logit transformed)	Back-transformed percentage, (Confidence intervals in parenthesis)
P21-50				
Sm	70	27	-0.53 ± 0.274	37.1 (35.7, 38.4)
M	95	36	-0.51 ± 0.228	37.6 (36.3, 38.8)
Ad	101	54	0.08 ± 0.211	51.9 (50.8, 53.2)
P50-140				
M	145	70	-0.07 ± 0.181	48.2 (47.2, 49.3)
Ad	121	47	-0.57 ± 0.213	36.2 (35.0, 37.3)
P21-50 × P50-140				
SmM	41	19	-0.32 ± 0.336	42.2 (40.2, 44.0)
SmAd	29	8	-0.74 ± 0.435	32.4 (30.2, 34.5)
MM	52	22	-0.19 ± 0.302	45.2 (43.5, 46.9)
MAd	43	14	-0.82 ± 0.356	30.5 (28.8, 32.2)
AdM	52	29	0.29 ± 0.297	57.3 (55.5, 58.9)
AdAd	49	25	-0.14 ± 0.307	46.5 (44.8, 48.3)

Table 4.4 Least squares means ± SEM of faecal egg counts (FEC); values shown are square root transformation of strongyloides (Stg) and nematodirus (Nem) egg/g faeces (back-transformed values shown in parenthesis), of twin-born ewe offspring born to dams fed either sub-maintenance (Sm), maintenance (M) or *ad libitum* (Ad) nutrition during early pregnancy (P21-50) and either pregnancy maintenance (M) or *ad libitum* (Ad) nutrition during mid-to-late pregnancy (P50-140).

	FEC Stg, square root egg/g				FEC Nem, square root egg/g	
Dam nutrition	*n*	D244	*n*	D277	*n*	D277
P21-50						
Sm	63	11.0[ab] (122.0) ± 1.00	56	32.5 (1056.1) ± 1.99	56	2.2 (4.9) ± 0.52
M	77	13.5[b] (182.5) ± 0.88	84	33.6 (1129.7) ± 1.62	84	1.9 (3.7) ± 0.43
Ad	95	10.1[a] (101.8) ± 0.79	88	33.3 (1110.0) ± 1.57	88	2.6 (6.8) ± 0.41
P50-140						
M	126	10.6 (113.1) ± 0.70	122	32.8 (1078.8) ± 1.38	122	2.0 (4.1) ± 0.36
Ad	109	12.5 (155.3) ± 0.77	106	33.4 (1118.2) ± 1.49	106	2.5 (6.1) ± 0.39
P21-50 × P50-140						
SmM	38	9.4 (87.9) ± 1.26	32	31.5 (992.3) ± 2.62	32	2.0 (4.1) ± 0.69
SmAd	25	12.7 (161.8) ± 1.55	24	33.5 (1121.8) ± 3.03	24	2.4 (5.6) ± 0.80
MM	40	13.8 (189.6) ± 1.23	46	34.2 (1170.7) ± 2.23	46	1.1 (1.2) ± 0.59
MAd	37	13.2 (175.5) ± 1.29	38	33.0 (1089.5) ± 2.43	38	2.7 (7.5) ± 0.64
AdM	48	8.8 (76.7) ± 1.10	44	32.8 (1076.9) ± 2.22	44	2.9 (8.5) ± 0.58
AdAd	47	11.4 (130.3) ± 1.14	44	33.8 (1143.5) ± 2.25	44	2.3 (5.3) ± 0.59

[a, b] Differing superscripts within columns, within treatments, indicate least squares means that are significantly different ($P < 0.05$).

4.5 Discussion

Numerous studies have demonstrated that differing levels of maternal nutrition during various stages of pregnancy can lead to foetal programming and ultimately, health and performance consequences for the adult offspring (Wu *et al*., 2004, Metges & Hammon, 2005). To date, there have been few longitudinal studies in sheep which have investigated and compared the effects of differing dam nutrition during multiple periods of pregnancy on productive performance of offspring (Kenyon, 2008, Ashworth *et al*., 2009).

In the present study there was an interaction between dam nutrition during P21-50 and P50-140 to affect LWG from weaning until six months of age in ewe offspring, although the minimal differences are unlikely to be of economic significance. Interaction between dam nutrition during both P21-50 and P50-140 did not affect any other production traits measured in ewe offspring which suggests that the effects of dam nutrition during early pregnancy are independent of those from mid-to-late pregnancy. The effect of dam nutrition during P50-140 on ewe-offspring LW varied over time, such that ewe offspring born to $Ad_{P50-140}$-dams were marginally lighter at birth, but not at any other time up to 18 months of age. This suggests that dam P50-140-nutrition altered the subsequent growth rates of the offspring. Indeed, ewes born to dams fed $M_{P50-140}$ had greater LWG from six to nine months of age while during 15 to 18 months, ewes born to $Ad_{P50-140}$-dams had greater LWG. In terms of implications for replacement breeding ewes, differing growth rates may influence reproductive traits, including puberty attainment (Dýrmundsson, 1981) and body composition (Bloomfield *et al*., 2006). Although, differences reported in the present study were minimal and, therefore, may not be of consequence to agriculture.

The BCS, BF and EMA measurements made at 13 to 14 months of age, were unaffected by dam nutrition during both P21-50 and P50-140. These findings are in agreement with that of Nordby *et al. (*1987), Krausgrill *et al. (*1999), Rae *et al. (*2002), Gopalakrishnan *et al. (*2004), and Daniel *et al. (*2007). However, the data on back-fat in the present study are in contrast to the studies of Gardner *et al. (*2005) and Ford *et al. (*2007) who reported increased fatness in 9- and 12-month old offspring born to dams which were allocated 50% NRC nutrient requirements during late- and early-gestation, respectively. In addition, Oliver *et al. (*2001) who observed the opposite in 30-month old offspring born to dams which were nutrient restricted during late-gestation. These inconsistencies are likely due to differences in the timing and level of nutritional insult as well as differences in timing of measurement on the offspring. In the previously mentioned studies, a nutritional restriction was applied to dams during late pregnancy while in the present study ewes were offered a minimum of pregnancy maintenance nutrition, thus the restriction was not severe.

The reproductive system is influenced by the level of dam nutrition during early gestation, with effects reported on ovarian development, steroid hormones and follicle development (Rhind, 2004). In the present study, however, the onset of puberty of ewe offspring was unaffected by dam nutrition during P21-50. Dam nutrition during P50-140 tended to affect puberty attainment of ewe offspring, such that a reduced proportion of ewe lambs born to $Ad_{P50-140}$-dams reaching puberty by nine months of age compared with ewe lambs born to $M_{P50-140}$-dams. The findings of the present study are in contrast with the findings of da Silva *et al. (*2001) and van der Linden *et al. (*2007) who found no effects from dam nutrition applied throughout gestation. In terms of production consequences, ewe offspring born to $Ad_{P50-140}$-dams may be less suited for breeding at a younger age. Breeding ewe lambs at eight to nine months of age, rather than at 18 months, is a management practice used to increase number and weight of lambs produced per ewe productive lifetime (Dýrmundsson, 1981), to increase rates of genetic gain, and to improve flock efficiency (Dýrmundsson, 1981, Gootwine *et al*., 2007, Kenyon *et al*., 2011b). Therefore, ewe offspring born to $Ad_{P50-140}$-dams may have reduced reproductive potential later in life.

There is evidence that maternal nutrition during pregnancy may lead to permanent changes in the immune system of the offspring, altering their resistance to disease (Cronje, 2003). A study conducted by Rooke *et al. (*2010) found FECs were increased in 3-month-old lambs that were born to nutrient-restricted ewes, fed 75% of energy requirements for maintenance and foetal growth, during early to mid-pregnancy (from conception to day 90 of pregnancy). In the present study, lambs born to M_{P21-50} dams had more strongyloides eggs than those born to H_{P21-50} dams at D244 with lambs born to Sm_{P21-50} not differing from either group. This effect was no longer apparent in the second sample on D277. While the results of the present study indicate that nutrition during early pregnancy may influence parasite susceptibility in the offspring, the differences between levels of infection did not persist. Before clear conclusions can be drawn, examining FECs at a younger age, before the onset of acquired immunity would be worthwhile.

4.6 Conclusions

Despite achieving relatively large differences of LWG in dams from nutritional management during the periods P21-50 and P50-139, there were minimal effects observed on the performance of twin-born female offspring from birth until 18 months of age. There was little influence of dam nutrition during P21-50 on the ewe offspring, with only a transient increase in FECs observed in ewes born to M_{P21-50}-dams at eight months of age. Dam nutrition during P50-140 appeared to be the more critical treatment period with $Ad_{P50-140}$-dam nutrition decreasing the proportion of ewe offspring which attained puberty by nine months of age compared with $M_{P50-140}$-dam nutrition. There were no interaction effects between dam nutritional treatments (P21-50×P50-140) on ewe-offspring postnatal growth, body composition, onset of puberty and parasite susceptibility. Overall, these results indicate that breeding-ewes can be subjected to

feeding-restriction during early pregnancy, providing they are allocated pregnancy maintenance nutrition or above during mid-to-late pregnancy, with little impact on twin-born ewe offspring to 18 months of age.

5 Maternal nutrition during early and mid-to-late pregnancy: comparative effects on milk production of twin-born ewe progeny during their first lactation [1]

Chapter based on the following publication:

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5.1 Foreword

The previous chapter (Chapter 4) reported that there were few effects of dam nutrition during early (P21-50) and mid-to-late (P50-140) pregnancy on various production traits in the twin-born ewe offspring up until 18 months of age. Dam maintenance nutrition during P21-50 resulted in a transient increase in faecal egg counts (FECs) of the ewe offspring at eight months of age, however, there was no difference at nine months of age. *Ad libitum* nutrition of the dam during P50-140 tended to decrease the proportion of ewe offspring that attained puberty by nine months of age. However, the difference was further reduced when live weight (LW) was included in the analysis as a covariate. There was a tendency for dam nutrition (P21-50×P50-140) to affect the average LW of ewe offspring from birth until 18 months of age. Ewe offspring born to dams that were fed sub-maintenance during P21-50 and fed maintenance during P50-140 (SmM) tended to be lighter, on average, than those born to dams fed maintenance during P21-140 (MM). There were no LW differences between the other treatment groups. The LW of ewe offspring at birth and at weaning did not differ among the treatment groups.

The effects of foetal programming may manifest later in life, which might have production implications, especially for ewe offspring retained as replacements for breeding (Ashworth *et al*., 2009; Kenyon, 2008; Greenwood *et al*., 2010). Indeed, there is evidence that lactational performance of offspring can be programmed by nutrition *in utero* (van der Linden *et al*., 2009). The influence of dam nutrition on the lactational performance of ewe offspring is particularly interesting as the consequences extend to growth of the subsequent generations. Using foetuses that were a cohort of the ewe offspring in the present study (background information reported in Chapter 3) Martin *et al. (*2012) found that early pregnancy was a critical period for programming of mammary gland morphology, whereby under-nourishment of the dam during this period reduced foetal mammary gland size. The objective of the present chapter was to extend the work of Martin *et al. (*2012), and Chapter 4. Chapter 5 will elucidate the effects of dam nutrition during both P21-50 and P50-140 on the first-lactation performance of a cohort of twin-born ewe offspring. This chapter is based on the version published in the *Journal of Animal Science* (Paten *et al*., 2013).

5.2 Abstract

Studies using sheep models indicate that the foetal mammary gland is sensitive to maternal nutrition during gestation; however, results have been inconsistent and do not identify critical feeding periods. This study aimed to clarify previous findings by partitioning the period of maternal nutritional manipulation into two stages: early and mid-to-late pregnancy. Sixty-six twin-born, twin-bearing ewes, born to dams that were fed either sub-maintenance, maintenance, or *ad libitum* (Ad_{P21-50}) during early pregnancy (day 21 to 50 of pregnancy; Sm_{P21-50}, M_{P21-50}, or Ad_{P21-50}, respectively) and then either maintenance, or *ad libitum* during mid-to-late pregnancy (day 50 to 140 of pregnancy; $M_{P50-140}$ or $Ad_{P50-140}$, respectively) were milked once a week, starting from day 7 ± 1 postpartum. Ewes were milked for seven subsequent weeks to enable estimation of daily milk yield and composition and lambs were weighed weekly. Ewes born to dams fed M_{P21-50} tended to have greater accumulated milk (P = 0.10), fat (P = 0.07), and net energy (P = 0.06) yields over 50 day compared with ewes born to dams fed Sm_{P21-50} and Ad_{P21-50}. In contrast, ewes born to dams fed $Ad_{P50-140}$ tended to have greater accumulated milk (P = 0.10) and lactose (P = 0.09) yields compared with ewes born to dams fed $M_{P50-140}$. Grand offspring birth weights were unaffected by granddam nutrition during pregnancy. Ewes born to dams fed Ad_{P21-50} weaned lighter lambs (P = 0.05) than ewes born to dams fed Sm_{P21-50} and tended to wean lighter lambs (P = 0.07) than ewes born to dams fed $M_{P21-50.}$ There were, however, no differences between the weaning weights of lambs (P = 0.43) from ewes born to dams fed $Ad_{P50-140}$ and $M_{P50-140}$. Maintenance nutrition of dams during early pregnancy appears to be associated with an improved lactation performance of ewe offspring. Higher levels of nutrition during mid-to-late pregnancy also appear to improve the first-lactation performance of ewe offspring. Interestingly, although grand-offspring birth weights were unaffected, weaning weight appears to be influenced by granddam early pregnancy nutrition in a manner discordant with the lactational performance of their dam. Results from this study indicate that dam nutrition during early pregnancy can influence the lactational performance of ewe offspring and the LW at weaning of their grand-offspring, which may ultimately affect farmer profits. This highlights the importance of nutritional management of breeding ewes during this period.

5.3 Introduction

The ability of the dam to provide milk for her offspring is a key driver of neonatal growth and survival (Morgan *et al*., 2007). Knowledge of the factors that influence lactational performance is of interest for both animal production and human

nutrition research. Nutrition during key stages of mammary gland development at puberty and pregnancy has been shown to influence subsequent lactation performance (Knight & Peaker, 1982; Sejrsen and Purup, 1997; Park, 2005; Capuco & Akers, 2010). However, relatively little is known about the influence of nutrition during foetal life on mammary development and lactation.

Sheep paradigms indicate that the foetal mammary gland is sensitive to the level of maternal nutrition. Blair *et al. (*2010) reported that maternal maintenance feeding during pregnancy negatively affected foetal mammary gland development, resulting in reduced duct area. In contrast, van der Linden *et al. (*2009) reported that foetuses from maintenance-fed dams had heavier mammary glands compared with foetuses from *ad libitum*-fed dams, with no difference in duct area. A cohort retained for milking showed that ewes born to maintenance-fed dams produced more milk with increased lactose and crude protein (CP) yields during their first lactation (van der Linden *et al*., 2009); however, this difference was no longer observed during the second lactation (Blair *et al*., 2010).

The aforementioned studies not only differ in apparent effects on the mammary gland development of the offspring, they also differ in the timing and level of nutritional insult. Therefore, a further study was designed to clarify previous findings by partitioning the period of gestational nutritional manipulation into two distinct stages; early and mid-to-late pregnancy (Kenyon *et al*., 2011a). Martín *et al.* (2012) reported that late-gestation foetuses, from dams fed at sub-maintenance levels during early pregnancy, had lighter mammary glands than foetuses from dams fed maintenance or *ad libitum*. This indicates a critical window for mammary gland development in sheep between foetal day 21 and 50. The present study examined the effects of maternal nutrition in early and mid-to-late pregnancy on the first lactation performance of a cohort of lambs born in the study reported by Kenyon *et al. (*2011a). Based on the previous findings, it was hypothesized that dam nutrition during early pregnancy, rather than mid-to-late pregnancy, would influence lactational performance of the offspring. In particular, we hypothesized that maintenance feeding, compared with sub-maintenance and *ad libitum* feeding, of the dam during early pregnancy would positively affect offspring milk yields and grand offspring weaning weights.

5.4 Materials and methods

5.4.1 Background

The experimental design has been published in full by Kenyon *et al. (*2011a); (Figure 1). Briefly, Romney ewes [(Group 0: dams fed nutrition treatments; G0); *n* = 1169, average LW = 66.3 ± 0.18 kg, and BCS = 2.96 ± 0.02] were randomly offered one of three pastoral nutritional treatments from day 21 to 50 of pregnancy (P21-50; sub-maintenance = $Sm_{P21–50}$, maintenance = $M_{P21–50}$, or *ad libitum* = $Ad_{P21–50}$). Ewe nutrition was controlled through forage intakes from grazing. The aim of the $Sm_{P21–50}$ treatment was to achieve a loss in total ewe LW (change achieved = –0.15 ± 0.02 kg/d) through restricting access to forage with a pre- and post-grazing mass of 996 ± 89.3 to 814 ± 54.2 kg DM/ha, respectively. The aim of the $M_{P21–50}$ treatment was to ensure there was no change in total ewe LW (change achieved = –0.02 ± 0.02 kg/d); through managing access to forage with a pre- and post-grazing mass of 1,479 ± 107.7 to 1,112 ± 59.4 kg DM/ha, respectively. The aim of the $Ad_{P21–50}$ treatment was to provide unrestricted access to pasture

forage and total ewe LW gain (change achieved = 0.15 ± 0.02 kg/d). This was achieved through providing ewes access to forage of no less than 1,200 kg DM/ha (pre- and post-grazing pasture mass = 2,331 ± 82.0 to 1,649 ± 54.2 kg DM/ha, respectively). Morris and Kenyon (2004) have previously shown that ewe intakes do not differ when pasture masses are above this level, therefore it can be assumed that ewes are *ad libitum* feeding.

Ewes from each P21-50 nutritional treatment group were then randomly reallocated to one of two pastoral nutritional treatments from day 50 to 140 of pregnancy (P50-140; maintenance = $M_{P50-140}$ vs. *ad libitum* = $Ad_{P50-140}$). The aim of the $M_{P50-140}$ treatment was to achieve total ewe LW change to match change in conceptus mass (change achieved = 0.19 ± 0.01 kg/d), through a pre- and post-grazing forage mass of 1,450 ± 83.9 to 1,011 ± 32.8 kg DM/ha, respectively. The $Ad_{P50-140}$ treatment offered unrestricted access to pasture forage (pre- and post-forage grazing mass: 1,828 ± 76.0 to 1,301 ± 37.8 kg DM/ha, respectively) and increased total ewe LW (LW change achieved: 0.26 ± 0.01 kg/d). Therefore, from day 50 of pregnancy, there was a 3 × 2 factorial arrangement of treatments, resulting in six dam nutrition groups: sub-maintenance-fed from day 21 to 50 of pregnancy, then maintenance-fed from day 50 to 140 of pregnancy (SmM); sub-maintenance-fed from day 21 to 50, then *ad libitum*-fed from day 50 to 140 of pregnancy (SmAd); maintenance-fed throughout pregnancy, from day 21 to 140 (MM); maintenance-fed from day 21 to 50 of pregnancy, then *ad libitum*-fed from day 50 to 140 of pregnancy (MAd), *ad libitum*-fed from day 21 to 50 of pregnancy, then maintenance-fed from day 50 to 140 of pregnancy (AdM); and *ad libitum*-fed throughout pregnancy (AdAd). From day 140 of pregnancy until weaning, all G0 dams and their offspring (Group 1, ewes who were foetuses at the time of G0 nutritional treatment, G1) were managed under commercial grazing conditions (average pasture cover of 1,435 ± 82 kg DM/ha). Post weaning, twin-born female G1 progeny were retained and managed as one group under commercial grazing conditions (Paten *et al*., 2011) until the present study.

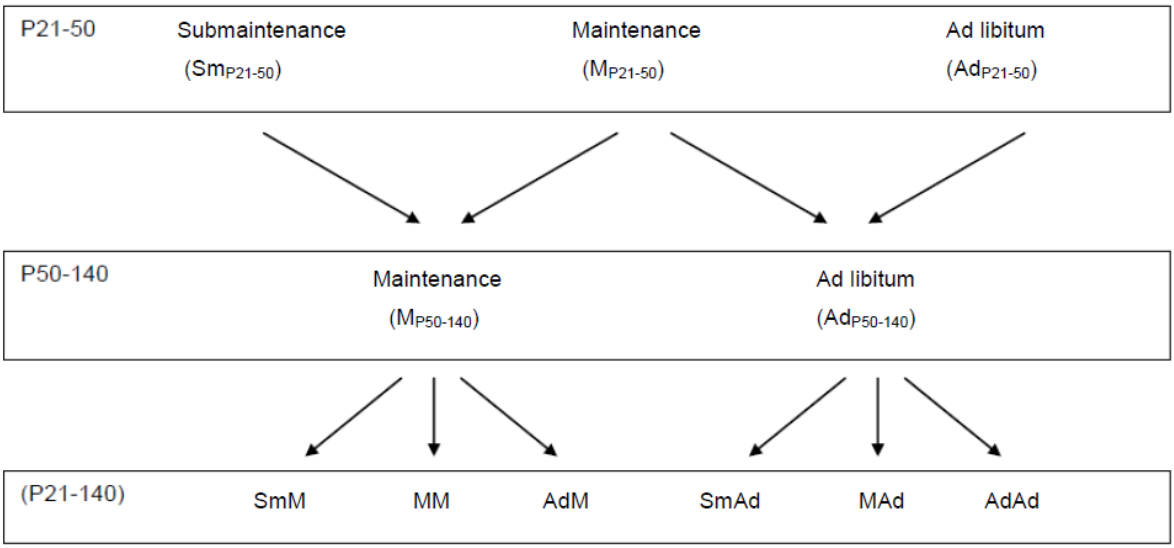


Figure 5.1 Experimental design: Dams (Group 0: dams fed nutrition treatments; G0) were fed sub-maintenance (SmP21-50), maintenance (M21-50) or *ad libitum* (AdP21-50) nutrition during early pregnancy, from day 21 to 50 of pregnancy (P21-50). Dams from each P21-50 nutrition group were then re-allocated to either pregnancy maintenance (MP50-140) or *ad libitum* (AdP50-140) nutrition during mid-to-late pregnancy, from day 50 to 140 of pregnancy (P50-140).

5.4.2 Current study

The present study used the twin-born female progeny (G1 ewes born to G0 dams: SmM, SmAd, MM, MAd, AdM and AdAd). Reproductive cycles of G1 ewes were synchronized at approximately 18 mo. of age using Controlled Internal-Drug-Release devices (CIDR; 0.3 g progesterone; Pharmacia & Upjohn, Auckland, New Zealand) for a period of 6 d. They were subsequently bred over a 17-d period, day 0 of pregnancy (P0) to day 17 of pregnancy, with 18 crayon-harnessed rams. Pregnancy diagnosis was undertaken on day 68 of pregnancy using ultrasound and the twin-bearing ewes were retained for the present study and managed under commercial conditions (average forage mass = 1440 ± 98.6 DM/ha) until weaning of their lambs (Group 2, offspring of G1 ewes, G2).

The LW and body condition score (BCS, scale 0 to 5, including half units; Jefferies, 1961) of G1 ewes were recorded on P0, day 73 of pregnancy (P73), day 139 of pregnancy (P139), day 38 of lactation (L38**;** 38 day after the midpoint of lambing), day 60 of lactation (L60), and day 102 of lactation (L102).

Sixty-six twin-bearing G1 ewes (SmM, SmAd, MM, MAd, AdM and AdAd; *n* = 11 for all treatment groups) were milked once per week. Starting from day 7 ± 1 postpartum (L7), for seven subsequent weeks, ewes were milked using the "oxytocin method" by machine followed by hand stripping, as previously described by McCance (1959), Peterson *et al. (*1997), and van der Linden *et al. (*2009). Ewes were milked in the morning and after an interval of approximately 5 h, in the afternoon. The time of each milking and weight of milk obtained was recorded. The G2 lambs were separated from the ewes between morning and afternoon milking to prevent suckling and were bottle-fed as required.

Daily milk yield was calculated using the formula:

$$\frac{24\text{ h}}{\text{time between milkings}} \times \text{milk yield at 2nd milking}$$

Milk obtained by machine and hand-stripping was mixed and subsampled for analysis of milk composition. Samples were preserved with bronopol and refrigerated at 4°C until composition analysis. Fat, CP, true protein (actual protein content, not including non-protein nitrogen; TP) casein and lactose percentages were determined using infrared spectroscopy using a Milkoscan FT120 (Foss, Hillerød, Denmark) calibrated for sheep milk (DairyNZ, Hamilton, New Zealand). The milk NE was calculated based on the percentage of CP and percentage of fat (Holmes *et al*., 2002 and used in Chapter 2).

On each milking occasion visual checks for mastitis were performed and somatic cell count estimates were obtained during composition analysis to ensure results were not confounded by infection. In addition, ewes were given a bionic capsule [Bionic Hi Mineral Combination Sheep Capsule (albendazole and abamectin); Merial, Auckland, New Zealand] at P139 to ensure milk production was not differentially affected by gastrointestinal parasite infections. Faecal egg counts were checked at day 49 of lactation to ensure the capsules were still performing adequately, which was the case.

5.4.3 Pasture quality

Based on parturition date, G1 ewes were divided into two milking subgroups and rotationally grazed over eight paddocks. Weekly pasture forage DM was estimated using a rising plate meter (50 readings per paddock; Filip's Folding Pasture Plate Meter, Jenquip, Palmerston North, New Zealand). The average grass cover was 1,614 ± 84.8 kg DM/ha.

5.4.4 Measurements of grand offspring

Within 24 h after birth, twin G2 lambs were identified to their dam and weighed (G2: SmM, SmAd, MM, MAd, AdM and AdAd; *n* = 22 for all groups). Lambs were weighed weekly for the 7-wk milking period and approximately every 4 weeks thereafter until weaning.

5.4.5 Statistical analyses

Individual animals (G1 ewes and G2 lambs) were used as the experimental unit for statistical analysis. However, to minimize variation from differing rearing rank, only data from G1 ewes with complete twin sets, and their respective G2 lambs were included. Eleven G2 lambs (SmM, *n* = 1; SmAd, *n* = 3; MAd, *n* = 3; AdM, *n* = 2; AdAd: *n* = 2) died during the milking trial period; therefore, their LW data and the data of their twin along with the milk yield and composition data of their dam were omitted from the trial. In addition, one ewe from the MAd treatment group was euthanized during the trial for necrotic mastitis. The data from this ewe and her twin lambs were also omitted. Therefore, the final numbers used for statistical analysis were as follows: G1 ewes: SmM, *n* = 10; SmAd, *n* = 8; MM, *n* = 11; MAd, *n* = 7; AdM,

n = 9; AdAd, *n* = 9 and G2 lambs: SmM, *n* = 20; SmAd, *n* = 16; MM, *n* = 22; MAd, *n* = 14; AdM, *n* = 18; and AdAd, *n* = 18.

5.4.5.1 Ewe LW and BCS

Univariate analyses were performed on ewe LW and BCS at mating (P0; 20 mo. of age), set stocking (P139; 25 mo. of age), docking (L38; 26 mo. of age), and weaning (L102; 28 mo. of age). Dam nutrition during P21-50, dam nutrition during P50-140, and the two-way interaction of dam nutrition during P21-50×P50-140 were fitted as fixed effects.

5.4.5.2 Calculation of ewe accumulated milk and composition yields

A third-degree orthogonal polynomial model was fitted to milk yield and composition data for each ewe milked:

$$yi = \alpha_0 \, \emptyset_{0i} + \alpha_1 \, \emptyset_{1i} + \alpha_2 \, \emptyset_{2i} + \alpha_3 \, \emptyset_{3i} + e_i,$$

in which y_i is the record of milk or composition taken at day *i*, α_n is the *n* regression and $\emptyset_{ni}$ is rescaled value of days in milk *i* calculated as:

$$\emptyset_{oi} = 1; \; \emptyset_{1i} = x; \; \emptyset_{2i} = \frac{(3x^2 - 1)}{2}; \emptyset_{3i} = \frac{(5x^3 - 3x)}{2},$$

in which $x = \{2[i - (50 + 1)]\}/(50 - 1)$ and e_i is the error term associated with each milk record. Accumulated milk and composition (fat, CP, TP, casein, NE and lactose) yields were calculated over day 1 to 50 of lactation for each animal using estimates calculated from the regression coefficients of the third-degree orthogonal polynomial model.

5.4.5.3 Ewe milk yields and milk composition

Accumulated milk, fat, CP, TP, casein, NE and lactose yields and percentages were analysed using PROC MIXED (SAS Inst., Cary, NC) with a linear model including fixed effects of dam nutritional treatment during P21-50 and P50-140 and the interaction of the two dam nutritional treatments.

A repeated measure analysis was performed on the daily recorded milk yields and milk components using PROC MIXED in SAS. Fixed effects included day of lactation (as the repeated time measure), dam nutrition during P21-50 and P50-140 and the two-way interaction of the two dam nutritional treatments, and three-way interaction of day of lactation×P21-50×P50-140. Milk yield and composition analyses were not adjusted for BCS or ewe LW as it was a component of the experimental effect.

5.4.5.4 Lamb LW and growth rates

A repeated measure analysis was performed on G2 lamb LW using PROC MIXED in SAS with fixed effects of G2 lamb age (as the repeated time measure), sex, granddam nutritional treatment during P21-50 and P50-140; two-way interactions of granddam nutritional treatment during P21-50×G2 lamb age, and P50-140×G2 lamb age, and granddam nutritional treatment during P21-50×P50-140. The three-way interaction of G2 lamb age×P21-50×P50-140 was also fitted. Growth rate to weaning was analysed using PROC MIXED in SAS with fixed effects of sex, granddam nutrition during P21-50 and P50-140, and the two-way interaction of granddam nutrition in both periods.

5.5 Results

5.5.1 Ewe LW and BCS

In the present study, there was no effect ($P > 0.05$) of dam nutrition during P21-50 or P50-140 on G1 ewe LW at P0 through to L102 (Table 5.1). However, BCS did significantly differ at P139 with ewes born to dams fed at sub-maintenance levels during early pregnancy (Sm_{P21-50}) having less ($P = 0.01$) body condition than ewes born to dams fed at maintenance (M_{P21-50}) or *ad libitum* (Ad_{P21-50}) levels during P21-50. At L102, ewes born to dams fed at pregnancy maintenance levels during P50-140 ($M_{P50-140}$) tended to have less ($P = 0.10$) body condition than ewes born to dams fed at *ad libitum* levels during P50-140 ($Ad_{P50-140}$). There were no interaction effects ($P > 0.05$) of dam nutrition during P21-50×P50-140 on either ewe LW or BCS from P0 to L102 (data not shown).

Table 5.1 Ewe LW (kg) and BCS (scale 0 to 5) at mating (20 mo. of age), set stocking (25 mo. of age), docking (26 mo. of age), and weaning (28 mo. of age) for ewes born to dams fed sub-maintenance (Sm; *n* = 18), maintenance (M; *n* = 18), or *ad libitum* (Ad; *n* = 18) from day 21 to 50 of pregnancy (P21-50), and pregnancy maintenance (M; *n* = 30) or *ad libitum* (Ad; *n* = 24) nutrition from day 50 to 140 of pregnancy (P50-140)[1]

	Dam nutritional treatment[2]								
	P21-50			SEM	P50-140		SEM	*P*-value	
Trait	Sm	M	Ad		M	Ad		P21-50	P50-140
LW (kg)									
Mating day 0 of pregnancy	63.2	65.2	63.7	1.34	64.0	64.0	1.09	0.60	0.77
Set stocking day 139 of pregnancy	81.8	83.6	81.6	1.42	81.4	83.3	1.15	0.54	0.29
Docking day 38 of lactation	63.2	66.5	64.5	1.43	63.5	66.0	1.16	0.29	0.15
Weaning day 102 of lactation	61.0	63.5	62.0	1.68	61.9	62.4	1.34	0.59	0.78
BCS									
Mating day 0 of pregnancy	3.9	3.7	3.8	0.11	3.8	3.8	0.09	0.66	0.80
Set stocking day 139 of pregnancy	2.5[b]	3.0[a]	2.9[a]	0.11	2.8	2.8	0.08	0.01	0.82
Docking day 38 of lactation	2.0	2.2	2.1	0.06	2.1	2.1	0.05	0.16	0.94
Weaning day 102 of lactation	2.4	2.6	2.5	0.10	2.4	2.6	0.08	0.23	0.10

[a, b] Within a row, within treatment periods, means without a common superscript differ ($P < 0.05$).

[1]Table shows least squares means ± pooled SEM.

[2]No interactions between dam nutrition during P21-50 and P50-140 were observed ($P > 0.05$); therefore, only the main effects have been reported.

5.5.2 Lactation curve and milk composition

There was no interaction ($P > 0.05$) of dam nutrition during early (P21-50) and mid-to-late (P50-140) pregnancy on ewe-offspring lactation performance (data not shown). A tendency, however, was observed for dam nutrition during early (P21-50) pregnancy to affect the lactation performance of ewe offspring. The average daily milk yields of M_{P21-50} ewes tended to be greater ($P = 0.07$), over the 49-day lactation period, compared with Sm_{P21-50} and Ad_{P21-50} ewes (2.6 ± 0.08, 2.4 ± 0.08 and 2.3 ± 0.08 L, respectively; Figure 5.2). These differences were more pronounced during early and late lactation than mid lactation, such that M_{P21-50} ewes tended ($P = 0.10$) to produce more milk at L7 and produced more ($P = 0.03$) milk at day 42 of lactation (L42) than Sm_{P21-50} ewes. In addition, M_{P21-50} ewes had greater milk yields at day 14 (L14) of lactation ($P = 0.03$), day 35 of lactation ($P = 0.01$), and L49 ($P = 0.05$) when compared with Ad_{P21-50} ewes (Figure 5.2). Overall, M_{P21-50} ewes tended to have greater accumulated milk ($P = 0.10$) and NE ($P = 0.06$) yields than Sm_{P21-50} and Ad_{P21-50} ewes (Table 5.2). Additionally, M_{P21-50} ewes tended to have greater ($P = 0.07$) accumulated fat yields when compared with Sm_{P21-50} ewes.

Mid-to-late pregnancy G0 dam nutrition did not appear to affect average daily milk yields of the G1 ewe offspring; however, $Ad_{P50-140}$ ewes produced more ($P = 0.04$) milk at L7 and tended to produce more milk at L49 ($P = 0.08$) than $M_{P50-140}$ ewes (Figure 5.2). Overall, $Ad_{P50-140}$ ewes tended to have greater accumulated milk ($P = 0.10$) and lactose ($P = 0.09$) yields than $M_{P50-140}$ ewes (Table 5.2).

Despite some differences observed in composition yields there were no differences ($P > 0.05$) in average milk composition percentages (fat, lactose, CP, TP, casein, and NE) for either P21-50 or P50-140 G0 dam nutritional treatments (Table 5.3). There were no time by G0 dam nutritional treatments interactions ($P > 0.05$) observed for G1 ewe milk and composition yields or composition percentages (data not shown).

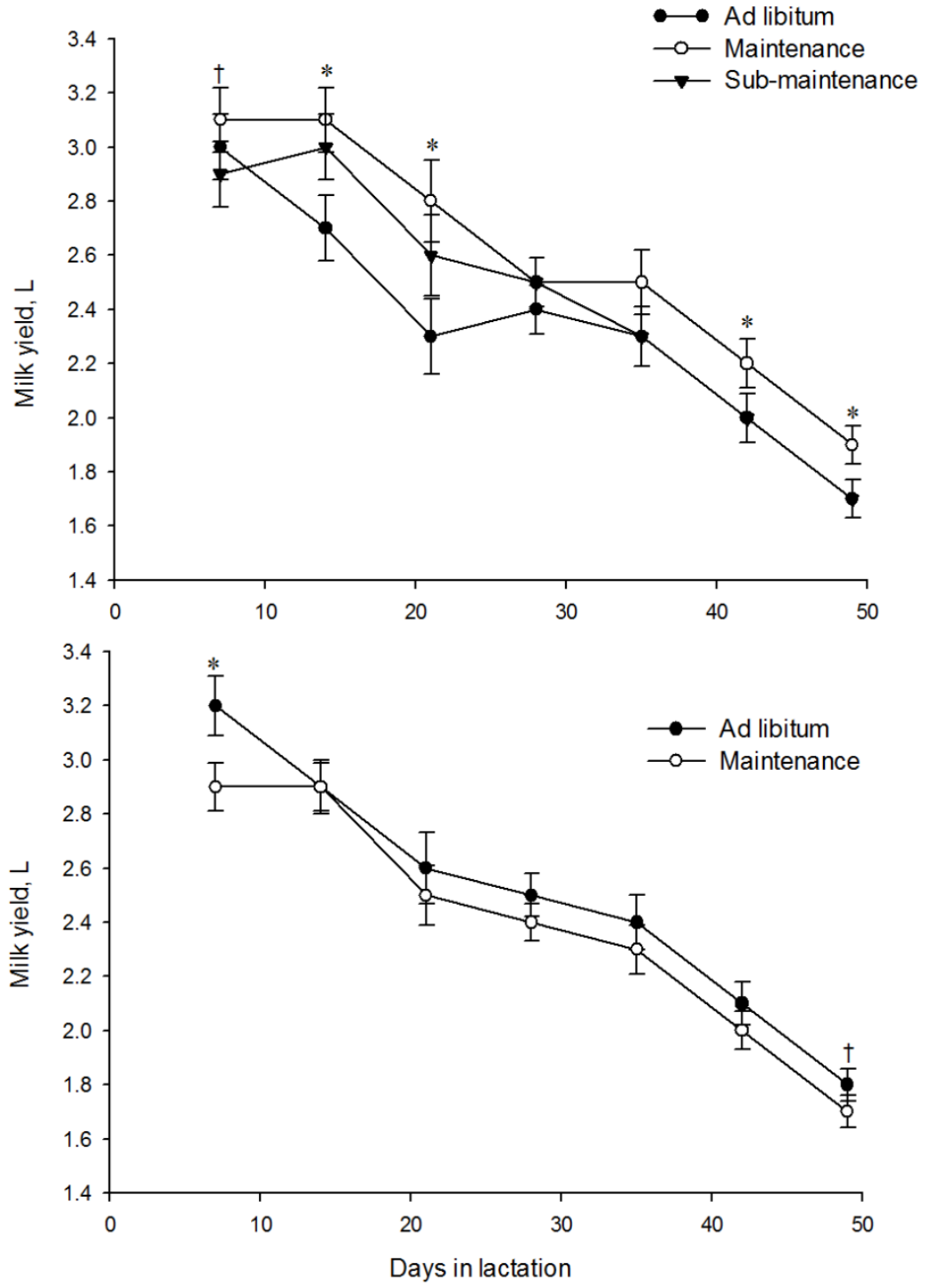


Figure 5.2 First 50 days of lactation daily milk yields of ewes born to dams fed sub-maintenance (*n* = 18), maintenance (*n* = 18), or *ad libitum* (*n* = 18) nutrition during early pregnancy, day 21 to 50 of pregnancy (top panel), and pregnancy maintenance (*n* = 30), or *ad libitum* (*n* = 24) nutrition during mid-to-late pregnancy, day 50 to 140 of pregnancy (bottom panel). Differences between means at individual time points are indicated with either * $P < 0.05$ or † $P < 0.10$.

Table 5.2 Accumulated milk, lactose, crude protein (CP), true protein (actual protein content, not including non-protein nitrogen, TP), casein, and fat yields (kg), and net energy (NE) yields (MJ) calculated over day 7 to day 50 of lactation, of ewes born to dams fed sub-maintenance (Sm; *n* = 18), maintenance (M; *n* = 18), or *ad libitum* (Ad; *n* = 18) from day 21 to 50 of pregnancy (P21-50), and pregnancy maintenance (M; *n* = 30) or *ad libitum* (Ad; *n* = 24) from day 50 to 140 of pregnancy (P50-140)[1].

	Dam nutritional treatment[2]								
	P21-50			SEM	P50-140		SEM	*P*-value	
Trait	Sm	M	Ad		M	Ad		P21-50	P50-140
Milk yield, kg	122.6	133.7	122.5	4.07	122.3	130.3	3.31	0.10	0.10
Lactose yield, kg	6.3	6.9	6.3	0.21	6.3	6.7	0.17	0.12	0.09
CP yield, kg	5.9	6.3	6.0	0.17	5.9	6.2	0.13	0.21	0.19
TP yield, kg	5.6	5.9	5.6	0.15	5.6	5.8	0.12	0.22	0.18
Casein yield, kg	4.7	5.0	4.7	0.13	4.7	4.9	0.10	0.25	0.18
Fat yield, kg	8.0	9.3	8.6	0.38	8.5	8.8	0.31	0.07	0.42
NE yield, MJ	546.2	613.8	561.8	20.13	562.1	585.8	16.33	0.06	0.32

[1]Table shows least squares means ± pooled SEM.

[2]No interactions between dam nutrition during P21-50 and P50-140 were observed ($P > 0.05$); therefore, only the main effects are reported.

Table 5.3 Average lactose, crude protein (CP), true protein (actual protein content, not including non-protein nitrogen; TP), casein, fat, and net energy (NE) percentages, calculated over a 49-day lactation period, of ewes born to dams fed sub-maintenance (Sm; *n* = 18), maintenance ($M_{21\text{-}50}G1$, *n* = 18), or *ad libitum* ($Ad_{21\text{-}50}G1$, *n* = 18) from day 21 to 50 of pregnancy (P21-50), and pregnancy maintenance ($M_{50\text{-}140}G1$, *n* = 30) or *ad libitum* ($Ad_{50\text{-}140}G1$, *n* = 24) from day 50 to 140 of pregnancy (P50-140)[1].

	Dam nutritional treatment[2]							
	P20-50			SEM	P50-140		SEM	*P*-value
Trait	Sm	M	Ad		M	Ad		P20-50	P50-140
Lactose, %	5.1	5.1	5.2	0.02	5.1	5.2	0.02	0.89	0.70
CP, %	4.8	4.7	4.9	0.07	4.9	4.8	0.06	0.58	0.32
TP, %	4.5	4.5	4.6	0.07	4.5	4.5	0.06	0.61	0.35
Casein, %	3.8	3.8	3.9	0.06	3.8	3.8	0.05	0.50	0.40
Fat, %	6.3	6.8	6.8	0.22	6.7	6.6	0.18	0.21	0.50
NE, MJ/L	4.3	4.5	4.5	0.09	4.5	4.4	0.07	0.35	0.34

[1]Table shows least squares means ± pooled SEM.

[2]No interactions between dam nutrition during P20-50 and P50-140 were observed ($P > 0.05$); therefore, only the main effects are reported.

5.5.3 Grand offspring birth weight and LW

There was no effect ($P > 0.05$) of G0 granddam nutrition during either P21-50 or P50-140 on G2 grand-offspring birth weights (Table 5.4). The LW of G2 lambs born to G1 ewes whose G0 dams were fed at *ad libitum* levels during early pregnancy (Ad_{P21-50}) were lighter ($P = <0.0001$) on average until weaning than G2 lambs born to G1 ewes whose G0 dams were fed at sub-maintenance (Sm_{P21-50}) or maintenance (M_{P21-50}) levels during P21-50. In contrast, G2 lambs with G0 granddams fed at *ad libitum* levels during P50-140 ($Ad_{P50-140}$) were heavier ($P = 0.01$) on average until weaning than G2 lambs with G0 granddams fed at maintenance levels during P50-140 ($M_{P50-140}$).

At weaning Ad_{P21-50} lambs were lighter ($P = 0.05$) than Sm_{P21-50} lambs and tended to be lighter ($P = 0.07$) than M_{P21-50} lambs whereas the weaning LW of Ad_{P21-50} and M_{P21-50} lambs did not differ from each other. There were no differences between G2 lamb growth rates during birth to weaning from either P21-50 or P50-140 granddam nutritional treatments (Table 5.4), and there were no time by G0 dam nutritional treatments interactions observed for G2 lamb LW or growth rates (data not shown).

Table 5.4 Lamb LW (kg), from birth (day 0) to weaning (day 102), average LW from birth to weaning, and growth rate to weaning (g/d), for lambs whose granddams were fed sub-maintenance (Sm; *n* = 36), maintenance (M; *n* = 36), or *ad libitum* (Ad; *n* = 36) during early pregnancy, from day 21 to 50 of pregnancy (P21-50), and pregnancy maintenance (M; *n* = 60) or *ad libitum* (Ad; *n* = 48) nutrition during mid-to-late pregnancy, from day 50 to 140 of pregnancy (P50-140)[1].

	Grand-dam nutritional treatment[2]						
	P21-50			SEM	P50-140		SEM
Grand-offspring trait	Sm	M	Ad		M	Ad	
Birth weight, kg	4.7	4.7	4.7	0.12	4.7	4.7	0.10
LW at day 21, kg	10.5	10.3	9.9	0.27	10.0	10.4	0.22
LW at day 49, kg	16.1	15.7	15.0	0.40	15.3	15.8	0.32
LW at weaning, kg	27.3[a]	27.1[ab]	25.2[b]	0.74	26.2	26.8	0.60
Average LW from birth to weaning, kg	14.0[a]	14.0[a]	13.3[b]	0.13	13.6[b]	14.0[a]	0.10
Growth rate to weaning, g/d	224.5	221.3	208.5	6.79	213.3	223.0	5.53

[a, b]Within a row, within treatment period, means without a common superscript differ (P <0.05).

[1]Table shows least squares means ± pooled SEM.

[2]No interactions between grand-dam nutrition during P21-50 and P50-140 were observed (P > 0.05); therefore, only the main effects are reported.

5.6 Discussion

This study aimed to identify the critical window in which the mammary gland and its future functionality can be programmed during foetal life. The effects of dam nutrition during early and mid-to-late pregnancy on the first lactation performance of the subsequent G1 ewe offspring and the LW to weaning of the G2 grand-offspring were investigated. No interaction effects were observed between the two treatment periods, P21-50 and P50-140; therefore, they will be discussed separately.

5.6.1 Dam nutrition during early pregnancy

Organogenesis of the mammary gland begins in early embryogenesis, when much of the mammary precursor tissue is formed (Forsyth *et al*., 1999; Hovey *et al*., 2002); therefore, it was hypothesized that nutritional perturbations during early gestation (P21-50) would affect mammary gland development and subsequent lactational performance of the G1 offspring. Indeed, Martín *et al. (*2012) observed that sub-maintenance feeding of dams during early pregnancy impaired foetal mammary gland growth. This indicates that early pregnancy is a "critical programming" period for the foetal mammary gland and that dam underfeeding during this period may be detrimental for offspring mammary gland development. This concept is supported by findings in the subsequent adult ewe cohort that Sm_{P21-50} ewes had lower milk, milk fat, and milk NE yields when compared with M_{P21-50} ewes. Additionally, Sm_{P21-50} ewes had lower BCS in late pregnancy, which may have contributed to reduced milk production (Gibb & Treacher, 1982; Kenyon *et al*., 2004) compared with M_{P21-50} ewes. Interestingly, despite the observed differences in milk composition and yields, there were no differences in the LW or growth rates of lambs born to M_{P21-50} and Sm_{P21-50} ewes from birth until weaning. Martín *et al. (*2012) found that foetuses from dams fed at *ad libitum* levels during early pregnancy had lighter mammary glands than foetuses from maintenance-fed dams although this difference did not reach statistical significance. In the present adult cohort, Ad_{P21-50} ewes produced less milk, milk fat, and milk NE yields and weaned lighter lambs when compared with M_{P21-50} ewes. There were no differences between the BCS of Ad_{P21-50} and M_{P21-50} ewes from mating to weaning. These findings are consistent with the findings of van der Linden *et al. (*2009).

There were no differences between the milk yields or composition of Ad_{P21-50} and Sm_{P21-50} ewes despite Ad_{P21-50}G1 ewes having greater BCS in late pregnancy. In contrast, Sm_{P21-50} ewes also weaned heavier G2 lambs when compared with Ad_{P21-50} ewes.

These findings indicate that overfeeding and underfeeding of ewes during early pregnancy may adversely affect offspring lactation performance. The differences in ewe milk production,

however, are inconsistent with differences in lamb weaning LW. One explanation could be that our technique used to measure ewe milk production may measure the potential milk production but not provide an accurate estimate of lamb milk intake. In a study comparing milk-estimation techniques with lamb growth rates van der Linden *et al. (*2010b) reported a poor correlation of ewe milk yield with singleton lamb growth rates. However, the present study investigated the LW of twin-born and reared lambs. An alternative explanation is a difference in an unmeasured milk component. A further alternative is that granddam gestational nutrition may cause an independent intergenerational programming effect on genes associated with growth and metabolism, which led to the observed differences in G2 LW (Blair *et al*., 2010; van der Linden *et al*., 2010a). However, further investigation is required to clarify this.

5.6.2 Dam nutrition during mid-to-late pregnancy

During mid-to-late gestation, foetal mammary gland development involves formation of the secondary ducts, which will give rise to the major collecting ducts of the adult gland (Forsyth *et al*., 1999; Hovey *et al*., 2002), and expansion of epithelial tissue and fat-pad from the mammary mesenchyme and fat-pad precursor tissue (Forsyth *et al*., 1999). Disruptions in development during this period may also impair future lactational performance (Hovey *et al*., 2002; Capuco and Akers, 2010). Martín *et al. (*2012) reported that dam nutritional treatment during mid-to-late pregnancy did not affect foetal mammary gland size. However, in the adult ewe cohort there was a tendency for $M_{P50\text{-}140}$ ewes to produce less accumulated milk and lactose yields over a 50-d period compared with $Ad_{P50\text{-}140}$ ewes. This indicates that foetal mammary gland size may not be a good indicator of function in adult life. There were, however, no differences in average daily milk or milk components yields. There were no differences from G0 dam P50-140 nutritional treatments on G1 ewe BCS at mating or late pregnancy. In addition, at weaning, G1 $M_{P50\text{-}140}$ ewes tended to have lower BCS, than G1 $Ad_{P50\text{-}140}$ ewes, indicating that they lost condition during lactation. It is possible that dam nutritional treatment during P50-140 may also affect metabolism and nutrient partitioning during lactation (Wilson *et al*., 1983). Further investigation into these effects may be warranted.

On average, from birth to weaning, G2 $Ad_{P50\text{-}140}$ lambs were heavier than G2 $M_{P50\text{-}140}$ lambs. However, the minimal differences observed in ewe milk production from the P50-140 dam nutritional treatments were not sufficient to alter G2 lamb LW at weaning.

5.7 Summary and conclusions

In summary, it appears there are minimal effects of dam nutrition during mid-to-late pregnancy on offspring milk production and grand-offspring LW. However, both under- and overfeeding during early pregnancy can be detrimental to milk production of offspring. This has

implications for management of production animals and human nutrition research. The cellular mechanisms driving the observed results are yet to be determined and further investigation is currently underway. Interestingly, it appears that G0 granddam sub-maintenance nutrition during P21-50 affects G2 grand offspring LW at weaning independently of G1 ewe lactation performance. This finding indicates there may be multigenerational foetal programming effects. Further research to better elucidate the apparent conflicting observations between G2 LW and G1 milk yield is warranted.

6 Effects of ewe nutrition during early and mid-to-late pregnancy on the performance of twin-born ewe offspring during their first parity pregnancy and lactation.

6.1 Abstract

The aim of this study was to investigate the effects of dam nutrition during early (P21-50) and mid-to-late (P50-140) pregnancy on the first parity pregnancy and lactational performance of the twin-born ewe offspring, and their offspring to weaning. The present study followed the productive performance, from 18 months of age, of ewes which were twin-born to dams which were allocated to either sub-maintenance (Sm_{P21-50}), maintenance (M_{P21-50}) or *ad libitum* (Ad_{P21-50}) nutrition during early pregnancy (day 21 to 50 of gestation), and then re-allocated to either pregnancy maintenance ($M_{P50-140}$) or *ad libitum* ($Ad_{P50-140}$) nutrition during mid-to-late pregnancy (day 50 to 140 of gestation). This resulted in six nutritional treatments groups: SmM, SmAd, MM, MAd, AdM and AdAd. At 18 months of age the offspring were bred (*n* = 265). Live weight and body condition scores of ewe offspring were recorded at breeding, during pregnancy, and during lactation. Live weight of grand-offspring was measured from birth until weaning. Ewe offspring born to SmM- and MAd-dams were lighter from late pregnancy, when compared to ewes born to MM-dams, which suggested they may be less physiologically adapted to cope with the stress of pregnancy and lactation, and potentially less able to provide for their lambs (the grand-offspring). However, there were no consequences for the grand-offspring, which may either suggest that there were minimal differences in productive performance of the ewe offspring, or that grand-offspring of SmM- and MAd-granddams are themselves programmed to overcome environmental stress. Further investigation into the growth and metabolic responses of grand-offspring to physiological stress may be warranted.

6.2 Introduction

The productivity of a ewe is measured by her ability to conceive and rear lambs that are heavy (15.5 to 17.4kg carcass weight (CW) is standard in New Zealand, ≥17.5kg is heavy) at weaning. Successful weaning of heavy lambs relies on the ewe's ability to provide nutrients to her conceptus during pregnancy (Morgan *et al*., 2007) and postnatal production of milk. The reproductive performance of a ewe may be influenced by her own intra-uterine experiences. Dam nutrition during pregnancy is known to affect development of the foetal mammary gland (van der Linden *et al*., 2009, Blair *et al*., 2010, Martín *et al*., 2012). Recently, van der Linden *et al. (*2009) reported that ewes born to dams which were fed pregnancy maintenance, compared to *ad libitum*, from days 21 to 140 of pregnancy gave birth to and weaned heavier lambs. These findings suggested that manipulation of the maternal environment during gestation could influence the productive performance of future generations of offspring and highlighted this as a potential management consideration for farmers.

The timing and levels of ewe nutrition treatments in the study of van der Linden *et al. (*2009) make it difficult to recommend specific management strategies to farmers. A follow up study was designed (described in Chapter 3; Kenyon *et al*., 2011a) to investigate and compare the effects and interactions between dam nutrition during early and mid-to-late pregnancy on the lactational performance of the progeny. In late gestation foetuses, Martin *et al. (*2012) reported that sub-maintenance dam nutrition during early pregnancy resulted in reduced mammary gland mass, compared with pregnancy maintenance or *ad libitum* nutrition, which suggested that there may be future differences in milk production as adults.

A cohort of twin-born, twin-bearing ewes were subsequently milked (Chapter 5). Ewes born to $Sm_{P21\text{-}50}$- and $Ad_{P21\text{-}50}$-dams produced less milk during their first lactation compared with those born to $M_{P21\text{-}50}$-dams (Paten *et al*., 2013). Differences in LW, however, of the grand-offspring were not consistent with that of dam milk production. The numbers of experimental animals in Chapter 5 were small, and only twin-bearing ewes and their lambs were studied.

The aim of the present study was to investigate the lambing performance of the entire cohort of offspring including single- and twin-bearing ewes. Based on the previous findings of van der Linden *et al. (*2009), Martin *et al. (*2012) and in Chapter 5 (Paten *et al*., 2013) it was hypothesized that both under-nutrition and *ad libitum* feeding of the granddam, specifically during early pregnancy, would have a negative effect on the weaning weights of grand-offspring.

6.3 Material and methods

6.3.1 Experimental design

The dam nutritional treatments and management have been described in Chapter 3. Briefly, ewes were fed either, sub-maintenance ($Sm_{P21\text{-}50}$), maintenance ($M_{P21\text{-}50}$), or *ad libitum* ($Ad_{P21\text{-}50}$) pasture allowance during early gestation (P21-50) and then reallocated to either a pregnancy maintenance ($M_{P50\text{-}140}$) or *ad libitum* ($Ad_{P50\text{-}140}$) pasture allowance during mid-to-late pregnancy (P50-140). Therefore, the offspring generated were from one of six dam nutritional treatment groups: SmM, SmAd, MM, MAd, AdM, and AdAd. Post-weaning, the offspring were managed together under commercial grazing conditions.

6.3.2 Experimental procedures and measurements

6.3.2.1 Management of ewes

Twin-born ewe offspring (*n* = 265) were synchronised at 18 months of age, using Controlled Internal Drug Releasing devices (CIDRs, 0.3 g progesterone; Pharmacia & UpJohn, Auckland, New Zealand), and subsequently bred with 18 crayon-harnessed Romney rams (Rams joined on 29th April (P0) and removed on 21st May (P22), 2011). Ram-harness-crayon marks on the rump of the ewes were recorded daily for 5-d as an indicator of breeding activity. The colour of the crayon was changed at P5 and a new crayon was placed in the harness for a further 17 days. Pregnancy diagnosis was carried out at P74 using trans-abdominal ultrasonography and ewes were identified as either: empty, single-, twin-, or triplet-bearing. A cohort of twin-bearing ewes (*n* = 66) was managed separately, from P140 (16th Sept, 2011) until day 49 of lactation (L49), to analyse milk production (reported in Chapter 5; Paten *et al*., 2013). Remaining ewes were allowed to lamb naturally on pasture. The LW and BCS of ewes were recorded on P0, P74, P139, L38 and L102.

6.3.2.2 Pasture quality

Ewes were managed, and allowed to lamb under commercial pastoral production conditions. Pasture quality and mass was monitored from P145 over the lambing period (21st Sept to 19th Oct, 2011) Weekly pasture DM mass was estimated using a rising plate meter (50 readings per paddock; Filip's Folding Pasture Plate Meter, Jenquip, Palmerston North, New Zealand). The average pasture mass on a sampling day was determined using the following calculation:

$Pasture\ mass\ (kg\ DM/ha) = (158 \times MR) + 200$, where MR is the average meter reading (Hodgson *et al*., 1999). The average pasture cover was 1328.3 ± 378.0 (SD) kg DM/ha.

To mimic ewe intake, pasture pluck samples were collected fortnightly and were immediately frozen at -20˚C until analysis of the nutritive value. Percentage of CP, lipid, neutral detergent fibre (NDF) and acid detergent fibre (ADF), and ME (MJ/kg) were estimated using near infrared spectroscopy (NIRS) using a Bruker MPA NIR spectrometer (Bruker, Ettigen, Germany) and OPUS software (version 5.0). Average pasture quality was as follows: CP = 17.3 ± 3.09%, lipid = 2.0 ± 0.37%, NDF = 46.0 ± 5.35%, ADF = 23.9 ± 3.06% and ME = 12.0 ± 0.85 MJ/kg.

6.3.2.3 Measurements of grand-offspring

Within 24 hours after birth, lambs were identified to their dam and ear-tagged. Measurements at this time included; lamb LW, thoracic girth, crown-rump length (CRL), right fore-leg (from elbow (cubital) joint to toe of the hoof, FL) length, and right hind-leg (from hip (coxofemoral) joint to toe of the hoof, HL) length. Lambs were re-weighed at L38 and at L102 and their growth (kg/d) from birth until weaning (L1-102) was calculated.

6.3.3 Statistical analysis

Individual animals were used as the experimental unit for statistical analyses. The ewe LW and BCS data recorded at P0 was analysed for the whole population of ewes, while from P74 only the data for ewes identified as single- or twin-bearing were analysed with pregnancy rank (PR: single = S, twi*n* = T) fitted as a fixed effect. In addition only data on single- or twin-born lambs was analysed with birth rank (BR: single = S and twi*n* = T) fitted as a fixed effect for measurements at birth, and rearing rank (RR: single = S; Twin-born, single-reared = TS, Twin-born, twin-reared = T) fitted as a fixed effect for measurements at L38 and L102. All data was analysed using proc mixed in SAS (SAS 9.2) and post-hoc comparisons of means were carried out using Tukey's HSD test. Data from non-pregnant (SmM, *n* = 4; MM, *n* = 5; MAd, *n* = 1; and AdAd, *n* = 4) and triplet-bearing (SmM, *n* = 1; AdM, *n* = 1; and AdAd, *n* =3), and data of two ewes (SmM, *n* = 1 and MM, *n* = 1) that died prior to PD, were excluded from all analyses after P0. Data from a further 5 ewes (SmM, *n* = 2; MM, *n* = 1; MAd, *n* = 1; and AdM, *n* = 1) which died, and 16 ewes (SmM, *n* = 3; SmAd, *n* = 1; MM, *n* = 4; MAd, *n* = 4; AdM, *n* = 3; and AdAd, *n* = 1) whose lambs died prior to docking were removed for analyses from L38. Data from another ewe (MM) whose lamb died prior to weaning was removed from the analyses at L102. Data from the ewe flock managed separately for milking (Chapter 5) were included in all analyses and lambing group was fitted as a random effect to account for any differences.

6.3.3.1 Ewe LW and BCS

Univariate analyses were performed on ewe LW and BCS at P0, P74, P139, L38 and L102. Dam nutrition during P21-50, dam nutrition during P50-140, PR (for measurements at P74 and P139) or RR (for measurements at L38 and L102), and the respective two- and three-way interactions were

fitted as fixed effects. Non-significant interactions were removed from the model, with the exception of dam nutrition during P21-50×P50-140, which was always retained to test the study design. Lambing group was fitted as a random effect for measurements at L38 and L102.

6.3.3.2 Lamb LW and growth rates

Univariate analyses were performed on body measurements and LW of lambs at L1, and LW of lambs at L38 and L102. The models included fixed effects of: sex, BR (for measurements at birth) or RR (for measures in lactation, L38 and L102), dam nutrition during P21-50, dam nutrition during P50-140 and their two- and three-way interactions. Non-significant interactions were removed from the model, with the exception of dam nutrition during P21-50×P50-140, which was always retained to test the study design. Date of birth was fitted as a covariate for all analyses and lambing group was fitted as a random effect. The analyses for measurements at birth and LW at L38 and L102 were run with and without adjustment for LW at birth.

6.4 Results

6.4.1 Ewe live weight and body condition scores

There was no interaction of dam nutrition during P21-50×P50-140 on ewe BCS at P0, P74, L38 or L102 (Table 6.2). There was also no interaction of dam nutrition during P21-50×P50-140 on ewe LW at P0 and at P74 and no independent effects of dam nutrition during P21-50 and P50-140 on ewe LW (Table 6.1) and BCS at mating or on ewe BCS at P74 (Table 6.2). At P74, however, ewes born to $Ad_{P50-140}$-dams tended (P = 0.06) to be lighter than ewes born to $M_{P50-140}$-dams. Twin bearing ewes were heavier (P = 0.001) than single bearing ewes at P74 and P140. The interaction between dam nutrition during P21-50×P50-140 affected ewe LW at P140 (P = 0.03), L38 (P = 001) and L102 (P = 0.001). At L38 and L102, ewes born to SmM-dams and MAd-dams were lighter than ewes born to MM-dams. Means of ewe LW at P140 were not found to differ when compared post-hoc using Tukey's test (Table 6.1).

Ewes born Sm_{P21-50}-dams had lower BCS at P140 than ewes born to M_{P21-50}-dams, while BCS of ewes born to Ad_{P21-50}-dams did not differ from either treatment. Twin-bearing ewes also had lower BCS at P140 than single-bearing ewes. Dam nutrition during P50-140 did not affect ewe BCS at P140. There were no effects of dam nutrition during P21-50 and P50-140 on ewe BCS at L38 and L102. Ewes which gave birth to twin lambs, reared as either twins or singles, had lower BCS and LW at L35 than ewes which had single-lambs. At L102, ewes which had reared twin-lambs had lower BCS and LW than ewes which had reared single lambs (both single- and twin-born) (Table 6.2).

Table 6.1 Least-squares means ± SEM of ewe live weight (kg) at breeding (P0), scanning (P74), pregnancy day 140 (P140), docking (L38) and weaning of their lambs (L102) of ewe offspring in their first parity born to dams fed either sub-maintenance (Sm), maintenance (M) or *ad libitum* (Ad) nutrition during early pregnancy (P21-50) and either pregnancy maintenance (M) or *ad libitum* (Ad) nutrition during mid-to-late pregnancy (P50-140). Ewes had a pregnancy rank (PR) of single- (S) or twin-bearing (T), and a rearing rank (RR) for their lambs which were single-born and reared (S), twin-born and reared, or twin-born and single-reared (TS)

	Ewe live weight									
	n	P0	*n*	P74	*n*	P140	*n*	L38	*n*	L102
PR / RR										
S			85	66.9 ± 0.81[a]	85	79.3 ± 0.76[a]	78	73.0 ± 0.78[b]	77	68.0 ± 0.70[b]
T			158	69.5 ± 0.59[b]	157	81.7 ± 0.56[b]	30	67.6 ± 0.96[a]	31	62.6 ± 0.70[a]
TS							113	67.1 ± 1.27[a]	107	66.3 ± 1.11[b]
P value				0.009		0.009		<0.0001		<0.0001
Trt P21-50										
Sm	97	63.2 ± 0.72	94	67.8 ± 0.94	94	80.3 ± 0.88	86	69.0 ± 1.04	82	65.4 ± 0.88
M	70	64.0 ± 0.61	63	69.1 ± 0.81	62	80.6 ± 0.76	54	69.3 ± 0.90	52	65.9 ± 0.77
Ad	91	63.4 ± 0.59	86	67.5 ± 0.79	86	80.6 ± 0.74	81	69.4 ± 0.85	81	65.6 ± 0.73
P value		0.678		0.318		0.964		0.937		0.897
Trt 50-140										
M	117	63.9 ± 0.49	107	69.1 ± 0.66	107	80.8 ± 0.62	91	69.9 ± 0.80	88	66.1 ± 0.67
Ad	141	63.2 ± 0.56	136	67.3 ± 0.73	135	80.2 ± 0.69	130	68.6 ± 0.81	127	65.2 ± 0.68
P value		0.404		0.062		0.478		0.180		0.330
Trt P21-50×P50-140										
SmM	47	62.3 ± 0.93	43	67.5 ± 1.24	43	79.0 ± 1.16	36	67.1 ± 1.37[a]	34	63.4 ± 1.22[a]
SmAd	50	64.1 ± 1.11	51	68.2 ± 1.39	51	81.7 ± 1.30	50	70.9 ± 1.38[ab]	48	67.5 ± 1.22[ab]
MM	29	65.3 ± 0.81	23	71.4 ± 1.09	23	82.3 ± 1.02	18	72.1 ± 1.16[b]	17	67.9 ± 1.01[b]
MAd	41	62.7 ± 0.91	40	66.9 ± 1.18	39	78.9 ± 1.11	36	66.6 ± 1.20[a]	35	63.9 ± 1.07[a]
AdM	41	64.0 ± 0.83	41	68.4 ± 1.05	41	81.2 ± 0.99	37	70.4 ± 1.08[ab]	37	66.9 ± 0.95[ab]
AdAd	50	62.9 ± 0.85	45	66.7 ± 1.17	45	80.0 ± 1.09	44	68.4 ± 1.15[ab]	44	64.3 ± 1.01[ab]
P value		0.073		0.104		0.029[1]		0.001		0.001

[a,b]Differing superscripts within columns, within treatment groups, indicate least-squares means that are significantly different ($P < 0.05$).

[1]A significant interaction effect of dam nutrition during P21-50 by P50-140 on ewe live weight at P140 was found, however, pairwise Tukey analysis indicated that at the $P < 0.05$ level there were no differences between treatment means.

Table 6.2 Least-squares means ± SEM of ewe BCS at breeding (P0), scanning (P74), pregnancy day 140 (P140), docking (L38) and weaning of their lambs (L102) of ewe offspring in their first parity born to dams fed either sub-maintenance (Sm), maintenance (M) or *ad libitum* (Ad) nutrition during early pregnancy (P21-50) and either pregnancy maintenance (M) or *ad libitum* (Ad) nutrition during mid-to-late pregnancy (P50-140). Ewes had a pregnancy rank (PR) of single- (S) or twin-bearing (T), and a rearing rank (RR) for their lambs which were single-born and reared (S), twin-born and reared, or twin-born and single-reared (TS).

Dam nutritional		Ewe body condition score								
treatments	*n*	P0	*n*	P74	*n*	P140	*n*	L38	*n*	L102
PR / RR										
S			85	3.7 ± 0.06	85	2.8 ± 0.05[b]	78	2.6 ± 0.04[b]	77	2.8 ± 0.04[b]
T			158	3.8 ± 0.04	157	2.7 ± 0.04[a]	30	2.2 ± 0.05[a]	31	2.4 ± 0.04[a]
TS							113	2.3 ± 0.06[a]	107	2.7 ± 0.06[b]
P value				0.190		0.028		<0.0001		<0.0001
P21-50										
Sm	97	3.8 ± 0.06	94	3.7 ± 0.07	94	2.6 ± 0.06[a]	86	2.3 ± 0.05	82	2.6 ± 0.05
M	70	3.7 ± 0.05	63	3.8 ± 0.06	62	2.9 ± 0.05[b]	54	2.4 ± 0.04	52	2.4 ± 0.04
Ad	91	3.8 ± 0.05	86	3.7 ± 0.06	86	2.8 ± 0.05[ab]	81	2.3 ± 0.05	81	2.3 ± 0.05
P value		0.932		0.558		0.014		0.254		0.340
P50-140										
M	117	3.7 ± 0.04	107	3.7 ± 0.05	107	2.8 ± 0.04	91	2.4 ± 0.04	88	2.6 ± 0.04
Ad	141	3.8 ± 0.05	136	3.7 ± 0.05	135	2.7 ± 0.04	130	2.4 ± 0.04	127	2.7 ± 0.04
P value		0.621		0.997		0.271		0.881		0.320
P21-50×P50-140										
SmM	47	3.8 ± 0.08	43	3.7 ± 0.09	43	2.7 ± 0.07	36	2.3 ± 0.07	34	2.6 ± 0.07
SmAd	50	3.8 ± 0.10	51	3.7 ± 0.10	51	2.6 ± 0.08	50	2.4 ± 0.07	48	2.6 ± 0.07
MM	29	3.7 ± 0.07	23	3.8 ± 0.08	23	2.9 ± 0.06	18	2.4 ± 0.06	17	2.7 ± 0.06
MAd	41	3.7 ± 0.08	40	3.8 ± 0.08	39	2.8 ± 0.07	36	2.4 ± 0.06	35	2.6 ± 0.06
AdM	41	3.7 ± 0.07	41	3.7 ± 0.07	41	2.8 ± 0.06	37	2.4 ± 0.05	37	2.7 ± 0.06
AdAd	50	3.8 ± 0.08	45	3.7 ± 0.08	45	2.8 ± 0.07	44	2.3 ± 0.06	44	2.6 ± 0.05
P value		0.626		0.611		0.758		0.892		0.902

[a,b] Differing superscripts within columns, within treatment groups, indicate least-squares means that are significantly different ($P < 0.05$).

6.4.2 Grand-offspring birth measurements

There were no interaction ($P > 0.05$) effects (Three-way: P21-50×P50-140×sex, P21-50×P50-140×BR; and two-way: P21-50×P50-140, P21-50×sex, P21-50×BR, P50-140×sex and P50-140×BR) on CRL, girth, FL and RL measurements made on grand-offspring at L1 (data not shown). Grand-offspring with Sm_{P21-50}-granddams had longer FL at L1 than grand-offspring with Ad_{P21-50}-granddams, while grand-offspring with M_{P21-50}-granddams did not differ from either treatment (Table 6.3). After the analysis was adjusted for LW at L1, grand-offspring with Sm_{P21-50}-granddams only tended ($P = 0.07$) to have longer FL than grand-offspring with Ad_{P21-50}-granddams (30.6 ± 0.31 vs. 30.1 ± 0.28 cm, respectively). Granddam nutrition during P21-50 did not affect any other measurements on grand-offspring at L1. There were also no effects of granddam nutrition during P50-140 on measurements on grand-offspring at L1 (Table 6.3).

Single-born grand-offspring had longer CRL ($P = 0.02$), FL ($P < 0.0001$), HL ($P < 0.0001$) and a larger girth ($P < 0.0001$) measurement than twin-born grand-offspring (Table 6.3). The difference in CRL between single- and twin-born grand-offspring was reversed when the analysis was adjusted for LW at L1 (P = 0.0001, 52.9 ± 0.58 vs. 54.9 ± 0.64 cm for single- and twin-born lambs, respectively). The differences in FL, HL and girth measurements between single and twin grand-offspring were no longer apparent ($P > 0.05$) when the analysis was adjusted for LW at L1 (data not shown).

Ram-lambs had longer FL ($P < 0.0001$) and HL ($P < 0.0001$) measurements and a larger girth ($P = 0.01$) than ewe-lambs (Table 6.3). When the analysis was adjusted for LW at L1, the difference in girth measurement was no longer significant (P > 0.05, 41.0 ± 0.42 vs. 41.1 ± 0.39 cm for ram- and ewe-lambs, respectively). The differences of FL ($P = 0.002$) and HL ($P = 0.02$) measurements between ram- and ewe-lambs were still significant after the analysis was adjusted for LW at L1 (FL: 30.6 ± 0.29 vs. 30.1 ± 0.27 and HL: 36.1 ± 0.32 vs. 35.6 ± 0.30 cm for ram- and ewe-lambs, respectively). There were no ($P > 0.05$) differences of CRL between ram- and ewe-lambs, when analysed both with (Table 6.3) and without (data not shown) LW at L1.

6.4.3 Grand-offspring live weight from birth until weaning

There were no interaction ($P > 0.05$) effects (Three-way: P21-50×P50-140×sex, P21-50×P50-140×BR/RR; and two-way: P21-50×P50-140, P21-50×sex, P21-50×BR/RR, P50-140×sex and P50-140×BR/RR) on the LW of grand-offspring at L1, L38 and L102 or on their LWG from L1 until L102 (data not shown). Granddam nutritional treatments during both periods (P21-50 and P50-140) also did not ($P > 0.05$) affect grand-offspring LW or LWG from L1 to L102 (Table 6.4).

At birth (L1), twin-born lambs were lighter ($P < 0.0001$) than single-born lambs. Twin-born lambs, reared as twins or as singles, remained lighter ($P < 0.0001$) through to weaning (L102) and had lower ($P < 0.0001$) LWG than single-born lambs. Twin-born and reared lambs were lighter ($P < 0.0001$) than twin-born, single-reared

lambs at L38 and L102, and they also had lower ($P < 0.0001$) LWG (Table 6.4). When the analyses for LW at L38 and L102, and LWG were adjusted for LW at L1, twin-born and reared lambs were still significantly lighter ($P < 0.0001$), with lower LWG ($P < 0.0001$) than single lambs (data not shown). However, after the analyses were adjusted for LW at L1, the differences between LW of single-born lambs compared with twin-born, single-reared lambs, at L38 and L102 was no longer present (L38: $P = 0.11$, 17.8 ± 0.34 vs. 16.9 ± 0.51 and L102: $P = 0.07$, 36.0 ± 0.68 vs. 34.0 ± 0.99.kg for single and twin-born, single-reared lambs, respectively). The difference in LWG between single-born and twin-born, single-reared lambs was also less, but still significant (P = 0.05; 310.2 ± 6.8 vs. 289.6 ± 9.8 g/day for single and twin-born, single-reared lambs, respectively).

Ram-lambs were born heavier ($P < 0.0001$), remained heavier to L102 ($P < 0.0001$), and had higher ($P < 0.0001$) LWG compared with ewe-lambs (Table 6.4). When the analyses were adjusted for LW at L1, ram lambs were still significantly heavier at L38 ($P = 0.005$) and L102 ($P < 0.0001$), and had greater LWG ($P < 0.0001$) than ewe-lambs (data not shown).

Table 6.3 Least-squares means ± SEM (cm) of first-parity single (S) and twin (T), ewe- (E) and ram-lamb (R) crown-rump length (CRL), thoracic girth width (girth), fore-leg length (FL), and hind-leg length (HL). Lambs were the grand-offspring of ewes fed sub-maintenance (Sm), maintenance (M) or *ad libitum* (Ad) nutrition during early pregnancy (P21-50) and either pregnancy maintenance (M) or *ad libitum* (Ad) nutrition during mid-to-late pregnancy (P50-140).

		Measurements at birth (cm)			
	n	CRL	Girth	FL	HL
BR					
S	86	55.8 ± 0.64[b]	43.5 ± 0.48[b]	31.5 ± 0.29[b]	37.3 ± 0.34[b]
T	303	54.6 ± 0.75[a]	40.5 ± 0.56[a]	30.4 ± 0.33[a]	35.7 ± 0.40[a]
P value		0.021	<0.0001	<0.0001	<0.0001
Sex					
E	203	55.0 ± 0.66	41.7 ± 0.49[a]	30.4 ± 0.30[a]	36.0 ± 0.35[a]
R	186	55.4 ± 0.69	42.4 ± 0.51[b]	31.4 ± 0.31[b]	37.0 ± 0.37[b]
P value		0.250	0.013	<0.0001	<0.0001
Trt P21-50					
Sm	105	55.6 ± 0.75	42.3 ± 0.56	31.2 ± 0.34[b]	36.7 ± 0.40
M	138	55.5 ± 0.68	42.2 ± 0.51	31.0 ± 0.31[ab]	36.6 ± 0.36
Ad	146	54.6 ± 0.68	41.7 ± 0.50	30.6 ± 0.30[a]	36.2 ± 0.36
P value		0.075	0.205	0.016	0.083
Trt P50-140					
M	181	55.4 ± 0.65	42.1 ± 0.49	30.9 ± 0.29	36.5 ± 0.35
Ad	208	55.1 ± 0.69	42.0 ± 0.52	30.9 ± 0.31	36.6 ± 0.37
P value		0.542	0.579	0.812	0.621
Trt P21-50×P50-140					
SmM	60	55.4 ± 0.81	42.4 ± 0.60	31.1 ± 0.37	36.7 ± 0.43
SmAd	45	55.8 ± 0.87	42.2 ± 0.65	31.3 ± 0.40	36.7 ± 0.47
MM	73	55.9 ± 0.73	42.2 ± 0.55	31.1 ± 0.33	36.6 ± 0.39
MAd	65	55.1 ± 0.78	42.1 ± 0.58	30.8 ± 0.35	36.7 ± 0.42
AdM	76	54.8 ± 0.73	41.7 ± 0.54	30.6 ± 0.33	36.1 ± 0.39
AdAd	70	54.4 ± 0.77	41.6 ± 0.57	30.6 ± 0.35	36.3 ± 0.41
P value		0.438	0.996	0.640	0.860

[a,b] Differing superscripts within columns, within treatment groups, indicate least-squares means that are significantly different ($P < 0.05$).

Table 6.4 Least-squares means ± SEM of first-parity single (S) and twin (T), ewe- (E) and ram-lamb (R) live weights (kg); at birth (L1), docking (L38), and weaning (L102), and live weight gain to weaning (LWG L1- L102, g/day). Lambs were the grand-offspring of ewes fed sub-maintenance (Sm), maintenance (M) or *ad libitum* (Ad) nutrition during early pregnancy (P21-50) and either pregnancy maintenance (M) or *ad libitum* (Ad) nutrition during mid-to-late pregnancy (P50-140).

		Live weight (kg)						
	n	L1	*n*	L38	*n*	L102	*n*	LWG L1-L102 (g/day)
BR								
S	86	5.9 ± 0.08[b]	78	19.4 ± 0.37[c]	77	37.9 ± 0.66[c]	77	319.8 ± 6.28[c]
T	301	4.8 ± 0.04[a]	227	14.2 ± 0.60[a]	212	28.9 ± 0.76[a]	212	237.8 ± 7.17[a]
TS			28	17.0 ± 0.42[b]	30	34.0 ± 1.06[b]	30	289.6 ± 10.06[b]
P value		<0.0001		<0.0001		<0.0001		<0.0001
Sex								
E	201	5.2 ± 0.06[a]	183	16.3 ± 0.41[a]	176	32.2 ± 0.74[a]	176	270.9 ± 6.98[a]
R	186	5.5 ± 0.06[b]	150	17.4 ± 0.43[b]	143	34.8 ± 0.77[b]	143	293.8 ± 7.33[b]
P value		<0.0001		<0.0001		<0.0001		<0.0001
Trt P21-50								
Sm	104	5.4 ± 0.07	87	16.9 ± 0.46	79	33.5 ± 0.83	79	282.2 ± 7.84
M	138	5.4 ± 0.06	114	16.8 ± 0.43	109	33.4 ± 0.77	109	280.7 ± 7.24
Ad	145	5.3 ± 0.06	132	16.9 ± 0.43	131	33.4 ± 0.76	131	281.4 ± 7.20
P value		0.373		0.859		0.938		0.895
Trt P50-140								
M	179	5.3 ± 0.06	174	17.0 ± 0.42	167	33.5 ± 0.74	167	282.2 ± 6.97
Ad	208	5.4 ± 0.05	159	16.8 ± 0.43	152	33.3 ± 0.59	152	280.7 ± 7.24
P value		0.231		0.468		0.702		0.834
Trt P21-50×P50-140								
SmM	59	5.5 ± 0.10	47	17.0 ± 0.51	42	33.3 ± 0.92	42	280.4 ± 8.75
SmAd	45	5.3 ± 0.11	40	16.8 ± 0.53	37	33.9 ± 0.96	37	287.3 ± 9.11
MM	73	5.4 ± 0.09	59	16.9 ± 0.46	57	33.9 ± 0.84	57	286.3 ± 7.96
MAd	65	5.4 ± 0.09	55	16.7 ± 0.48	52	33.0 ± 0.87	52	276.3 ± 8.30
AdM	75	5.4 ± 0.09	68	17.0 ± 0.46	68	33.5 ± 0.82	68	281.7 ± 7.82
AdAd	70	5.2 ± 0.09	64	16.8 ± 0.47	63	33.4 ± 0.85	63	282.2 ± 8.07
P value		0.597		0.996		0.427		0.299

[a, b, c] Differing superscripts within columns, within treatment groups, indicate least-squares means that are significantly different ($P < 0.05$).

6.5 Discussion

Previously, dam nutrition during both P21-50 and P50-140 was shown to have little impact on twin-born ewe-offspring growth and production parameters, including LW, body composition and puberty attainment, from birth until 18 months of age (Chapter 4). However, the effects of altered dam gestational-nutrition may manifest in offspring later in life, or under circumstances which may provoke a specific programmed phenotype (predictive adaptive response) (Gluckman & Hansen, 2004). For example, effects may be more likely to manifest, and affect offspring performance, during periods of physiological stress including pregnancy and lactation. This may, in-turn, have consequences for the grand-offspring. Indeed, in a subset of twin-bearing ewe offspring that were milked, ewes born to dams over- or under-nourished during early pregnancy produced less milk and of lower quality in their first lactation than ewes born to maintenance-fed dams (Chapter 5). In this subset of ewes, those born to over-nourished dams also weaned lighter lambs. The present study, therefore, examined the effects of dam nutrition on ewe-offspring LW and BCS at breeding (P0) and throughout pregnancy and lactation, and on grand-offspring measurements at birth and growth until weaning in the whole population.

6.5.1 Effects on ewe-offspring LW and BCS

Gestational nutrition of the dam had little impact on ewe-offspring LW and BCS from breeding until lactation. However, effects of dam gestational nutrition on ewe offspring became more apparent during lactation through to weaning such that, at L38 and L102 ewes born to SmM- and MAd-dams were lighter than ewes born to MM-dams. This finding raises the possibility that ewes born to SmM- and MAd-dams may be less able to tolerate physiological stressful situations such as lactation, or that ewes born to MM-dams may have a programmed advantage. In the study of van der Linden *et al. (*2010a) ewes born to dams which were maintenance-fed throughout pregnancy (P21-140) had higher blood glucose concentrations in response to an epinephrine challenge at 16 months of age. They speculated that ewes born to maintenance-fed dams may have increased gluconeogenic or glycolytic abilities enabling them to perform better under physiological stress. Indeed, ewes born to maintenance-fed dams in the study of van der Linden *et al. (*2009) had improved lactational performance compared with ewes born to *ad libitum*-fed dams, which is also consistent with the findings of Chapter 5, and improved lambing performance (van der Linden *et al.,* 2010b). The study of van der Linden *et al. (*2010b), however, did not report any differences between LW of ewe offspring born to maintenance and *ad libitum*-fed dams.

Unlike in the present study, van der Linden *et al. (*2010b) did not change dam nutrition during different stages of pregnancy. It is possible that the effects on ewe-offspring live weight may be associated with the moderate increase in dam nutrition from early to mid-to-late pregnancy, whereby foetal adaptations to dam nutrition during early pregnancy are inappropriate for the nutritional environment during mid-to-late pregnancy (Barker, 1997). It has also been previously shown that early nutritional insult can increase susceptibility to a second nutritional insult later in gestation (Harding & Johnston, 1995). As a consequence, growth and metabolism in adult offspring may be negatively affected.

6.5.2 Effects on grand-offspring size at birth and LW from birth until weaning

Effects of nutritional programming on ewe-offspring lactation performance may have consequences for growth of the grand-offspring (van der Linden *et al.,* 2009; van der Linden *et al.,* 2010b; Chapter 5; Paten *et al.*, 2012). In addition, granddam nutrition during pregnancy may exert direct programming effects on the grand-offspring through targeting the germline cells *in utero* (Painter *et al*., 2008). It was hypothesized that both under-nutrition and *ad libitum* feeding of the granddam, specifically during early pregnancy, would have a detrimental effect on the weaning weights of grand-offspring. This will be discussed based on the two dam feeding periods (early and mid-to-late pregnancy) in the following section.

6.5.2.1 Dam/granddam nutrition during early pregnancy

In the present study there were few effects of granddam nutrition, during P21-50, on the grand-offspring. Apart from a small increase in FL length at birth of lambs whose granddams were Sm_{P21-50}-fed, there were no other differences in LW or measurements of grand-offspring at birth. This is consistent with the findings from the smaller cohort, reported in Chapter 5. Grand-offspring LWGs until weaning were also unaffected by granddam nutritional treatments during P21-50. These findings are surprising given that lactation performance was found to be decreased in a cohort of ewes born to both Sm_{P21-50}- and Ad_{P21-50}-fed dams and weaning weight was lower in grand-offspring of Ad_{P21-50}-fed granddams (Chapter 5). These findings are also in contrast to those of van der Linden *et al. (*2009 and 2010b) who reported that ewes born to *ad libitum*-fed dams gave birth to, and weaned, lighter lambs compared with ewes born to maintenance-fed dams.

The lack of differences in LW and LWG of lambs from birth to weaning may indicate that overall there were no differences in lactational performance in the whole population or that the measure of milk production used was not a good indicator of milk intake of lambs

(van der Linden *et al.,* 2010c). Milk production of the ewes in the whole population may have differed but it is possible that lambs from granddams which were fed Sm_{P21-50} and Ad_{P21-50} may be programmed to overcome their dam's reduced lactational performance: e.g., through an intergenerational programming effects to improve growth and/or metabolic efficiency (Painter *et al*., 2008). This concept is further supported by the inconsistencies between dam milk and lamb LWs at weaning in the subset of ewes which were milked, reported in Chapter 5. In that subset the LW at weaning of grand-offspring of Sm_{P21-50}-granddams did not differ from that of grand-offspring of M_{P21-50}-granddams, despite differences in milk production of their dams. The LW at weaning of grand-offspring of Ad_{P21-50}-granddams, however, was reduced. Clearly, further investigation into intergenerational programming of growth and metabolism of the grand-offspring is required.

6.5.2.2 *Dam/ granddam nutrition during mid-to-late pregnancy*

The study reported in Chapter 5 demonstrated that milk production of the ewe may also be affected by dam nutrition during P50-140. In Chapter 5, twin-bearing ewes born to $Ad_{P50-140}$-dams had increased milk yields but did not wean heavier lambs (the grand-offspring). This may have been attributed to a lack in difference of the overall milk net energy yield (NEY), reported in Chapter 5. In the present study of the whole population of ewe offspring and grand-offspring, there were also no differences in the LW of grand-offspring at L1, L38 and L102, and no differences in the growth rates from birth to weaning. These results confirm the findings of Chapter 5 that there are minimal effects of dam nutrition during mid-to-late pregnancy on milk production of the offspring and LW of the grand-offspring. These findings demonstrate feeding dams above-maintenance nutrition during mid-to-late pregnancy confers no production advantage in the next generation in terms of lamb production. Thus, farmers looking to manipulate pregnancy nutrition to improve productivity of subsequent generations should focus on the early gestational period rather than mid-to-late pregnancy.

6.5.3 Summary and conclusions

The findings of the present chapter suggest little effect of dam nutrition on offspring pregnancy and lactational performance when the data is analysed on the whole flock, using lamb live weights as an indicator. The findings that dam nutrition during mid-to-late pregnancy does not improve lambing performance of offspring is consistent with the results of Chapter 5; the findings for early pregnancy nutrition, however, are inconsistent. In the subset of twin-bearing offspring (reported in Chapter 5), ewes born to Ad_{P21-50}-fed dams

weaned lighter lambs than ewes born to M_{P21-50}-fed dams, while in the whole population, reported in the present study, there were no differences. These different results may reflect a discrepancy between the technique used to measure ewe milk production and milk intake of lambs, and that other factors, additional to milk, influence lamb growth. Overall, the findings of this chapter demonstrate that there is very little effect of dam nutrition on ewe-offspring lambing performance, and that manipulating pregnancy nutrition may not be a feasible option to improve productivity of next generation in lamb production systems. The differences in ewe milk production and lamb growth reported in Chapter 5, however, do suggest that dam pregnancy nutrition can result in some productive gains in ewe offspring. Investigation into the mechanisms involved may help to explain the observed lactational differences and may be useful for developing interventions to gain a more consistent production advantage.

7 Effects of dam nutrition during pregnancy on gene expression in the late-pregnant and lactating mammary gland of ewe offspring

7.1 Abstract

In the present study, RNA-seq was used to examine the effect of maternal nutritional programming on gene expression in the mammary gland of adult ewe offspring. Mammary tissue was sampled during late pregnancy and lactation from ewes born to dams offered sub-maintenance (SmM), maintenance (MM) or *ad libitum* (AdM) nutrition during days 21 to 50 of gestation. Approximately 80% of all reads mapped back to the ovine genome (OARv.3.2). A total of 10,175 transcripts were detected in the ovine mammary gland. An expression change of greater than 1.2-fold and a false discovery rate *P* value < 0.05 was used as criteria to identify genes which were differentially expressed. Differentially expressed genes were detected between late pregnancy and lactation (2,750 genes), and among nutritional programming treatments in late pregnant and lactating ewe offspring (699 genes and 14 genes were differentially expressed among treatments during late pregnancy and lactation, respectively).

Gene ontology analysis using the Database for Annotation, Visualisation and Integrated Discovery (DAVID) revealed that cellular proliferation, translation, and beta-oxidation were down-regulated from late pregnancy to lactation, while protein processing and lipid metabolism were up-regulated. Gene ontology analysis of RNA-seq data also revealed that cell proliferation may be impaired in ewe offspring born to under- (SmM) or over- (AdM) nourished dams in comparison to ewes born to maintenance-fed (MM) dams. However, the mechanisms appear to be different, such that ewes born to SmM-fed dams had lower expression of genes associated with the extracellular matrix (which supports mammary epithelial cell proliferation), while ewes born to AdM-fed dams had lower expression of genes associated with cell cycle regulation. Differential expression of hormone receptors and genes associated with epigenetic mechanisms among programming treatments raises the possibility that transcriptional differences may be regulated by endocrine and/or epigenetic influences. Additional molecular studies are warranted to further investigate these potential mechanisms.

This is the first study to use RNA-seq to examine gene expression in the ovine mammary gland. This study provides new insights into the transcriptional regulation of ovine mammary gland development and function during late pregnancy and lactation, and possible molecular mechanisms underlying nutritional programming during foetal life of mammary development and lactational performance in ewe offspring.

7.2 Introduction

Maternal nutritional perturbations during early foetal life, when the mammary gland is developing, may influence the post-natal development and lactational function of the mammary gland in adult ewe offspring. It has been previously demonstrated that the foetal mammary gland is susceptible to maternal nutrition during pregnancy (van der Linden *et al*., 2009; Blair *et al*., 2010; Martin *et al*., 2012). Martin *et al. (*2012) identified that dam nutrition during early gestation could alter the mass of the foetal mammary gland, at day 140 of gestation, in sheep. In Chapter 5, dam nutrition during early gestation was found to influence lactation performance of ewe offspring in their first parity (Paten *et al*., 2013). Specifically, ewes born to dams fed under- (sub-maintenance) or over- (*ad libitum*) maintenance nutrition during early pregnancy tended to produce less milk in their first lactation compared with ewes born to dams fed maintenance. The underlying molecular mechanisms, however, are unknown.

The number of differentiated mammary epithelial cells (MECs), the cells responsible for milk synthesis and secretion, is known to correlate well with the amount of milk produced (Capuco *et al*., 2001; Boutinaud *et al*., 2004; Wall and McFadden, 2012). Proliferation, apoptosis and differentiation of theses cells are regulated by mammogenic hormone signalling pathways (Hennighausen and Robinson, 2001). Thus, if dam nutrition during pregnancy programmes these pathways in the offspring, the population of MECs and amount of milk produced during lactation may be altered.

Lactational performance may also be influenced by metabolic activity and secretory capacity of MECs. Cellular metabolism, as well as delivery of nutrients and lactogenic hormones, is important for the synthesis and secretion of milk components during lactation. Synthesis of milk fat, in particular, is sensitive to nutrition (Palmquist *et al*., 1993; Bauman and Griinari, 2003; Sanz Sampelayo *et al*., 2007). There is evidence in Chapter 5 that dam nutrition during pregnancy programmes the amount of milk fat synthesised (Paten *et al*., 2013). This finding highlights the possibility that metabolic pathways, particularly lipogenic pathways, may be influenced in adult ewe offspring subjected to nutritional programming during foetal life. Effects may be mediated through differential expression of genes involved metabolic and biosynthetic processes in mammary cells, through altered exposure and/or sensitivity to lactogenic hormones such as prolactin (Prl) and growth hormone (GH), or through metabolic adaptations in other tissues which support lactation (e.g., higher glucose

and fat metabolism in the liver and delivery of nutrients to the mammary gland (Wall and McFadden, 2012).

Functional genomics studies in other species (rodent, bovine and caprine) have highlighted the complex and coordinated networks of genes and molecular pathways involved in transcriptomic regulation of mammary development and lactation (Naylor *et al*., 2005; Ollier *et al*., 2007; Finucane *et al*., 2008; Casey *et al*., 2011; Bionaz *et al*., 2012). Recent advances in high-throughput transcriptome sequencing technologies, such as RNA-seq, provide a useful approach to examine differences in patterns of gene expression (Mortazavi *et al*., 2008; Wang *et al*., 2009; McCabe *et al*., 2012; Wickramasinghe *et al*., 2012; Cameron *et al*., 2013b). Such techniques may be used to gain insights into molecular mechanisms underlying observed physiological responses in the mammary gland of offspring subjected to nutritional programming during foetal life. In this chapter, RNA-seq was utilised to investigate whole-scale expression of genes in primiparous late-pregnant and lactating mammary glands of ewe offspring that had been subjected to nutritional programming during foetal life.

7.3 Materials and methods

7.3.1 Experimental animals

The present study utilised a subset of twin-bearing, twin-born ewes born to dams differentially fed during pregnancy. The dam nutritional treatments and management were described in detail in Chapter 3. Briefly, ewes were fed either a sub-maintenance ($Sm_{P21\text{-}50}$), maintenance ($M_{P21\text{-}50}$) or *ad libitum* ($Ad_{P21\text{-}50}$) pasture allowance during early (day 21 to 50 of pregnancy, P21-50) gestation and then reallocated to either a pregnancy maintenance ($M_{P50\text{-}140}$) or *ad libitum* ($Ad_{P50\text{-}140}$) pasture allowance during mid-to-late (day 50 to 140 of pregnancy, P50-140) gestation (Kenyon *et al*., 2011a). Therefore, the ewe offspring generated, were from one of six dam treatment groups; SmM, SmAd, MM, MAd, AdM, and AdAd.

7.3.2 Experimental design

Primiparous ewes that had been previously identified as twin-bearing at pregnancy diagnosis, as described in Chapter 5, were selected at random from each treatment group to be sampled for mammary gland tissue via biopsy procedure. A total of 10 ewes per treatment were biopsied over two days during late gestation (day 135 of pregnancy, P135 ± 2.4 SD). The same ewes were sampled again during early lactation (15 days post-partum, L15

± 1.27 SD). However, 16 ewes were excluded from the trial due to lamb deaths and missing data, leaving the following numbers of ewes in each treatment group: SmM, *n* = 6; SmAd, *n* = 9; MM, *n* = 7; MAd, *n* = 7; AdM, *n* = 7; and AdAd, *n* = 8.

7.3.3 Sample collection and processing

A Bard® Magnum® biopsy gun, with a 12 G needle (Bard biopsy systems, AZ, USA), was used to collect mammary gland tissue of ewe offspring during late pregnancy and lactation. The biopsy protocol used was based on the procedure described by Norgaard *et al. (*2008). Approximately 2- 3 ml of local anaesthetic (Lopaine®, 2% lignocaine HCl, Ethical Agents, Manukau, NZ) was administered to the biopsy site prior to the procedure. An analgesic (1 ml/30 kg LW, Ketoprofen 10%, Ethical Agents, Manukau, NZ) and an antibiotic (20mg/kg LW, Engemycin® (100mg oxytetracycline/ml), MSD Animal Health, Buckinghamshire, UK) were also administered to alleviate pain and reduce the risk of infection. To ensure biopsy sampling of lobulo-alveolar and ductal parenchyma tissue, and to avoid piercing major blood vessels, tissue was collected near the dorsal aspect and midline of the udder and ductal area, 15 to 20 mm away from the udder midline, at the point where the udder protrudes most caudally. Biopsied tissue was immediately snap-frozen in liquid nitrogen, then placed in a cryo-vial and stored at −80˚C until later use for RNA extraction and gene expression analyses.

7.3.4 RNA methods

7.3.4.1 RNA extraction

Cryo-vials containing mammary biopsy tissue were removed from -80˚C and stored on dry ice. Entire frozen biopsy samples were ground in 300 µl of Trizol reagent (Invitrogen, CA, USA) with a disposable, sterile pestle until partially homogenised. Trizol® reagent is a mono-phasic solution of phenol and guanidine isothiocyanate used to maintain the integrity of RNA while disrupting cells and dissolving cellular components. Once partially homogenised an additional 700 µl of Trizol® was added and further homogenisation was performed with a sterile 25 gauge needle and 1 ml syringe. The solution was left for 5 mins at room temperature (RT) to allow complete dissociation of nucleoprotein complexes, after which 200 µl of chloroform was added and mixed by vortex for 15 sec. The solution was left to incubate at RT for 5 mins. The samples were then centrifuged (Thermo Electron Corporation Heracus pico 17 centrifuge, Thermo Fisher Scientific, USA) at 12,000 g for 15 mins at 4˚C, to separate the solution into aqueous (RNA-containing) and organic phases. A total of 400 µl of the aqueous layer was removed and added to an equal volume of 70%

ethanol and mixed with the pipette tip. A Qiagen RNeasy kit (Qiagen, Netherlands) was used to further purify the RNA. RNeasy kits are designed for fast purification of high-quality RNA using silica-membrane RNeasy spin columns which are able to bind up to 100 µg of RNA. A total volume of 700 µl of the RNA-containing aqueous solution and 70% ethanol mixture was added to an RNeasy column which was then centrifuged at 12,000 g for 15 sec to bind the RNA. The flow through was discarded. The column was then washed with 350 µl of RW1 buffer using centrifugation at 12,000 g for 15 sec. This step was repeated and the flow-through was discarded. A volume of 500 µl of RPE buffer was added to the column and washed through using centrifugation at 12,000 g for 15 sec. Another 500 µl of RPE buffer was added to the RNeasy column and centrifuged for 2 mins at 12,000 g. The column was then placed in a sterile 1.5 ml eppendorf and 30 µl of diethylpyrocarbonate- (DEPC) treated H_20 was added to the centre of the column membrane and left to incubate for 1 min at RT. The samples were then centrifuged for 1 min at 10,000 rpm to elute the RNA into a final collection tube. An aliquot was taken to check the quantity and quality of RNA extracted. The remaining RNA was stored at -80˚C.

7.3.4.2 RNA quantification

The concentration of RNA was determined using either the NanoDrop ND-1000 spectrophotometer (Thermo Scientific, MA, USA) or the Qubit 2.0 fluorometer, using the Qubit RNA assay kit (Invitrogen).

The Nanodrop measures the absorbance of an RNA or DNA sample at 260 nm and provides the concentration in ng/µl. The NanoDrop also assess the purity of each sample by measuring the A_{260} nm/A_{230} nm and A_{260} nm/A_{280} nm ratios. An A_{260}:A_{280} nm ratio of approximately 2.0 is generally accepted for pure RNA. Pure DNA has a ratio of around 1.8. Samples which have ratios below 2.0 may have high levels of proteins or residual phenol. The A_{260}:A_{230} nm ratio should be between 2.0 and 2.2 for both RNA and DNA. Ratios below this suggest the presence of contaminants such as EDTA, carbohydrates or phenol, which absorb at 230 nm. Trizol® reagent will absorb at both 230 and 270 nm therefore any remaining Trizol reagent will affect these ratios.

The Qubit uses fluorescent dyes to quantify biomolecules of interest. The fluorescent dyes only emit a signal when they are bound to specific target molecules (e.g., RNA). The Qubit has an advantage over the Nanodrop in that the fluorescent dyes are specific to the type of molecule being measured (Bustin *et al.*, 2009). Therefore, even if there is DNA present in a sample, only the concentration of RNA will be given.

7.3.4.3 RNA agarose gels

The integrity of total RNA in samples was checked by running a small volume on a 1% agarose gel for quality control. Agarose gels were made with sodium boric acid buffer to which 50 µl of 10 mg/ml ethidium bromide stock solution was added giving a final concentration of 0.5 µg/ml. Approximately 1 µl of loading dye (25% bromophenol blue (1% solution), 25% xylene cyanol (1% solution), 30% glycerol and 20% MilliQ H_20) was added to each sample, then 10 µl of each sample was loaded on to the gel. A DNA ladder (1 kb^+, Invitrogen) was also loaded to one lane as a marker to determine the size of bands and as a control to check the quality of the gel. Gels were run for 10 to 20 mins at 2000 V (as RNA begins to degrade if run for longer periods). Gels were run with Owl B1A EasyCast Mini Gel systems (Thermo Scientific) using an EC250-90 power supply (Thermo Scientific). Gels were viewed under ultraviolet light and RNA checked for degradation. Intact total RNA should have sharp 28S and 18S rRNA bands; the 28S band should be approximately twice the intensity of the 18S band (indicating a 2:1 ratio). If RNA is partially degraded, the rRNA bands will be smeared in appearance, rather than sharp, or will not have the 2:1 ratio. Completely degraded RNA will appear as a low-molecular-weight smear.

7.3.4.4 RNA pools

The findings of Martin *et al*. (2012) and those reported in Chapter 5 demonstrated that dam nutrition during early pregnancy, as opposed to mid-to-late pregnancy, affected mammary gland development and first-lactation performance of offspring (Paten *et al*., 2013). Therefore, in the present study, only samples from ewes born to dams which were differentially fed during early pregnancy and fed maintenance during mid-to-late pregnancy (SmM, MM and AdM) were used for transcriptome analysis. For RNA-sequencing, RNA from multiple individuals within a treatment and time point was pooled in an attempt to minimise variation among samples (Kendziorski *et al*., 2003; Kendziorski *et al*., 2005; Konczal *et al*., 2014). Approximately 2 µg of RNA, subsampled from three randomly selected animals per treatment, was incorporated into pools. Three pools per treatment were generated for samples taken during late pregnancy and two pools were generated per treatment for samples taken during lactation, due to the reduced number of lactation samples. The pools were: late pregnancy; SmM, MM, and AdM (n = 3 pools sequenced for each treatment, with three samples per pool, n = 9 total samples for each treatment), and lactation; SmM, MM, and AdM (n = 2 pools sequenced for each treatment, with three samples per pool, n = 6 total samples per treatment). Therefore, the number of pools used to examine gene expression in late pregnancy versus lactation (irrespective of treatment) was as follows: n = 9 pools for

late pregnancy, with three samples per pool, *n* = 27 samples; and *n* = 6 pools for lactation, with three samples per pool, *n* = 18 samples. A diagram of the pooling strategy can be found in Chapter 8 (Figure 8.1).

7.3.4.5 Bioanalyzer

The quality of pooled RNA samples was checked using the Agilent 2100 Bioanalyzer with the Agilent RNA 6000 pico chip (Agilent Technologies, CA, USA). The bioanalyzer is a microfluidics-based system which utilises capillary electrophoresis to separate RNA molecules based on size. The RNA chip contains wells in which the samples are loaded. Micro-channels fabricated in glass create interconnected networks among the wells in the chip. When the chip is prepared, the micro-channels are filled with a fluorescence-gel-dye mix. Once the wells and channels are filled, the chip becomes an integrated electrical circuit. When the chip is placed in the bioanalyzer, a 16-pin electrode fits into the wells of the chip and charged biomolecules (e.g., RNA) are electrophoretically driven by a voltage gradient. Because of a constant mass-to-charge ratio and the sieve-like activity of the gel, the molecules are separated by size, with smaller fragments migrating faster than larger ones. Dye molecules complex with RNA strands and are detected by laser-induced fluorescence. Data is then translated into gel-like images (with bands) and electropherograms (peaks), which are interpreted much like a conventional gel electrophoresis (i.e., Intact samples have a 28S and 18S rRNA bands with a 2:1 ratio). The RNA integrity number (RIN) is derived through the bioanalyzer software and determines the amount of degradation present in each sample (Schroeder *et al*., 2006). A RIN of seven or greater (RIN ranges from 1, totally degraded, to 10, intact) is deemed acceptable for sequencing as it indicates little degradation.

7.3.5 High throughput sequencing (HTS)

RNA-seq data were generated from pooled RNA on an Illumina HiSeq 2000 (Illumina, CA, USA; service provided by New Zealand Genomics Limited, Dunedin). The Illumina system begins with generation of DNA libraries. DNA is sheared and adenylated, which allows ligation of adaptor molecules on both ends of the DNA fragment. DNA fragments are then selected on size and purified, and added to a flow cell. Flow cells are eight-channel sealed-glass microfabrication devices which have a lawn of oligonucleotides lining the bottom. Single-stranded DNA fragments hybridise to the oligonucleotides using the adaptor molecules, resulting in long single stranded fragments attached to the flow cell at one end. The DNA molecules are then amplified using DNA polymerase to form clusters of identical

sequences. After amplification, each cluster has around one million copies of the sequence. The reverse strands are removed and the clusters are simultaneously sequenced. Each nucleotide is given a unique fluorescent label, and all four nucleotides are washed over the cluster simultaneously, which creates competition for binding. After the nucleotide is incorporated, a laser is used to excite the fluorophore, and the colour is recorded for each individual cluster. Following detection, the fluorophore and blocking group are removed from the nucleotide, which allows binding of the next nucleotide and so on.

7.3.5.1 Alignment and analysis of high-throughput sequencing (HTS) data

Analysis of RNA-seq data was performed using CLC Genomics Workbench software (CLC bio, Denmark). Sequence reads (paired end reads) were mapped back to the *Ovis aries* genome version 3.2 (OARv.3.2): http://www.ncbi.nlm.nih.gov/genome?term=ovis%20aries (NCBI). Mapping parameters included a maximum number of two mismatches allowed (for short reads), a minimum length fraction of 0.9 (for long reads), a maximum similarity fraction of 0.8 (for long reads), a minimum and maximum paired distance of 1 and 550, respectively, minimum exon coverage fraction of 0.2, minimum number of reads of 10 and maximum length of putative exons of 50.

Mapping RNA-seq reads to the sheep genome generates a "count" for each gene, i.e., the number of reads mapping to each gene. This is normalised to the length of the gene (as longer genes will have higher counts) to generate a reads per kilobase per million reads (RPKM) statistic. These values were then normalised (to the total number of reads for the sample) and data transformed by adding +1 to the expression value, to avoid division by zero when calculating expression ratios.

7.3.5.2 Detection of differential gene expression

Using the normalised and transformed RPKM values, differences in gene expression were assessed using the Baggerly test, which is similar to a two sample t-test but the test statistic is weighted according to the number of reads in each sample (Baggerly *et al.*, 2003), as implemented by CLC Genomics Workbench. The *P* value generated by the Baggerly test was corrected for multiple testing using the False Discovery Rate (FDR) correction. Genes with a FDR corrected *P* value of less than 0.05 and a fold change greater than 1.5 were deemed significantly differentially expressed.

7.3.6 Quantitative reverse-transcriptase PCR

7.3.6.1 Overview

Despite the use of multiple testing corrections, false positive results may still exist in RNA-seq data-sets. Therefore, quantitative reverse transcription (or real-time) PCR (RT-qPCR) was performed on a subset of differentially expressed genes (DEGs), as an independent technique to validate the RNA-seq analysis (Table 7.4). RT-qPCR is considered the 'gold standard' for studies of gene expression (Bustin *et al*., 2009), however, the number of genes that can be feasibly examined is a limitation. Therefore, only a small subset of genes from the RNA-seq analysis was chosen for validation by RT-qPCR. Candidate genes were chosen from all comparisons and ranging in expression (i.e., low, medium and high transcript abundance), to examine the accuracy of gene expression variation reported by the RNA-seq analysis.

Real-time PCR, or RT-qPCR, is an adaptation of the standard reverse-transcriptase PCR (RT-PCR) approach that allows for real-time measurement of amplification of target sequences. In standard and semi-quantitative PCR, the product is run on a gel and visual comparisons between transcripts are performed (by eye). This approach does not accurately quantify the level of transcript in a sample. In RT-qPCR, fluorescence is used to track amplification to enable determination of the absolute or relative level of transcripts in a sample. The most commonly used approach is to use an intercalculating dye (e.g., SYBR green), which fluoresces when bound to double-stranded DNA (dsDNA). A modified PCR machine is used, which can detect changes in fluorescence across each cycle. Fluorescence is then plotted against the number of cycles on a log scale. When the product levels are high enough, the fluorescence in the system reaches a critical threshold above the level of background noise. The cycle at which the fluorescence levels cross this threshold is recorded and known as the threshold cycle or quantification cycle (Cq). The Cq is used to quantify levels of the transcript (Bustin *et al*., 2009).

Typically, gene expression is measured relative to stably expressed reference genes. Careful choice of reference genes is important; therefore, a study was conducted (detailed in Chapter 8), to identify appropriate reference genes to carry out RT-qPCR in the present study. Of the potential reference genes studied, *Prpf3* (pre-mRNA processing factor) and *Cul1* (cullin 1) were selected as the most stably expressed across all samples (Paten *et al.,*2014) and, therefore, these were used to normalise RT-qPCR data in the present study.

7.3.6.2 cDNA synthesis

The SuperScript VILO cDNA Synthesis Kit (Invitrogen) was used, as per the manufacturer's protocol, to produce high-quality cDNA for use in RT-qPCR. In brief; previously determined RNA concentrations were used to calculate the volume for 1 µg of RNA. This volume of RNA was then added to 4 µl of 5 × VILO reaction mix (contains random primers, MgCl2, dNTPs and a buffer solution), 2 µl of SuperScript enzyme mix (contains SuperScript III reverse transcriptase (an engineered version of M-MLV RT with reduced Rnase H activity and increased thermal stability), RNA inhibitors and a helper protein) and made up to a total volume of 20 µl with sterile nuclease-free water. Control reverse transcriptase negative (-RT) samples were made for each RNA sample by adding 1 µg of RNA to nuclease free water; this was for use in RT-PCR to confirm that the samples were free from contaminating genomic DNA (gDNA). The reactions were incubated at 25°C for 10 minutes, 42°C for 60 minutes and 85°C for 5 minutes to terminate the reaction. The incubations were carried out using a pre-programmed C1000 Thermal Cycler or S1000 Thermal Cycler (BioRad, CA, USA). cDNA was stored at -80°C until use.

7.3.6.3 Primer design for RT-qPCR target genes

The design of primers is important to ensure that only one PCR product is amplified in reactions. Primers were designed using laboratory standard protocols (Cameron *et al*., 2013a). The mRNA and gDNA sequences of target genes for *Ovis aries* were obtained from the NCBI database. The mRNA and gDNA sequences for each gene were aligned using Spidey (http://www.ncbi.nlm.nih.gov/spidey/) to identify the position of introns in the coding sequence. Primers were then designed either side of an intron (i.e., If there is an intron at position 120bp in the cDNA sequence, primers could be designed flanking the position by 2bp), to cross exons but not sit on exon-exon boundaries. This minimizes the likelihood of amplifying potential contaminating gDNA. Primers were designed using Primer3plus software (http://www.bioinformatics.nl/cgi-bin/primer3plus.cgi). Primers between 15 and 25 bp long were designed, as primers which are too short may compromise specificity of binding, while primers which are too long will limit reaction efficiency. Amplicon size was also limited, to between 80 and 120 bp in size, to optimise efficiency. Primers, in a pair, were designed to have no more than 1˚C difference in melting temperature between them, and an annealing temperature of approximately 60˚C. Primer self-complementarity was limited to four to minimize formation of secondary structures such as primer dimers. Primer dimers occur when primers prefer to bind to themselves or each other rather than to the cDNA template; this can reduce reaction efficiencies. Primer dimers are also double-stranded DNA

(dsDNA) which means they are able to bind the fluorophore, giving off fluorescence and interfering with the signal received by amplification of the template cDNA. As an additional measure of quality control, Beacon designer free edition (http://www.premierbiosoft.com/qpcr/index.html) was used to check the likelihood for formation of primer dimers. Primers were deemed acceptable if their delta G values (a negative value indicating the energy required for a spontaneous reaction to occur) for cross-dimer and self-dimer formation were no less than -3. Primers with delta G values closest to zero were selected. Finally, NCBI primer blast (www.ncbi.nlm.nih.gov/tools/primer-blast) was used to check specificity of primers (i.e., that they would not amplify any product other than the gene of interest).

Primers were synthesized by Invitrogen or Integrated DNA Technologies (IDT) and supplied lyophilised. They were re-suspended with nuclease free water to make up a concentration of 10 pmol/µl to use in RT-qPCR reactions. Primers used in this study are listed in Table 7.1.

7.3.6.4 Primer specificity (reverse transcriptase PCR)

RT-PCR was used to check the specificity of each primer pair to generate a single PCR product and indicate the presence of gDNA. Standard Taq (Invitrogen) RT-PCR reactions were made using 2 µl PCR buffer minus $MgCl_2$, 1.2 µl $MgCl_2$, 1 µl dNTPs, 2 µl forward primer, 2 µl reverse primer and 0.2 µl Taq, added to either 1 µl of cDNA (from a randomly selected sample), 1 µl of –RT control, 1 µl of nuclease free water or 100 ng gDNA (extracted from ovine muscle using a DNeasy Kit (Invitrogen) as per the manufacturers instruction), and made up to a total volume of 20 µl using nuclease-free water. The reactions were carried out on a pre-programmed C1000 Thermal cycler or S1000 Thermal cycler (BioRad) using the following protocol:

3 minutes at 94˚C

30 seconds at 94˚C
30 seconds at 58˚C
20 seconds at 72˚C
7 minutes at 72˚C
} Repeated for 35 cycles

Hold at 4˚C

Amplified products from the samples were then run on a 2% agarose gel (as per protocol for RNA gels) and visualised using a GelDoc (BioRad). Primers were deemed acceptable (i.e., specific) if a single band of expected product size (80 – 120 bp) was observed in the cDNA sample with no other bands, and no bands observed in the –RT and water controls and no band or a larger band (indicating gDNA) was observed in the gDNA sample.

7.3.6.5 Primer efficiency (quantitative real-time PCR)

In a RT-qPCR reaction, as the fluorescence levels rise above the threshold, the PCR becomes exponential, such that the amount of product is effectively doubled with each cycle. However, most reactions do not undergo perfect doubling due to varying efficiencies of the primers used. The optimal RT-qPCR efficiency should be 2 (100%); however, an efficiency of between 90 and 110% is acceptable (Bustin *et al*., 2009).

Primer efficiency of primers was measured by performing a RT-qPCR (as per the protocol described in the following section) using a five-point dilution series (1, 1:10, 1:100, 1:1000, 1:10000) to give a standard curve of cDNA.

Biorad CFX manager software was used to calculate the RT-qPCR efficiency for each primer tested. The amount of cDNA is plotted on the x axis (as a log value) against the Cq (threshold or quantification cycle, calculated by the software) on the Y axis. The slope of the line is calculated; a slope of between -3.6 and -3.1 equates to an efficiency of between 90 and 110%. Figure 7.1 is an example of the graphical output produced to calculate efficiency, and primer efficiencies are listed in Table 7.1.

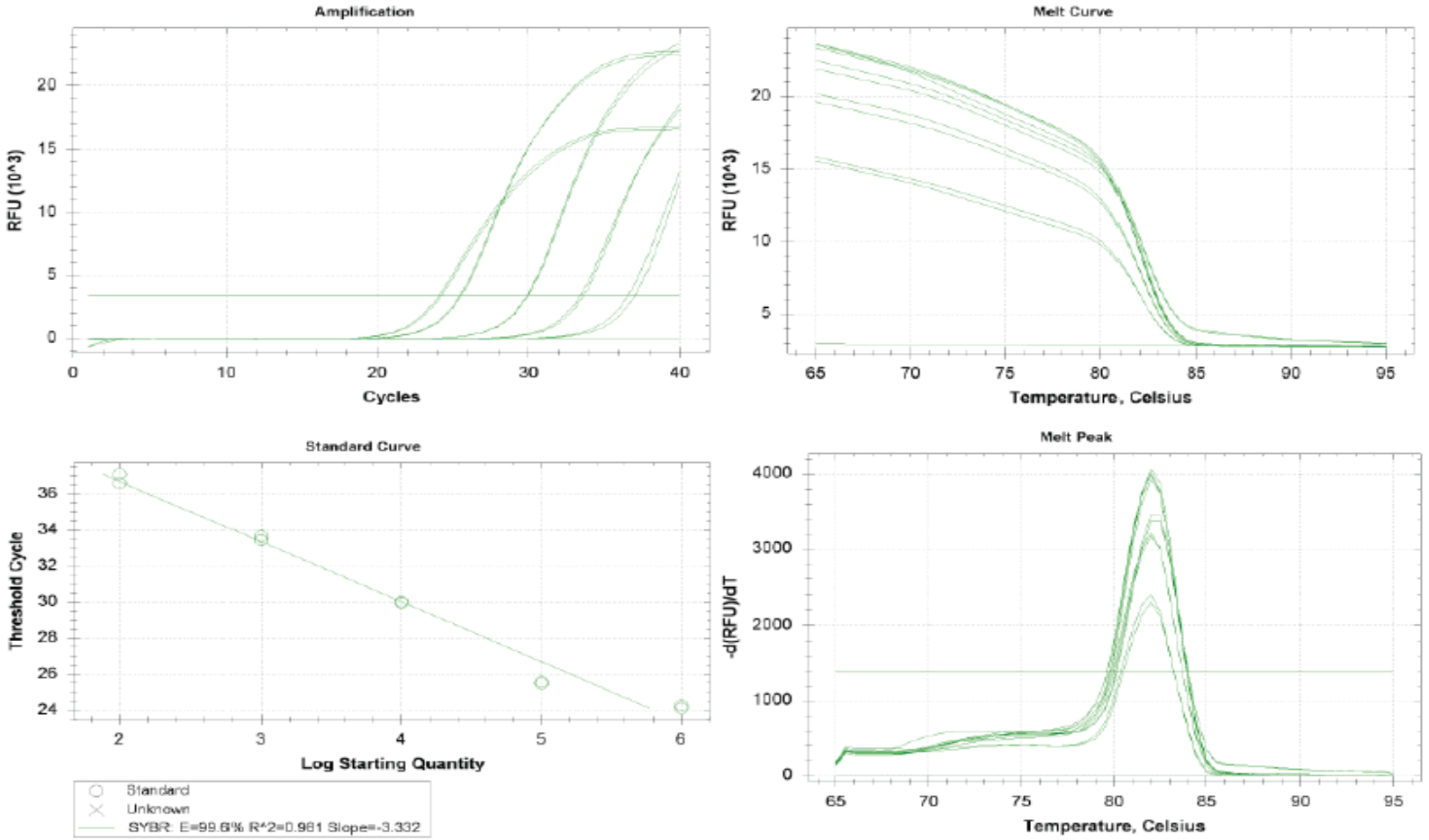


Figure 7.1 Example of the graphical output of a five-point dilution series reverse-transcription quantitative PCR (RT-qPCR) for measuring primer efficiency. Example shows the amplification over cycles (A.), the melt curve (B.), the standard curve, which shows the linear relationship and reaction efficiency (C.) and melt peak (D.) for the splicing factor 1 gene (*Sf1*) using 1:1, 1:10, 1:100, 1:1000 and 1:10,000 diluted cDNA. The efficiency, shown in the standard curve, in this example is acceptable at 99.6% and there is a strong linear relationship (i.e., $R^2 = 0.96$). There is also only one peak observed in the melt peak (bottom right), indicating only one PCR product has been amplified (i.e., good specificity). Therefore this primer would be acceptable to use for RT-qPCR to measure gene expression.

Table 7.1 Candidate genes tested by reverse-transcription quantitative PCR (RT-qPCR). Gene ID, NCBI accession number, forward and reverse RT-qPCR primer sequences, and primer efficiency (%) and amplicon sizes (base pairs, bp) are given.

Gene identifier[1]	NCBI accession	Forward primer sequence	Reverse Primer sequence	Efficiency (%)	Amplicon size (bp)
Flt4	XM_004009112.1	CTTCCTGTCCAACCCCTTC	TAGTTTTTCCCCAACCAGCA	98.4	103
Fyn	XM_004011196.1	ATGTGGCTCCAGTTGACTCC	GTGGGTTTCCAAAGGACAAA	95.4	99
Jak2	XM_004004358.1	AGCCTGGTGAAAGTCCCATA	TCCAAACATCTGAAGCCACA	100.9	81
Lalba	NM_001009797.1	AAAGACGACCAGAACCCTCA	TCTTGGCACACACAATGTCA	92.7	92
Lpo	NM_001009722.1	GACAACTGCTTCCCCATCAT	CGACTGGTAAGGTGGAGTGG	95.6	114
Map4k1	XM_004015231.1	GGCACCTATGGGGAAGTTTT	ATCATCGTCAGGCTCCATCT	92.8	90
Numbl	XM_004015260.1	TGTGGATGACAAGACCAAGG	CGGCAGATGTAGGAGAAAGC	105.9	113
Pdgfc	XM_004017535.1	GGGGACTTTGTGAAGAGCAG	GCGATGGTTTCCAATCTTTC	100.9	118
Loc443444	XM_004016530.1	GCTGGCATGGTTCTTGGA	TAGGGCTTGGCTTTCATTTG	104.0	120
Pth-rp	XM_004006757.1	CTGGGCTGGAAGAGGACTAC	TCTGAAGGTCTCTGCTGAAAAA	110.5	94
Tet1	XM_004021627.1	TTTCTCTGGGGTCACTGCTT	TGAGCGGTTATCTTCTCGTG	100.6	115
Tgfbi	XM_004008814.1	TGGCGATGAAATCCTGGT	GGCTCCTTATTGACACTCACC	106.4	117
Thbs4	XM_004010215.1	GTTCTTGGGGCAGATGTCAC	GCATTCGGCTATGGTGTTTC	106.8	109
Timp1	NM_001009319.2	CCAGAATCGCAGTGAGGAGT	TCCAGGGAGCCACAAAACT	101.9	89
Urgcp	XM_004018183.1	TTATGGAGAGGGTCCGAATG	AGGCTGAGTTTCTGTGTTTGG	93.3	120
Vegfc	XM_004021836.1	GCTGGATGTTTACAGACAAGTCC	GTAATCTGCGGGGCAAGTC	106.0	100

[1]Gene names: *Flt4* = fms-related tyrosine kinase 4, *Fyn* = FYN oncogene related to SRC, FGR, YES, *Jak2* = Janus kinase 2, *Lalba* = alpha-lactalbumin, *Lpo* = lactoperoxidase, *Map4k* = mitogen-activated protein kinase kinase kinase kinase 1, *Numbl* = numb-homolog-like, *Pdgfc* = platelet-derived growth factor C, *Loc443444* = progesterone receptor, *Pth-rp* = parathyroid hormone-related protein, *Tet1* = tet methylcytosine dioxygenase 1, *Tgfbl* = transforming growth factor beta-induced, *Thbs4* = thrombospondin 4, *Timp1* = TIMP metallopeptidase inhibitor 1, *Urgcp* = up-regulator of cell proliferation, and *Vegfc* = vascular endothelial growth factor.

7.3.6.6 Quantitative PCR reactions

RT-qPCR reactions were carried out using a C1000 thermal cycler adapted with the CFX96 Real-Time System (BioRad). Each reaction contained 10 µl SsoFast EvaGreen® Supermix reagent (BioRad), 0.6 µl forward primer, 0.6 µl reverse primer, 3.2 µl nuclease-free water and 5 µl cDNA template (or negative control i.e., –RT or nuclease free water). Reactions were made up in white optical plates (BioRad) and sealed with an optical seal (BioRad). Prior to carrying out the RT-qPCR protocols, the prepared plate was centrifuged up to 116 g (Centrifuge 5810 R, Eppendorf, Germany). The RT-qPCR program was as follows:

1 minute at 95°C (heat activates the PCR mix)

5 seconds at 95°C
30 seconds at 60°C
Data collected
10 seconds at 95°C

65°C to 95°C, by 0.5°C increments, collecting fluorescence data after every temperature increase (melt curve).

7.3.6.7 Statistical analysis of RT-qPCR data and correlation validation of RNAseq data

Relative gene expression, normalised to two reference genes (Chapter 8), was obtained using BioRad CFX manager software, for each gene tested. A linear regression plot showing the correlation between gene expression measured by RNA-seq and RT-qPCR was generated in the R statistical package. The expression values were represented as a fold-change between comparisons. This was a fold change in expression of genes between late pregnancy and lactation, and for the treatments during late pregnancy and lactation this was a fold change in comparison to the MM treatment (i.e., SmM/MM or AdM/MM). These expression values were used to calculate Spearman's correlation coefficient (R) and associated *P* values using R.

7.3.7 Gene ontology and pathways analysis

RNA-seq analysis generates lists of genes which are differentially expressed between two treatments e.g., late pregnancy vs. lactation, SmM vs. MM, AdM vs. MM and SmM vs. AdM. To put these differences into biological context, gene ontology (GO) analysis can be utilised. The Gene Ontology Project, managed by the Gene Ontology Consortium, is a collaborative effort that provides consistent descriptions of the manner in which gene

products behave in a cellular context. Annotations for genes are separated into three major categories: cellular compartment (identifies gene association with parts of the cell or extracellular environment), molecular function (identifies associated molecular activities of a gene product e.g., DNA binding, phosphorylation etc.), and biological process (identifies associated processes of a gene product essential to cell/tissue/organ/organism function e.g., cell cycle). Gene ontology is based on the findings that many genes involved in core biological functions are conserved among eukaryotes (Ashburner *et al*., 2000). Therefore, information about a gene product in one species may be transferable to other species. Online databases, such as the Database for Annotation, Visualisation and Integrated Discovery (DAVID; Huange *et al*., 2009a & b), can be used to investigate GO in transcriptomics gene lists. DAVID allocates GO terms to genes, which annotates genes with specific cellular compartments, biological process and molecular functions (e.g., mitochondria, cell cycle or protein binding). DAVID can perform a functional annotation clustering, whereby similar GO terms can be clustered together. Each cluster contains a list of GO terms, the genes contained in the terms and their *P* values. The cluster is allocated an enrichment score (ES) which is the geometric mean (in –log scale) of the individual GO term *P* values. DAVID can also be used to identify molecular pathways, that are differentially expressed from the Kyoto Encyclopaedia of Genes and Genomes (KEGG).

In the present study, DAVID was utilised to compare lists of DEGs to a background list to identify over-represented or enriched GO and KEGG terms. For the functional annotation cluster analysis an ES > 2, which is equivalent to a *P* value of 0.01 (Huang *et al*., 2009), was used as the significance cut-off. A Fisher's exact *P* value of < 0.01 was used as the cut-off for significance of KEGG pathways. The background list, to which the lists of DEGs were compared, was created by submitting a list of all genes detected in the mammary gland from RNA-seq (genes with RPKM expression value > 0). One limitation is that many sheep genes do not have assigned functional orthologies, therefore annotations were based on the mouse genome as it provided the most information. Another limitation is that uncharacterised or predicted genes in the RNA-seq dataset (genes with a Loc prefix) were not recognised by DAVID and could not be included in the GO analysis. Due to the preliminary nature of the sheep genome assembly, some genes with good orthologies (e.g., the progesterone receptor) have been assigned Loc gene identifiers rather than an official gene symbol; this may subsequently bias the results. GO analyses also tend to be biased towards genes involved in fundamental cellular processes (e.g., cell cycle). Additionally GO does not take into account genes which have diverged significantly with novel functions in

different species due to evolutionary change over time. Therefore, GO analysis results must be interpreted with caution, in the context of the cell, tissue, organ or species. Despite these limitations, GO analysis provides a useful method to analyse the large number of genes identified as differentially expressed by RNA-seq, and to formulate hypotheses which may be tested by further studies.

7.4 Results

7.4.1 RNA quality

All pooled RNA samples were of high quality, with RNA integrity numbers (RIN) of 7 or above (Table 7.2), and two clear bands (28S and 18S RNA) visible on electropherograms (Figure 7.2), indicating minimal degradation. Samples were, therefore, acceptable for RNA-sequencing.

Table 7.2 RNA integrity and RNA concentration of pooled[2] samples as determined by the Agilent 2100 bioanalyzer using the RNA 6000 Nano LabChip kit.

Pooled sample ID[1]	RNA integrity number (RIN)[3]	RNA concentration (ng/µl)
AdM_LP 1	8.30	399
AdM_LP 2	8.70	431
AdM_LP 3	8.80	322
MM_LP 1	8.10	439
MM_LP 2	8.00	298
MM_LP 3	6.50	463
SmM_LP 1	7.80	424
SmM_LP 2	7.90	337
SmM_LP 3	8.50	396
AdM_L 1	7.70	344
AdM_L 2	8.10	279
MM_L 1	7.90	384
MM_L 2	7.90	364
SmM_L 1	8.10	360
SmM_L 2	8.10	393

[1]Dam nutrition during early pregnancy (day 21 to 50 of gestation) with maintenance nutrition 'M' as control during mid-to-late pregnancy (day 50 to 140 of gestation) e.g., SmM = sub-maintenance, MM = maintenance, and AdM = *ad libitum*; physiological state e.g., LP = late pregnancy (day 135 of gestation) and L = lactation (day 15 postpartum); biological replicate e.g., 1, 2 or 3 for late pregnancy pools and 1 or 2 for lactation pools.

[2]Pooling for late pregnancy; SmM, MM, and AdM (n = 3 pools sequenced for each treatment, with three samples per pool, n = 9 total samples for each treatment), and lactation; SmM, MM, and AdM (n = 2 pools sequenced for each treatment, with three samples per pool, n = 6 total samples per treatment).

[3]A RIN of 7 or greater indicates samples are not degraded and are, therefore, acceptable for sequencing. MM_LP3 had a RIN of 6.5, however, inspection of the gel electropherogram indicated the sample was not degraded and of good quality for sequencing.

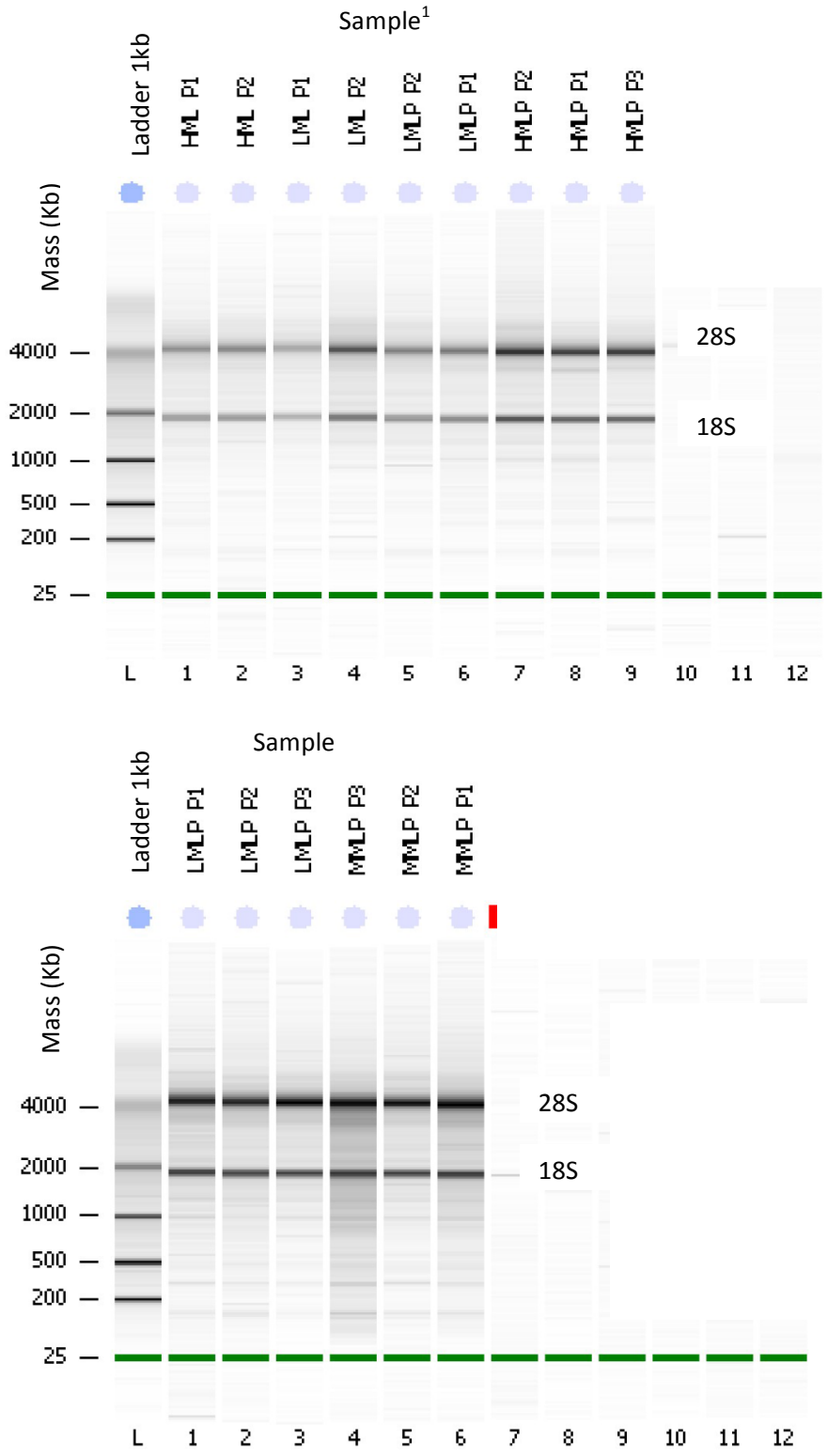


Figure 7.2 Electropherogram showing 28S and 18S rRNA, indicative of the 28S/18S ratio detected by the Agilent 2100 bioanalyzer for pooled[1] RNA samples from mammary tissue of late pregnant and lactating ewe offspring born to dams fed differentially during pregnancy. The lower band indicates the quantity of 18S RNA and the upper band represents the quantity of 28S RNA. Two intact bands indicates RNA is not degraded and can be used for RNA sequencing.

[1]Sample ID key: Dam nutrition during early pregnancy (day 21 to 50 of gestation) with maintenance nutrition 'M' as control during mid-to-late pregnancy (day 50 to 140 of gestation) e.g., LM = sub-maintenance, MM = maintenance, and HM = *ad libitum,* and physiological state e.g., LP = late pregnancy (day 135 of gestation) and L = lactation (day 15 postpartum), and biological replicate e.g., 1, 2 or 3 for late pregnancy pools and 1 or 2 for lactation pools. e.g., First pool for the late pregnancy sample from the sub-maintenance treatment = LMLP P1.

7.4.2 RNA-seq analysis

RNA sequencing of the ovine mammary gland produced an average of 41,528,368 paired-end reads during late pregnancy and 43,704,258 paired-end reads during lactation (Table 7.3). Generated reads mapped relatively well (approximately 80%) back to the ovine genome version 3.2 (OARv.3.2), with a total of 10,175 transcripts detected in ovine mammary tissue (10,132 during late pregnancy and 10,096 during lactation). This included 4,717 genes with either predicted or uncharacterised function (identified with a '*LOC*' prefix in the gene ID (i.e., *LOC101103639* which is an uncharacterised *Ovis aries* gene located on chromosome 3).

Table 7.3 Summary of RNA-seq reads and mapping statistics for each pooled[1] sample. Samples were generated from ovine mammary RNA collected during late pregnancy and lactation from ewe offspring subjected to nutritional programming during foetal life[2].

Pooled sample ID[2]	Total no. paired end reads (mapped and unmapped)	% reads mapped to genome (OARv.3.2)
AdM_LP 1	46,564,268	76.19
AdM_LP 2	21,764,810	75.63
AdM_LP 3	28,494,084	75.82
MM_LP 1	46,964,368	75.79
MM_LP 2	37,729,898	76.11
MM_LP 3	29,126,802	75.66
SmM_LP 1	57,023,110	75.25
SmM_LP 2	54,432,958	75.93
SmM_LP 3	51,655,012	75.10
AdM_L 1	29,123,882	77.67
AdM_L 2	35,132,680	84.89
MM_L 1	64,969,270	84.41
MM_L 2	55,143,000	84.77
SmM_L 1	45,849,024	84.17
SmM_L 2	32,007,694	84.43

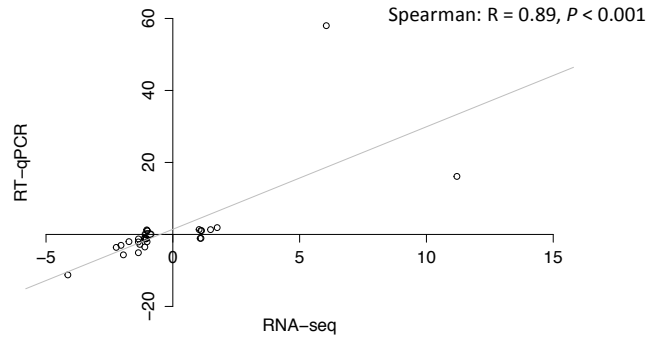
[1]Pooling for late pregnancy; SmM, MM, and AdM (*n* = 3 pools sequenced for each treatment, with three samples per pool, *n* = 9 total samples for each treatment), and lactation; SmM, MM, and AdM (*n* = 2 pools sequenced for each treatment, with three samples per pool, *n* = 6 total samples per treatment).

[2]Samples, from ewes subjected to nutritional programming treatments during foetal life, were pooled for RNA-seq. Pooled sample ID = dam nutrition during early pregnancy (day 21 to 50 of gestation) with maintenance nutrition 'M' as control during mid-to-late pregnancy (day 50 to 140 of gestation) e.g., SmM = sub-maintenance, MM = maintenance, and AdM = *ad libitum*, followed by physiological state of adult ewe offspring at time of tissue sampling e.g., LP = late pregnancy (day 135 of gestation) and L = lactation (day 15 postpartum), followed by biological replicate e.g., 1, 2 or 3 for late pregnancy pools and 1 or 2 for lactation pools.

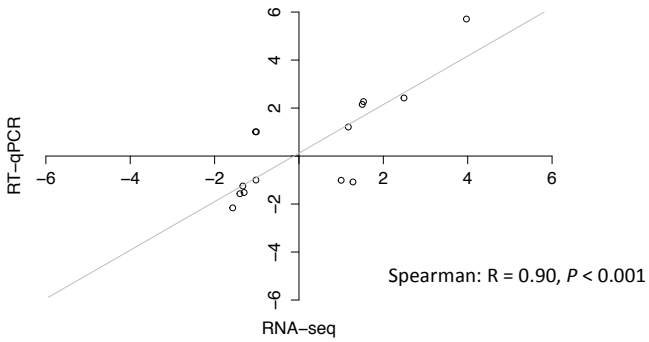
7.4.3 Validation of RNA-seq with RT-qPCR

Gene expression measured by RNA-seq followed a similar pattern in the subset of genes (Table 7.1) tested with RT-qPCR, such that there was a strong positive correlation for the comparison between late pregnancy and lactation (Figure 7.8A, LP vs. L: R value = 0.89, $P < 0.001$) and the comparison between nutritional programming treatments (AdM/SmM vs. MM: R value = 0.90 , $P < 0.001$) in ewe offspring during late pregnancy (Figure 7.3B). There was a much weaker, but still significant, correlation for the comparison between nutritional programming treatments (AdM/SmM vs. MM: R value = 0.46 , $P < 0.001$) in ewe offspring during lactation (Figure 7.3C). Overall, the results from RT-qPCR validate the RNA-seq analysis and provides evidence that the differences detected reflect real changes in expression of genes in response to environmental stimulus (i.e., physiological state or foetal programming by dam nutrition).

A. Late pregnancy vs. lactation



B. Nutritional programming (in ewe offspring during late pregnancy)



C. Nutritional programming (in ewe offspring during lactation)

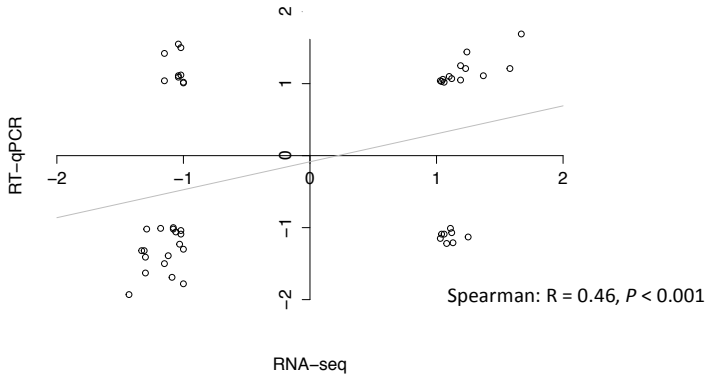


Figure 7.3 Correlation of gene expression fold-changes determined by RNA-seq and reverse-transcription quantitative PCR (RT-qPCR) for pooled[1] samples for *A:* late pregnancy vs. lactation (i.e., LP/L) *B*: in late pregnant ewes subjected to nutritional programming during foetal life (xM/MM fold change), and *C:* in lactating ewes subjected to nutritional programming during foetal life (xM/MM fold change); where x = either sub-maintenance (SmM) or *ad libitum* (AdM) nutrition of the dam during early pregnancy, day 21 to 50 of gestation, followed by maintenance nutrition during mid-to-late pregnancy, day 50 to 140 gestation (i.e., SmM or AdM), and MM = maintenance nutrition of the dam throughout pregnancy days 21 to 140.

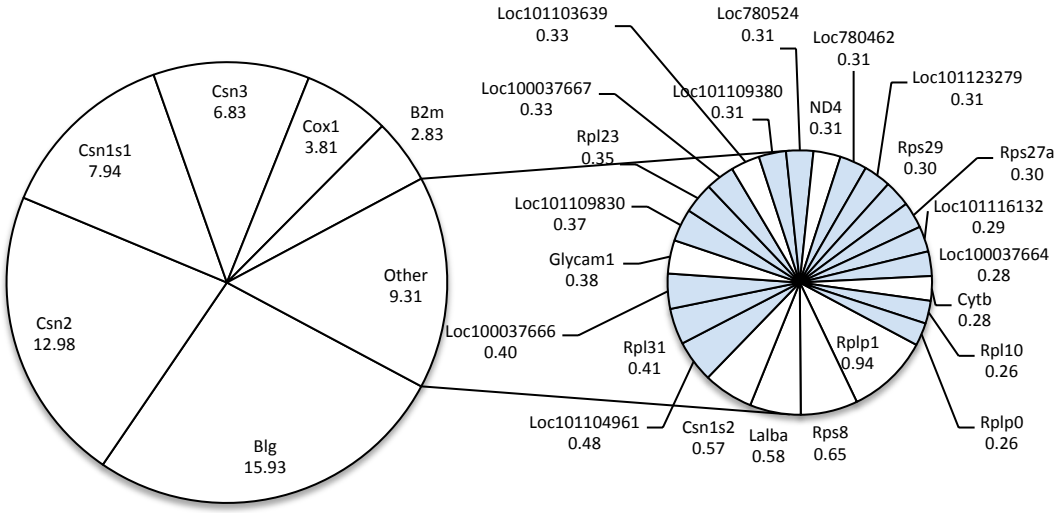
[1]Pooling was as follows: during late pregnancy, *n* = 3 pools sequenced for each treatment (SmM, MM, and AdM), with three samples per pool, *n* = 9 total samples for each treatment; during lactation, *n* = 2 pools sequenced for each treatment SmM, MM, and AdM, with three samples per pool, *n* = 6 total samples per treatment). Therefore the number of pools used to examine gene expression in late pregnancy versus lactation (irrespective of treatment) was as follows: *n* = 9 pools for late pregnancy, with 3 samples per pool, *n* = 27 samples; and *n* = 6 pools for lactation, with three samples per pool, *n* = 18 samples.

7.4.4 Highly expressed genes

During late pregnancy and lactation, a small sub-set of very highly expressed genes accounted for 60% of all RNA-seq reads (30 genes in late pregnancy and 24 genes in lactation; Figure 7.4). This list included genes encoding major milk protein (e.g., caseins, alpha-lactalbumin beta-lactoglobulin), genes encoding for components of large enzyme complexes involved in oxidative phosphorylation in the mitochondria (e.g., *Cox1, Cytb* and *Nd4*), genes encoding for ribosomal proteins, and genes which encode for proteins associated with immunity and inflammation (e.g., *Glycam1* and *B2m*).

During late pregnancy only, genes for an additional seven ribosomal proteins, and a further 11 genes predicted to encode for ribosomal proteins (e.g., *Loc101104961*, 40S ribosomal protein S24-like), were included in the list of most highly expressed. During lactation only, additional genes that were most highly expressed included: genes encoding other secreted proteins (e.g., *Spp1* and *Mfge8*), additional genes encoding components of the oxidative phosphorylation chain (e.g., *Cox3*, *Nd2*, *ATP6*), and genes with products involved in lipid metabolism and milk-fat secretion (e.g., *Fasn* and *Fabp3*).

A. Late pregnancy



B. Lactation

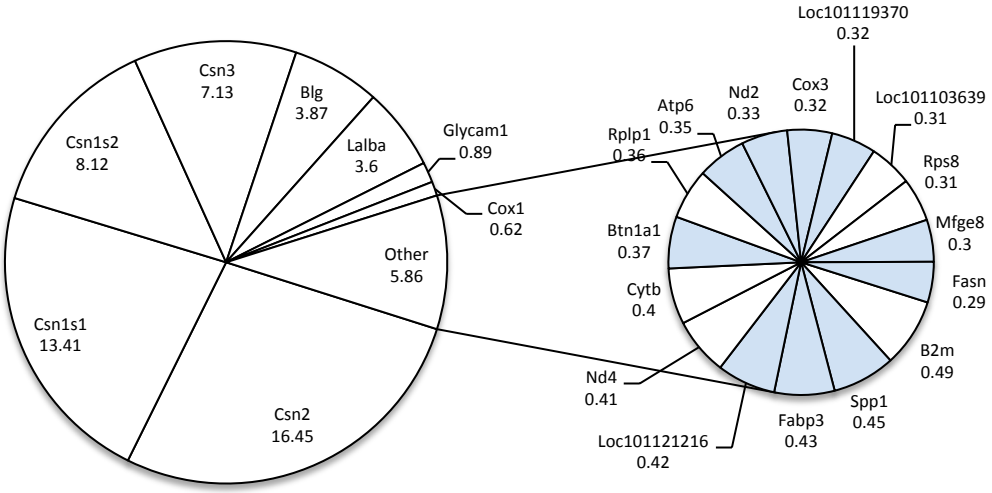


Figure 7.4 Genes[1] that were most highly expressed in the ovine mammary gland (accounting for 60% of the RNA-seq reads) in *A*: late pregnancy and *B:* lactation. Genes highlighted (in blue) represent genes highly expressed in only one physiological state. Graphs are shown out of 60%.

[1]Genes: *Atp6* = ATP synthase 6, *B2m* = beta-2-microglobulin, *Blg* = beta-lactoglobulin, *Btn1a1* = butyrophilin subfamily 1 member A1, *Cox1* = cytochrome C oxidase 1, *Cox3* = cytochrome C oxidase 3, *Csn1s1* = alpha-casein S1, *Csn1s2* = alpha-casein S2 , *Csn2* = beta-casein , *Csn3* = kappa-casein, *Cytb* = cytochrome B, *Fabp3* = fatty acid binding protein, *Fasn* = fatty acid synthase, *Glycam1* = glycosylation-dependent cell-adhesion molecule 1, *Lalba* = alpha-lactalbumin, *Loc100037664* = ribosomal protein L35a, *Loc100037666* = ribosomal protein S11, *Loc100037667* = ribosomal protein S12, *Loc101109380* = 60S ribosomal protein L 12-like, *Loc101104961* = 40S ribosomal protein S24-like, *Loc101109380* = 60S ribosomal protein L 12-like, *Loc101109830* = 40S ribosomal protein S17, *Loc101116132* = 40S ribosomal protein S3a-like, *Loc101119370* = cytochrome c oxidase subunit 3-like, *Loc101121216* = 60S ribosomal protein L27a-like, *Loc101123279* = 60S ribosomal protein L27a-like, *Loc780462* = ribosomal protein L4, *Loc780524* = ribosomal protein S2, *Mfge8* = milk-fat globule EGF-like 8 or lactadherin, *Nd2* = NADPH dehydrogenase 2, *Nd4* = NADH dehydrogenase 4, *Rpl0* = ribosomal protein L 10, *Rpl10* = ribosomal protein L10, *Rpl23* = ribosomal protein L23, *Rpl31* = ribosomal protein L31, *Rplp1* = ribosomal protein, large, P1, *Rps8* = ribosomal protein S8, *Rps27a* = ribosomal protein S27a, *Rps29* = ribosomal protein S29, and *Spp1* = secreted phosphoprotein 1.

Table 7.4 Summary table showing the number and proportion (out of the total genes detected in ovine mammary tissue) of differentially expressed genes (DEGs), and the number and proportion of DEGs of more-highly expressed genes in the ovine mammary gland for each RNA-seq comparison. Comparisons are between physiological state (late pregnancy vs. lactation), and in late pregnant and lactating ewe offspring between foetal programming (dam nutrition) treatments (SmM = sub-maintenance, MM = maintenance and AdM = *ad libitum* dam nutrition during pregnancy day (P) 21 to 50 with maintenance nutrition during P50-140). Pooled samples were used for RNA-sequencing[1].

	Differentially expressed genes		More-highly expressed genes	
Comparison	Number	%	Number	%
Late pregnancy vs. lactation	2750	11.14		
Late pregnancy			1510	54.91
Lactation			1240	45.09
Late pregnancy				
SmM vs. MM	120	0.49		
SmM			50	41.67
MM			70	58.33
AdM vs. MM	447	1.81		
AdM			50	11.19
MM			397	88.81
SmM vs. AdM	235	0.95		
SmM			202	85.96
MM			33	14.04
Lactation				
SmM vs. MM	5	0.02		
SmM			3	60.00
MM			2	40.00
AdM vs. MM	7	0.03		
AdM			3	42.86
MM			4	57.14
SmM vs. AdM	3	0.01		
SmM			1	33.33
MM			2	66.67

[1]Pooling was as follows: during late pregnancy, *n* = 3 pools sequenced for each treatment (SmM, MM, and AdM), with three samples per pool, *n* = 9 total samples for each treatment; during lactation, *n* = 2 pools sequenced for each treatment SmM, MM, and AdM, with three samples per pool, *n* = 6 total samples per treatment). Therefore the number of pools used to examine gene expression in late pregnancy versus lactation (irrespective of treatment) was as follows: *n* = 9 pools for late pregnancy, with 3 samples per pool, *n* = 27 samples; and *n* = 6 pools for lactation, with three samples per pool, *n* = 18 samples.

7.4.4.1 Differentially expressed genes

A large proportion (27.0%, 2,750 genes) of genes detected in the ovine mammary gland were differentially expressed (FDR $P < 0.05$, fold difference > 1.5) between late pregnancy and early lactation (Appendix A). A total of 1,510 genes (54.9% of DEGs) were more-highly expressed during late pregnancy compared with lactation. In lactation, compared with late pregnancy, 1,240 genes (45.1% of DEGs) were more-highly expressed (Table 7.4).

A total of 699 genes were differentially expressed among maternal nutritional programming treatments in late pregnancy mammary tissue from ewe offspring (Appendix A), while only 14 genes were differentially expressed among treatments in mammary tissue during lactation (Table 7.4). During late pregnancy, the greatest number of genes (447 genes) were differentially expressed between ewes born to MM- and AdM-fed dams, 235 genes were differentially expressed between ewes born to SmM- and AdM-fed dams, while 120 genes were differentially expressed between ewes born to SmM- and MM-fed dams (Table 7.4). Of the 14 genes which were differentially expressed among nutritional programming treatments during lactation, seven were differentially expressed between ewes born to MM- and AdM-fed dams (Table 7.4). Four of these genes, including genes encoding for the whey proteins: alpha lactalbumin (*Lalba*) and beta lactoglobulin (*Blg*), were more-highly expressed in ewes born to MM-fed dams, and three genes, including beta casein (*Csn2*), were more-highly expressed in ewes born to AdM-fed dams (Table 7.5). During lactation, five genes were differentially expressed between ewes born to MM- and SmM-fed dams (Table 7.4). Two mitochondrially encoded genes, cytochrome b (*Cytb*) and mitochondrially encoded NADH dehydrogenase 4L (*Nd4l*), involved in oxidative phosphorylation, were more-highly expressed in ewes born to MM-fed dams compared with ewes born to SmM-fed dams (Table 7.5). Three genes, lactoperoxidase (*Lpo*), secreted phosphoprotein 1 (*Spp1*) and glycosylation-dependent cell-adhesion molecule 1 (*Glycam1*), involved in immune function, were more-highly expressed in ewes born to SmM-fed dams, compared to ewes born to MM-fed dams. The expression of *Glycam1* was also higher in the lactating mammary gland of ewes born to SmM-fed dams compared with ewes born to AdM-fed dams. Two genes, insulin-induced gene 1 (*Insig1*) and stearoyl-CoA desaturase delta-9-desaturase (*Scd*), which are both regulated by the sterol regulatory element-binding protein (SREBP) pathway and have roles in fatty acid and cholesterol biosynthesis, were more highly expressed in ewes born to AdM-fed dams compared with ewes born to SmM-fed dams.

Table 7.5 Differentially expressed genes detected by RNA-seq in ovine mammary tissue sampled during lactation from ewes subjected to nutritional programming during foetal life (SmM = sub-maintenance, MM = maintenance, AdM = *ad libitum* dam nutrition during pregnancy day (P) 21 to 50 with maintenance nutrition during P50 -140). Gene expression shown as reads per kilobase per million reads (RPKM). Pooled RNA was used for RNA-sequencing[2].

Treatment	Gene ID	NCBI accession	Description	RPKM expression[1]		
MM vs. AdM				MM	SmM	AdM
MM	*SELENBP1*	XM_004002490.1	Selenium binding protein 1	103.5	91.9	59.3
	LALBA	NM_001009797.1	Alpha lactalbumin	62355.3	60550.0	48070.0
	COX1	NC_001941.1	Cytochrome c oxidase 1	4592.4	3771.2	4192.8
	BLG	NM_001009366.1	Beta lactoglobulin	62188.7	63505.9	58810.5
AdM	*CSN2*	NM_001009373.1	Beta casein	207817.0	216801.4	216858.1
	MFGE8	XM_004018049.1	Milk fat globule-EGF factor 8 protein	619.6	754.4	874.4
	RGS4	NM_001174111.1	Regulator of G-Protein signalling	137.8	179.0	286.2
MM vs. SmM						
MM	*CYTB*	NC_001941.1	Cytochrome B	1767.0	1506.8	1555.9
	ND4L	NC_001941.1	Mitochondrially encoded NADH dehydrogenase 4L	612.4	465.9	612.4
SmM	*LPO*	NM_001009722.1	Lactoperoxidase	233.2	367.4	163.0
	SPP1	NM_001009224.1	Secreted phosphoprotein 1	1838.2	2629.8	2231.8
	GLYCAM1	XM_004006268.1	Glycosylation-dependent cell adhesion molecule 1	9379.0	10586.2	9276.8
SmM vs. AdM						
SmM	*GLYCAM1*	XM_004006268.1	Glycosylation-dependent cell adhesion molecule 1	9379.0	10586.2	9276.8
AdM	*INSIG1*	XM_004008186.1	Insulin induced gene 1	226.6	189.4	257.6
	SCD	NM_001009254.1	Stearoyl-CoA desaturase (delta-9-desaturase)	606.5	626.2	889.2

[1] RPKM expression values are normalised to total reads and transformed by adding +1.

[2]Two pools were sequenced for each treatment (SmM, MM, and AdM) during lactation, with three samples per pool, *n* = 6 total samples per treatment.

7.4.5 Differences between late pregnancy and lactation in gene ontology (GO) and pathways expressed in the ovine mammary gland

Large lists of DEGs were generated by RNA-seq analysis for the comparisons between physiological state (i.e., between late pregnancy and lactation) and between foetal programming (dam nutrition) treatments in ewe offspring during late pregnancy (i.e., SmM vs. MM, AdM vs. MM and SmM vs. AdM). This allowed for the use of GO to analyse molecular pathways and biological functions which are differentially regulated at the transcriptome level. The GO and pathways analyses could not be carried out between foetal programming treatments in lactating ewe offspring due to the small number of DEGs identified.

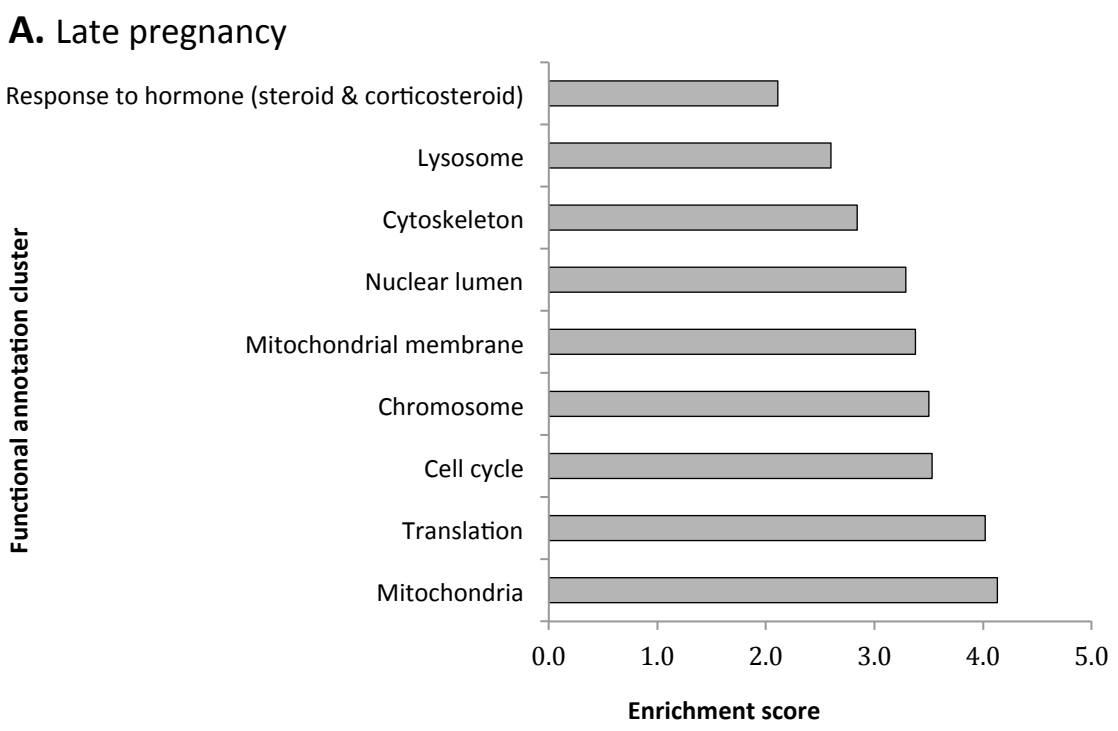
Genes more-highly expressed during late pregnancy, compared with lactation, were clustered into 198 functional GO groups (data not shown), of which nine (Figure 7.5A) were significant (ES > 2). Analysis of KEGG pathways in DAVID revealed that 11 pathways were differentially expressed (Fisher exact $P < 0.01$) during late pregnancy (Figure 7.6A). Genes that were more-highly expressed during lactation were clustered into 178 functional groups (data not shown). Of these groups, nine were significantly enriched (ES > 2, Figure 7.5B). There were five differentially expressed (Fisher exact $P < 0.01$) KEGG pathways during lactation (Figure 7.6B). Pathways and processes, which were differentially expressed between late pregnancy and lactation, were associated with cell proliferation, protein synthesis and metabolism (Appendix B). Specifically, genes associated with cell division, translation, beta-oxidation and epigenetics were more highly expressed during late pregnancy, while genes associated with protein processing and lipogenesis were more highly expressed during lactation. Different genes associated with hormone signalling pathways were enriched during both late pregnancy and lactation. During late pregnancy, a subset of DEGs were largely associated with growth factor activity, while during lactation a subset of DEGs were associated with lactogenic-hormone pathways and maintenance of lactation.

There were no enriched GO clusters (ES < 2) or KEGG pathways ($P > 0.01$) in mammary tissue from late pregnant ewes born to AdM-fed dams, compared with ewes born to SmM- or MM-fed dams (data not shown). There were also no enriched GO clusters (ES < 2) in ewes born to SmM-fed dams, compared with ewes born to AdM- and MM-fed dams, or in ewes born to MM-fed dam compared with ewes born to SmM-fed dams. However, using a cut-off of ES > 1 (which indicates a trend; equivalent to $P < 0.10$), cytokine and extracellular matrix (ECM) and cell adhesion-related GO clusters were enriched in ewes born to SmM-fed

dams, compared with ewes born to AdM-fed dams (Appendix B). There was also a trend (ES > 1) observed for enrichment of the ECM GO cluster in ewes born to MM-fed dams, compared with ewes born to SmM-fed dams (Appendix B).

Ewes born to MM-fed dams had six enriched (ES >2) functional GO clusters and four enriched ($P < 0.01$) KEGG pathways, when compared to ewes born to AdM-fed dams (Figure 7.7A and B, respectively). These clusters and pathways were largely associated with cellular proliferation and apoptosis and included: chromosome, mitotic cell division, regulation of the G2/M transition DNA damage check-point, and DNA processes (Figure 7.7A). Analysis of KEGG pathways also highlighted that ewes born to AdM-fed dams, compared with ewes born to MM-fed dams, had lower expression of genes involved in the cell cycle and p53 signalling pathways (Figure 7.7B and 7.8). Differentially expressed genes included genes encoding cyclins (e.g., cyclin A2: *Ccna2*), cyclin-dependent kinases (e.g., *Cdk1, Cdk2* and *Cdk6*), transcription factors (e.g., E2f transcription factor 1: *E2f1*), centromere proteins (e.g., *Cenpm, Cenpt,* and *Cenpl*), breast cancer 1 and 2 early onset genes (*Brca1* and *Brca2*), checkpoint kinase 1 (*Chek1*), anti-apoptotic oncogene *Bcl2* (B-cell lymphoma 2), and Janus kinase 2 (*Jak2*). These genes, and others contained in these clusters (Appendix B), have diverse functions such as involvement in cell cycle checkpoints, mitosis, DNA replication, spindle organisation, chromosome segregation, DNA repair and cytoskeleton organisation, which play a role in the cell cycle pathway (Figure 7.8). In particular, expression of genes associated with DNA replication (S phase) and the DNA damage (G2/M) check-point, which ensures that cells do not enter mitosis until DNA damage is repaired after replication, appears to be lower in ewes born to AdM-fed dams, compared with ewes born to MM-fed dams.

There were some unexpected categories, including: the small-cell lung cancer, the pathways in cancer and the progesterone-mediated oocyte maturation categories, which were also more enriched in ewes born to MM-fed dams, compared with ewes born to AdM-fed dams. The genes included in these KEGG pathway categories have roles in transcription, regulation of apoptosis, cellular growth, proliferation and differentiation (Appendix B).



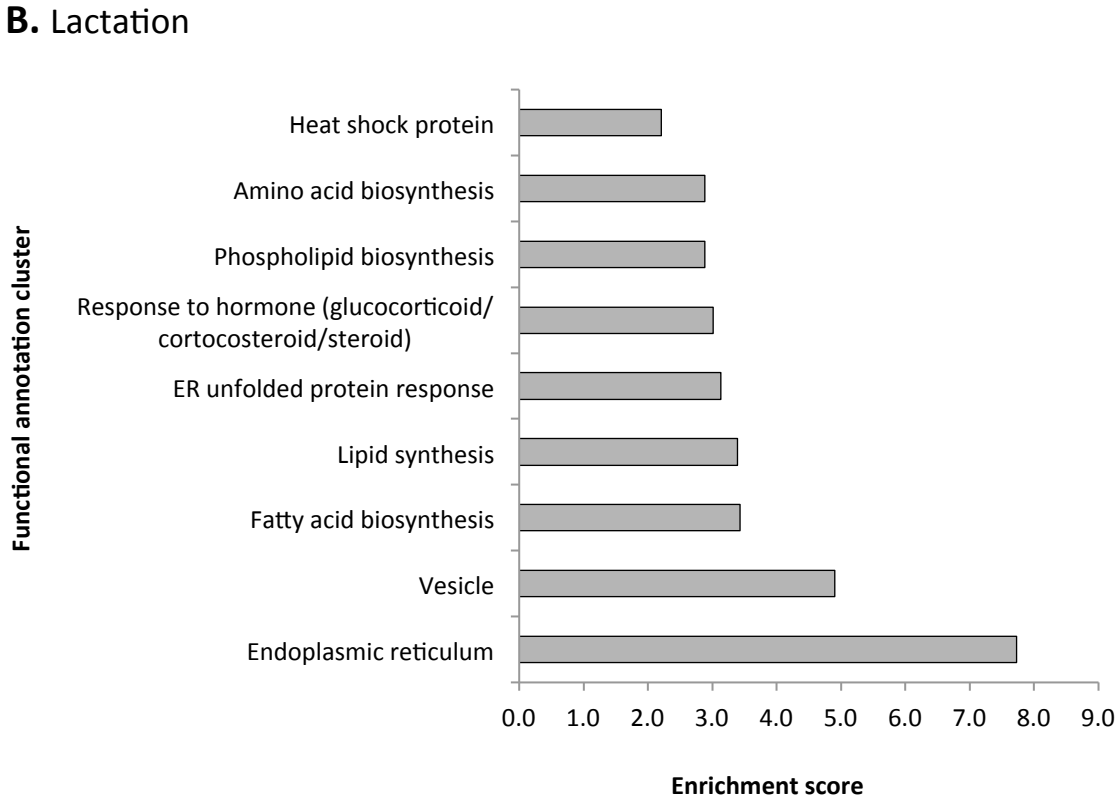


Figure 7.5 Functional annotation clusters significantly enriched (Enrichment score >2) in the ovine mammary gland during *A*: late pregnancy (*n* = 9 pools sequenced, with three animals per pool i.e., *n* = 27 ewes) and *B*: lactation (*n* = 6 pools sequenced, with three animals per pool i.e., *n* = 18 ewes).

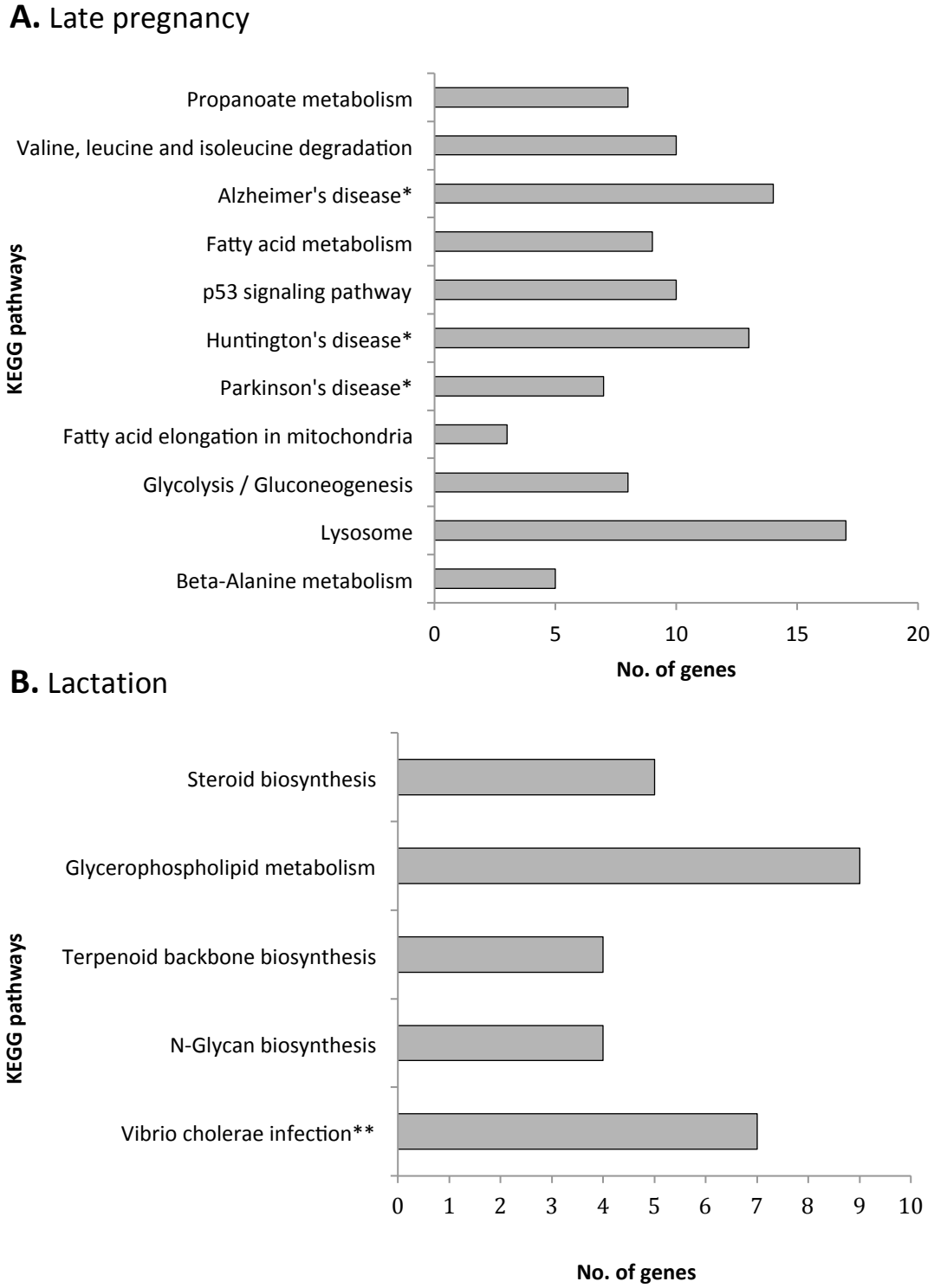


Figure 7.6 Kyoto Encyclopaedia of Genes and Genomes (KEGG) molecular pathways enriched ($P < 0.01$) in the mammary gland during *A*: late pregnancy ($n = 9$ pools sequenced, with three animals per pool i.e., $n = 27$ ewes) and *B:* lactation ($n = 6$ pools sequenced, with three animals per pool i.e., $n = 18$ ewes).

* The Alzheimer's, Huntington's and Parkinson's disease pathways enriched during late pregnancy predominantly contain differentially expressed genes involved in energy metabolism (*Atp5a1, Atp5c1, Atp5d, Atp5g2, Atp5h, Atp5o* and *Hsd17b10*) and apoptosis (*Bad, Calm2, Calm3, Htra2, Ift57* and *Itpr1*). It is the processes, rather than the disease pathway, which are likely to be of significance in the mammary gland.

** The vibrio cholerae infection pathway enriched in lactation contains genes related to ATP synthesis and protein processing in the endoplasmic reticulum. It is the processes, rather than the disease pathway, which are likely to be of significance in the mammary gland.

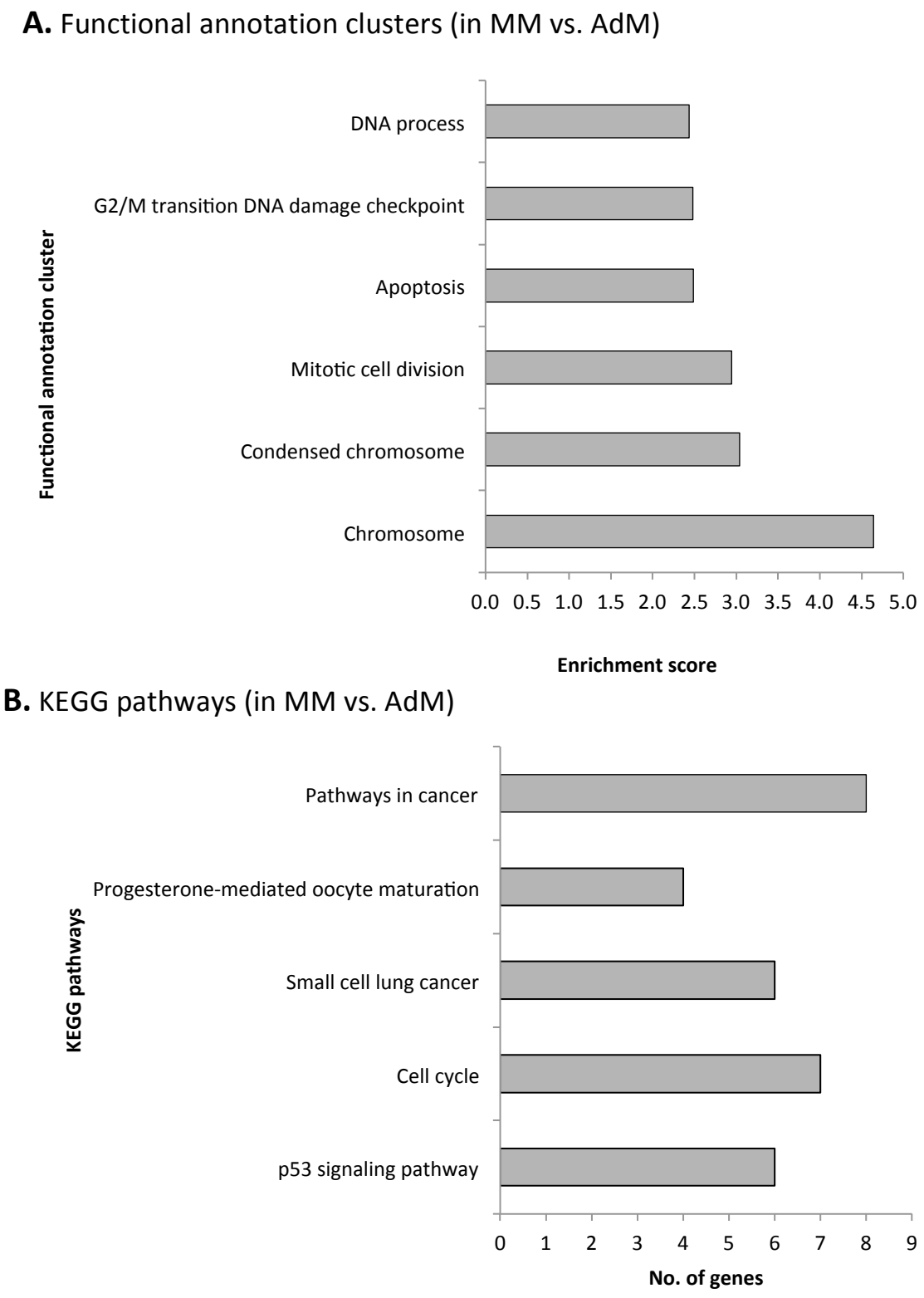


Figure 7.7 Significantly enriched gene ontology (GO) *A*: functional annotation clusters (enrichment score, ES > 2) and *B:* Kyoto Encyclopaedia of Genes and Genomes (KEGG) molecular pathways (Fisher exact $P < 0.01$), in the mammary gland of late-pregnant ewes which were born to dams fed maintenance (MM) throughout pregnancy, compared to ewes born to dams fed *ad libitum* during early pregnancy (day 21 to 50 of pregnancy) and maintenance during mid-to-late pregnancy (day 50 to 140 of pregnancy; AdM). RNA-seq data was generated from pooled RNA samples: $n = 3$ pools sequenced per treatment, with three animals per pool, $n = 9$ animals per treatment.

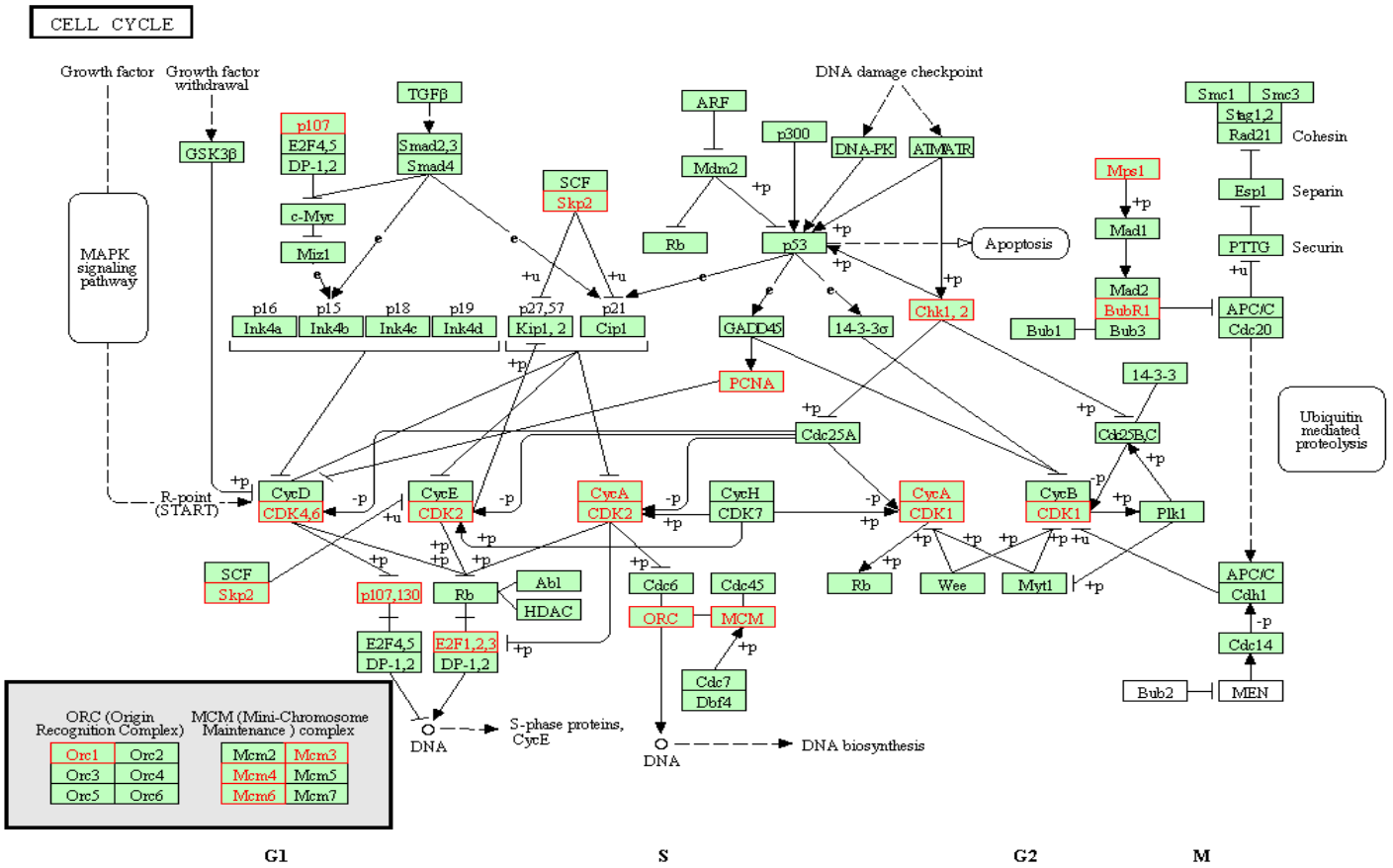


Figure 7.8 Diagram showing differentially expressed genes (in red) involved in cell cycle, which are more highly expressed during late pregnancy, in the mammary glands of ewes born to dams fed maintenance during pregnancy (MM), compared with ewes born to dams fed *ad libitum* during early pregnancy (day 21 to 50 of pregnancy) and maintenance during mid-to-late pregnancy (day 50 to 140 of pregnancy; AdM). RNA-seq data was generated from pooled RNA samples: n = 3 pools sequenced per treatment, with three animals per pool, n = 9 animals per treatment. Cell cycle phases: G1 = growth 1, S = DNA synthesis, G2 = growth 2, and M = mitosis. Diagram of cell cycle generated by Kyoto Encyclopaedia of Genes and Genomes (KEGG) using genes identified as differentially expressed (false discovery rate $P < 0.01$, expression fold difference > 1.5) by RNA-seq analysis.

7.5 Discussion

This study aimed to identify genes and molecular pathways involved in the regulation of development and function of the mammary gland, as a means to understand the influence of dam nutrition during pregnancy on foetal programming of lactational performance of ewe offspring. This is the first study to utilise RNA-seq to examine large-scale changes in gene expression in the ovine mammary gland that occur between late pregnancy and lactation and in response to nutritional programming during foetal life.

7.5.1 Usefulness of RNA-seq as a technique to study gene expression in the ovine mammary gland

The detection of a vast number of transcripts (10,175) in the ovine mammary gland highlights the large and complex network of genes involved in regulating development of the mammary gland and milk production. The strong, positive, correlation between RNA-seq and RT-qPCR data, for the comparison between physiological states (late pregnancy vs. lactation) and the comparison between maternal nutritional programming treatments in ewe offspring during late pregnancy, validates the transcriptome study. The weaker, but still significant, correlation for the comparison between nutritional programming treatments in ewe offspring during lactation is likely due to the smaller number of pools sequenced (i.e., only two pools per treatment were sequenced during lactation, compared with three pools during late pregnancy) as fewer biological replicates may increase the effect of errors in RNA-seq and/ or RT-qPCR analyses. This is particularly an issue in the mammary gland which has a heterogeneous cell distribution and heterogeneous function, potentially contributing to differences among individuals (Molenaar *et al.*, 1992; Casey *et al.*, 2011). There were also fewer genes which were differentially expressed between treatments in the mammary tissue collected during lactation, limiting the range for comparisons. In addition, a small sub-set of genes (approximately 30 genes during late pregnancy and lactation) made-up a large proportion (60%) of RNA-seq reads, effectively diluting the expression of other mammary genes (Bionaz & Loor *et al.*, 2007) and potentially limiting the detection of differences; smaller fold-changes in gene expression are difficult to detect by RT-qPCR. Overall, the RNA-seq analysis can be considered reliable, and provides useful insights into the molecular regulation of development and function of the mammary gland and mechanisms of developmental programming.

7.5.2 Gene expression in the mammary gland

RNA-seq analysis of mammary tissue collected during late pregnancy and lactation, revealed that approximately 27% of genes expressed in the ovine mammary gland were differentially expressed between the two physiological states. This finding, indicates that large-scale changes in gene expression, likely as a result of coordinated changes in the transcriptional-regulation, are required for the mammary gland to become functionally differentiated.

Findings reported in Chapter 5 showed that both over- and under-nutrition (compared with maintenance nutrition) of the dam during early pregnancy lowers lactation performance in twin-born twin-bearing ewe offspring in their first parity (Paten *et al*., 2013). These differences in lactational performance, as a result of nutritional programming during foetal life, may be a result of altered gene expression in the mammary glands of ewe offspring, which may alter tissue structure and function in adult life (Waterland and Garza, 1999). In the present study, RNA-seq analysis revealed that a vast number of genes were differentially expressed in the mammary gland, during late pregnancy, in ewes subjected to nutrition-driven developmental programming during foetal life. During late pregnancy, a total of 447 genes were differentially expressed between ewes born to AdM-fed and MM-fed dams, 120 genes were differentially expressed between ewes born to SmM-fed and MM-fed dams, and 235 genes were differentially expressed between ewes born to SmM-fed and AdM-fed dams. In contrast, during lactation just seven genes were differentially expressed among nutritional programming treatments. This finding suggests foetal programming (by dam nutrition during early pregnancy) influences mammogenesis in ewe offspring during late pregnancy, affecting the establishment of the gland prior to lactation, more so than influencing metabolic and secretory activity of the gland during lactation.

Nutritional programming effects on gene expression in the mammary gland of ewe offspring may be as a result of altered tissue development during foetal life (Waterland and Garza, 1999). With the onset of puberty, the mammary ducts, which are formed during foetal life, expand into the surrounding stroma to establish a ductal network which subsequently gives rise to the alveolar structures during pregnancy (Knight and Peaker, 1982). Therefore, stunted tissue development (e.g., mammary ducts or stroma) during foetal life may limit mammary growth and function in adult ewe offspring. Alternative mechanisms affecting gene expression in mammary tissue of offspring subjected to nutritional

programming during foetal life, may include modification of hormone responsiveness and epigenetic state (Waterland & Garza; 1999, Singh *et al*., 2010a).

7.5.2.1 Differential expression of genes involved in cell proliferation

While the rudiments of the mammary gland are formed during foetal life, the majority of mammary growth and development occurs postnatally. During pregnancy, the mammary gland undergoes extensive structural development and functional differentiation. The expansion of the mammary epithelium during the alveolar proliferative phase in late pregnancy, is particularly important as lactation potential is ultimately determined by the number of secretory epithelial cells (Capuco *et al*., 2001; Boutinaud *et al*., 2004; Wall & McFadden, 2012). Mammary growth continues into lactation for some species (e.g., in the goat, Anderson *et al*., 1981, and in rodents Tucker, 1987). In the bovine mammary gland there is very limited mammary growth during lactation (Sorensen *et al*., 2006; Finucane *et al*., 2008). In the present study, genes associated with cell proliferation were more highly expressed during late pregnancy and had lower expression during lactation. This finding is consistent with that of Anderson (1975), in demonstrating that the growth of the ovine mammary gland is limited during lactation, and that late pregnancy is critical for establishment of the mammary secretory cell population. Key genes associated with cell proliferation, that were more highly expressed during late pregnancy included cyclins and cyclin-dependent kinases, which form complexes to regulate transitions and progression through specific stages of the cell cycle (Sherr & Roberts, 2004). Genes associated with the cytoskeleton, which have functions in spindle formation and maintenance of chromosome structure and alignment during segregation in mitotic anaphase, were also more highly expressed during late pregnancy compared with lactation. These findings are consistent with those of previous studies, that show cell proliferation is enriched during late pregnancy and down-regulated during lactation, and that the increase in numbers of MECs in preparation for lactation is transcriptional regulated in the mammary gland (Finucane *et al*., 2008; Bionaz *et al*., 2012).

In the present study, transcriptional regulation of cellular proliferation appeared to be influenced by nutritional programming during foetal life. The abundance of transcripts for cyclins, cyclin-dependent kinases and various genes involved in cell cycle checkpoints (e.g., the G1/S and the G2/M checkpoints), were lower in ewes born to AdM-fed dams, compared with ewes born to MM-fed dams. Expression of *E2f1*, which encodes a cell cycle associated transcription factor involved in the Rb/E2F pathway, and up-stream activators and down-

stream targets (*Pcna, Ccna2, Cdk2, Brca1* and *2* and *Apaf1*) were reduced in ewes born to AdM-fed dams, compared with ewes born to MM-fed dams. The Rb/E2F pathway is important for progression through the G_1/S transition and the S phase (DNA replication) of the cell cycle (Bracken *et al*., 2002; Korenjak & Brehm, 2005). This finding may suggest that down-regulation of the Rb/E2F pathway may impair cell cycle progression and consequently cell proliferation in the mammary glands of ewes exposed to maternal over nutrition during foetal life.

The increased expression of genes associated with apoptosis, such as *Fos* (a component of the AP1 complex) and reduced expression of anti-apoptosis genes, such as *Bcl2* (Marti *et al*., 1994 & 2001), highlights the possibility of higher levels of cell death in mammary tissue of ewes born to AdM-fed dams, compared with ewe born to MM-fed dams. Apoptosis occurs in the mammary gland to facilitate cell-turnover, however if rates of apoptosis occur at greater levels than proliferation, numbers of MECs may be lowered (Capuco *et al*., 2001). Additionally, higher levels of apoptosis may be associated with failure of cells to progress through cell cycle checkpoints, thus undergoing cell death rather than replicating (Johnson & Walker, 1999).

In the present study, GO analysis revealed that genes associated with the extracellular matrix (ECM) were reduced in expression in mammary tissue of late-pregnant ewes born to SmM-fed dams, compared with ewes born to MM-fed dams. The ECM plays an important role in mediating mammary epithelial growth (reviewed by Maller *et al*., 2010), for example, *Fbln2* (fibulin 2) has been associated with ductal outgrowth during puberty and early pregnancy (Olinjnyk *et al*., 2014), and was more lowly expressed in ewes born to SmM-fed dams compared with ewes born to MM-fed dams. Tissue remodelling is a proposed mechanism of foetal programming, i.e., energy may be partitioned into development of vital organs, such as the brain, at the expense of other organs or tissues (Waterland and Garza, 1999). During foetal life, the rudimentary structures of the mammary gland are formed, and the tissue composition is largely stromal. Much of the functional parenchymal tissue is developed during later postnatal developmental stages, predominantly during pregnancy, but relies on the structural support, availability of substrates and signalling cues from the stromal tissue that was established during foetal life (Capuco & Akers, 2010; Maller *et al*., 2010). Therefore, it is possible that stunted mammary tissue development during foetal life may affect the developmental trajectory of the mammary gland later in life in ewe offspring born to SmM-fed dams. This hypothesis is supported by findings from Martin *et al*., 2012,

where late gestation foetuses from dams fed sub-maintenance during early gestation had lighter mammary glands in comparison to foetuses from maintenance-fed dams. Furthermore, their adult counterparts produced less milk in their first lactation (Chapter 5, Paten *et al*., 2013), providing a link between reduced mass in the foetal mammary gland, reduced function in adult life and decreased expression of ECM genes. Western blots or proteomics studies could be used to confirm that differences in expression of ECM genes translates to differences in abundance of ECM proteins. Then, additional experiments, using 3D cell culture models (O'Brien *et al*., 2010) for example, could be undertaken to investigate the role of these ECM proteins in mammary development.

Collectively, these results raise the possibility that alveolar proliferation in ewes during late pregnancy may be impaired as a result of both over nutrition (AdM) or under-nutrition (SmM) of the dam during early gestation. While the mechanisms appear to be different (i.e., cell cycle regulation in dam over nutrition and ECM in dam under-nutrition), decreased numbers of MECs may contribute to the reduced lactation performance observed in ewe offspring. Additional experiments, using histological and immunohistochemical techniques to measure cell numbers and cell proliferation and apoptosis indexes in mammary tissue of ewe offspring, could be used to further investigate and perhaps confirm this hypothesis (Knight, 2000).

7.5.2.2 Differential expression of genes involved in energy and fat metabolism

As previously discussed, mammary growth appears to be limited in the lactating mammary gland in sheep and in cows (Anderson, 1975; Sorensen *et al*., 2006; Finucane *et al*., 2008). Therefore, an increase in activity of secretory cells must contribute to the increase in milk yield to peak lactation (Knight and Peaker, 1984). Thus, an increase in genes associated with metabolism would be expected during lactation to accommodate the high levels of milk synthesis. Analysis of differentially expressed GO and KEGG pathways in RNA-seq data revealed that genes involved in energy metabolism were highly expressed in the mammary gland during late pregnancy and lactation. Many of these genes were associated with energy-related metabolic processes including oxidative phosphorylation (e.g., ATP synthases), the tricarboxylic acid (TCA) cycle, the glycolytic pathway, and fatty acid metabolism (including beta-oxidation, propionate metabolism, and valine, leucine and isoleucine degradation). These findings reflect the high energy demands of the mammary gland during late pregnancy and lactation required for growth and remodelling and for synthesis and secretion of milk components (Bauman & Currie, 1980).

Genes involved in beta-oxidation were more highly expressed in the mammary gland during late pregnancy, and therefore, could be considered down-regulated during lactation. Beta-oxidation is the process by which fatty acids are broken down to produce acetyl coA, a substrate for the TCA cycle, and NADH and FADH2, which are used by the electron transport chain (ETC) for energy production (Schulz, 1991). This finding is similar to that reported by Rudolph *et al. (*2007) in mice, and Bionaz *et al. (*2012) in cows, whereby expression of genes associated with fatty acid metabolism was decreased in the lactating mammary gland, indicating preferential partitioning of fatty acids taken up by the gland for milk-fat synthesis.

A reduction in fatty acid catabolism in the lactating mammary gland in preference for lipid synthesis, is further supported by the finding that lipogenic processes were enriched in the ovine mammary gland during lactation. This was evidenced by increased expression of genes associated with lipid synthesis, phospholipid biosynthesis, glycerophospholipid metabolism, steroid biosynthesis (i.e., cholesterol biosynthesis), terpenoid backbone synthesis (terpenoids or isoprenoids are important precursors for synthesis of sterols and steroids), and fatty acid biosynthesis: e.g., fatty acid synthase (*Fasn*); acetyl-CoA carboxylase alpha (*Acaca*), stearoyl CoA desaturase (*Scd*), and fatty acid desaturases 1 and 2 (*Fads1* and *Fads2*). This is consistent with the studies of both Finucane *et al. (*2008) and Bionaz *et al. (*2012), which also demonstrated an increase in expression of lipogenic genes in the bovine mammary gland during lactation.

Nutritional programming of ewes during foetal life did not appear to influence expression of energy metabolism-related genes during late pregnancy. There were also very few differences in expression of metabolism-related genes among nutritional programming treatments in the mammary glands of lactating ewe offspring. It is surprising that there were no differences in the expression of genes involved in fat metabolism as ewes born to dams which were over- (AdM) or under- (SmM) nourished during early pregnancy produced lower milk-fat yields in their first lactation compared with ewes born to MM-fed dams (Chapter 5). It is possible this lower milk-fat yield is simply related to their lower milk-yield – a result of less MEC development during pregnancy. Alternatively, during lactation, when genes are already very highly expressed, metabolic differences may be mediated at the post-translational level: e.g., lower protein (enzyme) abundance or activity (e.g., through altered phosphorylation state). For example, Naylor *et al. (*2005) used a rodent model to demonstrate that phosphorylated Prl (S179D Prl) did not reduced the amount of Stat5 protein, but did reduce the amount of phosphorylated (activated) Stat5 which, in-turn

caused failure of secretory activation. Thus, examination of protein abundance and phosphorylation state of candidate genes may be useful for investigating differences in mammary cell metabolism. Additionally, lower milk yield and milk-fat synthesis may be a result of lower metabolic activity in the liver, which has been shown to be susceptible to foetal programming (Desai & Hales, 1997; Vonnahme *et al*., 2003). If metabolism in the liver is impaired, and less substrates (e.g., glucose) are supplied, milk synthetic capacity of the mammary gland may be reduced. Indeed, van der Linden *et al. (*2010a) demonstrated that ewes born to dams fed *ad libitum* during pregnancy, compared with ewes born to dams fed maintenance, had lower glucose concentrations in response to an epinephrine challenge, and may have, therefore, had lower gluconeogenic capacity and ability to supply glucose under physiological stress (e.g., lactation). Further investigation into gene expression in the liver of ewe offspring exposed to nutritional programming in early foetal life may be warranted.

7.5.2.3 Differential expression of genes involved in translation and protein packaging

Milk protein synthesis begins during late pregnancy and greatly increases during lactation. Therefore, it is unsurprising that during both late pregnancy and lactation, there was increased expression of genes associated with different aspects of protein synthesis. Genes associated with translation, predominantly translation initiation factors and translation elongation factors, were more highly expressed during late pregnancy compared with lactation. This is likely representative of the large amount of milk proteins and enzymes being synthesised in preparation for lactation. In contrast, during lactation, there was no further increase in expression of genes involved in protein translation. This finding is likely reflective of the fact that protein synthesis pathways (e.g., mTOR) are largely regulated by phosphorylation, rather than by transcription (Bionaz & Loor, 2011). Rather, during lactation, genes associated with post-translational processing of proteins were more highly expressed compared with late pregnancy. In particular, the endoplasmic reticulum unfolded protein response (ERUPR), heat-shock protein (HSP) activity and the N-glycan biosynthesis pathway appeared to be enriched during lactation. The ERUPR occurs as quality control, degrading un-folded proteins in-response to high levels of protein synthesis, heat-shock proteins function as molecular chaperones involved in protein folding, and N-glycans play a role in assisting chaperones in protein folding through protein binding (Fiedler & Simons, 1995; Feder and Hofmann, 1999; Clarke *et al*., 2012). The increase in transcripts associated with protein folding is likely related to the high levels of proteins being synthesised for lactation, and the increased need to properly process them for milk secretion.

Translation during late pregnancy, and protein processing during lactation in the mammary gland of ewe offspring appeared to be unaffected by nutritional programming during foetal life. This finding is not surprising as there were no differences detected in protein yield measured in milk produced by ewe offspring during their first lactation. Additionally, if differences in protein synthesis are mediated by phosphorylation state (Bionaz & Loor, 2011) they would not be detected by the transcriptomics analysis of the present study. Interestingly, during lactation the expression of genes encoding for two key milk whey-proteins (alpha-lactalbumin and beta-lactoglobulin) was lower in ewes born to AdM-fed dams compared with MM-fed dams. This lower expression in ewes born to AdM-fed dams may be a consequence of decreased numbers of differentiated MECs. Alpha-lactalbumin, in addition to being a whey protein, forms part of the enzyme complex lactose-synthase which is required for the synthesis of lactose, the main carbohydrate and osmotic component in milk responsible for milk volume (Stelwagen, 2011b). If the lower expression of *Lalba*, the gene encoding for alpha-lactalbumin, in ewes born to AdM-fed dams translates to lower protein abundance, this may have contributed to the lower milk yield observed in their first lactation.

7.5.2.4 Differential expression of genes involved in hormone signalling pathways

The role of hormones and growth factors in the mammary gland is well known (Tucker, 2000; Hennighausen and Robinson, 2001; Hovey *et al.*, 2002; Neville *et al*., 2002). Therefore, unsurprisingly, during both late pregnancy and lactation there was enrichment of genes associated with hormone signalling pathways. Genes associated with oestrogen and corticosteroid, in particular, were more highly expressed during late pregnancy compared with lactation. However, abundance of gene transcripts for hormones and hormone receptors were not higher during late pregnancy compared to lactation. Rather, genes associated with growth factor activity, such as IGF binding proteins 2, 4 and 7 (*Igfbp2, Igfbp4* and *IGFbp7*), and V-Erb-B2 Avian Erythroblastic Leukaemia Viral Oncogene Homolog 2 (*Erbb2*), which encodes a member of the epidermal growth factor (EGF) family, were more highly expressed during late pregnancy. Both the IGFs and EGFs have been implicated in mammary development, and have potent effects on growth and cell proliferation which are important for establishment of secretory tissue during pregnancy (Turkington 1969; Forsyth, 1996; Stull *et al*., 2002; Akers, 2006). Expression of Stat5a and Stat5b, which act in response to various cytokines (e.g., Prl, EGF and GH) to promote alveolargenesis and expression of milk protein genes (Hennighausen and Robinson, 2001; Naylor *et al*., 2005; Akers, 2006), were also more highly expressed during late pregnancy.

A sub-set of genes which were more highly expressed during lactation compared with late pregnancy, were associated with glucocorticoid, steroid, peptide and corticosteroid hormones. A number of genes encoding for receptors were found to be more highly expressed during lactation e.g., oestrogen type 1 receptor (*Esr1*), thyroid hormone receptor (*Erba beta1*), growth hormone receptor (*Ghr*), insulin receptor substrate 1 (*Irs1*), glucocorticoid receptor subfamily 3, group c, members 1 and 2 (*Nr3c1* and *Nr3c2*), transforming growth factor beta receptor type 1 (*Tgfbr1*), as well as parathyroid hormone-related protein (*Pth-rp*). Many of these genes encode for receptors which mediate effects of lactogenic and galactopoietic hormones (e.g., insulin, GH and glucocorticoids) and are important for the initiation and maintenance of milk production (Tucker, 2000; Akers, 2006).

A number of genes encoding hormone receptors and growth factors were differentially expressed in mammary tissue of late-pregnant ewes subjected to nutritional programming during foetal life. Expression of *Igf2* (insulin-like growth factor 2) was lower in ewes born to AdM-fed dams, compared with ewes born to MM-fed dams. Insulin-like growth factor 2 (IGF-II) is usually implicated in embryonic development. The identification of IGF-II mRNA in parenchymal and stromal tissue in the ovine mammary gland by Hovey *et al. (*1998) supports a role in post-natal mammary development. Indeed, using mouse models and cell culture, Hovey *et al. (*2003) demonstrated that insulin-like growth factor 2 (IGF-II) acts as a mediator of Prl action to stimulate ductal and alveolar development in the mammary gland. Prl and IGF-II are mitogenic for MECs in the mouse, and over-expression of IGF-II in the mammary gland has been shown to result in alveolar hyperplasia and is associated with pregnancy-related mammary tumours (Bates *et al*., 1995; Huynh *et al*., 1996). IGF-II has also been shown to regulate anti-apoptotic proteins, including Bcl-2, leading to a potential role in cell survival (Singh *et al*., 2010b). Therefore, it is possible that nutritional programming during foetal life may affect cell proliferation in ewe offspring through altered Prl signalling and IGF-II activity.

Late-pregnant ewes born to AdM-fed dams had lower expression of *Loc443444* (ovine progesterone receptor), compared with ewes born to MM-fed dams. Progesterone mediates mammary cell proliferation and differentiation during late pregnancy, acting through the progesterone receptor (PR) which is a nuclear transcription factor (Shyamala, 1999; Hennighausen and Robinson, 2001). Binding of progesterone to PR stimulates transcription of down-stream targets involved in regulation of cell cycle, including cyclins and cyclin-dependent kinases, which were also found to be differentially expressed. Thus

altered progesterone signalling may play a role in foetal programming of mammary development in ewe offspring.

Leptin receptor mRNA has previously been identified in the ovine mammary gland (Laud *et al*., 1999; Chilliard *et al*., 2001). High concentrations of serum leptin are usually associated with over nutrition and impaired mammary development, for example in heifers reared on high-energy diets (Silva *et al*., 2002; Block *et al*., 2003). Interestingly, in the present study, late-pregnant ewes born to dams which were over-nourished during early pregnancy (AdM) had lower expression of *Lepr* in their mammary tissue compared with ewes born to maintenance-fed (MM) dams. Despite having higher expression of *Lepr*, ewes born to MM-fed dams appeared to have higher levels of mammary development, and produced more milk during lactation (Chapter 5). Insulin and IGF-I, which are both mitogenic in the mammary gland, have been shown to increase expression of *Lepr* in the mammary gland (Block *et al*., 2001; Akers, 2006). In addition, the leptin receptor has been shown to activate Jak2, which was also more highly expressed in ewes born to MM-fed dams compared to ewes born to AdM-fed dams, and the insulin-receptor substrate 1 and mitogen activated kinase (MAPK) signalling pathways, which regulate growth in the mammary gland (Akers, 2006). This raises the possibility that the leptin receptor may play a role in promoting mammary development, potentially through activation of the Jak2 signalling pathway. Further investigation into the role of leptin, in the development of the ovine mammary gland, and in foetal programming, is warranted.

The findings of the present study identify altered hormonal and growth factor regulation as a possible mediator of foetal programming effects in the mammary glands of ewe offspring. Immunolocalisation of hormone proteins, receptors and growth factors (e.g., for Prl, progesterone, leptin and IGF-II), to identify which cell types are likely to be more responsive to these hormone signals, and western-blots, to confirm that there is less protein (e.g., receptors) present in ewes born to AdM-fed dams, compared with ewes born to MM-fed dams, may be useful to test this hypothesis. It would also be of interest to examine if dam nutrition during pregnancy altered the endocrine environment of the foetus and, as a result, the responsiveness to hormones in mammary tissue of ewe offspring in adult life (Hilakivi-clarke and de Assis, 2006). Additionally, endocrine influences may originate from altered HPA-axis function through nutritional programming during foetal life (Hawkins *et al*., 2001; Fowden *et al*., 2005). Analyses of serum hormone levels in foetal and adult ewe offspring could be used to further investigate this.

7.5.2.5 Differential expression of genes associated with epigenetic reprogramming

Epigenetic mechanisms may be involved in mediating gene expression changes in the mammary gland of ewe offspring in response to foetal programming by dam nutrition (Capuco and Akers, 2010). Epigenetic mechanisms such as DNA methylation and histone modification may be responsible for gene expression patterns later in life, which may affect development and function of the mammary gland (Singh *et al*., 2010a). In the present study, a large proportion of DEGs between ewes born to dams fed AdM versus MM were associated with chromosome components, such as chromatin. While many of these genes may simply be related to increased cell division (chromosome replication and segregation), some DEGs may be involved in epigenetic regulation of gene expression. Expression of two genes, *H2afz* and *Cbx5*, which are associated with chromatin remodelling, was lower in ewes born to AdM-fed dams, compared with ewes born to MM-fed dams. Expression of *H2afz* was also lower in ewes born to SmM-fed dams, compared with ewes born to MM-fed dams. The *H2afz* gene encodes a variant of the H2 histone protein; histone variants may affect how tightly DNA is wrapped, and, depending on the positioning near transcription start sites, may affect the expression of genes (Jenuwein & Allis, 2001; Valdés-Mora *et al*., 2012). The *Cbx5* (chromobox homolog 5) gene encodes a non-histone protein associated with heterochromatin and epigenetic silencing of gene expression (Azzaz *et al*., 2014). Little is known about the role of these genes in normal mammary gland function, however their role in chromatin remodelling and the effect of nutritional programming on their expression make them interesting targets for further investigations. Modern techniques, such as whole-genome bisulphite sequencing (WGBS) or reduced representation bisulphite sequencing (RRBS), to investigate DNA methylation marks (Doherty and Couldrey, 2014), and chromatin immuno-precipitation sequencing (CHIPseq), to investigate chromatin marks (Rijnkels *et al*., 2010), could be used to examine genome-wide epigenetic differences in the mammary tissue of foetuses at the time of nutritional programming.

7.6 Summary and conclusions

The findings of the present study demonstrates that apparent, coordinated, transcriptional regulation of a large number of genes is required to guide processes involved in mammary gland development and secretory activation. Dam nutrition during early pregnancy appeared to influence the expression of genes in the mammary gland of ewe offspring during late pregnancy, while there were very few differences in expression of genes during lactation. This suggests that mammary development and establishment prior to lactation is likely responsible for observed differences in lactation performance of ewe

offspring i.e., the reduced lactation performance of ewes born to AdM- or SmM-fed dams, compared with ewes born to MM-fed dams (Chapter 5), may be related to reduced numbers of secretory cells during lactation as a result of impaired development during late pregnancy. However, despite both treatments resulting in a similar phenotype, based on gene expression differences, it appears the underlying mechanisms may be quite different. Maternal over nutrition during early pregnancy appears to affect cell cycle regulation in ewe offspring, while the effects of maternal under-nutrition during early pregnancy on lactation performance of ewe offspring may be mediated through an altered ECM. Differences in gene expression may be due to direct mechanisms (e.g., hormonal influences) or indirect mechanisms (e.g., through epigenetic modifications).

The present study has identified a large number of differentially expressed genes in ewe offspring which may be implicated in nutritional programming of the mammary gland during foetal life. These novel findings may provide a platform for future studies, to examine the functionality and role of genes in developmental programming of lactation performance.

8 Identification of reference genes for RT-qPCR in ovine mammary tissue during late pregnancy, lactation and in response to maternal nutritional programming.

Chapter based on the following publication:

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8.1 Foreword

In Chapter 5, it was reported that both under- and over-nourishment of dams during early pregnancy may impair the first-lactation performance of twin-born ewe offspring (Paten *et al*., 2013). Mammary tissue was subsequently collected from late-pregnant and lactating ewe offspring, via biopsy, and transcriptomics (RNA-seq) analysis was undertaken in order to investigate the molecular mechanisms underlying the observed programming effects (Chapter 7). Analysis of RNA-seq data generated a list of genes which were differentially expressed among the nutritional programming treatments. This valuable data-set enabled further analyses, using gene ontology, to examine the types of genes, biological functions and molecular pathways which were impacted in ewes as a result of nutritional programming during foetal life. In order to validate the accuracy of the RNA-seq data-set, the expression of a sub-set of genes was examined by RT-qPCR, which is the gold standard for gene expression studies (Chapter 7). Reference genes, which are stably expressed across experimental treatments and physiological states, are typically used to normalise gene expression data generated by RT-qPCR. Therefore, the present chapter is a method chapter that describes an experiment that was conducted to examine the stability of expression of candidate reference genes across nutritional programming treatments, and late pregnancy and lactation. These findings are presented in this chapter, and the identified reference genes were used to normalise the RT-qPCR data reported in Chapter 7. This chapter is based on the version published in *Physiological Genomics* (Paten *et al*., 2014).

8.3 Abstract

The mammary gland is a complex tissue consisting of multiple cell types which, over the lifetime of an animal, go through repeated cycles of development associated with pregnancy, lactation and involution. The mammary gland is also known to be sensitive to maternal programming by environmental stimuli such as nutrition. The molecular basis of these adaptations is of significant interest, but requires robust methods to measure gene expression. Reverse-transcription quantitative PCR (RT-qPCR) is commonly used to measure gene expression, and is currently the method of choice for validating genome-wide expression studies. RT-qPCR requires the selection of reference genes that are stably expressed over physiological states and treatments. In this study we identify suitable reference genes to normalise RT-qPCR data for the ovine mammary gland in two physiological states; late pregnancy and lactation. Biopsies were collected from offspring of ewes that had been subjected to different nutritional paradigms during pregnancy to examine effects of maternal programming on the mammary gland of the offspring. We evaluated eight candidate reference genes and found that two reference genes (*Prpf3* and *Cul1*) are required for normalising RT-qPCR data from pooled RNA samples, but five reference genes are required for analysing gene expression in individual animals (*Senp2, Eif6, Mrpl39, Atp1a1, Cul1*). Using these stable reference genes, we showed that *Tet1*, a key regulator of DNA methylation, is responsive to maternal programming and physiological state. The identification of these novel reference genes will be of utility to future studies of gene expression in the ovine mammary gland.

8.4 Introduction

The mammary gland is a dynamic organ that undergoes repeated cycles of development during the physiological stages of pregnancy, lactation and involution. Dramatic developmental changes and metabolic adaptations occur in the mammary gland during the transition from late pregnancy to lactation, in order to synthesise and secrete milk. These processes are carefully regulated by complex signalling networks, involving hormones of the endocrine system and local factors, and are influenced by the health and nutritional status of the animal (Hovey *et al*., 2002; Neville *et al*., 2002; Capuco & Akers, 2010). Development and function of the mammary gland may also be programmed by experiences *in utero*, including the level of nutrition of the dam (Hilakivi-Clarke *et al*., 1997; van der Linden *et al*., 2009; Blair *et al*., 2010; Martin *et al*., 2012; Paten *et al*., 2013). In sheep, *ad libitum* nutrition of the dam has been shown to reduce the size of the foetal mammary gland and reduce the amount of milk produced during the first lactation of adult offspring (van der Linden *et al*., 2009; Paten *et al*., 2013). In rodents, a maternal diet high in fat has been linked to increased breast cancer risk in offspring (Hilakivi-Clarke *et al*., 1997). Understanding the molecular mechanisms that underpin maternal programming will benefit animal production, and is of the utmost importance in human and animal health research.

The use of high-throughput sequencing (HTS) technologies, such as RNA-seq, has enabled analysis of the mammary transcriptome, providing insights into the patterns of gene expression involved in mammary gland development and function (Ferreira *et al*., 2013). Transcriptomic tools allow for further exploration into molecular mechanisms that may modulate effects in the mammary gland from external influences. To ensure accuracy of results, HTS data must be validated. This is typically done by correlation with expression data generated by RT-qPCR (reverse-transcription quantitative PCR), a highly sensitive and specific technique for measuring gene expression (Bustin *et al*., 2009). RT-qPCR is considered to be the gold standard for gene expression analysis as it is able to specifically detect transcript expression over a wide dynamic range (Valasek & Repa, 2005). RT-qPCR is, however, subject to technical variation introduced during RNA extraction, cDNA synthesis or during reverse-transcriptase reactions. To combat this, internal controls, such as reference genes, must be used to normalise data (Vandesompele *et al*., 2002). Ideal reference genes are expressed at levels similar to the gene(s) of interest, and are stably-expressed across all samples. Fluctuations in reference gene expression across physiological states can significantly skew the measurement of target gene expression (Cameron *et al*., 2013a).

Selection of appropriate reference genes for studies of mammary gland development during late pregnancy and lactation may be difficult as changes in cell numbers, differences in ratios of cell types, as well as changes in cell metabolism and biological processes leads to variation in the expression of genes (Bionaz *et al*., 2012). Potential modulation of gene expression through maternal nutritional programming may also contribute to variation in expression of reference genes. Studies in other species have identified reference genes for bovine and porcine mammary tissue during pregnancy and lactation (Bionaz & Loor, 2007; Tramontana *et al*., 2008). There are no studies, to date, for the ovine mammary gland, and no studies investigating stability of reference genes in offspring of maternal nutritional programming studies.

In this study we identify, in a non-biased way, candidate reference genes for normalising RT-qPCR data in the ovine mammary gland during late pregnancy and lactation and in response to maternal nutritional programming.

8.5 Material and methods

8.5.1 Animals and sampling

Ovine mammary gland tissue was sampled from a sub-set of twin-bearing, twin-born ewe offspring of a previously published maternal nutritional programming study (Kenyon *et al*., 2011a; Paten *et al*., 2013). In brief, Romney ewes (G0 dams) were fed a sub-maintenance ($Sm_{P21\text{-}50}$), maintenance ($M_{P21\text{-}50}$) or *ad-libitum* ($Ad_{P21\text{-}50}$) pasture allowance during early gestation (P21-50), and were reallocated to either a maintenance ($M_{P50\text{-}140}$) or *ad libitum* ($Ad_{P50\text{-}140}$) pasture allowance during mid-to-late gestation (P50-140) (Figure 8.1A). The ewe offspring generated were utilised as the experimental animals of the present study and were therefore from one of six dam nutritional treatment groups: SmM, SmAd, MM, MAd, AdM, and AdAd (Figure 8.1B, Table 8.1). All ewe offspring (G1 offspring) were managed under the same New Zealand commercial pastoral farming conditions and received the same level of nutrition (average intakes). Mammary parenchymal tissue (30-50 mg) was sampled from 10 ewes per treatment (n = 60) via needle biopsy (Bard Magnum reusable core biopsy gun and 12G, 10cm core biopsy needles, Bard Biopsy Systems) during late pregnancy (135 ± 2.4 SD days of gestation) and again during lactation (15 ± 1.27 SD days post partum). Tissue samples were immediately frozen in liquid nitrogen and stored at -80°C until RNA extraction. Ewes were ~2 yrs. of age at the time of the study. Late pregnancy biopsies were collected in September 2011 [ewe age 733.9 ± 1.66 days (SD)], and lactation biopsies were collected in October 2011 [ewe age 761.0 ± 2.11 days (SD)]. The study was conducted at the Massey

University Keeble Sheep and Beef farm, 5 km south of Palmerston North, New Zealand. The study was approved by the Massey University Animal Ethics Committee, Palmerston North, New Zealand.

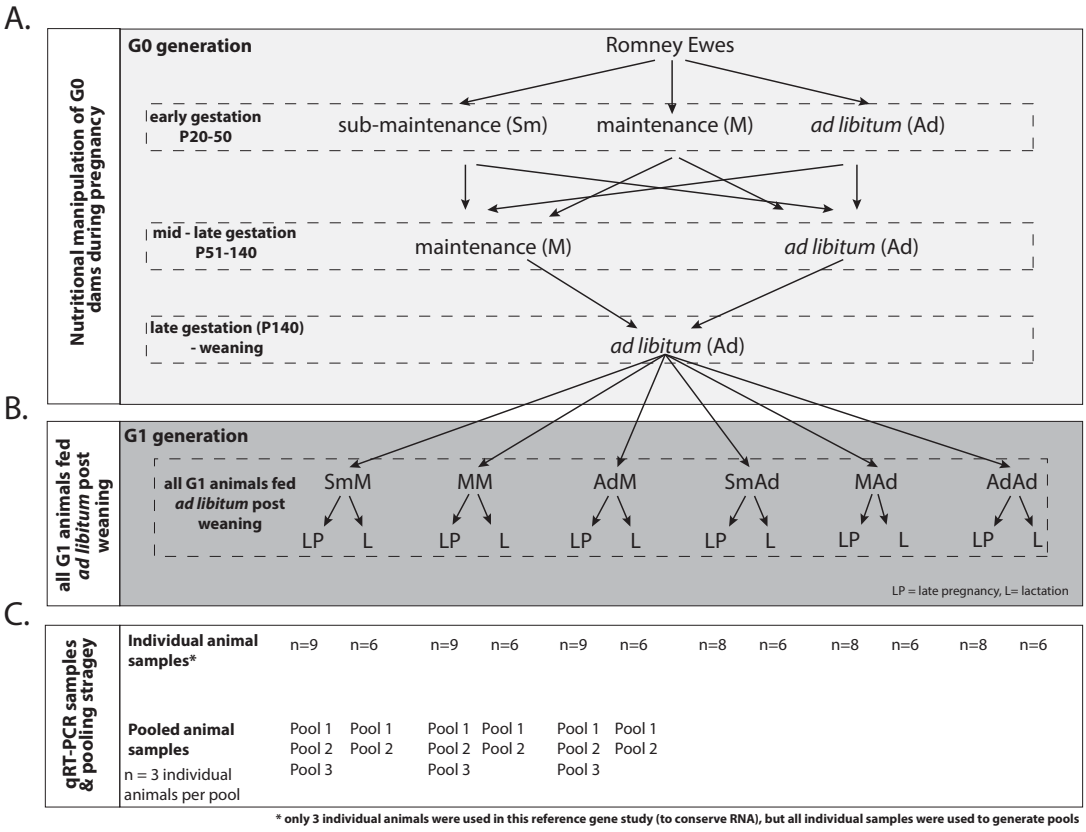

Figure 8.1 Experimental design and RNA-pooling strategy used for this reference gene study. *A:* Maternal-feeding paradigm. Romney ewes (G0) were fed *ad libitum* until day 21 of pregnancy when animals were randomly allocated to a sub-maintenance (Sm), maintenance (M) or *ad libitum* (Ad) diet. At day 50 of pregnancy, ewes were randomly reallocated to either a maintenance (M) or *ad libitum* (Ad) diet until day 140 of pregnancy when all ewes were switched to an *ad libitum* diet. *B:* The offspring (G1) exposed to maternal nutritional programming treatment are identified according to the nutrition that their G0 mothers received during pregnancy i.e., the SmM groups' mothers were allocated a sub-maintenance diet in early gestation and a maintenance diet in mid-late gestation as detailed in Table 8.1. All G1 offspring were fed *ad libitum*. RNA was extracted from G1 mammary biopsies collected during late pregnancy (LP) or lactation (L), and the number of individual RNA samples isolated are indicated in the diagram (C). For RT-qPCR of individual animals only three RNA samples were used for each group to conserve RNA for future experiments. For pooling, RNA samples were randomly allocated to one of three pools for LP and one of two pools for L; each pool consisted of RNA isolated from three individual animals.

Table 8.1 Summary of maternal nutritional treatments used in this study.

Treatment	Pasture allowance during early gestation (P21-50)	Pasture allowance during mid-late gestation (P50-140)
SmM	Sub-maintenance	Maintenance
SmAd	Sub-maintenance	*Ad-libitum*
MM	Maintenance	Maintenance
MAd	Maintenance	*Ad-libitum*
AdM	*Ad-libitum*	Maintenance
AdAd	*Ad-libitum*	*Ad-libitum*

8.5.2 RNA extraction and cDNA synthesis

Total RNA was isolated from mammary tissue samples using Trizol (Invitrogen) and purified using RNeasy mini kit (Qiagen). Genomic DNA contamination was eliminated via on-column digestion with DNase (Qiagen), as per the manufacturer's protocol. The concentration and quality of RNA was measured on a Nanodrop ND-1000 spectrophotometer (Nanodrop), and integrity was assessed with an Agilent 2100 Bioanalyzer (Agilent Technologies). Only RNA with RNA integrity numbers > 7 was used in this study. We used 1 µg of total RNA as template to perform cDNA synthesis using the SuperScript VILO cDNA Synthesis Kit (Invitrogen) as per the manufacturer's protocol. Controls with no reverse transcriptase were used to assess the possibility of genomic DNA contamination in both RT-PCR and RT-qPCR.

8.5.3 Pooling of samples

One of the aims of this study was to identify candidate reference genes that could be used to validate RNA-seq data (Chapter 7) by RT-qPCR. For RNA-sequencing we attempted to minimise individual variation between animals within the treatments by pooling RNA from multiple individuals (Kendziorski *et al*., 2003; Kendziorski *et al*., 2005; Konczal *et al*., 2014). RNA from samples within the same treatment group were pooled separately for the two time points, late pregnancy and lactation. We incorporated 2 µg of RNA, subsampled from three randomly selected animals per treatment, into pools (Figure 8.1C). Three pools per treatment were generated for late pregnancy samples, and two pools were generated per treatment for lactation samples. The pools were *1)* late pregnancy [SmM, MM, and AdM (n = 3 for each treatment, total samples n = 9)] and *2)* lactation [SmM, MM, and AdM (n = 2 for each treatment, total samples n = 6)]. To assess variation in expression of candidate genes between individuals, we also carried out RT-qPCR analysis on a subset of samples from individual animals from all six treatment groups: SmM, MM, AdM, SmAd, MAd, and AdAd (n = 3 for each treatment) (Figure 8.1B).

8.5.4 Selection of potential reference genes

Candidate reference genes were selected from the RNA-seq data reported in Chapter 7. RNA-seq data were generated from pooled RNA (as detailed above) on an Illumina Hi-Seq 2000 (service provided by New Zealand Genomics Limited). Reads were mapped to the *Ovis aries* genome (version 3.2) using CLC Genomics Workbench (CLC Bio). To identify candidate reference genes from the RNA-seq data, genes were initially ranked based on the SD of total gene relative to their overall expression (i.e., SD/total gene reads). This relative SD accounts for the fact that genes with high expression will have a higher SD than genes with low expression. By ranking genes on their relative SD we were attempting to determine the variation in gene expression irrespective of expression level. The genes with the lowest standard deviation (relative to their overall expression: SD% range = 0 – 1.03%) were analysed for expression stability using geNorm (Vandesompele *et al*., 2002) and NormFinder software (Andersen *et al*., 2004). Genes were allocated a ranking from 1 to 100 for expression stability (1 representing most stable and 100 representing least stable) for each of the three methods for measuring expression stability (SD%, geNorm, and NormFinder). The sum of the ranking numbers were calculated and used to create an overall ranking of expression stability (with lower numbers representing less variable genes). Genes which ranked well for high expression stability, and which had low to medium expression based on the RNA-seq data (total gene reads approximating the mean), were chosen for evaluation as reference genes via RT-qPCR (refer to Table 8.2 for genes and expression stability rankings). Four genes were selected from the RNA-seq data; *Cul1* (part of the E3 ubiquitin ligase complex), *IPO9* (nuclear transport receptor), *Prpf3* (U4/U6 small nuclear ribonucleoprotein) and *Sf1* (RNA splicing). Two additional candidate reference genes (*Mrpl39*, *Eif6*), which were stably expressed in the RNA-seq data, were selected from the literature (Bionaz & Loor, 2007; Tramontana *et al*., 2008) and compared with *Atp1a1* (Calcagno *et al*., 2006), which had been previously used as a reference gene in our laboratory. Co-regulation of reference genes is known to bias the calculations for gene expression stability using geNorm (Vandesompele *et al*., 2002). Possible co-regulation was detected between *Cul1* and *EIF6*, and *Cul1* and *Atp1a1* [determined using Ingenuity Pathway Analysis software (Ingenuity Systems, www.ingenuity.com)].

Table 8.2 Ranking of candidate reference genes

Gene code	Gene description	SD% rank	geNorm rank	NormFinder rank	Overall rank
Sf1	Splicing factor 1 isoform 2	2	4	5	2
Senp2	Sentrin-specific protease 2 isoform 1	6	2	4	3
Cul1	Cullin 1	4	7	3	5
Prpf3	U4/U6 small nuclear ribonucleoprotein *PRP3*	14	17	14	12
IPO9	Importin 9	10	19	6	10
Mrpl39	Mitochondrial ribosomal protein L39	From literature			
Eif6	Eukaryotic translation initiation factor 6	From literature			
Atp1a1	ATPase, Na+/K+ transporting, alpha 1 polypeptide	Previously used in laboratory			

8.5.5 Primer design

RT-qPCR Primers were designed using Primer3Plus (Untergasser *et al*., 2012) (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/). Where possible primers were designed to span intron / exon boundaries to allow detection of amplification from contaminating genomic DNA. *In silico* specificity of the primers was assessed using primer-BLAST (Ye *et al*., 2012).

Primer sequences and their amplicon lengths are listed in Table 8.3. Primers were highly specific as shown by a single band when PCR product was run on a 2% agarose gel, and a single peak observed in melt curve (data not shown). PCR products were also sequenced to confirm their specificity. The efficiency of primers was calculated from RT-qPCR of a 10 x dilution series of the cDNA**.** The RT-qPCR reaction efficiency was between 90 and 110% for all primer pairs (Table 8.3).

Table 8.3 Gene name, primer sequences, amplicon length (base pairs, bp) and PCR efficiency for reference genes evaluated.

Gene	NCBI accession	Forward Primer 5' → 3'	Reverse Primer 5' → 3'	Amplicon length (bp)	PCR efficiency (%)
Mrpl39	XM_004002812.1	CCCTGGAAGTTGAAGCAAAA	GGTTCTGGGATGCCTTCTCT	90	98.1
Eif6	NM_001162563.1	AATTGAGGACCAGGATGAGC	GCACACCAGTCATTCACCAC	114	103.8
Atp1a1	NM_001009360.1	GAGATTGTGTTCGCCAGGAC	CGTCTCCAGTTACAGCCACA	94	95.9
Cul1	XM_004008343.1	AAAAATACAACGCCCTGGTG	CTGAGCCATCTTGGTGACTG	116	95.9
Ipo9	XM_004014142.1	ACTACGAGGACGACGAGGAG	GGCAGAGGAAGTCTGTGAGG	93	98.3
Prpf3	XM_004002449.1 XM_004002450.1	ACAGATGATGGAAGCAGCAA	GGTTGGGAGGATGAAGGAGT	105	101.0
Sf1	XM_004019657.1	GAGAGTTGGCTCGCTTGAAT	CCCCTCCACACTTGGTACAC	120	99.6
Senp2	XM_004003073.1 XM_004003074.1	GAGGTGTTCAAAGGGGAAAA	TCTTCAGACAGGTCGGGTTC	105	101.0
Tet1 (*target gene)*	XM_004021627.1	TTTCTCTGGGGTCACTGCTT	TGAGCGGTTATCTTCTCGTG	115	100.6

8.5.6 Quantitative PCR reactions

RT-qPCR reactions were carried out on a Bio-Rad C1000 Thermal cycler (Bio-Rad CFX96 Real-Time System) using SsoFast EvaGreen Supermix (BioRad) with 10 × diluted cDNA template and 300 nM of oligonucleotide primers. The following PCR program was used: 1 min initial incubation at 95°C followed by 40 cycles of 5 seconds at 95°C and 30 seconds at 60°C. On completion the reactions were held at 95°C for 10 seconds, reduced to 65°C and incrementally raised by 0.5°C until reaching 95°C for a melt curve analysis. In all cases the quantitation cycle (Cq) measured for no-template controls and –RT controls was greater than 40. Reactions were carried out in duplicate for each sample to minimise effects of technical errors, duplicates that differed by more than 0.5 cycles were repeated.

8.5.7 Data analysis

RT-qPCR data was analysed using the Bio-Rad CFX Manager™ software. For the samples tested, raw Cq values were obtained and used to determine gene expression stability with geNorm^{PLUS}. Gene expression stability analysis was carried out using the geNorm algorithm (Vandesompele *et al*., 2002) implemented in qbase+ (version 2.6) (Hellemans *et al*., 2007). geNorm calculates the average pairwise variation of a candidate reference gene with all other control genes, reported as the 'M' value. The lower the M value the more stably expressed the gene. An M value of less than 0.05 is considered highly stable. The use of a single reference gene for data normalisation is not recommended (Vandesompele *et al*., 2002), thus two or more reference genes should be used to normalise RT-qPCR data. geNorm was, therefore, also used to perform a pairwise variation analysis (V value), based on the geometric mean of all the candidate reference genes, to identify the optimal number of reference genes required. The V value analysis is a stepwise exclusion model that calculates pairwise variation for every series NF_n and NF_{n+1} Normalisation Factors, reflecting the effect of adding an n+1 gene. For example it calculates the variation of two compared with three reference genes (V2/3), and three compared with four reference genes (V3/4) etc. and generates a V value. A V value of 0.15 is considered the upper limit for selecting an adequate combination of reference genes.

For analysis of TET1 expression, raw Cq values were obtained using the Bio-Rad CFX Manager™ software and imported into qbase+ (version 2.6)(Hellemans *et al*., 2007). Outliers were identified in RT-qPCR data using Grubbs' test (Burns *et al*., 2005) as implemented by the outliers package in R. TET1 expression was normalised by the geometric mean of the

relative quantities for the selected reference genes. Differences in TET1 gene expression were determined using ANOVA with a Tukey HSD post-hoc test implemented in R.

8.6 Results

8.6.1 Reference gene stability in pooled samples

Our aim was to identify appropriate reference genes for the mammary gland in late pregnancy and lactation that did not change as a result of maternal nutritional programming in order to validate RNA-seq data (Chapter 7). For the RNA-seq analysis we pooled RNA samples in an attempt to minimise individual variation (Kendziorski *et al*, 2003; Kendziorski *et al*., 2005; Konczal *et al*., 2014). We therefore examined the expression of our candidate reference genes across our pooled samples, for both late pregnancy and lactation, which were derived from the three maternal nutritional programming groups (SmM, MM and AdM) (Figure. 8.2A) during late pregnancy and lactation. Expression data derived from RT-qPCR was used to carry out the gene stability analysis with geNorm (Figure 8.2B). The gene expression stability measures (M) of these genes indicate that all of the candidate reference genes are stably expressed across physiological time points (lactation and late pregnancy) and amongst the nutritional programming groups [M values < 0.5 is indicative of highly stable expression in homogenous tissue samples (Hellemans *et al*., 2007; Vandesompele *et al*., 2002)]. The results showed that *Prpf3*, *Cul1* and *Sf1*, which were all candidate reference genes selected from the RNA-seq data, had the highest expression stability across pooled samples (M = 0.183, 0.190, 0.195, respectively) (Figure 8.2B). *Mrpl39*, selected from literature, had an intermediate expression stability ranking (M = 0.234), while the other two candidate genes selected from literature, *Eif6* and *Atp1a1A*, were ranked the least stable (M = 0.308, 0.327, respectively). The remaining genes, *Senp2* and *Ipo9*, selected from RNA-seq, had an intermediate expression stability ranking (M = 0.259, 0.273, respectively). In general, reference genes selected from RNA-seq data were more stably expressed than those chosen from the literature.

Pairwise variation analysis suggests that two genes, *Prpf3* and *Cul1*, would be acceptable to accurately normalise expression data [Figure. 8.2C, V value < 0.15 (Vandesompele, 2002; Hellemans, 2007)]. The addition of a third gene would have no significant effect, as the V2/3 value was less than the suggested cut-off of 0.15 (Vandesompele *et al*., 2002).

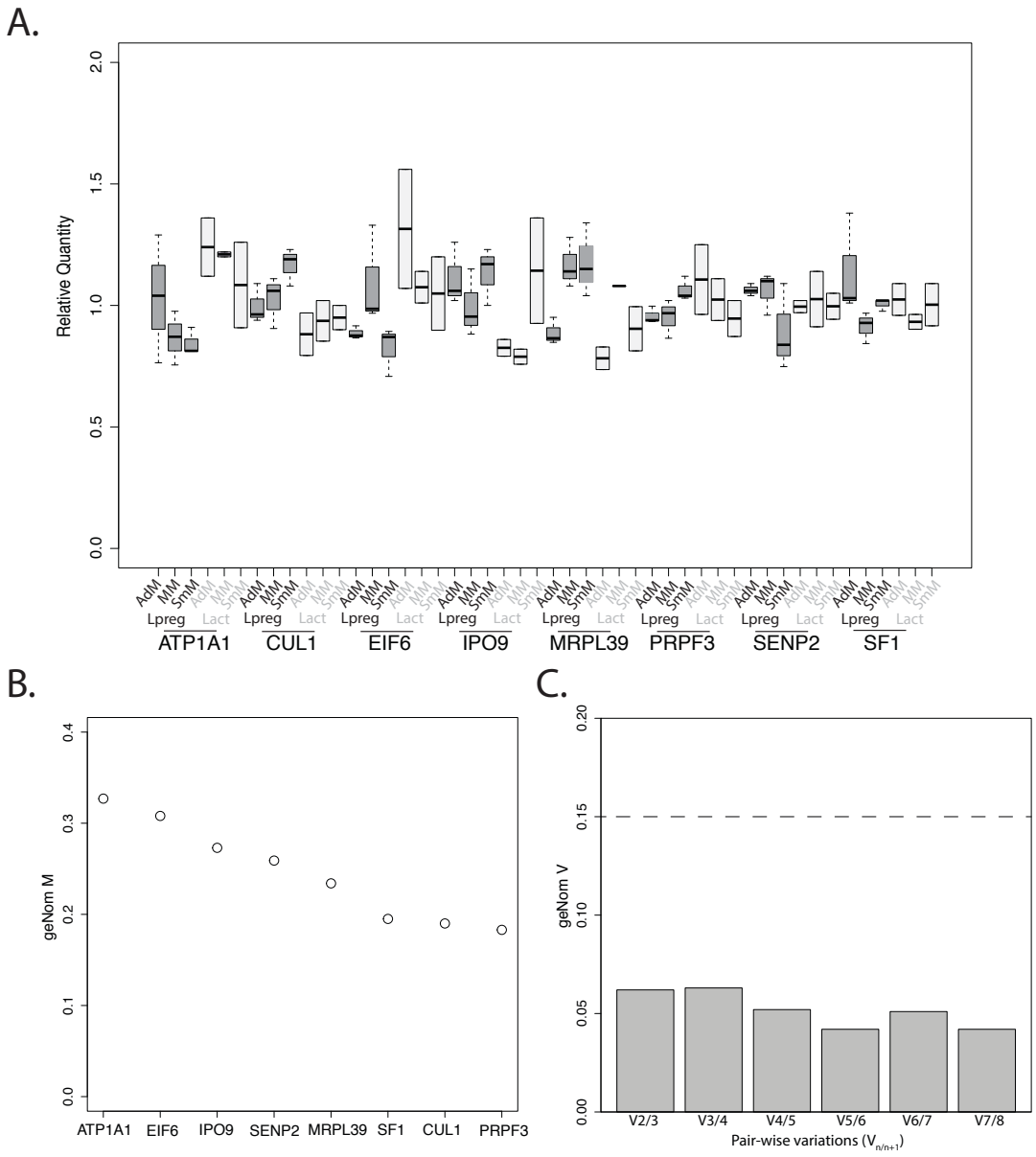

Figure 8.2 Expression and stability analysis of the eight candidate genes in pooled RNA samples. *A:* Relative quantity of the eight candidate reference genes in pooled RNA samples across the two physiological states [late pregnancy (Lpreg) and lactation (Lact)] and three maternal programming treatment groups, *ad libitum*/maintenance (AdM), maintenance/maintenance (MM), sub-maintenance/maintenance (SmM). *B:* geNorm stability analysis (M value) of the candidate reference genes. Low M values indicate more stable expression. All M values < 0.5 which is considered highly stable. *C:* geNorm pairwise variation analysis (V value) of the candidate reference genes. V < 0.15 (marked by dashed line) is considered as the upper limit for selecting an adequate combination of reference genes, all combinations of pairwise variation meet this criteria and two reference genes are recommended.

8.6.2 Reference gene stability in individual animal samples

Our rationale for pooling samples for our RNA-seq analysis was to minimise individual variation between animals within the treatments (Kendziorski *et al*., 2003; Kendziorski *et al*., 2005; Konczal *et al*., 2014). In order to determine the levels of individual variation in gene expression, and also to extend our search for reference genes to include analyses performed on individual animals, we also performed expression stability of potential reference genes for individual animal samples (from all maternal nutrition treatment groups: SmM, SmAd, MM, MAd, AdM, AdAd, and just those that comprised the pools).

Compared with the pools (Figure 8.2A), variation in expression of reference genes was much greater for the individual animal samples (from all six treatments, Figure 8.3A). During late pregnancy (Figure 8.3B) 7/8 reference genes had an acceptable stability value (M < 1) (Vandesompele *et al*., 2002; Hellemans *et al*., 2007), while during lactation only six reference genes had acceptable stability values (Figure 8.3D). The higher M values of the reference genes in all individuals (8.3B), compared with the pooled samples (8.2B), indicated greater variation in gene expression between the individual animals. Analysis of the V values indicated that, for both late pregnancy and lactation, the top five most stably expressed reference genes (*Atp1a1, Cul1*, *Eif6*, *Mrpl39* and *Senp2*) would need to be used to normalise expression data for all the individual animals (Figure 8.3B-E).

To examine if the additional variation in the individual animal samples arose from the incorporation of the remaining treatments (i.e., SmAd, MAd and AdAd) not included in the pools, an additional analysis was carried out using just the individual animals that comprised the pools. For this analysis, across both late pregnancy and lactation, none of the genes had an M value of less than 0.5 (Figure 8.4A), which is considered to represent stable expression in a homogenous sample (Vandesompele *et al*., 2002; Hellemans *et al*., 2007). The mammary biopsies were standardised as much as possible for this study but are still likely to comprise of different proportions of cell types. In a heterogeneous sample, such as this, M-values of less than 1 can be considered stable (Vandesompele *et al*., 2002; Hellemans *et al*., 2007) and four of the genes sampled (*Cul1, Atp1a1, Ipo9* and *Senp2*) met these criteria (Figure 8.4A). The V value analysis, however, indicated that in the individuals used to comprise the pools, no combination of the reference genes could normalise expression data across both late pregnancy and lactation. If a slightly higher cut-off of V < 0.2 is used then five reference genes may be used for normalisation (Figure 8.4B) of RT-qPCR

data generated from individuals (*Cul1, Atp1a1, Ipo9, Senp2* and *Mrpl39*, Figure 8.4A). However, because our aim was to identify reliable and robust reference genes within each physiological state (rather than reference genes that were stable over time), the two physiological states were also analysed separately (Figure 8.4C-F).

In addition to having more variation, the individual samples also had differences in the ranking of reference genes, compared with the pools. For example, the least stable reference gene in the pools (*Atp1a1*, Figure 8.2B) was ranked as a stable gene amongst all the individual animal samples (Figure 8.3B and D), and ranked as one of the most stable genes in the individuals that comprised the pools (Figure 8.4A, C and E). Additionally, unlike the pooled samples (Figure 8.2 B), reference genes chosen from RNA-seq data (*Cul1*, *Prpf3* and *Sf1*) were less stably expressed in both analyses of individual animals (all groups, Figure 8.3B and D, and just the pooled samples, Figure 8.4A, C and E) than those chosen from literature (*Eif6* and *Mrpl39*) and *Atp1a1*, which was a previously used reference gene. The exceptions to this are *Senp2*, selected from RNA-seq data, which ranked as the most stably expressed gene for all individual animal samples for late pregnancy (Figure 8.3B), and *Cul1*, also selected from RNA-seq, which ranked as most stable across late pregnancy and lactation for individuals that comprised the pools (Figure 8.4A).

Using Ingenuity pathway analysis software possible co-regulation was identified between *Cul1* and *Atp1a1*, and *Cul1* and *Eif6*. This has the potential to bias calculations of gene expression stability (Vandesompele *et al*., 2002). The correlation coefficients for expression of these genes are relatively low (r = 0.32 – 0.55), with the exception of *Cul1* and *Atp1a1* for the individual animals (r = 0.89, Figure 8.3). This indicates, at least for the pooled RNA samples, that there is no evidence for co-regulation amongst these genes. However, this, together with the fact that five reference genes are required for the normalisation of RT-qPCR data from individual animals, may justify selection and testing of additional reference genes in individual animals.

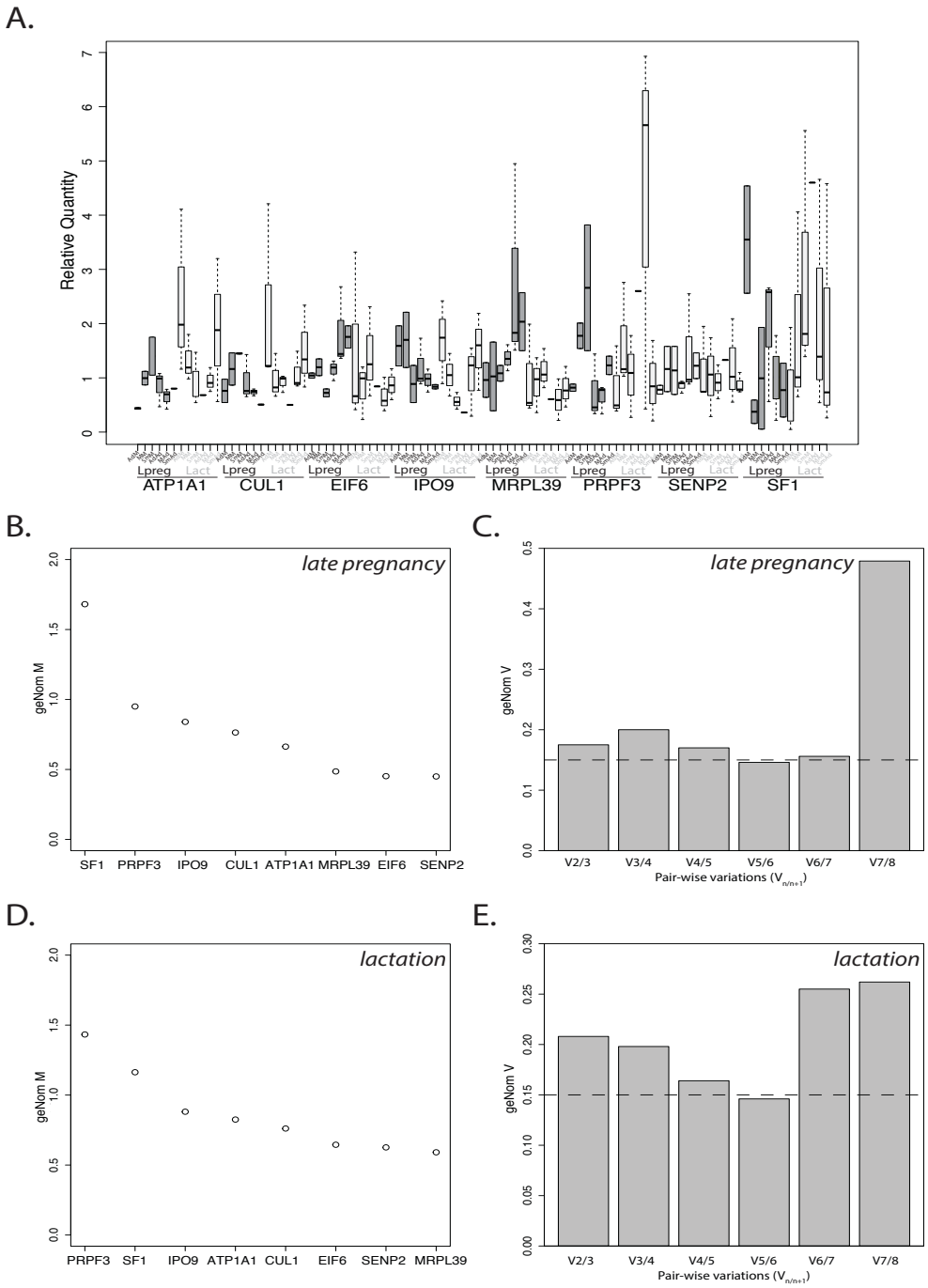

Figure 8.3 Expression and stability analysis of the eight candidate genes in individual RNA samples. *A*: Relative quantity of the eight candidate reference genes in individual RNA samples across the two physiological states (Lpreg and Lact) and in the six maternal programming treatment groups: *ad libitum*/maintenance (AdM), maintenance/maintenance (MM), sub- maintenance/maintenance (SmM), *ad libitum*/ *ad libitum* (AdAd), maintenance / *ad libitum* (MAd), sub-maintenance/*ad libitum* (SmAd). *B*: geNorm stability analysis (M value) of the candidate reference genes in late pregnancy. Low M values indicate more stable expression. All M values, with the exception of *Sf1*, are < 1 which is considered moderately stable. *C*: geNorm pairwise variation analysis (V value) of the candidate reference genes in late pregnancy. V < 0.15 (marked by dashed line) is considered as the upper limit for selecting an adequate combination of reference genes and only the inclusion of five reference genes meets this criteria. *D:* geNorm stability analysis (M value) of the candidate reference genes in lactation. Low M values indicate more stable expression. All M values, with the exception of *Prpf3* and *Sf1*, are < 1 which is considered moderately stable. *E*: geNorm pairwise variation analysis (V value) of the candidate reference genes in lactation. V < 0.15 (marked by dashed line) is considered as the upper limit for selecting an adequate combination of reference genes and only the inclusion of five reference genes meets this criteria.

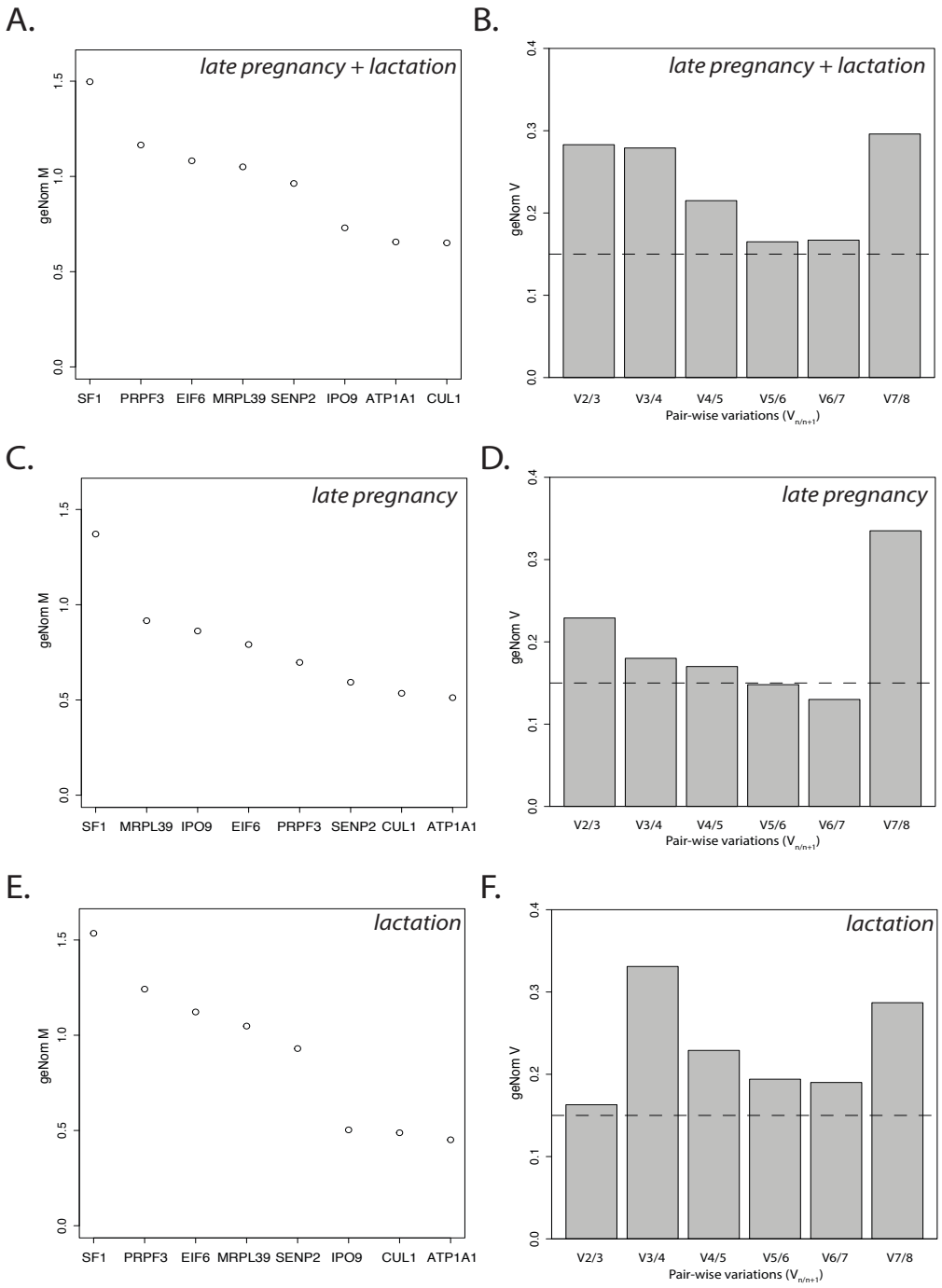

Figure 8.4 Expression and stability analysis of the eight candidate genes in the individual RNA samples that were used to constitute the RNA pools. *A*: geNorm stability analysis (M value) of the candidate reference genes in both physiological states. Low M values indicate more stable expression. All M values, with the exception of *Sf1* and *Prpf3*, are < 1 which is considered moderately stable. *B*: geNorm pairwise variation analysis (V value) of the candidate reference genes in late pregnancy. V < 0.15 (marked by dashed line) is considered as the upper limit for selecting an adequate combination of reference genes and no combination of reference genes satisfied this criteria. *C*: geNorm stability analysis (M value) of the candidate reference genes in late pregnancy. Low M values indicate more stable expression. All M values, with the exception of *Sf1*, are < 1 which is considered moderately stable. *D*: geNorm pairwise variation analysis (V value) indicates that the most stable five or six genes would be appropriate for normalizing RT-qPCR data. *E*: geNorm stability analysis (M value) of the candidate reference genes in lactation. Low M values indicate more stable expression. Only four of the tested genes have moderately stable expression (M < 1). *F:* geNorm pairwise variation analysis (V value) indicates that no combination of reference genes can be used for normalizing RT-qPCR data.

8.6.3 Sensitivity analysis of selected reference genes in RT-qPCR analysis

As there is substantial individual variation in expression of our candidate reference genes (Figure 8.3A) we wanted to determine if the candidate genes we determined to be the most stable (*Senp2*, *Eif6*, *Mrpl39*, *Atp1a1* and *Cul1*) provided more sensitivity to detect differences in transcript abundance of a gene of interest, compared with two of the less stable reference genes (*Sf1* and *Prpf3*). For this analysis we examined the expression of *Tet1* (Tet methylcytosine dioxygenase 1).

DNA methylation, the addition of a methyl group to cytosine residues, is a well-studied epigenetic mechanism. DNA methylation has been associated with imprinting (reviewed in Abramowitz & Bartolomei, 2012), X-inactivation (Wutz & Gribnau, 2007), repression of gene expression (Jones, 2012) and, more recently, repressing intragenic promoter activity (Maunakea *et al*., 2010), alternative splicing (Lyko *et al*., 2010; Shukla *et al*., 2011; Sati *et al*., 2012; Foret *et al*., 2012) and controlling transcriptional elongation (Lorincz *et al*., 2004; Sati *et al*., 2012). The TET enzymes convert 5-methylcytosine to 5-hydroxymethyl cytosine (Tahiliani *et al*., 2009), which is then further processed to result in the regeneration of a non-methylated cytosine (He *et al*., 2011; Maiti & Drohat, 2011). The biological functions of the derivatives of 5-methylcytosine are unknown, but they may also act as epigenetic marks that recruit transcriptional regulators (Spruijt *et al*., 2013). Loss of 5-hydroxymethyl cytosine has been observed in different cancers, including breast cancer, and is associated with decreased expression of *Tet1* (Wielscher *et al*., 2013).

Using stable reference genes (*Senp2*, *Eif6*, *Mrpl39*, *Atp1a1* and *Cul1)* decreases expression of *Tet1* from late pregnancy to lactation (63% reduction), and using the sub-optimal reference genes (*Sf1* and *Prpf3*) yields a similar result (60% reduction) (Figure 8.5A). Using the sub-optimal reference genes does increase variation in gene expression (range = 0.19-3.6 with appropriate reference genes and 0.03-7.28 with sub-optimal reference genes). If the difference in *Tet1* expression was less marked it would be unlikely to be detected using sub-optimal reference genes.

This is indeed what we see when we compare the effect of late pregnancy maternal nutrition on the expression of *Tet1* in the mammary gland of offspring (Figure 8.5B). Irrespective of physiological state, *ad libitum* maternal nutrition in late pregnancy results in a decrease of 35% in *Tet1* expression in offspring (maintenance = 1.48, *ad libitum* = 0.95) when using appropriate reference genes. If the same data is analysed with sub-optimal reference genes, no significant difference in gene expression is reported and the mean

expression value is higher in offspring from dams fed an *ad libitum* diet during late pregnancy (maintenance = 1.44, *ad libitum* = 2.01).

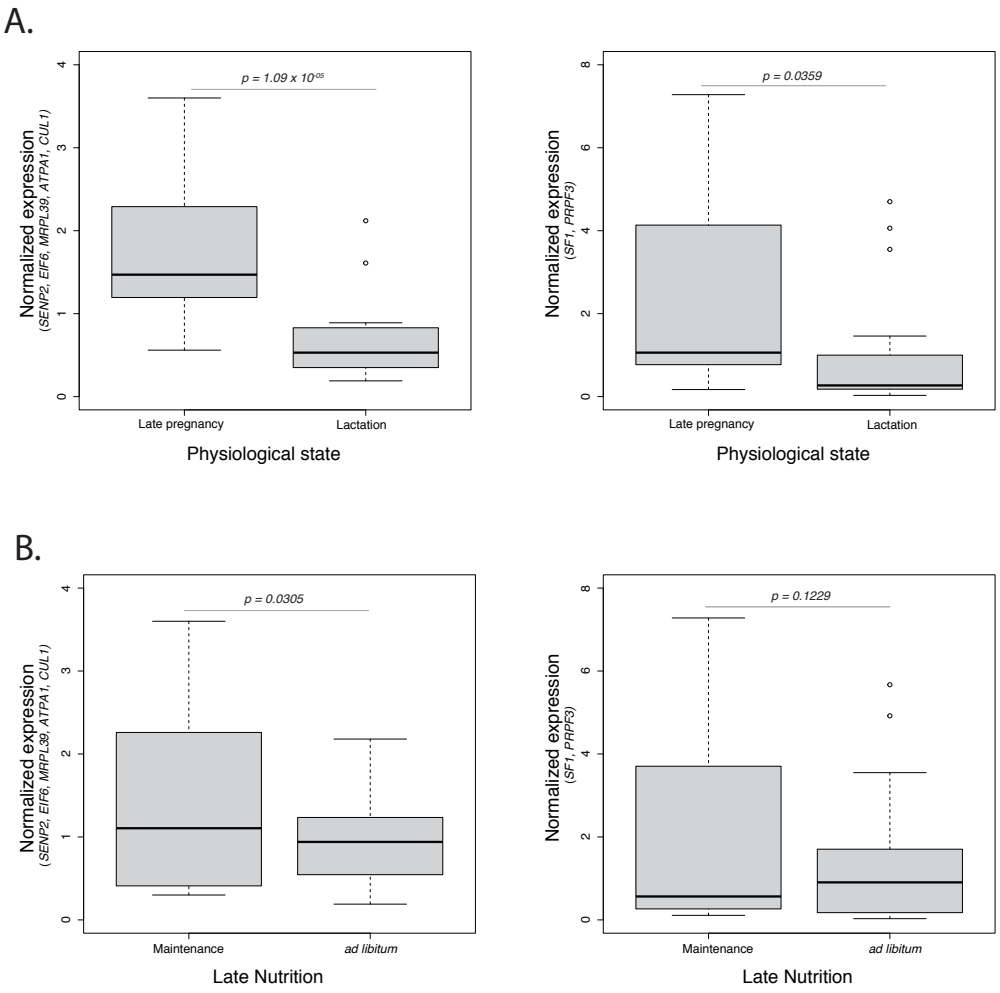

Figure 8.5 Normalization of *Tet1* expression with stable reference gens and sub-optimal reference genes. *A: Tet1* expression differs significantly between late pregnancy and lactation when using stable reference genes (*Senp2*, *Eif6*, *Mrpl39*, *Atp1a1* and *Cul1*). When *Tet1* expression is normalised to sub-optimal reference genes (right), a significant difference in gene expression is observed, but there is more variation in the normalised expression values. *B*: *Tet1* expression is responsive to maternal nutritional programming. *Ad libitum* feeding in late pregnancy results in lower levels of *Tet1* expression in the mammary glands of the adult offspring when data is normalised to the expression of stable reference genes (left). When the same data is normalised to sub-optimal reference genes (right), no difference in *Tet1* expression is observed.

8.7 Discussion

Transition from late pregnancy to lactation requires extensive physiological and metabolic adaptation in the mammary gland. These adaptations are regulated by endocrine hormones and local factors and may be altered by external environmental events such as maternal nutritional programming. To understand the molecular basis of these processes and adaptations we need to accurately and sensitively monitor differences in gene expression. The ability of RT-qPCR to accurately detect changes in gene expression relies upon the selection of stably expressed reference genes. Studies in other species have shown that the expression of commonly used reference genes may vary between physiological and nutritional states and experimental treatments (Bionaz & Loor, 2007; Tramontana *et al*., 2008; Kadegowda *et al*., 2009; Aggarwal *et al*., 2013). Variation in expression of reference genes may limit the ability to detect and verify changes in expression of target genes, thus reducing the percentage of genes that validate. In a recent study RT-qPCR validation of microarray data was improved by 13% (from 33% to 46%) when less stable reference genes were changed to more stable ones (Cameron *et al*., 2013). In the present study we also observed a marked difference in the detection of a differentially expressed gene, *Tet1*, when analysed with poor and high quality reference genes (Figure 8.5). The use of poor reference genes introduced significant variation in the analysis, which masked detection of more subtle gene expression differences. These findings highlight the importance of choosing appropriate internal controls for RT-qPCR studies.

To date there are no studies which compare expression stability of reference genes in the ovine mammary gland. Therefore, in the present study candidate reference genes were selected from RNA-seq expression data (*Prpf3*, *Cul1*, *Sf1*, *Senp2* and *IPO9*) and from studies conducted in other species [*Mrpl39*: bovine (Bionaz & Loor, 2007; Kadegowda *et al*., 2009), porcine (Tramontana *et al*., 2008); *Eif6* (Bionaz & Loor, 2007) and *Atp1a1* (Calcagno, 2006; Leth-Larsen *et al*., 2009)]. These genes were evaluated across pooled and individual RNA samples.

RNA samples may be pooled for gene expression analysis when samples are limited, to reduce costs, or in an attempt to reduce the effects of biological variation between individuals, particularly when the focus is on identifying expression patterns across the population (Kendziorski *et al*., 2003; Kendziorski *et al*., 2005; Konczal *et al*., 2014). Consistent with this, there was considerably less variation in expression of candidate reference genes in the pooled samples (Figure 8.2) compared with the individual animal samples (Figure 8.3).

geNorm analysis indicated that all of the genes tested had high stability in the pooled samples and that the geometric mean of the two most stable genes (*Prpf3* and *Cul1*) could be used to normalise expression data in mammary gland tissue samples, across late pregnancy and lactation, of ewes subjected to maternal nutritional programming.

In contrast to the pooled RNA samples, gene expression was less stable when tested across all the individual animal samples (of all six treatment groups), implying that the pooling strategy we have employed is effectively reducing the individual variation in gene expression. When both physiological states (late pregnancy and lactation) were analysed together, no combination of the candidate genes could be used to normalise the RT-qPCR data. Analysed separately, the same five reference genes were recommended for normalisation of RT-qPCR data (*Senp2*, *Eif6*, *Mrpl39*, *Atp1a1* and *Cul1*), but the order in which these genes were ranked differs between the physiological states.

The high levels of variation in gene expression observed between individuals (Figure 8.3A) may be, at least partially, attributed to limitations in the sampling method used in this study. Biopsy sites were standardised as much as practical, but the mammary gland is a mixed tissue type (containing mammary epithelial cells, fibroblasts, blood vessels, connective and adipose tissue) and it is likely that individual biopsy samples contained different proportions of these cell types. In addition, gene expression in the mammary gland is known to be patchy, with not all epithelial cells actively expressing genes for milk synthesis and secretion (Molenaar *et al*., 1992). It may be possible to use cell sorting and labelling to obtain more homogenous samples. Increasing sample sizes would also reduce the effect of individual variation, and it is likely that the relatively small sample sizes in this study were insufficient to account for biological variation arising from the heterogeneous nature of the mammary tissue (Molenaar *et al.*, 1992).

Analysis of pooled RNA samples revealed *Prpf3* and *Cul1* as the most stable reference genes, but *Prpf3* was ranked least stable in the analysis of all the individual animals (of all six treatments) and *Cul1* was ranked as moderately stable. It is unknown why genes that ranked highly for stability among the pooled samples ranked so poorly when analysed in individual animals and *vice versa*. When we compare analysis of pooled samples (Figure 8.2; AdM, MM, SmM) with just the individual animals that comprised those pools (Figure 8.4A), over late pregnancy and lactation *Cul1* is the most stable gene but *Prpf3* continues to rank poorly. This indicates that *Cul1* (and to a lesser degree *Prpf3)* may be more variable amongst the treatments that were not included in the pooled experiment (i.e.,

AdAd, MAd, SmAd). This reinforces the importance of determining appropriate references genes for each tissue and experimental paradigm.

We used *Tet1*, a key gene involved in epigenetic remodelling, to validate the quality of the reference genes identified in this study (Figure 8.5). Here we show that when using high-quality reference genes we are able to detect accurately the decrease in *Tet1* between late pregnancy and lactation. When using low-quality reference genes we were still able to detect a difference in *Tet1* expression; however a greater level of variation was introduced into the analysis. *Tet1* expression has been shown to correlate with lower levels of 5-hydroxymethylcytosine (Wielscher *et al*., 2013) and raises the possibility that epigenetic remodelling is required for maturation of the mammary gland prior to lactation. Unexpectedly, when using high-quality reference genes, we were also able to detect that the expression of *Tet1* is responsive to maternal nutritional programming, as *ad libitum* feeding of dams late in pregnancy results in offspring with significantly lower levels of *Tet1* expression in the mammary gland. When low-quality reference genes were used this difference could not be detected, highlighting the importance of using high-quality, stably expressed reference genes for data normalisation, particularly for detection of more subtle differences in expression of genes. The physiological significance of *Tet1* expression in the ovine mammary gland and the role of 5-hydroxymethylcytosine in maternal programming are yet to be determined.

8.8 Conclusions

This study demonstrates that reference gene expression can vary between physiological states, treatments (such as maternal gestational nutrition), and even between individual samples within the same treatment group and physiological state. We have identified novel reference genes for the mammary gland (i.e., *Prpf3* and *Cul1*), and we show that using stable reference genes (*Senp2*, *Eif6*, *Mrpl39*, *Atp1a1* and *Cul1*) increases the sensitivity of RT-qPCR analyses using *Tet1* as an example. These findings highlight the importance of confirming stability of expression of reference genes, under specific experimental conditions, for RT-qPCR.

9 General discussion

9.1 Overview and discussion of key findings

Previous research has demonstrated that the level of dam nutrition during pregnancy can affect development and function of the mammary gland in ewe offspring in their first parity lactation (van der Linden *et al*., 2009). Specifically, ewes born to dams exposed to *ad libitum* compared to maintenance nutrition during pregnancy had reduced lactation performance and weaned lighter lambs. This interesting developmental-programming effect could have implications for animal production enterprises, as *ad libitum* feeding of the pregnant dam appears to disadvantage the offspring. The aim of this thesis was to build on these previous results by identifying the critical period of pregnancy in which this programming effect could be influenced by dam nutrition, the underlying molecular mechanisms affecting mammary tissue of ewe offspring and the longer-term (lifetime) effects on their lactation performance.

Ewe offspring from the study of van der Linden *et al. (*2009) were retained by our research group, and additional milk production and lambing data were collected from the second through to the fifth parities to investigate lifetime productivity (Chapter 2). The results demonstrated that while first-lactation performance of ewe offspring could be programmed by dam nutrition during pregnancy, differences did not persist in subsequent lactations. Understanding the mechanisms that underpin this transient difference in lactation performance, and identification of the critical programming period, may provide an opportunity for intervention. Therefore, a new study was designed (Chapter 3), which separated the period of dam nutritional treatment into early (day 21 to 50 of gestation) and mid-to-late pregnancy (day 50 to 140 of gestation, Kenyon *et al*., 2011a).

In Chapter 4, the effects of dam nutrition during either early or mid-to-late pregnancy on production traits in ewe offspring up to 18 months of age were reported. Production traits measured included growth, live weight, body condition and composition, and attainment of puberty. There were some differences caused by dam nutrition during pregnancy, for example, ewes born to dams which were fed *ad libitum* during mid-to-late pregnancy were lighter at birth and tended to be less likely to attain puberty by nine months of age in comparison to ewes born to dams which were offered maintenance nutrition. Further investigation into reproductive performance is underway but is not part of this thesis. There was also a small, transient, difference in faecal egg counts (FECs) of ewes as a result of dam nutrition during early pregnancy. Maintenance, compared with *ad libitum*, nutrition of the dam during early pregnancy resulted in ewe offspring with higher FECs at

eight months of age, but not at nine months. This difference, however, is unlikely to be detrimental to ewes. The finding that different levels of dam nutrition, during both early and mid-to-late pregnancy, resulted in only minor productive differences between ewe offspring, suggests that farmers can manipulate ewe feeding during pregnancy at levels utilised in the present study, while incurring only minimal economic consequences in the ewe offspring to two-years of age.

At 24 months of age, first-parity lactation performance was investigated in a cohort of twin-bearing ewe offspring (Chapter 5). Ewes born to dams fed either sub-maintenance or *ad libitum* during early gestation tended to produce lower milk, milk-fat and milk net-energy yields, compared with ewes born to dams fed maintenance. This finding indicates that lactational performance of offspring may be impaired by both under- and over-feeding of dams during the critical window of early gestation. Production of milk is influenced by the number and metabolic activity of mammary epithelial cells (Capuco *et al*., 2001, Boutinaud *et al*., 2004, Wall & McFadden, 2012). Therefore, the programming-mechanism may be related to factors which affect cell proliferation and metabolic processes in the mammary glands of ewe offspring.

Twin-bearing (and rearing) ewes born to dams fed *ad libitum* during early gestation also tended to wean lighter lambs than ewes born to maintenance-fed dams, which could be of economic importance (Chapter 5). An interesting and unexpected finding was that, despite no differences in their milk yields, twin-bearing ewes born to sub-maintenance-fed dams also weaned heavier lambs than ewes born to *ad libitum*-fed dams. This indicates the possibility of a multigenerational programming effect on lamb growth. Lambing performance was also examined in the whole population of ewe offspring, including single- and twin-bearing (and rearing) ewes (Chapter 6). Despite the differences in milk production observed in the above-mentioned subset of twin-rearing ewes (Chapter 5), dam nutrition in both early and mid-to-late pregnancy did not affect the weaning weights of lambs (grand-offspring) in the whole population. This finding does not exclude differences in milk production, but rather highlights the influence of other factors additional to milk availability, including rearing rank of lambs, stress (i.e., from human interference), and lamb consumption of pasture, on the growth and weaning weights of lambs. Additionally, these conflicting findings may indicate that the technique used to measure milk production may not be a good indicator of milk intake by lambs.

These findings may suggest that management of dam nutrition during pregnancy to alter lactational performance of the next generation may not be a robust option to improve lamb production systems. However, the results of altered lactational performance of ewe offspring (reported in Chapter 5) may be of more relevance to farming systems where milk is harvested (e.g., dairy sheep, goat and cattle enterprises). Increased understanding of the mechanisms that alter milk yield and/or composition may contribute to the development of specific production manipulations, for example, to change the milk composition for improved efficiency of some food manufacturing systems.

To investigate molecular mechanisms potentially involved in nutritional programming of the mammary gland, transcriptomics analysis was carried out on biopsied mammary tissue, collected from primiparous ewe offspring during late pregnancy and lactation (Chapter 7). Whole-scale gene expression analysis of RNA-seq data revealed that 447 genes were differentially expressed during late pregnancy, while only seven genes were differentially expressed during lactation. This result suggests that impaired mammary development (mammogenesis), rather than secretory activity of mammary cells during lactation, may be underlying reduced lactational performance in ewes born to dams which were fed *ad libitum* and sub-maintenance during early pregnancy. In particular, molecular pathways involved in proliferation of mammary secretory cells may be impaired in ewes, as a result of exposure to *ad libitum* or sub-maintenance nutrition of their dam during early pregnancy. This hypothesis is supported by evidence of lower expression of genes associated with regulation of cell cycle in ewes born to *ad libitum*-fed, compared with maintenance-fed dams, including genes involved in the G1/S and G2/M cell cycle checkpoints, which regulate progression of DNA replication and mitosis. Proliferation of mammary epithelial cells may also be impaired in ewes born to sub-maintenance-fed, compared with maintenance-fed dams, contributing to poorer lactation performance. However, the mechanism may be related to altered extra-cellular matrix (ECM) remodelling, as the expression of genes encoding for ECM components, with roles in cell-cell signalling and mediation of epithelial outgrowth, was lower in ewes born to dams fed sub-maintenance, compared with ewes born to dams fed maintenance during early pregnancy. These results are important, as impaired regulation of cellular proliferation in the mammary gland may result in lower numbers of secretory epithelial cells and, therefore, lower milk yield (Boutinaud *et al*., 2004). These findings may also be of relevance for studies of foetal programming of breast cancer in humans. Maternal diet has been linked with breast cancer risk in daughters

(Hilakivi-clarke and de Assis, 2006), and dysregulation of cell cycle in breast epithelial cells is implicated as a potential mechanism.

In contrast to late pregnancy, very few genes were differentially expressed in mammary tissue during lactation among the nutritional programming treatments. Genes which were differentially expressed during lactation, were very highly expressed and diverse in function (e.g., genes encoding milk proteins and genes associated with metabolic pathways, including oxidative phosphorylation (energy metabolism), fat metabolism and lactose synthesis). With such high expression, translation to protein may be limited (i.e., by saturation of ribosomes, Reuveni *et al*., 2011), thus differences in transcript abundance may not necessarily reflect differences in abundance of proteins, or biological effects. However, the possibility that over- and under-, compared to maintenance, nutrition of the dam during early pregnancy may reduce metabolic capacity in the mammary glands of ewe offspring cannot be ruled out. Studies undertaken at the protein level (e.g., proteomics) may be useful to investigate this further (Chevalier, 2011).

9.2 Future research

The design of the present study, through allocation of nutrition treatments to dams during early and mid-to-late pregnancy separately, allowed identification of early pregnancy as the critical period for maternal nutritional programming of lactation performance in ewe offspring. A limitation of the study design is that it does not rule out the possibility that differences in neonatal nutrition may have arisen from altered milk production of G0 dams, as a consequence of the nutritional treatments (i.e., this was not a programmed effect). The present study, however, was designed to be representative of what would happen on farm and therefore the chosen experimental design was deemed appropriate. Mechanistic studies, in future, could utilise either a cross-fostering or hand-rearing approach to ensure neonatal nutrition was the same for all treatments.

This study was the first to utilise RNA-seq to examine the ovine mammary transcriptome, and to begin unravelling the mechanisms behind maternal nutritional programming of lactational performance. The use of needle biopsy allowed collection of mammary tissue, from ewe offspring during late pregnancy and lactation, for RNA-seq experiments, with little damage or disruption to the mammary gland and therefore milk production. However, the mammary gland is a heterogeneous tissue, comprised of mixed cell types including mammary epithelial cells, myoepithelial cells, fibroblasts, adipose and endothelial cells, which are known to have differences in their gene expression profiles

(Casey *et al*., 2011). The small physical size of samples increased the chances of variation in tissue composition sampled between animals. Thus variation in gene expression between animals within treatments was also increased. Furthermore, the expression of genes is known to be patchy in the mammary gland with not all mammary epithelial cells simultaneously actively expressing genes for milk synthesis (Molenaar, 1992). This may explain why in the present study high variation in expression of reference genes between individuals was observed (Chapter 8) and why RNA-seq data during lactation, which had the lowest number of replicates, was less reliable (e.g., lower correlation between RT-qPCR and RNA-seq data) than other comparisons. The use of more animals, or more samples per animal, may help to alleviate this problem. Future studies may, therefore, benefit from a more focused approach, targeting fewer comparisons (less treatments and/or time periods included); this may allow for increased sampling of replicates to improve representation of gene expression in the mammary gland. In the present study, pooling was used to overcome the effects of variation in samples, which enabled generation of high-quality gene-expression data which provided useful insights into maternal nutritional programming mechanisms.

RNA-seq analysis in this study revealed that, through differential expression of genes associated with cell cycle (in ewes born to dams fed *ad libitum* vs. maintenance during early pregnancy) and the extracellular matrix (in ewes born to dams fed sub-maintenance vs. maintenance during early pregnancy), cellular proliferation may be compromised in the mammary gland of ewes subjected to nutritional programming during foetal life. Although, as with all transcriptomics studies, there is a limitation in that there is not a 1:1 ratio of mRNA transcript to protein abundance, and the function of proteins may also be affected by post-translational modifications. Thus, differences at the transcriptional level may not necessarily be biologically significant. Therefore further experiments could include histological counts of cells, and immunohistochemical analysis of cell proliferation (Ki67 or PCNA) and apoptosis (TUNEL) markers, to validate these results. The transcriptomics analysis could also be paired with a proteomics analysis to examine differences at the protein level; although, the high proportion of milk proteins (which accounted for ~60% of RNA-seq reads in the present study) may limit the ability to detect differences in abundance of other proteins.

It must also be acknowledged that regulation of lactation is complex, requiring regulation of the endocrine system, in addition to autocrine and paracrine mechanisms, to

coordinate activities in a variety of tissues, including the mammary gland and the liver, to support milk production (Wall & McFadden, 2012). Differential expression of hormone receptors (e.g., leptin and progesterone receptor) in the mammary gland, as well as associated down-stream genes (e.g., *Jak2* and *Eif5a*), raises the possibility that hormone signalling pathways may be involved. Endocrine effects may be mediated at a higher level, e.g., through altered activity of the HPA-axis (Fowden *et al*., 2009), with consequences for mammary development and function. This could be investigated through analysis of hormone levels in blood collected during foetal life, and at the time of biopsy.

The present study provided novel insights into the molecular mechanisms underlying maternal nutritional programming of lactation performance of ewe offspring. Interestingly, both over- and under-nutrition of the dam, compared with maintenance nutrition, during early pregnancy resulted in reduced lactation performance of ewe offspring. The mechanisms, however, mediating the effects of dam over-nutrition and under-nutrition (compared with maintenance nutrition) appear to be different. In particular, it appears that regulation of the cell cycle in mammary cells is a developmental programming target, susceptible to over-nutrition of the dam during early pregnancy. In addition, in the case of maternal under-nutrition during early pregnancy, the extracellular matrix appears to be a target of developmental programming. In both cases, nutritional programming during foetal life appears to influence the expression of genes in ewes during late pregnancy, potentially affecting tissue development, and resulting in lactational differences. Exactly how expression of genes associated with the cell cycle and the extracellular matrix is controlled by nutritional programming is, however, still unknown. A possible hypothesis is that epigenetic mechanisms, such as histone modification or DNA methylation, may be involved (Singh *et al*., 2010a). The use of next-generation sequencing technologies could be utilised to identify epigenetic marks in foetal mammary tissue, to establish a link between the early-life cause, and effect later in life (e.g., whole-genome or reduced-representation bisulphite sequencing to investigate differentially methylated DNA, and chromatin immuno-precipitation sequencing (ChIP-seq), to investigate chromatin modifications). Gene knock-out and/or knock-down (RNAi) models could also be used to test functions of specific candidate genes identified from RNA-seq.

Another question deserving further study is why programming effects are not observed beyond the first lactation. It could be that differences are only present in cells that undergo apoptosis during involution, and thus programming is not retained. A parity

hypothesis is proposed in which initial mammary development is not the same as mammary re-development (Miller *et al*., 2006), and that only molecular processes involved in initial development are affected by maternal nutritional programming. The lack of differences in second parity, and lifetime, lactation performances may be due to the ewe offspring being on the same level (*ad libitum*) of post-natal nutrition when they undergo mammary re-development in subsequent pregnancies. If the mismatch hypothesis (Gluckman and Hanson, 2004) is correct, differences might be induced in subsequent lactations by applying different environmental conditions, i.e., restricted nutrition or stress. Future research could focus on identifying the specific cell types programmed in ewe offspring by dam nutrition during pregnancy, e.g., mammary stem cells or stromal cells, identifying the mechanism acting during foetal life, e.g., epigenetics or hormones, and testing responses of 'programmed' ewe offspring in different post-natal environments, e.g., through altering nutrition of adult ewe offspring during subsequent pregnancies and lactations.

Finally, the question about the potential of multigenerational effects arises. The findings from the milking study reported in Chapter 5, that twin-bearing ewes born to sub-maintenance-fed dams weaned the heaviest lambs, despite having reduced milk yields, raises the possibility that grand-dam nutrition may programme growth and metabolism in grand-offspring. It is possible that germ-line cells may be programmed by grand-dam nutrition, a phenomenon that has been suggested by epidemiological studies, such as the Dutch famine (Painter *et al*, 2008). Further investigation into growth, metabolism and lactation performance of grand-offspring and great-grand-offspring generations is warranted, as there may be relevant long-term implications for productivity of ewes in New Zealand.

9.3 Summary

This is the first study to examine the long-term effects of nutritional programming during foetal life on lactation performance of ewe offspring. Although differences did not persist beyond the first lactation, increased understanding of the mechanisms underlying programming of first-lactation performance will be important for successful management of milk production. The discovery that dam under- and over nutrition in early gestation can be detrimental for lactation performance in ewe offspring may suggest that farmers should consider management of ewe nutrition early in gestation, even before pregnancy scanning. However, this may be difficult for farmers, as the stage of pregnancy may vary greatly within a sheep flock in which mating is not synchronised. The present examination of the molecular

mechanisms involved in programming lactational performance of offspring, identified that cell cycle and extra-cellular matrix remodelling genes are targets of nutritional programming. These affected genes may improve milk production in offspring through promoting alveolar proliferation during late pregnancy, and may be suitable genes to target for development of interventions. This study has contributed to knowledge about the gene networks and pathways involved in normal mammary development, and which respond to developmental programming. These are important findings which provide a useful platform for future studies aiming to manipulate mammary development and milk production.

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Appendices

Appendix A: Differentially expressed genes from RNA-seq analysis

Table A. 1 Genes identified by RNA-seq as differentially expressed in the ovine mammary gland between late pregnancy and lactation. Genes shown were more highly expressed during late pregnancy. Samples were pooled for RNA-seq analysis: *n* = 9 pools for late pregnancy, with 3 samples per pool, *n* = 27 samples.

Genes more highly expressed during late pregnancy								
A2ml1	Ccng2	Erh	Katna1	Loc101114909	Nab1	Qdpr	Sltm	Vgll1
Aamdc	Ccni	Esm1	Kdm5b	Loc101115136	Naca	R3hdm2	Smarcd2	Vim
Abca3	Cct2	Etaa1	Khdrbs1	Loc101115302	Nans	Rab12	Smarce1	Vta1
Abcd3	Cct3	Etfa	Khnyn	Loc101115388	Nap1l1	Rab14	Smc2	Vti1b
Abcd4	Cct4	Etfb	Kiaa0101	Loc101115438	Nasp	Rab19	Smc3	Wbp1
Abhd12	Cct8	Ewsr1	Kiaa1033	Loc101115527	Nat9	Rab24	Smc4	Wdr13
Abhd14b	Cd2	Exosc5	Kif11	Loc101115685	Nbeal2	Rabep1	Smim7	Wdr17
Abi3bp	Cd244	F11r	Kif1c	Loc101115694	Ncapg	Rac1	Smox	Wdr38
Acaa2	Cd247	Fam100b	Kif20a	Loc101115926	Ncapg2	Rac2	Snrnp70	Wdr6
Acadvl	Cd2bp2	Fam162a	Kif22	Loc101116099	Ncf4	Racgap1	Snrpa	Wdr90
Acat1	Cd34	Fam165b	Kif23	Loc101116121	Nck2	Rad51	Snrpb	Wrb
Acer3	Cd3e	Fam180a	Kif2c	Loc101116132	Ncl	Rad54l	Snrpb2	Wwc1
Acot8	Cd3g	Fam213a	Kif4a	Loc101116450	Ndc80	Raly	Snrpd2	Wwc2
Acsl5	Cd44	Fam3b	Kifc1	Loc101116631	Ndrg1	Ran	Snrpe	Wwtr1
Acsm5	Cd47	Fam82b	Kifc3	Loc101116687	Ndst1	Ranbp1	Snw1	Xcl1
Actl6a	Cd52	Fam84a	Kl	Loc101117013	Ndufb10	Rangap1	Snx4	Xrn2
Actr2	Cd53	Fam96a	Klf10	Loc101117107	Ndufb9	Rapgef3	Snx6	Ybx1
Actr3b	Cd7	Fancd2	Klf11	Loc101117272	Ndufs2	Rarres2	Sostdc1	Ypel3
Ada	Cd74	Farp1	Klhdc2	Loc101117285	Ndufs5	Rasgef1a	Sox4	Ypel5
Adam10	Cd96	Farsb	Klrg2	Loc101117300	Ndufv2	Rbbp4	Sox9	Zadh2
Adam15	Cdc20	Fau	Krt5	Loc101117395	Neil2	Rbbp7	Sp17	Zbed5
Adck3	Cdca3	Fbl	Krt7	Loc101117414	Nek2	Rbbp9	Spag5	Zbp1

Genes more highly expressed during late pregnancy								
Adi1	Cdca7l	Fbxo32	Lamp1	Loc101117481	Nek6	Rbm3	Spag7	Zc3h14
Adtrp	Cdca8	Fbxo7	Lamp2	Loc101117590	Nenf	Rbm39	Sparc	Zcrb1
Aebp1	Cdh3	Fcer1g	Laptm4a	Loc101117657	Net1	Rbm5	Sparcl1	Zdhhc1
Ahr	Cdipt	Fcf1	Laptm5	Loc101117682	Nfe2l3	Rbmx	Spats2l	Zfand5
Aif1	Cdk1	Fcgr2b	Las1l	Loc101117765	Ngrn	Rce1	Spc24	Zmiz2
Aimp1	Cdk4	Fcgr3a	Lasp1	Loc101117885	Nhp2l1	Rcn1	Spg21	Znf185
Ak2	Cdk5rap2	Fcgrt	Lbh	Loc101118057	Nit2	Reep6	Sphk1	Znf22
Akip1	Cds1	Fcho2	Ldha	Loc101118100	Nkain2	Rfc3	Sphk2	Znf226
Akr1a1	Cebpa	Fdx1	Ldhb	Loc101118130	Nkd1	Rfc4	Spink2	Znf277
Aktip	Cebpz	Fermt2	Lect1	Loc101118138	Nkg7	Rfng	Spint1	Znf3
Alad	Cecr5	Fes	Leprot	Loc101118150	Nme3	Rgl2	Spint2	Znf32
Alas1	Cenpa	Fgl1	Lgals9	Loc101118190	Nob1	Rgs2	Sptlc1	Znf34
Alcam	Cenpf	Fh	Lig1	Loc101118252	Nono	Rhbdl1	Sptssb	Znf503
Aldh1a1	Cenpn	Flad1	Lima1	Loc101118459	Nop56	Rhno1	Srd5a1	Znf532
Aldh1a2	Cenpw	Flnb	Lipg	Loc101118476	Npm1	Rhoj	Srd5a2	Znhit6
Aldh6a1	Cers5	Fmr1	Lmo4	Loc101118481	Npm3	Rhpn2	Srgn	Zswim7
Aldh7a1	Cetn3	Fnta	Lmo7	Loc101118825	Npy3r	Rin2	Sri	
Aldh9a1	Cfi	Foxs1	Loc100037661	Loc101118944	Nrep	Ripk3	Srpk2	
Aldoc	Chchd10	Frg1	Loc100037664	Loc101119027	Nsmce1	Rit1	Srsf1	
Amdhd2	Chd3	Fstl1	Loc100037665	Loc101119050	Nsmce4a	Rnase12	Srsf10	
Amfr	Chi3l1	Ftl	Loc100037666	Loc101119111	Nt5c	Rnd3	Srsf11	
Amica1	Chmp4a	Fubp1	Loc100037667	Loc101119342	Nt5c2	Rnf114	Srsf3	
Angptl4	Chmp5	Fuca1	Loc100037674	Loc101119447	Nt5c3	Rnf13	Srsf5	
Ankrd35	Chn2	Furin	Loc100037680	Loc101119517	Ntn3	Rnf145	Srsf6	
Anks6	Chrac1	Fuz	Loc100037687	Loc101119545	Nub1	Rnf180	Srsf7	
Ano1	Chrdl2	Fxr1	Loc100101228	Loc101119803	Nudt2	Rnf187	Ss18	

Genes more highly expressed during late pregnancy							
Anp32a	Cinp	Fxyd6	Loc100101231	Loc101120072	Nudt6	Rnpep	Ssrp1
Anpep	Cirbp	G0s2	Loc100101238	Loc101120118	Nuf2	Rnps1	St13
Anxa1	Cited1	G3bp1	Loc100125628	Loc101120148	Nusap1	Rogdi	St8sia1
Anxa2	Cited4	Gabarapl1	Loc100137070	Loc101120322	Oaf	Rom1	Stat1
Anxa3	Ckap2	Gadd45a	Loc101101911	Loc101120392	Obsl1	Rorc	Stat5a
Anxa4	Cks1b	Gadd45g	Loc101102096	Loc101120470	Ociad2	Rp9	Stat5b
Ap4b1	Cks2	Gal-1	Loc101102142	Loc101120478	Olfml3	Rpia	Steap1
Apbb2	Clcn7	Galk1	Loc101102165	Loc101120615	Osr1	Rpl10	Steap2
Apex1	Cldn4	Galm	Loc101102278	Loc101120701	Ovol2	Rpl11	Stk17b
Aph1a	Cldn8	Galnt12	Loc101102527	Loc101120815	Ovubq-L40	Rpl12	Stmn1
Apod	Clec14a	Gata3	Loc101102537	Loc101120819	Pa2g4	Rpl13a	Stoml2
App	Clic1	Gatm	Loc101102690	Loc101120860	Pabpc1	Rpl14	Stradb
Arf5	Clint1	Gbas	Loc101103182	Loc101120874	Pabpc4	Rpl15	Stx7
Arglu1	Clk1	Gbp5	Loc101103780	Loc101121365	Pacsin2	Rpl18	Suclg1
Arhgap26	Clk4	Gca	Loc101103787	Loc101121371	Paics	Rpl18a	Suclg2
Arhgef28	Clta	Gck	Loc101103957	Loc101121379	Paip2	Rpl19	Sumo2
Arhgef39	Cmtm7	Gcnt7	Loc101104079	Loc101121413	Pak1	Rpl21	Sun2
Arl3	Cnbp	Gdf10	Loc101104162	Loc101121454	Palmd	Rpl22	Syngr3
Armc10	Cndp1	Gdi2	Loc101104348	Loc101121630	Pan3	Rpl22l1	Sypl1
Arpc1b	Cnih4	Gfm2	Loc101104441	Loc101122014	Parp12	Rpl23	Tacc2
Arpc3	Cnn2	Gga2	Loc101104504	Loc101122112	Parp14	Rpl24	Tacc3
Arpc5	Cnn3	Ggh	Loc101104530	Loc101122242	Parp2	Rpl26	Taf1d
Arrdc3	Cnot8	Ggt6	Loc101104700	Loc101122261	Pbxip1	Rpl27	Taf7
Art3	Col16a1	Ggta2p	Loc101104787	Loc101122400	Pc	Rpl28	Tagln2
Asah1	Col18a1	Gipc1	Loc101104866	Loc101122468	Pcbp2	Rpl29	Tap1
Atp1b1	Col1a1	Gltp	Loc101104961	Loc101122577	Pcbp4	Rpl3	Tap2

Genes more highly expressed during late pregnancy							
Atp2b1	Col5a2	Gltscr2	Loc101105065	Loc101123213	Pccb	Rpl30	Tapbp
Atp5a1	Colec12	Gm2a	Loc101105144	Loc101123219	Pced1a	Rpl31	Tasp1
Atp5c1	Commd1	Gmds	Loc101105179	Loc101123279	Pcmtd1	Rpl35	Tax1bp3
Atp5d	Commd2	Gna14	Loc101105235	Loc101123290	Pcmtd2	Rpl36	Tbc1d14
Atp5g2	Copg2	Gnao1	Loc101105316	Loc101123329	Pcna	Rpl36a	Tbc1d16
Atp5h	Coq2	Gng11	Loc101105437	Loc101123453	Pcolce	Rpl37	Tbc1d24
Atp5o	Coro1a	Gng5	Loc101105609	Loc101123533	Pdcd4	Rpl37a	Tbl1xr1
Atp6v0e1	Cotl1	Gnl2	Loc101105810	Loc443070	Pdcd5	Rpl38	Tceal1
Atp6v1d	Cox1	Gnly	Loc101105990	Loc443239	Pde7a	Rpl39	Tceal4
Aurkb	Cox20	Gnpda1	Loc101105992	Loc443340	Pdgfc	Rpl5	Tcp1
B2m	Cpq	Gns	Loc101106058	Loc443512	Pdk4	Rpl6	Tctn3
B3galt4	Cpsf3l	Golim4	Loc101106083	Loc554317	Pdlim1	Rpl7	Tdp2
B4galnt3	Cpt1a	Golm1	Loc101106144	Loc780446	Pdxk	Rpl7a	Tes
B4galt3	Crabp2	Gpa33	Loc101106243	Loc780462	Pdzk1ip1	Rpl8	Tex33
B9d1	Crispld1	Gpc4	Loc101106245	Loc780467	Pdzrn3	Rpl9	Tex9
Bad	Crls1	Gpr1	Loc101106288	Loc780525	Pebp1	Rplp0	Tfap2c
Barx2	Cryab	Gpr125	Loc101106313	Lrp2	Pecam1	Rplp2	Tfpi2
Bbc1	Csde1	Gpr39	Loc101106384	Lrpap1	Pef1	Rps13	Tgfbi
Bcam	Csnk1e	Gpsm2	Loc101106419	Lrpprc	Pepd	Rps14	Tgfbr3
Bckdha	Cstb	Gpx8	Loc101106722	Lrrc31	Pept1	Rps15	Tgif1
Bcl2a1	Ctgf	Grb7	Loc101106730	Lsm14a	Perp	Rps15a	Tgm1
Bcl7c	Cth	Grhl1	Loc101106919	Lsm2	Pex5	Rps16	Tia1
Bdh2	Ctns	Grn	Loc101107006	Lsm3	Pfdn5	Rps18	Tial1
Birc5	Ctps1	Gss	Loc101107098	Lsm6	Pfkfb3	Rps19	Timm44
Blg	Ctsb	Gstk1	Loc101107389	Lsm7	Pfn1	Rps21	Timp2
Blmh	Ctsc	Gtf2h5	Loc101107504	Lsp1	Pgap2	Rps23	Tinagl1

Genes more highly expressed during late pregnancy							
Bloc1s1	Ctsf	Gtf2i	Loc101107641	Lta4h	Pgls	Rps26	Tkt
Bmp4	Ctsk	Gtf3c1	Loc101107700	Ltbp1	Pglyrp1	Rps27	Tm4sf1
Bnipl	Ctss	Gtpbp6	Loc101107729	Luc7l	Phb	Rps27a	Tm4sf18
Bphl	Ctsw	Gypc	Loc101107772	Luc7l3	Phb2	Rps28	Tma7
Brd8	Ctu2	Gzmm	Loc101107908	Lum	Phf23	Rps29	Tmco4
Bri3	Cuedc1	H3f3a	Loc101108033	Lxn	Phkg2	Rps3	Tmem110
Bst-2a	Cuta	H3f3b	Loc101108110	Lypd1	Phlda1	Rps5	Tmem139
Btbd2	Cxcl10	H6pd	Loc101108171	Lzts2	Phospho2	Rps7	Tmem150a
Btd	Cxcl14	Hacl1	Loc101108292	M6pr	Picalm	Rps8	Tmem150c
Btf3	Cxcl16	Hadha	Loc101108339	Mad2l1	Pigq	Rps9	Tmem176b
Btg1	Cxcl9	Hadhb	Loc101108538	Maf1	Pik3c2g	Rrm1	Tmem42
Bub1	Cyclophilin B	Hbp1	Loc101108558	Mal2	Pin1	Rrm2	Tmem50a
Bub1b	Cyp24	Hcar3	Loc101108599	Mall	Pla2g7	Rsl1d1	Tmem59l
Bub3	Dazap2	Hdac2	Loc101108633	Manbal	Pla2r1	Rspo1	Tmem80
C-met	Dbndd2	Hes1	Loc101108696	Mansc1	Plekha1	Rtkn	Tmem87a
C11h17orf49	Dbp	Hfe	LOC101108870	Map1lc3b	Plekhd1	Ruvbl2	Tmem9
C11h17orf61	Dcaf12	Hibch	LOC101108962	Map3k1	Plekhh3	S100a14	Tmod3
C12h1orf115	Dcaf4	Hmg20b	LOC101108963	Map4k4	Plekhs1	S100a16	Tmsb4x
C12h1orf174	Dcn	Hmgb2	LOC101108990	Map4k5	Plscr3	Sae1	Tnfrsf21
C13h20orf111	Dcps	Hmgb3	LOC101109078	Mapk3	Pltp	Sarnp	Tom1
C13h20orf27	Dctn2	Hmgn2	LOC101109080	Mar-02	PLVAP	SAT1	Tomm22
C13h20orf72	Dctn4	Hmgn3	LOC101109105	Mar-07	PMF1	SATB1	Top2a
C14h16orf80	Dctpp1	Hnmt	LOC101109132	Mast	Pmp22	Sav1	Top2b
C15h11orf52	Ddah2	Hnrnpa0	LOC101109212	Mat2a	Pnisr	Scand1	Tp53
C15h11orf58	Ddb2	Hnrnpa3	LOC101109220	Mat2b	Pnn	Scara3	Tpgs1
C16h5orf34	Ddr1	Hnrnpd	LOC101109246	Matn2	Pnpt1	Scarb2	Tpi1

Genes more highly expressed during late pregnancy							
C1h1orf54	Ddx17	Hnrnpf	LOC101109380	Matn3	Pnrc2	Scd5	Tpm3
C1h21orf2	Ddx39b	Hnrnph3	LOC101109421	Matr3	Pogk	Sdc1	Tpm4
C1qa	Decr1	Hnrnpm	Loc101109492	Maz	Pole3	Sdha	Tppp3
C1qb	Dek	Hnrnpu	Loc101109545	Mbd3	Polr2g	Sdhaf2	Tpx2
C1qbp	Denr	Hnrpdl	Loc101109634	Mbnl1	Pop7	Sdhb	Tra2a
C1qc	Dhrs3	Hnrpll	Loc101109655	Mcm2	Ppa2	Sec11a	Traf4
C1qtnf5	Dlg3	Hp1bp3	Loc101109701	Mcm3	Ppap2b	Sema4c	Trappc5
C1s	Dlgap5	Hprt1	Loc101109746	Mcm4	Ppap2c	Sep-02	Trim28
C20h6orf136	Dlk1	Hs3st1	Loc101109747	Mcm5	Ppic	Sep-07	Trim34
C20h6orf62	Dnajc10	Hsd17b10	Loc101109830	Mcm6	Ppid	Sep-09	Trim38
C21h11orf48	Dnajc7	Hsd17b4	Loc101110095	Mcm7	Ppih	Serbp1	Trim7
C23h18orf21	Dock10	Hsp90ab1	Loc101110151	Mcu	Ppm1f	Serf2	Trip10
C24h16orf13	Drap1	Hspa12b	Loc101110176	Mdp1	Ppp1cb	Serpinb1	Trip6
C25h10orf10	Dsn1	Hspb1	Loc101110408	Med17	Ppp1cc	Serpinh1	Trpm2
C26h8orf4	Dtd1	Hspd1	Loc101110438	Med4	Ppp1r14b	Sertad4	Trps1
C2h9orf3	Dtnbp1	Htra1	Loc101110529	Metap2	Ppt1	Sesn1	Tsc22d4
C3h2orf68	Dtwd1	Htra2	Loc101110543	Mettl10	Prc1	Sesn3	Tsen15
C4h7orf55	Dtx3	Iah1	Loc101110546	Mettl12	Prdx1	Sestd1	Tsen34
C4h7orf73	Dtymk	Id2	Loc101110593	Mff	Prdx2	Set	Tspan12
C5h19orf53	Dut	Id3	Loc101110657	Mfn1	Prdx3	Sf3b1	Tspan33
C5h19orf60	Dync1i2	Idh2	Loc101110710	Mgll	Prdx6	Sf3b5	Tspan7
C5h5orf15	Dync2li1	Idh3b	Loc101110918	Mgst1	Prickle1	Sfpq	Tst
C6h4orf19	Dynll2	Ifi35	Loc101111150	Mia	Prim1	Sfr1	Ttc14
C7h14orf166	Dynlt3	Ifi6	Loc101111367	Mier1	Prkag2	Sfrp2	Ttc29
C8h6orf225	Ech1	Ifitm1	Loc101111388	Mitd1	Prpf39	Sfxn5	Ttll1
C9h8orf59	Eci2	Ifngr1	Loc101111412	Mlec	Prpf40b	Sgms1	Tubb2a

Genes more highly expressed during late pregnancy							
Ca2	Ecscr	Ift52	Loc101111694	Mlf1	Prr15l	Sh3bgrl	Tufm
Cacnb3	Ect2	Ift57	Loc101111804	Mlph	Psap	Sh3d19	Txndc17
Cadm1	Eef1b2	Igfbp2	Loc101112018	Mmd	Psma1	Sh3d21	Tyrobp
Calcoco1	Eef1d	Igfbp4	Loc101112038	Mme	Psma2	Sh3glb1	U2af1
Cald1	Eef1g	Igfbp7	Loc101112071	Mmp23b	Psma3	Shf	Ubash3b
Calm2	Eef2	Igsf9	Loc101112245	Mns1	Psma4	Shfm1	Ube2c
Calm3	Eef2k	Ik	Loc101112246	Morf4l1	Psma5	Shisa2	Ube2e1
Cap1	Efna1	Ikbke	Loc101112251	Mpeg1	Psma6	Shisa6	Ube2l6
Carhsp1	Efs	Il2rb	Loc101112253	Mpst	Psmb10	Shkbp1	Ube2t
Casp8	Egfl7	Il2rg	Loc101112287	Mrcl3	Psmb2	Slan	Ubl3
Cat	Ehf	Il34	Loc101112300	Mrpl10	Psmb3	Slbp	Ublcp1
Cav1	Eif1ax	Ilf2	Loc101112330	Mrpl12	Psmb4	Slc12a2	Ubxn1
Cav2	Eif2s3	Ilvbl	Loc101112406	Mrpl19	Psmb7	Slc13a2	Ubxn11
Cbx3	Eif3d	Impdh2	Loc101112491	Mrpl3	Psmb8	Slc16a2	Ucp2
Cby1	Eif3e	Ing4	Loc101113111	Mrpl45	Psmb9	Slc18b1	Uhrf1
Ccdc109b	Eif3f	Ino80b	Loc101113217	Mrpl9	Psmd13	Slc22a23	Unc45a
Ccdc12	Eif3g	Ipo5	Loc101113219	Mrps17	Psmd4	Slc24a3	Ung
Ccdc13	Eif3h	Ipo7	Loc101113250	Mrps21	Psme1	Slc25a10	Upf3b
Ccdc23	Eif3k	Irf1	Loc101113344	Msh2	Psme2	Slc25a28	Uqcrh
Ccdc28b	Eif3l	Irx3	Loc101113604	Msrb1	Psmg3	Slc25a3	Urod
Ccdc3	Eif3m	Ism1	Loc101113705	Msrb2	Ptk7	Slc25a39	Usp16
Ccdc57	Eif4b	Ist1	Loc101113715	Mtss1	Ptn	Slc25a5	Utp14a
Ccdc80	Eif4ebp1	Itga6	Loc101113965	Mtss1l	Ptpmt1	Slc25a6	Uxt
Ccdc82	Elk3	Itgb1	Loc101114032	Muc20	Ptprc	Slc27a1	Vamp3
Ccl14	Elmo3	Itgb6	Loc101114092	Mvp	Ptprcap	Slc28a3	Vamp5
Ccl21	Emcn	Itgb7	Loc101114093	Mxd3	Ptprf	Slc29a1	Vamp8

Genes more highly expressed during late pregnancy							
Ccl5	Eml3	Itpa	Loc101114335	Mybl2	Ptrf	Slc35a1	Vasp
Ccna2	Eno1	Itpr1	Loc101114342	Myc	Pttg1	Slc37a3	Vav3
Ccnb1	Epb41l2	Jup	Loc101114533	Myl6	Pus1	Slc40a1	Vdac3
Ccnd1	Erbb2	Kank1	Loc101114582	Mylip	Pvrl4	Slc43a1	Vdup1
Ccnf	Erbb3	Kars	Loc101114599	Naa10	Pyroxd2	Slc44a1	Vegfb
Ccng1	Ercc1	Kat2b	Loc101114839	Naaa	Qars	Slc48a1	Vezf1

Table A. 2 Genes identified by RNA-seq as differentially expressed in the ovine mammary gland between late pregnancy and lactation. Genes shown were more highly expressed during lactation. Samples were pooled for RNA-seq analysis: *n* = 6 pools for lactation, with three samples per pool, *n* = 18 samples.

Genes more highly expressed during lactation						
Actb	Chpf	Gbf1	Loc101108663	Myo1e	Rnd2	Tceb3
Spp1	Cidea	Gcat	Loc101109268	Mzb1	Rnf130	Tcirg1
Aacs	Cish	Gch1	Loc101109397	Naa20	Rnf217	Tcn2
Aars	Cit	Gcn1l1	Loc101109657	Nabp1	Rnft1	Tecr
Aass	Cited2	Gda	Loc101109877	Nars	Rnls	Tectb
Abcd1	Ckmt1b	Gem	Loc101110099	Nbas	Rora	Tff3
Abcg2	Clcn5	Gfpt1	Loc101110341	Ncoa2	Rpn2	Tfg
Abhd3	Clgn	Ggcx	Loc101110672	Ncoa3	Rpp14	Tfrc
Abr	Clic2	Ghitm	Loc101110679	Ncoa4	Rps27l	Tgfbr1
Acaca	Clip2	Ghr	Loc101110797	Ncoa7	Rps6ka3	Thbd
Aco1	Cltc	Gk	Loc101110855	Ncr3lg1	Rrad	Thyn1
Aco2	Cmpk1	Gk5	Loc101110998	Ncstn	Rras	Timm8b
Acot11	Cmtm6	Glrx	Loc101111169	Nd1	Rrbp1	Tlr2
Acp5	Cmtm8	Glut-1	Loc101111174	Nd2	Rtn4	Tm7sf3
Acsl1	Cnn1	Glycam1	Loc101111669	Nd3	S100a10	Tm9sf1
Acsm4	Cnnm4	Gmppb	Loc101111697	Nd4	S100a2	Tm9sf4
Acss2	Cobll1	Gna12	Loc101111847	Nd4l	Saa1	Tmbim1
Acss3	Copa	Gna13	Loc101111915	Nd5	Sar1a	Tmc4
Adamts1	Copb2	Gnai1	Loc101111922	Nd6	Sar1b	Tmc5
Add3	Cops5	Gne	Loc101112356	Ndel1	Sardh	Tmcc3
Adh5	Cops7a	Gnmt	Loc101112362	Ndrg2	Sars	Tmco1
Adipor2	Coq10b	Golga2	Loc101112590	Ndrg4	Sc5dl	Tmed10
Adm	Cox3	Golga3	Loc101112867	Ndufa5	Sccpdh	Tmed2
Adora2b	Cpt1b	Golt1b	Loc101113137	Ndufa7	Scd	Tmed3

Genes more highly expressed during lactation						
Adpgk	Crat	Gorasp2	Loc101113302	Ndufb1	Scgb2a2	Tmed4
Adrb1	Creb3l1	Gp2	Loc101113398	Ndufs1	Scn1b	Tmed8
Agpat1	Creg1	Gpaa1	Loc101113430	Necap1	Scyl1	Tmem109
Agpat4	Creld2	Gpam	Loc101113583	Nfe2l1	Sdc2	Tmem119
Agpat6	Cry1	Gpcpd1	Loc101113754	Nfil3	Sdf2l1	Tmem120a
Agr2	Csda	Gpd1	Loc101113795	Nhp2	Sdf4	Tmem120b
Ahcyl2	Csdc2	Gpd1l	Loc101113893	Nmb	Sdsl	Tmem125
Aif1l	Csn1s1	Gpr116	Loc101114008	Nme2	Sec11c	Tmem167a
Aifm1	Csn1s2	Gpr146	Loc101114126	Notch1	Sec13	Tmem170a
Aim1	Csn2	Gpr160	Loc101114192	Nov	Sec16b	Tmem171
Ak3	Csrnp1	Gpr68	Loc101114930	Nqo1	Sec23b	Tmem181
Ak4	Cst6	Gprc5b	Loc101114941	Nr3c1	Sec23ip	Tmem184a
Aldh18a1	Ctnnal1	Gpx1	Loc101114959	Nr3c2	Sec24a	Tmem2
Aldh2	Cxhxorf38	Gpx3	Loc101114987	Nr4a1	Sec24d	Tmem205
Alg1	Cyp17a1	Gpx4	Loc101115115	Nr4a2	Sec61a1	Tmem208
Alg14	Cyp2j	Gramd2	Loc101115154	Nrip1	Sec61g	Tmem214
Alg3	Cysltr2	Grina	Loc101115432	Nsdhl	Sec62	Tmem25
Alg5	Cystm1	Gsdmb	Loc101115614	Nt5e	Sel1l	Tmem33
Alox12	Cytb	Gspt1	Loc101115724	Nucb2	Sema3a	Tmem39a
Alpl	Cyth3	Gyltl1b	Loc101115772	Nudcd3	Sema3c	Tmem41a
Angpt1	Cytip	Hapln3	Loc101115978	Nudt12	Sema4a	Tmem53
Ankh	Dbi	Hax1	Loc101115979	Nudt19	Sema6d	Tmem66
Ankrd22	Dcdc2	Hbs1l	Loc101116003	Nudt4	Sema7a	Tmem68
Ano3	Ddah1	Hdlbp	Loc101116026	Nup188	Sepw1	Tmem70
Ap1m1	Ddhd2	Hebp2	Loc101116156	Nupr1	Serinc2	Tmod4
Ap2s1	Ddost	Herpud1	Loc101116298	Nus1	Serinc5	Tmsb10

Genes more highly expressed during lactation						
Aqp3	Ddrgk1	Hhipl2	Loc101116302	Nxf1	Serpina1	Tmtc4
Arcn1	Ddx1	Hibadh	Loc101116327	Nxpe3	Sertad1	Tmx2
Arf4	Degs1	Hid1	Loc101116448	Oaz1	Sertm1	Tnfrsf12a
Arfgef2	Dennd3	Hlf	Loc101116800	Odc1	Sesn2	Tnfrsf13b
Arfip2	Dennd4a	Hm13	Loc101116973	Ogdh	Setd7	Tnfsf13
Arg2	Desi1	Hmgcr	Loc101117120	Orai1	Sft2d2	Tnip1
Arhgap35	Dgat1	Hmgcs1	Loc101117129	Ormdl3	Sh3bp4	Tnrc6c
Arhgap6	Dhcr24	Hmgxb4	Loc101117221	Osbpl10	Sh3bp5	Tns4
Arhgdia	Dhcr7	Hpn	Loc101117287	Osbpl1a	Sh3glb2	Tom1l1
Arhgef16	Dhdds	Hsd17b12	Loc101118164	Osbpl8	Shisa5	Tp53inp2
Arhgef19	Dhrs1	Hsd17b14	Loc101118256	Oxct1	Shmt1	Tpd52
Arhgef4	Dhrs7	Hsd17b7	Loc101118495	P2ry1	Sigmar1	Tpm1
Arl1	Dio2	Hsp70.1	Loc101118516	P4hb	Sik1	Tpt1
Arl15	Dkkl1	Hspa13	Loc101119078	Palld	Sil1	Traf3ip3
Arl4a	Dnajb1	Hspa5	Loc101119269	Pank3	Slc10a7	Tram1
Arl4d	Dnajb11	Hspa6	Loc101119296	Park7	Slc12a8	Trappc6a
Armcx3	Dnajb2	Hspa8	Loc101119335	Parp4	Slc16a1	Trappc8
Asap3	Dnajb9	Hspb8	Loc101119338	Pcdh1	Slc16a5	Trex1
Ascc2	Dnajc12	Hsph1	Loc101119370	Pck2	Slc17a9	Trib1
Asgr2	Dnajc3	Hyou1	Loc101119576	Pcyt2	Slc1a4	Trib3
Asns	Dolk	Idh1	Loc101119616	Pde10a	Slc1a5	Trim25
Ass1	Dpm2	Idi1	Loc101119731	Pdhb	Slc20a1	Trim29
Atf3	Dqx1	Ier2	Loc101119889	Pdia6	Slc20a2	Trip11
Atf4	Dusp1	Ifngr2	Loc101120455	Pdp2	Slc22a16	Trpm3
Atf5	Dusp4	Ift46	Loc101120481	Pdrg1	Slc23a1	Tspan1
Atg12	Dusp5	Igsf5	Loc101120775	Pdxdc1	Slc25a1	Tspan13

Genes more highly expressed during lactation						
Atox1	Dusp6	Il1r1	Loc101120900	Pex2	Slc25a17	Tspan3
Atp13a2	Dync1li2	Il6r	Loc101120945	Pgd	Slc25a21	Tspan6
Atp13a4	Dynll1	Insig1	Loc101121082	Pgm3	Slc25a4	Tspyl2
Atp13a5	Eaat2	Irf6	Loc101121134	Phf19	Slc2a9	Ttc23l
Atp2a2	Eaat3	Irs1	Loc101121199	Phyhd1	Slc30a2	Ttc37
Atp2b2	Ebp	Irx1	Loc101121216	Pi4k2a	Slc30a6	Ttc39a
Atp2c2	Echdc1	Isoc1	Loc101121235	Piezo1	Slc30a9	Tuft1
Atp6	Edem3	Itm2b	Loc101121418	Pigk	Slc31a1	Txndc11
Atp6v0a1	Eef1e1	Itm2c	Loc101121506	Pigr	Slc33a1	Tyr
Atp6v0d1	Efhd1	Itpr2	Loc101121718	Pigw	Slc34a2	Uba5
Atp6v1c1	Efnb2	Itsn1	Loc101121764	Pigx	Slc35a2	Ubc
Atp6v1f	Efr3a	Ivd	Loc101121779	Pim1	Slc35b1	Ube2f
Atp8	Egr1	Ivns1abp	Loc101122003	Pink1	Slc35c1	Ube2j1
Atrn	Ehd4	Jak1	Loc101122296	Pkd2	Slc35d2	Ube2q2
Atrnl1	Eif1	Junb	Loc101122398	Pkdcc	Slc35e1	Ube4a
Aven	Eif2ak2	Jund	Loc101122575	Pkm	Slc36a1	Ubfd1
Avpi1	Eif2ak3	Kansl2	Loc101122661	Pkp2	Slc38a2	Ubn1
Azin1	Eif2s2	Kcnj15	Loc101123118	Pla2g16	Slc38a3	Ufm1
B4galt1	Eif4ebp2	Kcnk1	Loc101123437	Pla2g2c	Slc38a6	Uggt1
Bag1	Eif4g2	Kcnk5	Loc101123546	Pld2	Slc39a11	Ugp2
Bag3	Eif4g3	Kcnk6	Loc443178	Plekhb1	Slc39a14	Unc13a
Bag5	Eif4gi	Kctd14	Loc443255	Plgrkt	Slc39a3	Unc13b
Batf3	Eif5	Kdelr2	Loc443535	Plin2	Slc39a7	Unc93a
Bcap31	Elf5	Kdm5a	Loc780464	Plk2	Slc39a8	Uso1
Bcar1	Ell2	Kel	Loc780773	Plk3	Slc39a9	Usp31
Bcat2	Ell3	Kiaa1279	Lonrf3	Pllp	Slc41a1	Usp54

Genes more highly expressed during lactation						
Bcl2l15	Elmod3	Kiaa1324	Lpar4	Plod2	Slc4a1ap	Uxs1
Bcl6	Elovl1	Kiaa1467	Lpcat4	Plod3	Slc50a1	Vamp1
Bdh1	Elovl6	Kiaa1598	Lpin1	Plp2	Slc5a1	Vegfa
Bex2	Emc3	Klf15	Lpl	Pm20d1	Slc5a5	Vimp
Bin2	Emc7	Klf5	Lpo	Pmm2	Slc7a6	Vipr1
Bpifa1	Emp1	Klf6	Lrat	Pmvk	Slc7a6os	Vldlr
Brd2	Epas	Klhdc5	Lrig1	Pnpla3	Slc9a3r1	Vmac
Bscl2	Epb41	Kras	Lrp3	Pnpo	Slco4a1	Vps35
Bsg	Ephb3	Krt19	Lrp8	Polr2a	Slit3	Wars
Bspry	Ephx1	Krtcap2	Lrrc16a	Polr3d	Smim3	Wdr1
Btc	Epm2aip1	Krtcap3	Lrrc20	Polr3k	Smpd1	Wdr26
Btg2	Eprs	Lalba	Lrrc59	Por	Smtn	Wdr37
Btg3	Epx	Lama3	Lrrc8a	Ppa1	Smyd2	Wdr47
Btn1a1	Erba Beta1	Lamtor2	Lrrc8c	Ppard	Snap23	Wdr83
Bzw2	Ergic1	Large	Lsm12	Ppfia1	Snap29	Wdr85
C-Jun	Erlec1	Larp4b	Lss	Ppm1a	Snapc5	Wfdc2
C11h17orf89	Ero1l	Lat1	Ly96	Ppm1k	Snd1	Wisp3
C12h1orf21	Erp44	Lbp	Lyn	Ppp1r10	Snta1	Wnk4
C12h1orf27	Esr1	Lcat	Lysmd3	Ppp1r15b	Sntb1	Wnt5a
C15h11orf31	Esyt1	Lcn2	Magix	Ppp1r2	Sntb2	Xdh
C1h1orf210	Etnk1	Lcp2	Maml3	Pprc1	Snx10	Yars
C1h1orf228	Ets2	Lifr	Man1a1	Pqlc1	Snx19	Yif1a
C1h2orf82	Extl1	Lig3	Man2a1	Pradc1	Socs1	Yipf2
C20h6orf89	Extl3	Lin7a	Manf	Prkab1	Sod3	Yipf4
C21h11orf24	Eya2	Liph	Mansc4	Prkar2b	Sowahb	Yipf5
C21h11orf75	Ezr	Lipm	Map1lc3a	Prkch	Sp140	Ykt6

Genes more highly expressed during lactation						
C24h16orf72	F2r	Litaf	Map2k1	Prkg1	Spata9	Zbtb16
C25h10orf54	F2rl1	Lman1	Map2k3	Prom2	Spcs2	Zc3h7a
C2cd2	Fabp3	Lman2	Map7	Prps2	Spcs3	Zdhhc6
C2h9orf152	Fabp4	Lmtk2	Mapk12	Prrc1	Sppl2a	Zfp36
C3h12orf23	Fads1	Loc100037677	Mapk13	Psat1	Sptbn2	Znf135
C3h22orf32	Fads2	Loc100037686	Mapk14	Psd3	Sptlc2	Znf592
C4h7orf25	Fam104a	Loc100037692	Mapkapk5	Psd4	Sptlc3	Znf664
C5	Fam107a	Loc100037702	Mar-01	Psme3	Sqle	Znf76
C5h19orf71	Fam110a	Loc100137069	Marcksl1	Psph	Sqstm1	
C9	Fam118a	Loc100885760	Mars	Ptbp3	Sra1	
Cachd1	Fam134b	Loc101102019	Mast4	Ptgr1	Srebf1	
Cad	Fam198a	Loc101102218	Matk	Pth-Rp	Srek1ip1	
Cage1	Fam20a	Loc101102223	Mcfd2	Pvrl2	Srm	
Calm1	Fam46a	Loc101102284	Mdfic	Pycr1	Srp54	
Camkk2	Fam46c	Loc101102390	Me1	Rab11fip2	Srpr	
Capn2	Fam59a	Loc101102403	Mea1	Rab18	Srprb	
Capn5	Fam73b	Loc101102407	Mfge8	Rab1a	Ssbp1	
Cars	Fam78b	Loc101102550	Mfsd12	Rab25	Ssbp3	
Cat-1	Fam83f	Loc101102588	Mfsd2a	Rab26	Ssfa2	
Catsperg	Fam84b	Loc101102794	Mfsd4	Rab2a	Ssr3	
Cbr4	Fam92a1	Loc101102861	Mgat1	Rab3d	St3gal1	
Cbs	Fasn	Loc101102999	Mgat4a	Rab3gap1	St6galnac2	
Cbx4	Fbp1	Loc101103238	Mgmt	Rab3ip	St7	
Ccbl1	Fbxl19	Loc101103427	Mib1	Rab6b	Stard4	
Ccdc107	Fchsd2	Loc101103477	Mical2	Rabac1	Stard8	
Ccdc115	Fdft1	Loc101103591	Mid2	Rai14	Stim2	

Genes more highly expressed during lactation					
Ccdc149	Fdps	Loc101103614	Miox	Ramp2	Stom
Ccdc167	Fgfbp1	Loc101104051	Mknk1	Rap1b	Ston2
Ccdc47	Fgr	Loc101104146	Mknk2	Rapgef2	Stt3a
Ccdc64	Ficd	Loc101104536	Mks1	Rapgef5	Stx3
Ccdc88c	Fkbp11	Loc101104661	Mocs1	Raph1	Stxbp2
Ccdc91	Fkbp1a	Loc101104790	Mocs2	Rasef	Stxbp6
Ccl28	Fkbp5	Loc101105022	Mov10	Rasgef1b	Suco
Ccna1	Flvcr2	Loc101105274	Mpc1	Rasgrf2	Sulf2
Ccny	Fndc3a	Loc101105290	Mpc2	Rassf3	Sult1c2
Ccnyl1	Fos	Loc101105315	Mpo	Rassf4	Suox
Cd14	Fosb	Loc101105484	Mpp5	Rbm25	Supt6h
Cd164	Foxj3	Loc101105787	Mpp6	Rbm47	Surf1
Cd55	Foxn2	Loc101105822	Mpv17l2	Rcc2	Surf2
Cd59	Foxo3	Loc101106014	Mrap2	Rdh10	Surf4
Cd63	Frrs1	Loc101106024	Mrpl14	Rdh11	Susd1
Cd81	Fst	Loc101106041	Mrpl17	Rela	Swi5
Cd82	Fth1	Loc101106093	Mrto4	Relt	Syne2
Cdcp1	Ftsj1	Loc101106123	Msh3	Rer1	Synj1
Cdk10	Fxc1	Loc101106160	Msi2	Ret	Sytl2
Cdk18	Fzd4	Loc101106227	Msmo1	Retsat	Syvn1
Cdkn1a	Fzd5	Loc101106347	Mst1r	Rexo2	Tab2
Cdo1	Gabarapl2	Loc101106646	Mtch1	Rgag4	Taf15
Cdr2	Gadd45b	Loc101106651	Mthfd1	Rgs4	Tars
Ceacam1	Gale	Loc101106806	Mtmr4	Rhob	Tax1bp1
Chac1	Galnt3	Loc101106871	Mtus1	Rhobtb3	Tbc1d30
Chchd7	Galnt5	Loc101107030	Muc1	Rhof	Tbc1d8

Genes more highly expressed during lactation					
Chka	Ganab	Loc101107522	Muc15	Rnase1	Tbc1d9b
Chkb	Gars	Loc101107770	Mvd	Rnase4	Tbl2
Chmp1a	Gas2l2	Loc101107954	Myd88	Rnasek	Tbrg1
Chp1	Gbe1	Loc101108062	Myo18a	Rnd1	Tc2n

Table A. 3 List of genes identified by RNA-seq as differentially expressed between nutritional programming treatments in mammary tissue sampled from late-pregnant ewe offspring. Dam nutritional treatments: SmM = sub-maintenance, MM = maintenance and AdM = *ad libitum* nutrition during early pregnancy (day 21 to 50 of gestation) followed by maintenance nutrition during mid-to-late pregnancy (day 50 to 140 of gestation). Samples were pooled for RNA-seq analysis. For late pregnancy; SmM, MM, and AdM (*n* = 3 pools sequenced for each treatment, with three samples per pool, *n* = 9 total samples for each treatment).

Differentially expressed genes during late pregnancy					
MM vs. AdM		SmM vs. MM		SmM vs. AdM	
MM	AdM	SmM	MM	SmM	AdM
Abcc4	Abca3	Acp5	Bdh2	Acp5	Abp1
Acmsd	Art3	Anxa3	Bnip1	Anxa3	Btn1A1
Acp2	Asb11	Bex5	Bspry	Bex5	Ccdc167
Acp6	C12h1orf105	C5	C1Qtnf3	C5	Csn1S1
Actr6	C14h19orf12	C5h5orf63	Cd248	C5h5orf63	Dbi
Adam19	C3h9orf116	Cdrt4	Ddit4L	Cdrt4	Fam134B
Aen	C5h5orf63	Cers5	Dhcr7	Cers5	Fkbp1A
Aif1L	Ccdc64	Cldn11	Efemp2	Cldn11	Gadd45A
Akap17A	Cdh16	Creg2	Emc10	Creg2	Inpp4B
Alg1	Csn1S1	Cspp1	Fbln2	Cspp1	Lalba
Als2Cr12	Csn3	Epb41L4A	Fen1	Epb41L4A	Loc101102223
Antxr2	Fam134B	Fbp1	Fhl1	Fbp1	Loc101104199
Apaf1	Fos	Fcgr3A	Fkbp4	Fcgr3A	Loc101105104
Apitd1	Gch1	Gca	Ftsj2	Gca	Loc101107532
Arf4	Gfra4	Hook3	Grip2	Hook3	Loc101108870
Arhgap11A	Gjb5	Hrsp12	Gtpbp4	Hrsp12	Loc101110099
Asb13	Gpr110	Kiaa1143	H2Afz	Kiaa1143	Loc101110442
Asf1B	Kiaa1984	Loc101101856	Hirip3	Loc101101856	Loc101110663
Atp11C	Loc100037663	Loc101104348	Hspb6	Loc101104348	Loc101118976
Axin2	Loc101103658	Loc101106597	Kera	Loc101106597	Loc101119740
Bcas1	Loc101104866	Loc101110799	Lhfpl2	Loc101110799	Loc101122201
Bche	Loc101105998	Loc101113279	Loc101105484	Loc101113279	Mical2
Bcl2	Loc101107532	Loc101115089	Loc101107031	Loc101115089	Pank3
Bcl2L14	Loc101109850	Loc101119090	Loc101107436	Loc101119090	Sema7A
Bco2	Loc101115109	Loc101121149	Loc101109608	Loc101121149	Slc25A27
Bdh2	Loc101118000	Lyrm1	Loc101109692	Lyrm1	Slc25A4
Bhlhb9	Loc101118976	Mcl1	Loc101110442	Mcl1	Snph
Bhlhe41	Loc101119090	Mospd1	Loc101110679	Mospd1	Sqle
Bin1	Loc101119740	Mrpl50	Loc101111927	Mrpl50	Tgfbi
Bmf	Loc780442	Mrps17	Loc101111936	Mrps17	Tlr5
Bnip1	Man1C1	Ncs1	Loc101117673	Ncs1	Tmem184A
Bpnt1	Mical2	Ndrg2	Loc101118597	Ndrg2	Tppp3
Brca1	Napb	Pcmtd2	Loc101122718	Pcmtd2	Trabd2B
Brca2	Nit2	Pgm1	Ltbp4	Pgm1	Tyr
Bri3Bp	Npm1	Pik3C2G	Mcm6	Pik3C2G	
Btbd3	Polr1D	Pogk	Me2	Pogk	
Bub1B	Prss27	Rem1	Mfap4	Rem1	
C12h1orf112	Rab17	Rgmb	Nbl1	Rgmb	

Differentially expressed genes during late pregnancy					
MM vs. AdM		SmM vs. MM		SmM vs. AdM	
MM	AdM	SmM	MM	SmM	AdM
C13h20orf112	Rab18	Sh3Bp5	Nfe2L3	Sh3Bp5	
C17h22orf31	Rims4	Slc16A7	Nkain1	Slc16A7	
C24h16orf71	Rnase12	Slc18B1	Nt5C	Slc18B1	
C2h2orf69	Sdsl	Slc22A5	Oaf	Slc22A5	
C2h9orf156	Sec62	Slc7A6Os	Olfml3	Slc7A6Os	
Ca11	Sema7A	Stap2	Papss1	Stap2	
Camk1	Serpina1	Stk3	Paqr4	Stk3	
Cbr4	Slc25A21	Surf1	Phgdh	Surf1	
Cbx5	Syne2	Sycp3	Pigb	Sycp3	
Ccbe1	Tmem184A	Syne2	Pla2G4F	Syne2	
Ccbl2	Tnfrsf21	Tcn1	Plekhd1	Tcn1	
Ccdc57	Znf22	Tgm1	Podn	Tgm1	
Ccna2		Tmc4	Ppa1	Tmc4	
Cd248			Prkcdbp		
Cd3D			Prss23		
Cd3G			Rcn3		
Cd6			Rmi2		
Cdc42Se2			Rnaseh2C		
Cdca8			Sfrp4		
Cdk1			Slc25A22		
Cdk2			Slc46A1		
Cdk6			Socs4		
Cdr2L			Sult6B1		
Cecr1			Tbc1D14		
Cela1			Tgm2		
Cenpl			Thy1		
Cenpm			Tmem119		
Cenpt			Tppp3		
Cep41			Tuba1C		
Chaf1A			Unc93A		
Chek1			Wtip		
Chml			Znf185		
Chrne			Zwilch		
Chtf18					
Ckap2L					
Cks2					
Cnp					
Cobl					
Cog5					
Commd5					
Crabp2					
Crlf2					
Ctns					

Differentially expressed genes during late pregnancy					
MM vs. AdM		SmM vs. MM		SmM vs. AdM	
MM	AdM	SmM	MM	SmM	AdM
Cxcl10					
Cyp24					
Cyyr1					
Ddx39A					
Dpysl3					
Dstyk					
Dtl					
Dtx3L					
Dusp16					
E2F1					
E2F8					
Efemp2					
Eif2Ak2					
Eif5A					
Elmo2					
Emp1					
Entpd1					
Epb41L3					
Epsti1					
Fa2H					
Fam105A					
Fam108C1					
Fam149A					
Fam71E1					
Fam78A					
Fam83F					
Fam86A					
Fam98C					
Fat1					
Fbf1					
Fcrla					
Fen1					
Fn1					
Foxp4					
Frk					
Fut11					
Fzd7					
Gal3St4					
Gda					
Gfra3					
Glut8					
Grem1					
Grip2					

Differentially expressed genes during late pregnancy					
MM vs. AdM		SmM vs. MM		SmM vs. AdM	
MM	AdM	SmM	MM	SmM	AdM
Gtse1					
H2Afz					
Hcst					
Hells					
Hfe					
Hirip3					
Hk2					
Hmga1					
Homer2					
Hr					
Hsd3B7					
Hspa1A					
Hspa1L					
Hspa2					
Hspb6					
I-Mf					
Id2					
Ier5L					
Ifi44					
Igf2					
Igfbp6					
Incenp					
Invs					
Irak1					
Itgb2					
Itpk1					
Jak2					
Jam3					
Kcnk5					
Kcnn4					
Kctd10					
Kera					
Kiaa0930					
Kin					
Kntc1					
L2Hgdh					
Lbr					
Lclat1					
Lepr					
Lgals3					
Lhfpl2					
Lmnb2					
Loc100037680					

Differentially expressed genes during late pregnancy					
MM vs. AdM		SmM vs. MM		SmM vs. AdM	
MM	AdM	SmM	MM	SmM	AdM
Loc100171393					
Loc101102142					
Loc101102169					
Loc101102411					
Loc101102820					
Loc101103163					
Loc101103296					
Loc101103726					
Loc101104089					
Loc101104132					
Loc101104346					
Loc101104687					
Loc101105425					
Loc101105810					
Loc101105937					
Loc101106040					
Loc101106192					
Loc101106750					
Loc101107840					
Loc101108048					
Loc101108333					
Loc101108934					
Loc101109021					
Loc101109578					
Loc101109608					
Loc101110168					
Loc101110507					
Loc101110679					
Loc101111343					
Loc101111642					
Loc101111710					
Loc101111936					
Loc101112300					
Loc101112480					
Loc101112864					
Loc101114535					
Loc101114552					
Loc101114667					
Loc101115359					
Loc101116003					
Loc101116071					
Loc101116336					
Loc101116756					

Differentially expressed genes during late pregnancy					
MM vs. AdM		SmM vs. MM		SmM vs. AdM	
MM	AdM	SmM	MM	SmM	AdM
Loc101117673					
Loc101117960					
Loc101118156					
Loc101118195					
Loc101118252					
Loc101118883					
Loc101120002					
Loc101120065					
Loc101120732					
Loc101121006					
Loc101122689					
Loc101122718					
Loc101123541					
Loc443444					
Lrrc8B					
Lrrn2					
Lynx1					
Lzts1					
Magi3					
Maoa					
Mcm3					
Mcm4					
Mcm6					
Mcoln2					
Mdk					
Mdm1					
Mettl15					
Mfsd9					
Mki67					
Mmp2					
Mrc2					
Mrpl1					
Msc					
Mta3					
Muc20					
Mxra5					
Myef2					
Nav2					
Nbl1					
Ncald					
Ncapd3					
Nckipsd					
Nfe2L3					

Differentially expressed genes during late pregnancy					
MM vs. AdM		SmM vs. MM		SmM vs. AdM	
MM	AdM	SmM	MM	SmM	AdM
Nfic					
Nfix					
Nhlrc1					
Nkain1					
Nme4					
Nol3					
Nt5C					
Nt5M					
Ntf4					
Nudt1					
Nuf2					
Nup37					
Oaf					
Oas1					
Orc1					
Pag1					
Papss1					
Pcdhga12					
Pcna					
Pdzd9					
Pet112					
Phgdh					
Pla1A					
Pmaip1					
Pole2					
Polr2D					
Ppa1					
Ppp1R14D					
Ppp1R1A					
Prex1					
Prkacb					
Prkcdbp					
Prkrip1					
Psmb9					
Psmc3Ip					
Ptpn20B					
Ptpn22					
Rab30					
Racgap1					
Rad51					
Rad51D					
Rapgefl1					
Rasgrp1					

Differentially expressed genes during late pregnancy					
MM vs. AdM		SmM vs. MM		SmM vs. AdM	
MM	AdM	SmM	MM	SmM	AdM
Rassf6					
Rbbp9					
Rbl1					
Rccd1					
Rcn3					
Recql4					
Reep4					
Rfc3					
Rin3					
Rmi2					
Rpain					
Rrm2					
Rsl1D1					
Rspo1					
Rsrc1					
Rsu1					
S100A16					
Saal1					
Samd4A					
Scd5					
Setbp1					
Sept6					
Sfrp4					
Sfxn1					
Sfxn2					
Sfxn5					
Sh2B2					
Shcbp1					
Sirt5					
Skp2					
Slc25A19					
Slc25A22					
Slc29A1					
Slc35B4					
Smc2					
Snip1					
Snrnp27					
Socs4					
Spag5					
Spata5					
Spc25					
Spon1					
Spr					

Differentially expressed genes during late pregnancy					
MM vs. AdM		SmM vs. MM		SmM vs. AdM	
MM	AdM	SmM	MM	SmM	AdM
Sptssb					
Srd5A2					
Srfbp1					
St3Gal1					
St3Gal4					
Steap3					
Stk10					
Stk17B					
Strip2					
Sufu					
Sult2B1					
Susd3					
Tapt1					
Tbc1D14					
Tcf19					
Tdrkh					
Tes					
Tgm2					
Thnsl2					
Tk1					
Tmem106A					
Tmem144					
Tmem30B					
Tmem48					
Tnrc18					
Tor3A					
Tpx2					
Traf3					
Trim37					
Trmt61A					
Tstd3					
Ttk					
Tuft1					
Uhrf1					
Urgcp					
Vmac					
Vsnl1					
Wdhd1					
Wdr17					
Wdr25					
Wdr34					
Wdr35					
Wdr46					

Differentially expressed genes during late pregnancy					
MM vs. AdM		SmM vs. MM		SmM vs. AdM	
MM	AdM	SmM	MM	SmM	AdM
Wdr66					
Wscd2					
Yars2					
Zbtb12					
Zbtb38					
Zcchc2					
Znf200					
Znf250					
Znf345					
Znf383					
Znf507					
Znf575					
Znf618					
Znf683					
Zwilch					

Appendix B: Gene ontology

Table B. 1 Gene Ontology (GO) analysis carried out on ovine mammary genes identified as differentially expressed by RNA-seq. This analysis was carried out using the DAVID functional annotation clustering which groups similar annotations together under a parent GO term, and calculates an enrichment score (ES). Results shown for comparison between late pregnancy and lactation (showing GO terms enriched in late pregnancy or lactation), and for comparison during late pregnancy between offspring born to dams fed maintenance (MM) or *ad libitum* (AdM) during early pregnancy (no GO terms were significantly enriched in AdM, therefore only MM is shown)[1].

Physiological state/ treatment[2]	GO cluster term	ES[3]	Count	Genes
Late pregnancy				
Mitochondria		4.14		
	Mitochondrion	1.73	59	*Atp5d, Gm2a, Bphl, Alas1, Acot8, Htra2, Gbas, Casp8, Atp5o, Cat, Atp5h, Brd8, Aldh6a1, Acaa2, Crls1, Gatm, Decr1, Acadvl, Aldh7a1, Chrdl2, C1qbp, Grn, Atp5c1, Ctsb, Hsd17b10, Cav2, Cav1, Ech1, Fdx1, Aldoc, Kiaa0101, Atp5g2, Elk3, Acat1, Kars, Hadha, Hadhb, Dtd1, Gfm2, Gstk1, Csde1, Hsd17b4, Etfb, Etfa, Acsl5, Fh, Bckdha, Idh3b, Ak2, Bad, Cpt1a, Chchd10, Gck, Hspd1, Atp5a1, Hibch, Ifi6, Acsm5, Dut*
	Mitochondrial part	2.09	35	*Atp5d, Hsd17b10, Fdx1, Atp5g2, Acat1, Kars, Hadha, Dtd1, Hadhb, Alas1, Htra2, Gstk1, Casp8, Csde1, Atp5o, Cat, Atp5h, Etfb, Acsl5, Etfa, Fh, Bckdha, Acaa2, Crls1, Gatm, Ak2, Idh3b, Bad, Cpt1a, Acadvl, C1qbp, Atp5c1, Hspd1, Atp5a1, Acsm5*
	Mitochondrial lumen	2.72	20	*Atp5d, Bckdha, Fdx1, Idh3b, Acat1, Hadha, Kars, Hadhb, Dtd1, Acadvl, Alas1, C1qbp, Gstk1, Atp5c1, Atp5a1, Hspd1, Etfb, Acsm5, Fh, Etfa*
	Mitochondrial matrix	2.72	20	*Atp5d, Bckdha, Fdx1, Idh3b, Acat1, Hadha, Kars, Hadhb, Dtd1, Acadvl, Alas1, C1qbp, Gstk1, Atp5c1, Atp5a1, Hspd1, Etfb, Acsm5, Fh, Etfa*
	Transit peptide	2.19	30	*Atp5d, Ech1, Fdx1, Atp5g2, Acat1, Hadha, Kars, Dtd1, Hadhb, Gfm2, Alas1, Htra2, Atp5o, Fh, Etfa, Bckdha, Aldh6a1, Acaa2, Gatm, Idh3b, Decr1, Acadvl, Chchd10, C1qbp, Atp5c1, Hspd1, Atp5a1, Hibch, Acsm5, Dut*
	Mitochondrion	1.85	39	*Atp5d, Hsd17b10, Ech1, Fdx1, Kiaa0101, Atp5g2, Acat1, Kars, Hadha, Dtd1, Hadhb, Gfm2, Alas1, Htra2, Atp5o, Atp5h, Etfb, Acsl5, Etfa, Fh, Bckdha,*

Physiological state/ treatment[2]	GO cluster term	ES[3]	Count	Genes
Late pregnancy				
	Transit peptide: Mitochondrion	2.09	28	*Aldh6a1, Acaa2, Crls1, Gatm, Idh3b, Ak2, Bad, Decr1, Cpt1a, Acadvl, Chchd10, C1qbp, Atp5c1, Hspd1, Atp5a1, Hibch, Acsm5, Dut Atp5d, Ech1, Fdx1, Atp5g2, Acat1, Hadha, Dtd1, Hadhb, Gfm2, Alas1, Htra2, Atp5o, Fh, Etfa, Bckdha, Aldh6a1, Gatm, Idh3b, Decr1, Acadvl, Chchd10, C1qbp, Atp5c1, Atp5a1, Hspd1, Hibch, Acsm5, Dut*
Translation initiation		4.02		
	Protein biosynthesis	3.42	21	*Eef1b2, Aimp1, Eif2s3, Eef2, Kars, Dtd1, Eif4b, Eif3d, Gfm2, Eif3g, Eif3h, Eif3e, Eif1ax, Eif3f, Farsb, Eif3k, Fau, Eif3l, Eef1g, Eef1d, Eif3m*
	Translation	2.66	24	*Rpl36a, Eef1b2, Aimp1, Eif2s3, Eef2, Denr, Kars, Dtd1, Eif4b, Eif3d, Gfm2, Eif3g, Eif3h, Eif3e, Eif1ax, Eif3f, Farsb, Eif3k, Eef2k, Fau, Eif3l, Eef1g, Eef1d, Eif3m*
	Eukaryotic translation initiation factor 3 complex	6.90	8	*Eif3d, Eif3g, Eif3h, Eif3e, Eif3f, Eif3k, Eif3l, Eif3m*
	Translation factor activity, nucleic acid binding	2.77	18	*Eef1b2, Eif2s3, Eef2, Denr, Eif4b, Eif3d, Gfm2, Eif3g, Eif3h, Eif3e, Eif1ax, Eif3f, Eif3k, Eef2k, Eif3l, Eef1g, Eef1d, Eif3m*
	Translational initiation	4.09	11	*Eif3d, Eif3g, Eif3h, Eif1ax, Eif3e, Eif3f, Eif3k, Eif2s3, Eif3l, Denr, Eif3m*
	Initiation factor	3.23	11	*Eif4b, Eif3d, Eif3g, Eif3h, Eif1ax, Eif3e, Eif3f, Eif3k, Eif2s3, Eif3l, Eif3m*
	Translation initiation factor activity	2.77	12	*Eif4b, Eif3d, Eif3g, Eif3h, Eif1ax, Eif3e, Eif3f, Eif3k, Eif2s3, Eif3l, Denr, Eif3m*
Cell cycle		3.53		
	Cell cycle	2.20	36	*Kif23, Cks1b, Ing4, Kifc1, Cetn3, Aurkb, Ccng1, Ccng2, Kif2c, Cdca8, Katna1, Ccna2, Cdca3, Khdrbs1, Ckap2, Cdk1, Erh, Kat2b, Kif11, Lig1, Dlgap5, Cinp, Ccnf, Cenpf, Birc5, Hmg20b, Pmf1, Cdk4, Ahr, Ccnb1, Ccnd1, Fancd2, Cks2, Bub1b, G0s2, Gadd45a*
	Cell division	2.63	26	*Kif23, Kifc1, Cks1b, Cetn3, Aurkb, Ccng1, Ccng2, Kif2c, Cdca8, Katna1, Ccna2,*

Physiological state/ treatment[2]	GO cluster term	ES[3]	Count	Genes
Late pregnancy				
				Cdca3, Cdk1, Kif11, Lig1, Ccnf, Cinp, Cenpf, Birc5, Pmf1, Cdk4, Ccnb1, Ccnd1, Cks2, Bub1b, Cdca7l
	Spindle	2.89	19	*Ckap2, Kif23, Kifc1, Kif22, Cdk1, Kif4a, Kif11, Dlgap5, Cenpf, Cbx3, Birc5, Aurkb, App, Cdca8, Fam82b, Katna1, Calm3, Bub1b, Hspb1, Calm2*
	Mitosis	2.41	22	*Kif23, Kifc1, Kif22, Cdk1, Kif11, Dlgap5, Ccnf, Cetn3, Cenpf, Birc5, Pmf1, Aurkb, Ccng1, Ccng2, Dctn2, Ccnb1, Kif2c, Cdca8, Katna1, Bub1b, Ccna2, Cdca3*
	M phase of mitotic cell cycle	2.41	22	*Kif23, Kifc1, Kif22, Cdk1, Kif11, Dlgap5, Ccnf, Cetn3, Cenpf, Birc5, Pmf1, Aurkb, Ccng1, Ccng2, Dctn2, Ccnb1, Kif2c, Cdca8, Katna1, Bub1b, Ccna2, Cdca3*
	Nuclear division	2.41	22	*Kif23, Kifc1, Kif22, Cdk1, Kif11, Dlgap5, Ccnf, Cetn3, Cenpf, Birc5, Pmf1, Aurkb, Ccng1, Ccng2, Dctn2, Ccnb1, Kif2c, Cdca8, Katna1, Bub1b, Ccna2, Cdca3*
	Mitosis	2.62	19	*Kif23, Kifc1, Cdk1, Kif11, Ccnf, Cetn3, Cenpf, Birc5, Pmf1, Aurkb, Ccng1, Ccng2, Ccnb1, Kif2c, Cdca8, Katna1, Bub1b, Ccna2, Cdca3*
	Cell cycle	1.71	47	*Kif23, Cks1b, Ing4, Kif22, Kifc1, Dtymk, Cetn3, Aurkb, Ccng1, Itgb1, Ccng2, Kif2c, App, Cdca8, Cenpa, Krt7, Katna1, Hbp1, Ccna2, Cdca3, Khdrbs1, Ckap2, Cdk1, Erh, Kat2b, Kif11, Lig1, Dlgap5, Cinp, Ccnf, Anxa1, Cenpf, Birc5, Hmg20b, Pmf1, Cdk4, Ahr, Dctn2, Ccnb1, Ccnd1, Fancd2, Cks2, Calm3, Bub1b, G0s2, Apbb2, Gadd45a, Calm2*
	Organelle fission	2.36	22	*Kif23, Kifc1, Kif22, Cdk1, Kif11, Dlgap5, Ccnf, Cetn3, Cenpf, Birc5, Pmf1, Aurkb, Ccng1, Ccng2, Dctn2, Ccnb1, Kif2c, Cdca8, Katna1, Bub1b, Ccna2, Cdca3*
	Mitotic cell cycle	2.02	29	*Kif23, Kifc1, Kif22, Cetn3, Aurkb, Ccng1, Ccng2, Itgb1, Kif2c, Cdca8, App, Cenpa, Katna1, Ccna2, Cdca3, Khdrbs1, Cdk1, Kif11, Dlgap5, Ccnf, Cenpf, Birc5, Pmf1, Cdk4, Dctn2, Ccnb1, Ccnd1, Bub1b, Gadd45a*
	Cell cycle phase	1.94	31	*Kif23, Kifc1, Kif22, Cetn3, Aurkb, Ccng1, Itgb1, Ccng2, Kif2c, App, Cdca8,*

Physiological state/ treatment[2]	GO cluster term	ES[3]	Count	Genes
Late pregnancy				
				Katna1, Krt7, Ccna2, Cdca3, Khdrbs1, Cdk1, Kif11, Dlgap5, Ccnf, Cenpf, Birc5, Pmf1, Cdk4, Dctn2, Ccnb1, Ccnd1, Fancd2, Cks2, Bub1b, Gadd45a
	M phase	2.13	24	*Kif23, Kifc1, Kif22, Cdk1, Kif11, Dlgap5, Ccnf, Cetn3, Cenpf, Birc5, Pmf1, Aurkb, Ccng1, Ccng2, Dctn2, Ccnb1, Kif2c, Cdca8, Fancd2, Katna1, Cks2, Bub1b, Ccna2, Cdca3*
	Cell cycle process	1.79	36	*Kif23, Kifc1, Kif22, Ing4, Cetn3, Aurkb, Ccng1, Itgb1, Ccng2, Kif2c, App, Cdca8, Cenpa, Krt7, Katna1, Hbp1, Ccna2, Cdca3, Khdrbs1, Cdk1, Kif11, Kat2b, Dlgap5, Ccnf, Cenpf, Birc5, Pmf1, Cdk4, Dctn2, Ccnb1, Ccnd1, Fancd2, Cks2, Bub1b, Apbb2, Gadd45a*
	Cell division	2.02	25	*Kif23, Kifc1, Cks1b, Cetn3, Aurkb, Ccng1, Ccng2, Cdca8, Katna1, Ccna2, Cdca3, Cdk1, Kif11, Lig1, Ccnf, Cinp, Cenpf, Birc5, Pmf1, Cdk4, Ccnb1, Ccnd1, Cks2, Bub1b, Cdca7l*
	Microtubule cytoskeleton	1.55	39	*Kif23, Kifc1, Kif22, Kif4a, Dync2li1, Cetn3, Cbx3, Aurkb, Kifc3, Kif2c, Cdca8, App, Dcaf12, Fnta, Dynll2, Katna1, Cdk5rap2, Apex1, Ckap2, Cdk1, Gabarapl1, Kif11, Cryab, Dlgap5, Dynlt3, Cenpf, Birc5, Dctn4, Dctn2, Ccnb1, Kif1c, Eml3, Fam82b, Ift57, Calm3, Bub1b, Hspb1, Calm2, Dync1i2, Kif20a*
	Regulation of cell cycle	1.57	24	*Bmp4, Cks1b, Cdk1, Cav2, Dlgap5, Cenpf, Birc5, Cdk4, Ccng1, Itgb1, Ccng2, Ccnb1, App, Ccnd1, Fnta, Hdac2, Id2, Gadd45g, Cks2, Bub1b, Id3, Apbb2, Ccna2, Gadd45a*
Chromosome		3.50		
	Chromosomal protein	4.32	14	*Cenpn, Hmgb2, Hmgn2, Hmgb3, Hp1bp3, Cenpf, Birc5, Aurkb, Kif2c, Cdca8, Fancd2, Cenpa, Hnrnpd, H3f3a, H3f3b*
	Chromosome	2.12	27	*Kif22, Hmgn3, Hmgb2, Hmgb3, Hmgn2, Hp1bp3, Cbx3, Aurkb, Calcoco1, Kif2c, Cdca8, Cenpa, Eif3e, Hnrnpd, Ercc1, Chd3, Cenpn, Cenpf, Birc5, Pmf1, Dctn2, Chrac1, Hdac2, Id2, Fancd2, Bub1b, H3f3a, H3f3b*
	Chromosomal part:	2.12	23	*Cenpn, Kif22, Hmgn3, Hmgn2, Hp1bp3, Cenpf, Cbx3, Birc5, Pmf1, Aurkb,*

Physiological state/ treatment[2]	GO cluster term	ES[3]	Count	Genes
Late pregnancy				
	chromosome, centromeric region	2.53	12	*Calcoco1, Dctn2, Chrac1, Kif2c, Cdca8, Hdac2, Id2, Cenpa, Eif3e, Bub1b, H3f3a, H3f3b, Ercc1, Chd3* *Kif2c, Cenpn, Kif22, Cdca8, Cenpa, Bub1b, Cenpf, Cbx3, Birc5, Pmf1, Aurkb, Dctn2*
Cytoskeleton		2.84		
	Non-membrane-bounded organelle	1.35	116	*Kifc1, Rpl36a, Hmgn3, Hmgn2, Btd, Dync2li1, Cbx3, Aurkb, C1qc, Kifc3, Cdca8, App, Fnta, Fau, Gnl2, Ftl, Cryab, Fmr1, Grhl1, Dctn4, Farp1, Dctn2, Chrac1, Jup, Arpc1b, Cstb, Hspb1, Kif4a, Hmgb2, Hmgb3, Actr3b, Aldoc, Elk3, Arpc5, Hadha, Fuz, Hadhb, Hnrnpa3, Hnrnpm, Arpc3, Frg1, Katna1, Hnrnpf, Eif3e, Hnrnpd, Eif3l, Ckap2, Gabarapl1, Pmf1, Fxr1, Coro1a, Hdac2, Lasp1, H3f3a, H3f3b, Kif23, Gypc, Kif22, Lima1, Hp1bp3, Fermt2, Fubp1, Actr2, Kif2c, Dcaf12, Dynll2, Casp8, Cdk5rap2, Ifngr1, Ino80b, Brd8, Cdk1, Adam10, Kif11, Exosc5, Dynlt3, Las1l, Flnb, Elmo3, Arhgap26, Kif1c, Epb41l2, Acadvl, Eml3, Fam82b, Fancd2, Ipo5, Bub1b, Aldh9a1, Dync1i2, Gltscr2, Cnn3, Cald1, Cetn3, Calcoco1, Krt5, Cenpa, Krt7, Gpsm2, Cnn2, Apex1, Ercc1, Chd3, Cenpn, Dlgap5, Anxa1, Cenpf, Birc5, Cotl1, Itpr1, Fbl, Ccnb1, Id2, Ilf2, Ift57, Calm3, Calm2, Kif20a*
	Intracellular non-membrane-bounded organelle	1.35	116	*Kifc1, Rpl36a, Hmgn3, Hmgn2, Btd, Dync2li1, Cbx3, Aurkb, C1qc, Kifc3, Cdca8, App, Fnta, Fau, Gnl2, Ftl, Cryab, Fmr1, Grhl1, Dctn4, Farp1, Dctn2, Chrac1, Jup, Arpc1b, Cstb, Hspb1, Kif4a, Hmgb2, Hmgb3, Actr3b, Aldoc, Elk3, Arpc5, Hadha, Fuz, Hadhb, Hnrnpa3, Hnrnpm, Arpc3, Frg1, Katna1, Hnrnpf, Eif3e, Hnrnpd, Eif3l, Ckap2, Gabarapl1, Pmf1, Fxr1, Coro1a, Hdac2, Lasp1, H3f3a, H3f3b, Kif23, Gypc, Kif22, Lima1, Hp1bp3, Fermt2, Fubp1, Actr2, Kif2c, Dcaf12, Dynll2, Casp8, Cdk5rap2, Ifngr1, Ino80b, Brd8, Cdk1, Adam10, Kif11, Exosc5, Dynlt3, Las1l, Flnb, Elmo3, Arhgap26, Kif1c, Epb41l2, Acadvl, Eml3, Fam82b, Fancd2, Ipo5, Bub1b, Aldh9a1, Dync1i2, Gltscr2, Cnn3, Cald1, Cetn3, Calcoco1, Krt5, Cenpa, Krt7, Gpsm2, Cnn2, Apex1, Ercc1, Chd3, Cenpn,*

Physiological state/ treatment[2]	GO cluster term	ES[3]	Count	Genes
Late pregnancy				
				Dlgap5, Anxa1, Cenpf, Birc5, Cotl1, Itpr1, Fbl, Ccnb1, Id2, Ilf2, Ift57, Calm3, Calm2, Kif20a
	Cytoskeleton	1.68	43	*Kif23, Kifc1, Lima1, Kif4a, Actr3b, Cald1, Dync2li1, Fermt2, Cetn3, Aurkb, Arpc5, Fuz, Kif2c, Actr2, Cdca8, Dcaf12, Arpc3, Krt5, Katna1, Itgb7, Itgb6, Cdk5rap2, Ckap2, Dlgap5, Cenpf, Birc5, Cotl1, Dctn4, Flnb, Arhgap26, Dctn2, Jup, Ccnb1, Epb41l2, Arpc1b, Coro1a, Fam82b, Itga6, Lasp1, Ift57, Calm3, Bub1b, Hspb1, Calm2*
	Cytoskeletal part	1.47	55	*Kif23, Kif22, Kifc1, Lima1, Dync2li1, Fermt2, Cbx3, Aurkb, Kifc3, Kif2c, Actr2, Dcaf12, App, Cdca8, Fnta, Dynll2, Cdk5rap2, Ifngr1, Cdk1, Kif11, Adam10, Cryab, Dynlt3, Dctn4, Dctn2, Epb41l2, Kif1c, Arpc1b, Eml3, Fam82b, Bub1b, Hspb1, Dync1i2, Kif4a, Cnn3, Cald1, Cetn3, Arpc5, Krt5, Arpc3, Krt7, Katna1, Apex1, Ckap2, Gabarapl1, Dlgap5, Cenpf, Birc5, Itpr1, Ccnb1, Coro1a, Lasp1, Ift57, Calm3, Calm2, Kif20a*
	Microtubule cytoskeleton	1.55	39	*Kif23, Kifc1, Kif22, Kif4a, Dync2li1, Cetn3, Cbx3, Aurkb, Kifc3, Kif2c, Cdca8, App, Dcaf12, Fnta, Dynll2, Katna1, Cdk5rap2, Apex1, Ckap2, Cdk1, Gabarapl1, Kif11, Cryab, Dlgap5, Dynlt3, Cenpf, Birc5, Dctn4, Dctn2, Ccnb1, Kif1c, Eml3, Fam82b, Ift57, Calm3, Bub1b, Hspb1, Calm2, Dync1i2, Kif20a*
	Cytoskeleton	1.25	70	*Kif23, Kif22, Gypc, Kifc1, Lima1, Rpl36a, Fermt2, Dync2li1, Cbx3, Aurkb, Kifc3, Actr2, Kif2c, Dcaf12, App, Cdca8, Fnta, Dynll2, Casp8, Cdk5rap2, Ifngr1, Cdk1, Kif11, Adam10, Cryab, Dynlt3, Flnb, Dctn4, Elmo3, Arhgap26, Farp1, Dctn2, Kif1c, Epb41l2, Jup, Eml3, Arpc1b, Fam82b, Bub1b, Hspb1, Aldh9a1, Dync1i2, Kif4a, Cnn3, Actr3b, Aldoc, Cald1, Cetn3, Arpc5, Fuz, Krt5, Arpc3, Katna1, Krt7, Cnn2, Apex1, Ckap2, Gabarapl1, Dlgap5, Anxa1, Cenpf, Birc5, Cotl1, Itpr1, Ccnb1, Coro1a, Lasp1, Ift57, Calm3, Calm2, Kif20a*
Response to hormone		2.11		2.11
	Response to organic substance	1.62	53	*Hsp90ab1, Atp6v0e1, Igfbp7, Dek, Cited1, Asah1, B2m, Eif4ebp1, Fnta, Cd44, Gata3, Casp8, Ddah2, Ccna2, Gng5, Adam10, Gatm, Cryab, Colec12, Clic1,*

Physiological state/ treatment[2]	GO cluster term	ES[3]	Count	Genes
Late pregnancy				
				C1qb, Ddr1, Ccnd1, Hspb1, Ctsc, Col1a1, Ca2, Amfr, Cav2, Cav1, Hmgb2, Erbb3, Aldoc, Erbb2, Gng11, C1s, Ccl5, Hprt1, Aldh1a2, Bmp4, Bckdha, Gnao1, Kat2b, Klf10, Ggh, Coro1a, Hdac2, Gck, Id2, Cxcl16, Hspd1, Id3, Igfbp2
	Response to steroid hormone stimulus	2.15	18	*Bckdha, Bmp4, Cav2, Cav1, Hmgb2, Cryab, Erbb2, Igfbp7, Ccl5, Cited1, Aldh1a2, C1qb, Ccnd1, Gata3, Ca2, Col1a1, Igfbp2, Ccna2*
	Response to oestrogen stimulus	2.72	11	*Bmp4, Cav2, Aldh1a2, Cav1, Ccnd1, Cryab, Gata3, Ca2, Igfbp2, Ccna2, Cited1*
	Response to endogenous stimulus	1.57	29	*Cav2, Hmgb2, Cav1, Atp6v0e1, Erbb3, Erbb2, Aldoc, Igfbp7, Gng11, Hprt1, Ccl5, Cited1, Aldh1a2, Eif4ebp1, Gata3, Ccna2, Gng5, Bckdha, Bmp4, Gnao1, Kat2b, Gatm, Cryab, Ggh, C1qb, Ccnd1, Col1a1, Ca2, Igfbp2*
	Response to hormone stimulus	1.53	25	*Cav2, Cav1, Hmgb2, Erbb3, Erbb2, Igfbp7, Gng11, Ccl5, Cited1, Aldh1a2, Eif4ebp1, Gata3, Ccna2, Gng5, Bmp4, Bckdha, Kat2b, Gatm, Cryab, Ggh, C1qb, Ccnd1, Col1a1, Ca2, Igfbp2*
	Response to corticosteroid stimulus	1.99	9	*Bckdha, Bmp4, C1qb, Cav1, Ccnd1, Igfbp7, Col1a1, Igfbp2, Ccl5*

Physiological state/ treatment[2]	GO cluster term	ES[3]	Count	Genes
Lactation				
	Endoplasmic reticulum	7.73		
	Endoplasmic reticulum	3.01	53	*Hm13, Hmgcr, Bscl2, Erlec1, Fdft1, Elovl1, Agpat6, Dnajb11, Insig1, Creb3l1, Elovl6, Dnajc3, Ddost, Dhcr24, Kdelr2, Aifm1, Ergic1, Bcap31, Dgat1, Eif2ak3, Degs1, Ggcx, Extl3, Ganab, Creld2, Hax1, Hsd17b12, Gpaa1, Lman2, Edem3, Extl1, Lman1, Epm2aip1, Acsl1, Dhcr7, Ero1l, Hspa5, Ebp, Herpud1, Fads1, Fads2, Ephx1, Crat, Itpr2, Dolk, Hyou1, Erp44, Cyp17a1, Clgn, Atp2a2, Dpm2, Hspa13, Agr2*
	Endoplasmic reticulum	2.39	65	*Copa, Hm13, Hmgcr, Bscl2, Erlec1, Fdft1, Elovl1, Atp2b2, Agpat6, Dnajb11, Insig1, Creb3l1, Elovl6, Dnajc3, Ddost, Ifngr2, Dhcr24, Agpat1, Kdelr2, Golt1b, Aifm1, Ccdc47, Krtcap2, Ergic1, Bcap31, Dhrs1, Dgat1, Eif2ak3, Degs1, Ggcx, Extl3, Ganab, Creld2, Hax1, Hsd17b12, Gpaa1, Fkbp1a, Lman2, Edem3, Extl1, Lman1, Epm2aip1, Acsl1, Dhcr7, Ero1l, Hspa5, Hsd17b7, Ehd4, Ebp, Herpud1, Fads1, Fads2, Ephx1, Crat, Itpr2, Dolk, Erp44, Hyou1, Cyp17a1, Dnajb9, Clgn, Atp2a2, Dpm2, Hspa13, Agr2*
	Endoplasmic reticulum part	3.03	30	*Extl3, Hmgcr, Bscl2, Gpaa1, Fkbp1a, Edem3, Lman1, Extl1, Erlec1, Fdft1, Dnajb11, Dhcr7, Hspa5, Elovl6, Ero1l, Ddost, Hsd17b7, Dhcr24, Herpud1, Aifm1, Krtcap2, Bcap31, Itpr2, Dolk, Erp44, Hyou1, Cyp17a1, Atp2a2, Dpm2, Eif2ak3*
	Endoplasmic reticulum membrane	3.39	25	*Extl3, Hmgcr, Bscl2, Gpaa1, Fkbp1a, Lman1, Extl1, Fdft1, Dhcr7, Elovl6, Ero1l, Hspa5, Ddost, Hsd17b7, Dhcr24, Herpud1, Krtcap2, Dolk, Itpr2, Bcap31, Erp44, Cyp17a1, Atp2a2, Dpm2, Eif2ak3*
	Nuclear envelope-endoplasmic reticulum network	3.22	25	*Extl3, Hmgcr, Bscl2, Gpaa1, Fkbp1a, Lman1, Extl1, Fdft1, Dhcr7, Elovl6, Ero1l, Hspa5, Ddost, Hsd17b7, Dhcr24, Herpud1, Krtcap2, Dolk, Itpr2, Bcap31, Erp44, Cyp17a1, Atp2a2, Dpm2, Eif2ak3*
	Organelle membrane	1.93	57	*Copa, Ckmt1b, Hmgcr, Ap2s1, Gcat, Bscl2, Cltc, Fdft1, Copb2, Efhd1, Gbf1, Fxc1, Gpx4, Elovl6, Atp6v0d1, Fndc3a, Ddost, Golga3, Dhcr24, Bsg, Krtcap2, Bcap31, Atp6v1f, Dhrs1, Chpf, Arcn1, Gpam, Eif2ak3, Extl3, Ap1m1,*

Physiological state/ treatment[2]	GO cluster term	ES[3]	Count	Genes
Lactation				
				Aldh18a1, Hax1, Chkb, Abcd1, Gpaa1, Cytb, Fkbp1a, Extl1, Lman1, Arfgef2, Acsl1, Dhcr7, Hspa5, Ero1l, Hsd17b7, Gabarapl2, Cpt1b, Herpud1, Crat, Cd63, Itpr2, Dolk, Erp44, Cyp17a1, Atp2a2, Large, Dpm2, Gk
	Endomembrane system	1.82	43	*Copa, Extl3, Ap1m1, Hax1, Hmgcr, Ap2s1, Gpaa1, Bscl2, Fkbp1a, Extl1, Lman1, Cltc, Arfgef2, Itsn1, Fdft1, Copb2, Gbf1, Dhcr7, Gpx4, Hspa5, Ero1l, Elovl6, Fndc3a, Ddost, Golga3, Hsd17b7, Dhcr24, Gabarapl2, Bsg, Herpud1, Krtcap2, Bcap31, Itpr2, Dolk, Erp44, Chmp1a, Cyp17a1, Atp2a2, Large, Chpf, Arcn1, Dpm2, Eif2ak3*
Vesicle		4.90		
	Cell fraction	1.88	62	*Copa, Hmgcr, Bcar1, Btc, Foxo3, Itsn1, Fdft1, Fos, Atp2b2, Agpat6, Dynll1, Gpx4, Gpx3, Atp6v0d1, Fndc3a, Ddost, Ceacam1, Aifm1, Ccdc88c, Aars, Ccdc47, Irs1, Atp6v1f, Dgat1, Adrb1, Adm, Aven, Emp1, Degs1, Galnt3, Ggcx, Hax1, Chkb, Hmgcs1, Cytb, Asns, Fkbp1a, Cyth3, Lman1, Gch1, Acsl1, Kras, Ero1l, Actb, Gabarapl2, Cpt1b, Ebp, Fads1, Acaca, Gars, Fads2, Ephx1, Eprs, Capn2, Itpr2, Dolk, Lin7a, Cd55, Atp2a2, Cd59, Fabp4, Hspa13, Dusp6*
	Vesicular fraction	3.05	21	*Cpt1b, Ebp, Copa, Hmgcr, Aifm1, Fads1, Chkb, Ephx1, Ccdc47, Lman1, Irs1, Itpr2, Fdft1, Atp2b2, Adrb1, Dgat1, Acsl1, Atp2a2, Hspa13, Ero1l, Ddost, Fndc3a*
	Microsome	2.97	20	*Cpt1b, Ebp, Copa, Hmgcr, Aifm1, Fads1, Chkb, Ephx1, Ccdc47, Lman1, Irs1, Itpr2, Fdft1, Atp2b2, Adrb1, Dgat1, Acsl1, Atp2a2, Hspa13, Ero1l, Ddost*
	Insoluble fraction	1.86	46	*Galnt3, Ggcx, Copa, Hmgcr, Chkb, Bcar1, Cytb, Fkbp1a, Foxo3, Cyth3, Lman1, Itsn1, Fdft1, Atp2b2, Fos, Agpat6, Kras, Acsl1, Dynll1, Ero1l, Atp6v0d1, Fndc3a, Ddost, Ceacam1, Gabarapl2, Cpt1b, Ebp, Aifm1, Ccdc88c, Fads1, Fads2, Ephx1, Ccdc47, Capn2, Irs1, Itpr2, Dolk, Lin7a, Atp6v1f, Adrb1, Dgat1, Atp2a2, Cd59, Aven, Hspa13, Emp1, Degs1*
	Membrane fraction	1.85	44	*Galnt3, Ggcx, Copa, Hmgcr, Chkb, Bcar1, Cytb, Fkbp1a, Foxo3, Cyth3, Lman1, Itsn1, Fdft1, Atp2b2, Fos, Agpat6, Kras, Acsl1, Dynll1, Ero1l, Atp6v0d1,*

Physiological state/ treatment[2]	GO cluster term	ES[3]	Count	Genes
Lactation				
				Fndc3a, Ddost, Ceacam1, Gabarapl2, Cpt1b, Ebp, Aifm1, Fads1, Fads2, Ephx1, Ccdc47, Irs1, Itpr2, Dolk, Lin7a, Atp6v1f, Adrb1, Dgat1, Atp2a2, Cd59, Aven, Hspa13, Emp1, Degs1
	Fatty acid biosynthesis	3.43		
	Organic acid biosynthetic process	3.41	15	*Aldh18a1, Bcat2, Ass1, Fads1, Acaca, Fads2, Asns, Cdo1, Elovl1, Fasn, Elovl6, Agpat1, Alox12, Degs1, Cbs*
	Carboxylic acid biosynthetic process	3.41	15	*Aldh18a1, Bcat2, Ass1, Fads1, Acaca, Fads2, Asns, Cdo1, Elovl1, Fasn, Elovl6, Agpat1, Alox12, Degs1, Cbs*
	Fatty acid biosynthesis	5.54	8	*Elovl1, Fads1, Acaca, Fasn, Fads2, Cbr4, Elovl6, Degs1*
	Fatty acid biosynthetic process	3.14	8	*Elovl1, Fads1, Acaca, Fasn, Fads2, Elovl6, Alox12, Degs1*
	Lipid synthesis	3.39		
	Lipid synthesis	5.23	17	*Ebp, Hmgcr, Fads1, Hsd17b12, Fdps, Acaca, Hmgcs1, Fads2, Cbr4, Fdft1, Elovl1, Dhcr7, Fasn, Elovl6, Hsd17b7, Dhcr24, Degs1*
	Cholesterol biosynthesis	12.12	7	*Ebp, Hmgcr, Dhcr7, Hmgcs1, Fdps, Dhcr24, Fdft1*
	Sterol biosynthesis	10.77	7	*Ebp, Hmgcr, Dhcr7, Hmgcs1, Fdps, Dhcr24, Fdft1*
	Steroid biosynthesis	6.23	9	*Ebp, Hmgcr, Dhcr7, Hsd17b12, Hmgcs1, Fdps, Hsd17b7, Dhcr24, Fdft1*
	Cholesterol biosynthetic process	7.55	7	*Ebp, Hmgcr, Dhcr7, Hmgcs1, Fdps, Dhcr24, Fdft1*
	Steroid biosynthetic process	4.19	11	*Ebp, Cyp17a1, Adm, Hmgcr, Dhcr7, Hsd17b12, Hmgcs1, Fdps, Hsd17b7, Dhcr24, Fdft1*
	Sterol biosynthetic process	6.97	7	*Ebp, Hmgcr, Dhcr7, Hmgcs1, Fdps, Dhcr24, Fdft1*
	Steroid metabolic process	2.90	15	*Ebp, Hdlbp, Hmgcr, Hsd17b14, Hsd17b12, Fdps, Hmgcs1, Fdft1, Cyp17a1, Adm, Lcat, Dhcr7, Insig1, Hsd17b7, Dhcr24*
	Steroid biosynthesis	7.82	5	*Ebp, Dhcr7, Hsd17b7, Dhcr24, Fdft1*
	Cholesterol metabolic	3.41	10	*Ebp, Hdlbp, Hmgcr, Dhcr7, Lcat, Insig1, Hmgcs1, Fdps, Dhcr24, Fdft1*

Physiological state/ treatment[2]	GO cluster term	ES[3]	Count	Genes
Lactation				
	process			
	Sterol metabolic process	3.24	10	*Ebp, Hdlbp, Hmgcr, Dhcr7, Lcat, Insig1, Hmgcs1, Fdps, Dhcr24, Fdft1*
	Isoprenoid biosynthetic process	4.98	5	*Hmgcr, Hmgcs1, Fdps, Dolk, Fdft1*
	Terpenoid backbone biosynthesis	5.47	4	*Dhdds, Hmgcr, Hmgcs1, Fdps*
	Isoprenoid metabolic process	3.60	5	*Hmgcr, Hmgcs1, Fdps, Dolk, Fdft1*
	NADP	1.95	9	*Aldh18a1, Hmgcr, Dhcr7, Hsd17b12, Fasn, Aass, Cbr4, Hsd17b7, Fdft1*
	ER unfolded protein response	3.13		
	ER-nuclear signalling pathway	6.13	9	*Herpud1, Atp2a2, Insig1, Aars, Ccdc47, Ero1l, Hspa5, Eif2ak2, Eif2ak3*
	Response to unfolded protein	3.70	14	*Herpud1, Aars, Edem3, Hsph1, Erp44, Hspa6, Creb3l1, Dnajb2, Ero1l, Dnajb1, Dnajc3, Eif2ak2, Eif2ak3, Hspa8*
	Response to endoplasmic reticulum stress	5.18	8	*Herpud1, Atf4, Aars, Ccdc47, Ero1l, Hspa5, Eif2ak2, Eif2ak3*
	Endoplasmic reticulum unfolded protein response	4.98	5	*Herpud1, Aars, Ero1l, Eif2ak2, Eif2ak3*
	Cellular response to unfolded protein	4.98	5	*Herpud1, Aars, Ero1l, Eif2ak2, Eif2ak3*
	Response to hormone	3.01		
	Response to organic substance	2.13	56	*Bcar1, Fos, Hsph1, Eif4ebp2, Gpx4, Lcat, Creb3l1, Angpt1, Lbp, Dnajc3, Ddost, Ghr, Egr1, Bsg, Aars, Adipor2, Esr1, Lifr, Il6r, Cdo1, Irs1, Junb, Krt19, Btg2, Adm, Eif2ak2, Eif2ak3, Alpl, Il1r1, Hmgcs1, Acp5, Asns, Fkbp1a, Edem3, Gch1, Acsl1, Kras, Hspa6, Ero1l, Hspa8, Herpud1, Fads1, Acaca, Ephx1, Dusp4, Erp44, Cdkn1a, Dusp1, Aldh2, Fabp3, Fabp4, Dnajb2, Dnajb1, Aacs,*

Physiological state/ treatment[2]	GO cluster term	ES[3]	Count	Genes
Lactation				
				Cd14, F2r
	Response to glucocorticoid stimulus	3.61	12	*Alpl, Fos, Cdkn1a, Kras, Dusp1, Adm, Lcat, Fabp4, Il6r, Cdo1, Junb, Ghr*
	Response to endogenous stimulus	1.96	29	*Alpl, Bcar1, Hmgcs1, Asns, Fos, Eif4ebp2, Kras, Gpx4, Lcat, Angpt1, Ghr, Bsg, Fads1, Aars, Adipor2, Esr1, Il6r, Cdo1, Irs1, Junb, Krt19, Cdkn1a, Dusp1, Adm, Btg2, Aldh2, Fabp3, Fabp4, Aacs*
	Response to corticosteroid stimulus	3.31	12	*Alpl, Fos, Cdkn1a, Kras, Dusp1, Adm, Lcat, Fabp4, Il6r, Cdo1, Junb, Ghr*
	Response to steroid hormone stimulus	2.53	17	*Alpl, Esr1, Il6r, Cdo1, Junb, Fos, Krt19, Cdkn1a, Kras, Dusp1, Adm, Lcat, Gpx4, Aldh2, Fabp4, Angpt1, Ghr*
	Response to hormone stimulus	1.90	25	*Alpl, Bcar1, Fos, Eif4ebp2, Kras, Gpx4, Lcat, Angpt1, Ghr, Bsg, Fads1, Adipor2, Esr1, Il6r, Cdo1, Irs1, Junb, Krt19, Cdkn1a, Dusp1, Adm, Btg2, Aldh2, Fabp3, Fabp4*
	Response to peptide hormone stimulus	2.43	12	*Bsg, Eif4ebp2, Btg2, Adm, Fads1, Bcar1, Fabp3, Il6r, Cdo1, Irs1, Junb, Ghr*
	Response to cAMP	4.09	6	*Fos, Bsg, Dusp1, Il6r, Cdo1, Junb*
	Response to radiation	1.04	7	*Fos, Cdkn1a, Kras, Dusp1, Hmgcr, Il6r, Junb*
Phospholipid biosynthesis		2.88		
	Lipid biosynthetic process	3.64	36	*Chka, Hmgcr, Gne, Chkb, Hsd17b12, Hmgcs1, Gpaa1, Acss2, Fdft1, Elovl1, Agpat6, Dhcr7, Lcat, Fasn, Etnk1, Elovl6, Agpat4, Hsd17b7, Agpat1, Dhcr24, Cpt1b, Ebp, Fads1, Acaca, Fdps, Fads2, Dolk, Cyp17a1, Dgat1, Large, Adm, Cd81, Fabp3, Dpm2, Gpam, Degs1, Alox12*
	Glycerolipid biosynthetic process	6.76	12	*Cpt1b, Chka, Chkb, Gpaa1, Agpat6, Dgat1, Lcat, Cd81, Fabp3, Etnk1, Dpm2, Gpam, Agpat1*
	Organophosphate metabolic process	4.09	18	*Chka, Cpt1b, Fads1, Chkb, Gpaa1, Fdft1, Gpd1l, Agpat6, Lcat, Gpx4, Cd81, Fabp3, Dpm2, Etnk1, Gk, Gk5, Agpat4, Gpam, Agpat1*

Physiological state/ treatment[2]	GO cluster term	ES[3]	Count	Genes
Lactation				
	Glycerophospholipid biosynthetic process	6.81	10	*Chka, Cpt1b, Agpat6, Chkb, Lcat, Cd81, Fabp3, Gpaa1, Dpm2, Etnk1, Agpat1*
	Phospholipid biosynthetic process	4.95	13	*Chka, Cpt1b, Fads1, Chkb, Gpaa1, Agpat6, Lcat, Cd81, Fabp3, Etnk1, Dpm2, Agpat4, Gpam, Agpat1*
	Phospholipid metabolic process	3.81	15	*Chka, Cpt1b, Chkb, Fads1, Gpaa1, Fdft1, Agpat6, Lcat, Gpx4, Cd81, Fabp3, Etnk1, Dpm2, Agpat4, Gpam, Agpat1*
	Glycerophospholipid metabolic process	4.32	10	*Chka, Cpt1b, Agpat6, Chkb, Lcat, Cd81, Fabp3, Gpaa1, Dpm2, Etnk1, Agpat1*
	Glycerolipid metabolic process	3.45	12	*Cpt1b, Chka, Chkb, Gpaa1, Agpat6, Dgat1, Lcat, Cd81, Fabp3, Etnk1, Dpm2, Gpam, Agpat1*
	O-acyltransferase activity	4.95	8	*Cpt1b, Agpat6, Dgat1, Chkb, Lcat, Crat, Agpat4, Gpam, Agpat1*
	Cellular amino acid derivative metabolic process	2.62	16	*Chka, Cpt1b, Ccbl1, Ckmt1b, Chkb, Azin1, Cdo1, Gch1, Atp2b2, Agpat6, Lcat, Gpx4, Gpx3, Fabp3, Etnk1, Gnmt, Ghr*
	Phosphatidylcholine biosynthetic process	8.09	5	*Chka, Cpt1b, Agpat6, Chkb, Lcat, Fabp3*
	Phospholipid biosynthesis	4.62	7	*Chka, Cpt1b, Agpat6, Chkb, Etnk1, Agpat4, Gpam, Agpat1*
	Glycerophospholipid metabolism	3.28	9	*Gpd1l, Chka, Cpt1b, Agpat6, Chkb, Lcat, Etnk1, Agpat4, Gpam, Agpat1*
	Phosphatidylcholine metabolic process	6.47	5	*Chka, Cpt1b, Agpat6, Chkb, Lcat, Fabp3*
	Ethanolamine and derivative metabolic process	4.32	6	*Chka, Cpt1b, Agpat6, Chkb, Lcat, Fabp3, Etnk1*
	Biogenic amine metabolic process	2.59	9	*Chka, Atp2b2, Cpt1b, Agpat6, Chkb, Lcat, Fabp3, Etnk1, Azin1, Gch1*
	PlsC	5.92	4	*Agpat6, Agpat4, Gpam, Agpat1*

Physiological state/ treatment[2]	GO cluster term	ES[3]	Count	Genes
Lactation				
	Choline/ethanolamine kinase	10.17	3	*Chka, Cpt1b, Chkb, Etnk1*
	Acylglycerol O-acyltransferase activity	5.45	4	*Agpat6, Dgat1, Agpat4, Agpat1*
	Acyltransferase	2.27	9	*Cpt1b, Agpat6, Dgat1, Chkb, Lcat, Gcat, Crat, Agpat4, Gpam, Agpat1*
	Short sequence motif: HXXXXD motif	4.61	4	*Agpat6, Agpat4, Gpam, Agpat1*
	Phospholipid/glycerol acyltransferase	4.52	4	*Agpat6, Agpat4, Gpam, Agpat1*
	Glycerolipid metabolism	2.32	7	*Agpat6, Dgat1, Aldh2, Gk, Agpat4, Gpam, Agpat1*
	1-acylglycerol-3-phosphate O-acyltransferase activity	5.11	3	*Agpat6, Agpat4, Agpat1*
	Ether lipid metabolism	2.99	3	*Agpat6, Agpat4, Agpat1*
	Lipid localization	1.24	7	*Chka, Cpt1b, Hdlbp, Chkb, Lcat, Fabp3, Cidea, Lbp*
	Lipid transport	1.16	6	*Chka, Cpt1b, Hdlbp, Chkb, Lcat, Fabp3, Lbp*
	Amino-acid biosynthesis	2.88		
	Amine biosynthetic process	4.32	9	*Aldh18a1, Bcat2, Ass1, Etnk1, Azin1, Asns, Cdo1, Cbs, Gch1*
	Cellular amino acid biosynthetic process	6.47	6	*Aldh18a1, Bcat2, Ass1, Asns, Cdo1, Cbs*
	Amino-acid biosynthesis	7.70	5	*Aldh18a1, Bcat2, Ass1, Asns, Cbs*
	Heat shock protein	2.21		
	Heat shock protein 70	6.78	6	*Hyou1, Hsph1, Hspa6, Hspa13, Hspa5, Hspa8*
	Heat shock protein Hsp70	6.78	6	*Hyou1, Hsph1, Hspa6, Hspa13, Hspa5, Hspa8*
	Heat shock protein 70, conserved site	5.43	6	*Hyou1, Hsph1, Hspa6, Hspa13, Hspa5, Hspa8*
	Chaperone HSP70	6.88	5	*Abr, Hspa6, Hspa13, Hspa5, Hspa8*

Physiological state/ treatment[2]	GO cluster term	ES[3]	Count	Genes
Lactation				
	Antigen processing and presentation	1.43	3	*Hspa6, Hspa5, Hspa8*

Physiological state/ treatment[2]	GO cluster term	ES[3]	Count	Genes
Late pregnancy MM (vs. AdM)				
Chromosome		4.64		
	Chromosomal part	5.66	16	*Cenpm, Cenpl, Kntc1, Chek1, Hmga1, Cdk2, Cbx5, Cdca8, Apitd1, Id2, Incenp, H2afz, Bub1b, Cenpt, Asf1b, Hells*
	Chromosome	5.11	17	*Cenpm, Cenpl, Kntc1, Chek1, Hmga1, Cdk2, Brca1, Cbx5, Cdca8, Apitd1, Id2, Incenp, H2afz, Bub1b, Cenpt, Asf1b, Hells*
	Chromosome, centromeric region	8.09	10	*Cdca8, Cenpm, Cenpl, Apitd1, Incenp, Kntc1, Bub1b, Cenpt, Hells, Cbx5*
	Centromere	11.58	6	*Cdca8, Cenpl, Apitd1, Incenp, Cenpt, Cbx5*
	Chromosomal protein	7.47	7	*Cdca8, Cenpm, Cenpl, Apitd1, H2afz, Cenpt, Hmga1*
	Intracellular non-membrane-bounded organelle	1.61	36	*Lmnb2, Hsd3b7, Kntc1, Chek1, Gtse1, Cbx5, Cdca8, Actr6, Aen, Incenp, Aif1l, H2afz, Asf1b, Bmf, Hells, Cdk1, Cenpm, Cenpl, Brca2, Dpysl3, Homer2, Hmga1, Foxp4, Brca1, Cdk2, Elmo2, Epb41l3, Kctd10, Id2, Invs, Apitd1, Cdc42se2, Bub1b, Cenpt, Jak2, Bin1*
	Non-membrane-bounded organelle	1.61	36	*Lmnb2, Hsd3b7, Kntc1, Chek1, Gtse1, Cbx5, Cdca8, Actr6, Aen, Incenp, Aif1l, H2afz, Asf1b, Bmf, Hells, Cdk1, Cenpm, Cenpl, Brca2, Dpysl3, Homer2, Hmga1, Foxp4, Brca1, Cdk2, Elmo2, Epb41l3, Kctd10, Id2, Invs, Apitd1, Cdc42se2, Bub1b, Cenpt, Jak2, Bin1*
Condensed chromosome		3.04		
	Chromosome, centromeric region	8.09	10	*Cdca8, Cenpm, Cenpl, Apitd1, Incenp, Kntc1, Bub1b, Cenpt, Hells, Cbx5*
	Condensed chromosome	7.93	8	*Cenpm, Incenp, Kntc1, Bub1b, Cenpt, Chek1, Brca1, Cdk2*
	Kinetochore	8.81	5	*Cenpm, Incenp, Kntc1, Bub1b, Cenpt*
	Kinetochore	6.84	5	*Cenpm, Kntc1, Bub1b, Cenpt, Cbx5*
	Condensed chromosome kinetochore	6.89	4	*Cenpm, Kntc1, Bub1b, Cenpt*
	Condensed chromosome,	6.34	4	*Cenpm, Kntc1, Bub1b, Cenpt*

Physiological state/ treatment[2]	GO cluster term	ES[3]	Count	Genes
Late pregnancy MM (vs. AdM)				
	centromeric region			
Cell cycle		2.95		
	Cell cycle phase	4.42	17	*E2f1, Cdk1, Hsd3b7, Kntc1, Brca2, Chek1, Cdk6, Gtse1, Cdk2, Cdca8, Hspa2, Bcl2, Incenp, Cks2, Bub1b, Ccna2, Hells*
	Cell cycle process	3.72	18	*E2f1, Cdk1, Hsd3b7, Kntc1, Brca2, Cdk6, Chek1, Gtse1, Cdk2, Brca1, Cdca8, Hspa2, Bcl2, Incenp, Cks2, Bub1b, Ccna2, Hells*
	Cell cycle	3.17	21	*E2f1, Cdk1, Hsd3b7, E2f8, Kntc1, Brca2, Cdk6, Chek1, Brca1, Gtse1, Cdk2, Cdca8, Hspa2, Bcl2, Incenp, Cks2, Bub1b, Chtf18, Chaf1a, Ccna2, Hells*
	M phase	4.80	13	*Cdk1, Hsd3b7, Kntc1, Brca2, Chek1, Cdk2, Cdca8, Hspa2, Incenp, Cks2, Bub1b, Ccna2, Hells*
	Regulation of cell cycle	4.08	15	*E2f1, Cdk1, Kntc1, Brca2, Igf2, Chek1, Cdk6, Gtse1, Cdk2, Brca1, Id2, Bcl2, Cks2, Bub1b, Ccna2*
	Regulation of mitotic cell cycle	6.37	9	*Cdk1, Bcl2, Kntc1, Bub1b, Brca2, Igf2, Ccna2, Gtse1, Cdk2*
	Cell cycle	3.38	16	*E2f1, Cdk1, E2f8, Kntc1, Cdk6, Chek1, Cdk2, Brca1, Cdca8, Incenp, Cks2, Bub1b, Chtf18, Chaf1a, Ccna2, Hells*
	Mitotic cell cycle	3.77	13	*E2f1, Cdk1, Kntc1, Chek1, Cdk6, Gtse1, Cdk2, Cdca8, Bcl2, Incenp, Bub1b, Ccna2, Hells*
	Cell cycle checkpoint	7.38	7	*Cdk1, Kntc1, Bub1b, Chek1, Ccna2, Gtse1, Brca1*
	Mitotic cell cycle checkpoint	12.71	5	*Cdk1, Kntc1, Bub1b, Ccna2, Gtse1*
	Cell division	3.68	11	*Cdk1, Cdca8, Incenp, Kntc1, Cks2, Bub1b, Brca2, Cdk6, Ccna2, Hells, Cdk2*
	Cell division	3.49	10	*Cdk1, Cdca8, Incenp, Kntc1, Cks2, Bub1b, Cdk6, Ccna2, Hells, Cdk2*
	Cell cycle	4.71	7	*E2f1, Cdk1, Bub1b, Cdk6, Chek1, Ccna2, Cdk2*
	Mitosis	3.81	8	*Cdk1, Cdca8, Incenp, Kntc1, Bub1b, Ccna2, Hells, Cdk2*
	Mitosis	3.64	8	*Cdk1, Cdca8, Incenp, Kntc1, Bub1b, Ccna2, Hells, Cdk2*

Physiological state/ treatment[2]	GO cluster term	ES[3]	Count	Genes
Late pregnancy MM (vs. AdM)				
	M phase of mitotic cell cycle	3.64	8	*Cdk1, Cdca8, Incenp, Kntc1, Bub1b, Ccna2, Hells, Cdk2*
	Nuclear division	3.64	8	*Cdk1, Cdca8, Incenp, Kntc1, Bub1b, Ccna2, Hells, Cdk2*
	Organelle fission	3.56	8	*Cdk1, Cdca8, Incenp, Kntc1, Bub1b, Ccna2, Hells, Cdk2*
	Spindle	3.50	6	*Cdk1, Cdca8, Invs, Incenp, Kntc1, Bub1b*
	Cytoskeleton	1.43	21	*Cdk1, Lmnb2, Kntc1, Brca2, Chek1, Dpysl3, Homer2, Brca1, Gtse1, Elmo2, Epb41l3, Cdca8, Invs, Actr6, Cdc42se2, Incenp, Aif1l, Bub1b, Jak2, Bin1, Bmf*
	Cytoskeletal part	1.43	14	*Cdk1, Lmnb2, Kntc1, Brca2, Chek1, Homer2, Gtse1, Brca1, Cdca8, Invs, Incenp, Aif1l, Bub1b, Bmf*
	Microtubule cytoskeleton	1.52	10	*Cdk1, Cdca8, Invs, Incenp, Kntc1, Bub1b, Brca2, Chek1, Gtse1, Brca1*
	Cytoskeleton	1.48	11	*Epb41l3, Cdca8, Invs, Actr6, Cdc42se2, Incenp, Kntc1, Aif1l, Bub1b, Chek1, Itgb2*
	Microtubule	1.55	5	*Cdk1, Invs, Incenp, Kntc1, Gtse1*
Cell death		2.49		
	Cell death	2.52	17	*E2f1, Bcl2l14, Fa2h, Itgb2, Igf2, Grem1, Elmo2, Brca1, Bnip1, Bcl2, Aen, Bub1b, Jak2, Apaf1, Axin2, Eif2ak2, Bmf*
	Death	2.51	17	*E2f1, Bcl2l14, Fa2h, Itgb2, Igf2, Grem1, Elmo2, Brca1, Bnip1, Bcl2, Aen, Bub1b, Jak2, Apaf1, Axin2, Eif2ak2, Bmf*
	Apoptosis	2.47	14	*E2f1, Bcl2l14, Itgb2, Grem1, Elmo2, Brca1, Bnip1, Bcl2, Aen, Bub1b, Jak2, Apaf1, Eif2ak2, Bmf*
	Programmed cell death	2.44	14	*E2f1, Bcl2l14, Itgb2, Grem1, Elmo2, Brca1, Bnip1, Bcl2, Aen, Bub1b, Jak2, Apaf1, Eif2ak2, Bmf*
	Apoptosis	2.38	9	*E2f1, Bcl2l14, Bnip1, Bcl2, Aen, Bub1b, Apaf1, Bmf, Elmo2*
Cell cycle G2/M checkpoint		2.48		
	DNA damage response, signal transduction	7.96	7	*Cdk1, Aen, Brca2, Chek1, Ccna2, Gtse1, Brca1*

Physiological state/ treatment[2]	GO cluster term	ES[3]	Count	Genes
Late pregnancy MM (vs. AdM)				
	Cell cycle checkpoint	7.38	7	*Cdk1, Kntc1, Bub1b, Chek1, Ccna2, Gtse1, Brca1*
	DNA damage checkpoint	9.00	5	*Cdk1, Chek1, Ccna2, Gtse1, Brca1*
	DNA integrity checkpoint	8.64	5	*Cdk1, Chek1, Ccna2, Gtse1, Brca1*
	G2/M transition DNA damage checkpoint	14.40	3	*Cdk1, Ccna2, Brca1*
	G2/M transition checkpoint	12.96	3	*Cdk1, Ccna2, Brca1*
	Cell Cycle: G2/M Checkpoint	7.71	3	*Cdk1, Chek1, Brca1*
DNA process		2.43		
	DNA replication	7.48	9	*Dtl, Chtf18, Brca2, Chek1, Chaf1a, Kin, Hmga1, Brca1, Cdk2*
	DNA damage response, signal transduction	7.96	7	*Cdk1, Aen, Brca2, Chek1, Ccna2, Gtse1, Brca1*
	Cellular response to stress	3.00	15	*Cdk1, Dtl, Hfe, Brca2, Chek1, Kin, Gtse1, Brca1, Hspa1l, Bcl2, Aen, Jak2, Chaf1a, Eif2ak2, Ccna2*
	DNA metabolic process	3.19	13	*Dtl, Hsd3b7, Brca2, Chek1, Hmga1, Kin, Cdk2, Brca1, Hspa1l, Chtf18, Apaf1, Chaf1a, Hells*
	Response to DNA damage stimulus	3.49	11	*Hspa1l, Cdk1, Dtl, Aen, Brca2, Chek1, Chaf1a, Kin, Ccna2, Gtse1, Brca1*
	DNA recombination	9.39	5	*Brca2, Chek1, Kin, Hmga1, Brca1*
	DNA damage	3.95	8	*Dtl, Aen, Dtx3l, Brca2, Chek1, Chaf1a, Kin, Brca1*
	Role of BRCA1, BRCA2 and ATR in Cancer Susceptibility	6.52	3	*Brca2, Chek1, Brca1*
	DNA repair	2.62	6	*Hspa1l, Brca2, Chek1, Chaf1a, Kin, Brca1*
	DNA repair	2.78	5	*Brca2, Chek1, Chaf1a, Kin, Brca1*
	Microtubule organizing center	1.25	4	*Bub1b, Brca2, Chek1, Brca1*

[1] There were no significantly enriched GO terms during late pregnancy for the analyses of offspring born to dams fed sub-maintenance (SmM) vs. MM or SmM vs. AdM; therefore, these results are not included in the table. There were not enough differentially expressed genes between treatments in lactation to conduct a GO analysis.

[2] Denotes the physiological state (eg. Late pregnancy or lactation) or treatment (eg. MM during lactation) in which GO terms are enriched.

[3] ES = enrichment score; calculated as the –log of the geometric mean of all the p-values for all members of the cluster. The higher the enrichment score the lower the *P*-value. A parent GO term enrichment score of 2, equivalent to *P* 0.001, was used as the significance cut-off for this analysis.

Table B. 2 Analysis of enriched Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathways, carried out on ovine mammary genes identified as differentially expressed by RNA-seq. This analysis was carried out using the Database for Annotation, Visualisation and Integrated Discovery (DAVID) using a Fisher Exact *P* value < 0.01 as the significance cut-off. Results shown for comparison between late pregnancy and lactation[1].

Physiological state/ treatment	KEGG pathway	Fisher Exact P value	Count	Genes
Late pregnancy				
	Beta-Alanine metabolism	1.10E-02	5	*ALDH7A1, CNDP1, HIBCH, HADHA, ALDH9A1*
	Lysosome	7.20E-03	17	*CLTA, LAPTM4A, GM2A, CTSS, FUCA1, ASAH1, CTSW, GNS, LAMP1, LAMP2, CTSK, LAPTM5, CTSC, CTSB, CTNS, AP4B1, CTSF*
	Glycolysis / Gluconeogenesis	6.00E-03	8	*LDHB, LDHA, GALM, ALDH7A1, GCK, AKR1A1, ALDOC, ALDH9A1*
	Fatty acid elongation in mitochondria	5.20E-03	3	*ACAA2, HADHA, HADHB*
	Parkinson's disease*	3.30E-03	7	*ATP5D, HTRA2, ATP5C1, ATP5G2, ATP5O, ATP5A1, ATP5H*
	Huntington's disease*	3.20E-03	13	*ATP5D, CLTA, ATP5G2, DCTN4, ITPR1, DCTN2, HDAC2, IFT57, CASP8, ATP5C1, ATP5O, ATP5A1, ATP5H*
	p53 signalling pathway	3.10E-03	10	*CCNB1, CDK1, CCND1, GADD45G, CASP8, DDB2, CDK4, CCNG1, CCNG2, GADD45A*
	Fatty acid metabolism	2.60E-03	9	*ACADVL, ACAA2, ALDH7A1, ACAT1, HADHA, CPT1A, ALDH9A1, ACSL5, HADHB*
	Alzheimer's disease*	1.90E-03	14	*ATP5D, HSD17B10, ADAM10, APH1A, ATP5G2, BAD, ITPR1, APP, CASP8, ATP5C1, CALM3, ATP5O, ATP5A1, ATP5H, CALM2*
	Valine, leucine and isoleucine degradation	1.90E-03	10	*BCKDHA, HSD17B10, ACAA2, ALDH6A1, ALDH7A1, HIBCH, ACAT1, HADHA, ALDH9A1, HADHB*
	Propanoate metabolism	3.80E-04	8	*ALDH6A1, LDHB, LDHA, ALDH7A1, HIBCH, ACAT1, HADHA, ALDH9A1*

Lactation				
	Vibrio cholerae infection**	1.30E-02	7	*ATP6V1C1, ACTB, KDELR2, ATP6V0A1, ERO1L, ATP6V0D1, ATP6V1F*
	N-Glycan biosynthesis	1.30E-02	4	*B4GALT1, GANAB, DPM2, DDOST*
	Terpenoid backbone biosynthesis	3.50E-03	4	*DHDDS, HMGCR, HMGCS1, FDPS*
	Glycerophospholipid metabolism	9.50E-04	9	*GPD1L, CHKA, CPT1B, AGPAT6, CHKB, LCAT, ETNK1, AGPAT4, GPAM, AGPAT1*
	Steroid biosynthesis	1.10E-04	5	*EBP, DHCR7, HSD17B7, DHCR24, FDFT1*
Late pregnancy MM (vs. AdM)				
	p53 signalling pathway	0.00E+00	6	*CDK1, CDK6, CHEK1, APAF1, GTSE1, CDK2*
	Cell cycle	0.00E+00	7	*E2F1, CDK1, BUB1B, CDK6, CHEK1, CCNA2, CDK2*
	Small cell lung cancer	0.00E+00	6	*E2F1, BCL2, CDK6, APAF1, CDK2, FN1*
	Progesterone-mediated oocyte maturation	2.00E-02	4	*CDK1, IGF2, CCNA2, CDK2*
	Pathways in cancer	4.00E-02	8	*E2F1, BCL2, BRCA2, CDK6, AXIN2, CDK2, FZD7, FN1*
Late pregnancy Sm (vs. AdM)				
	Cell adhesion molecules (CAMs)	7.70E-04	5	*ITGB8, ITGB2, CD4, CLDN11, CDH3*
	Natural killer cell mediated cytotoxicity	5.40E-03	3	*ITGB2, FCGR3A, HCST*