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The molecular and cellular effects of maternal dietary intervention on fetal development

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Abstract

Previous studies have provided a basis of knowledge of The Developmental Origins of Health and Disease (DOHaD), and the effects of maternal dietary protein during early gestation on fetal gene expression and development. Dietary protein is an essential macronutrient during pregnancy as it supports fetal growth.

Insufficient levels of maternal dietary protein may lead to sub-optimal growth and development of offspring. In beef production, the effects of low maternal dietary protein on the placentome, and the fetal mammary gland are not fully understood. The aims of the current study were to investigate the effects of maternal dietary protein on fetal gene expression and development in the mammary gland, and protein localisation in the bovine placentome.

Twelve-month-old Santa Gertrudis heifers were individually fed either low (L) (9% crude protein (CP) or high (H) (14.6% CP) protein during periconception (60 days preconception to 23 days postconception (dpc). At 23 dpc, heifers were split equally and either entered the low (7.6% CP) or high (12.4% CP) protein diet during postconception (23 dpc until the end of the first trimester, 98 dpc) in a 2x2 factorial design, yielding four maternal dietary interventions (HH, HL, LH and LL). At 98 dpc n=48 heifers were culled and placentomes and female fetal mammary tissues (n=20) were collected for analysis. At term, cotyledons were collected for analysis. Bovine fetal mammary tissues (n=4 per fetus) were designated for RNA-seq (n=2 per fetus) or histological (n=2 per fetus) analysis. The current study investigated placentomes and term cotyledons via immunohistochemistry.

To our knowledge this is the first study to quantify parenchymal structures and produce transcriptomic data of the bovine fetal mammary gland at 98 dpc. Total mammary epithelial and collagen area was unaffected by maternal diet ($p>0.05$). Duct lumen size was 45.9% greater in tissues exposed to HPost versus LPost ($p<0.05$). Fetal mammary stromal blood vessel number was 59% greater ($p<0.05$) in tissues exposed to LPeri (9% CP), versus HPeri (14.6% CP). Transcriptomics analysis of (n=2) LH and (n=2) HH exposed mammary tissues indicated up-regulation of 45 genes related to immune function, and down-regulation of 46 genes involved predominantly in branching morphogenesis, cell-adhesion, and future milk production in LH exposed

mammary tissues. In 98 dpc placentomes, the distribution of trophoblast binucleate cells within feto-maternal junctions differed between fetal sex and maternal dietary exposure ($p<0.05$). In 98 dpc placentomes, SerpinB1 protein expression was observed to be high (96.5% of observed cells) in trophoblast binucleate cells (BNCs) bordering the maternal epithelial cell wall. While SerpinB1 expression was low (9.2% of observed cells) in BNCs localised centrally to the fetal cotyledons.

Results indicate that low maternal dietary protein increased fetal mammary expression of genes associated with the immune system, and decreased expression of genes associated with structural development. Histological results suggest that maternal diet did not affect total area, size, or number of fetal mammary ducts. Fetal mammary glands exposed to HPost diet had greater lumen size compared with LPost counterparts. This study provides a basis for future investigation of the fetal mammary gland. The long-term effects of maternal dietary intervention on fetal mammary development in beef cows are yet to be elucidated.

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Table of Abbreviations

Abbreviation	Description	Abbreviation	Description
AI	Artificial insemination	M	Male
BNC	Trophoblast binucleate cell	mg	milligram
CDH1	*E-cadherin*	ml	millilitre
CP	Crude Protein	MMP	Matrix metalloproteinase
Cx43 / GJA1	*Connexin 43 / gap junction protein alpha 1*	mRNA	messenger RNA
DEG	Differentially expressed gene	n	Number of samples
dH2O	distilled water	NICD	Notch intracellular domain
DNA	Deoxyribonucleic acid	*Notch1*	*Notch receptor 1*
DOHaD	Developmental Origins of Health and Disease	NRC	Nutritional Research Council
dpc	Days postconception	PBS	Phosphate buffered saline
ECM	Extracellular matrix	PR3	Proteinase 3
F	Female	QC	Quality Control
FGFR2	*Fibroblast Growth Factor Receptor 2*	RBP	Retinol binding protein
FOXO4	*Forkhead box O4*	RIN	RNA integrity value
g	gram	RNA	Ribonucleic acid
GATM	*glycine amidinotransferase*	RNA-Seq	RNA-sequencing
GO	Gene Ontology	RT-qPCR	Real Time quantitative Polymerase Chain Reaction
HPeri	maternal high protein during periconception (-60 preconception to 23 days postconception)	SEM	Standard Error of the Mean
HPost	maternal high protein during postconception (23 to 98 dpc)	*SERPINB1*	*Serine peptidase inhibitor, clade B, member 1*
IL33	*Interleukin 33*	*STRA6*	*Stimulated by retinoic acid 6*
ITGAL	*Integrin subunit alpha L*	*TFAP2*	*AP-2 transcription factor*
ITGB2	*Integrin subunit beta 2*	*TWIST1*	*Twist family bHLH transcription factor 1*
kg	kilogram	UTC	Uninuclear trophoblast
KRT80	*Keratin 80*	µg	microgram
LPeri	maternal low protein during periconception (-60 preconception to 23 dpc)	µl	microlitre
LPost	maternal low protein during postconception (23 to 98 dpc)		

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1 Introduction

1.1 The Beef Industry

Over the years, the beef industry has been affected by challenges such as increased consumer demands, and rising animal feed costs (Godfray et al., 2010). There is pressure to produce and source natural, sustainably produced animal feed which is affordable, meets ethical standards, whilst using less land to produce more meat at a quicker rate, at the cheapest price (Connor, 2015, Gerber et al., 2015, Müller et al., 2018). Understandably, beef production has adapted to challenges to remain sustainable and profitable.

Profitability is defined as the ability of a business to make a profit after costs (Ball et al., 2015). Annual farm income of UK grazing livestock including beef farming is variable. The Department for Environment, Food and Rural Affairs (DEFRA) reported the average 2013/14 farm income (£/farm/year) for grazing livestock in lowland, and less favoured areas as £15,100 and £14,500 respectively (DEFRA, 2015). These figures were 21 and 44% lower than forecasted respectively (DEFRA, 2015). Since 2013/14, the percentage change of average farm incomes have been incredibly variable for lowland (+23% 2014/15, -35% 2015/16, +34% 2016/17) and less favoured areas (+0.7% 2014/15, +30% 2015/16, +42% 2016/17) (DEFRA, 2018). This highlights the financial vulnerability to a multitude of factors which influence farm management decisions (DEFRA, 2015, DEFRA, 2017). Beef production profitability is directly linked to the productivity of mature cows, in terms of health, fertility and production (Amer et al., 1996, Ash et al., 2015, Crosson et al., 2006, De Haas et al., 2013, Summers and Funston, 2013). In beef cattle, productivity is associated with the ability to produce a calf at 2 years of age, maintain a 365-day calving interval, and wean one saleable calf per year (Amer et al., 1996, De Haas et al., 2013, Summers and Funston, 2013). Arguably, a vital organ in achieving productivity is the bovine placenta. Secondly, early calf nutrition relies on the proper formation and function of the adult mammary gland. This study focuses on the role of the placenta and mammary gland as vital organs in beef cattle production (Chapters 1.4 and 1.5). The link between sustainability and production performance supports further study of the effect of maternal nutrition on offspring. Further investigation into the effects of maternal dietary protein on offspring is important for the sustainability of the industry as dietary protein is a major

cost in cattle production. Feed costs for beef production account for approximately 60% to 90% of the total cost of production, depending on the production system (Martello et al., 2015, Meteer et al., 2015). Nutritional management of cows therefore has a crucial role in the economic viability of beef farming.

1.1.1 Animal Feed Prices

Feed is the largest cost in animal production, including beef production (Arthur et al., 2004, Arthur et al., 2001, Arthur et al., 1996). This cost includes forages and grass produced on farm and purchased feeds. The price of protein feed sources is greater than energy feed sources (Table 1.1) (Agriculture and Horticulture Development Board, 2015, Agriculture and Horticulture Development Board, 2016). The global animal feed market is partly affected by climate, subsequent yields and prices (Ray et al., 2015). Temperature and precipitation are large environmental factors affecting crop yields (Ray et al., 2015). The cost of protein sources supports research into the level of protein inclusion in cattle diets (Table 1.1). If maternal dietary protein levels are not favourable to profitability, the sustainability of beef farming may be negatively impacted. Additionally, the increasing demand for meat in developing countries, due to population and income growth, and a shift towards globalisation of the Western diet is an added pressure to increase food sustainability (Obersteiner et al., 2016). Developments in nutrition, breeding and animal health will contribute towards increased production and efficiency (reviewed by Thornton, 2010). Therefore, further investigation of the effect of maternal dietary protein on fetal development, and the implications for future cattle health and production, is highly relevant to global beef farming and food security.

Table 1.1 UK feed prices of protein and energy sources

Feed straights	Price (£/tonne) 2015[a]	Price (£/tonne) 2016[b]
Hipro (high protein) soybean meal	276-279	362-364
Soybean meal (Argentina)	268-270	352-354
Rapeseed meal (imported)	200-203	201-203
Feed Wheat	114-121	160-161
Feed Barley	105-106	106.5

[a]Agriculture and Horticulture Development Board (2015). [b]Agriculture and Horticulture Development Board (2016).

1.1.2 Protein Levels in Grass and Feedlot Diets

Grass growth and its nutritive value, including crude protein (CP), is affected by climate and annual rainfall (Boschma et al., 2017, Müller et al., 2018). Within the same grass species, the CP varies dependent on rainfall and nitrogen availability (Boschma et al., 2017). Australian grassland is subject to large fluctuations in rainfall (Boschma et al., 2017). This is the basis for investigation of maternal dietary CP and the effects on fetal development. However, this study is also relevant to protein inclusion in cattle feedlot diets.

In addition to grass protein content, the current study is applicable to feedlot beef production systems. The producer has more control over the nutrient content of a total mixed ration fed in a feedlot in comparison to grazed beef production. Feedlot production involves feeding a total mixed ration (TMR), or a partial mixed ration (PMR). A TMR includes every feed component of the cattle diet, such as silage, premix (vitamins and minerals), and concentrates (carbohydrates), whereas concentrates are fed separately from a PMR (Keane et al., 2006, Daigle et al., 2016). Silage analysis of individual cuts allows producers to measure nutritive contents, and subsequent diet formulation to ensure protein and energy requirements of the herd are met. Therefore, the current study is of interest to both pasture-fed and feedlot beef and dairy production. As protein is an expensive feed, research into protein levels required by cattle is ongoing.

Further understanding gained from the current study will benefit all aforementioned production systems. An increased knowledge of maternal

dietary protein level on fetal development can be applied to level of rainfall in addition to nutrient content of a TMR and or PMR. Therefore, the pasture production system can be more informed of the effect of timing of gestation in wet and dry seasons and make management decisions beneficial to fetal development. Similarly, the feedlot production system can be more informed of the effect of protein levels leading up to conception or artificial insemination (AI), and during the first trimester of gestation. This may not be practical if calving is year-round, whereas a block calving system would benefit because the breeding herd will be in the same stage of gestation, and therefore the diet is easier to manage on a herd level rather than grouping by gestational stage, which may not be practical. Therefore, this study is industrially relevant, with economic and environmental advantages to support future industrial adaptation to new practices (Kuehne et al., 2017).

1.1.3 Dietary Protein Digestion in Ruminants

The major protein sources to the ruminant are microbial protein and digestible, rumen undegradable protein (DUP) (Firkins and Reynolds, 2005). The majority of dietary protein is converted into amino acids, ammonia and peptides, and utilised by the rumen microbiota (Figure 1.1) (Firkins and Reynolds, 2005). The rumen microbes then pass into the abomasum, where they are termed microbial protein (Figure 1.1), and are degraded by enzymes to amino acids and absorbed by the ruminant (Firkins and Reynolds, 2005).

Previous studies have shown beneficial effects of rumen undegradable protein (UDP) feeding on calf growth, lactation performance and improved reproductive success in cows (Wankhede and Kalbande, 2001, Tandon and Siddique, 2016, Kumar et al., 2005, Garg et al., 2003). Regarding the fetus, the source of amino acids is maternal, derived from microbial protein and DUP (Figure 1.1). The proportion of DUP and UDP alter the amino acid profile available to the cow, and potentially alter the *in utero* environment (Santos et al., 1998). It is therefore relevant to consider how maternal dietary protein may affect the supply of amino acids to the fetus.

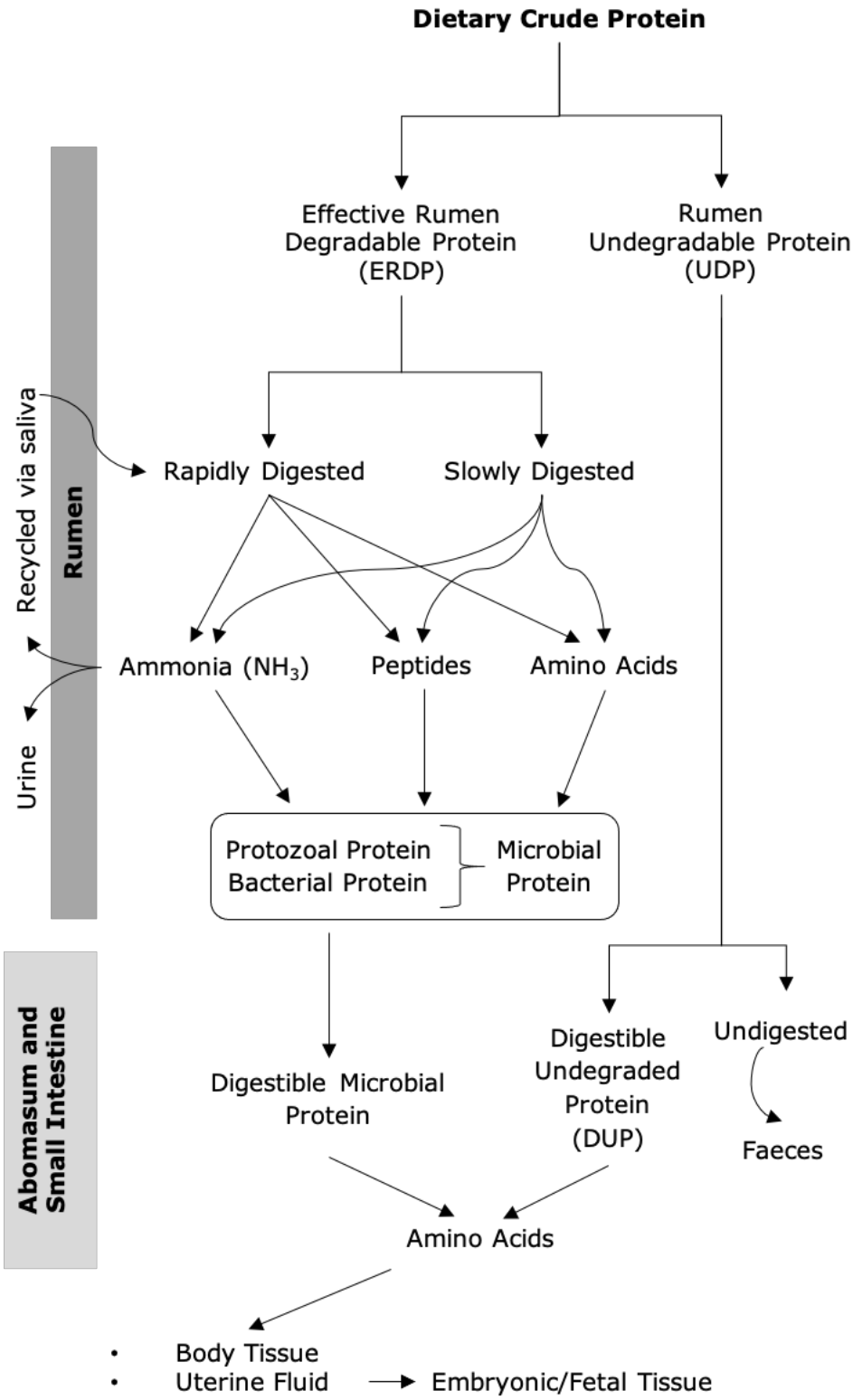


Figure 1.1 A brief outline of dietary protein digestion in ruminants. Adapted from (McDonald et al., 2011).

1.1.4 The Effects of Protein Supply to the Gestating Heifer on Pregnancy and Metabolism

During gestation, protein and the subsequent supply of amino acids are required for normal fetal growth and development, and protein deficiencies during gestation can affect fetal growth, such as reduced fetal bodyweight and organ weight at 98 dpc and calf birth weight (Micke et al., 2011; Copping et al., 2014; Hernandez-Medrano et al., 2015). Gestation increases the demand upon the heifer or cow for amino acids to support protein synthesis of the fetus (Bell et al., 2000). The metabolism of the gestating heifer was not within the scope of the current study.

1.1.5 Amino Acid Supply to the Bovine Conceptus and Fetus

Soybean meal is a source of protein and amino acids. The current study focused on dietary crude protein, although CP content is inextricably linked with the supply of amino acids. Amino acids are an end product of protein digestion, which are subsequently transported to the fetus (Figure 1.1). Individual amino acids were not analysed in maternal dietary interventions in the current study. CP is a common measure in silage and grass analyses, thus making this study practical and applicable to the beef production industry.

In summary, the current study has potential to benefit global cattle production. In order to achieve these benefits, an in-depth understanding of the bovine placenta (Chapter 1.4.1), mammary gland (Chapter 1.5), and the effects of the intrauterine nutrition (Chapter 1.2) on development is required. Previous studies have focused on heifer nutrition and growth performance, and the correlation with future production, and feed efficiency (Connor, 2015, Kertz et al., 2017). More recent research has focused on the earlier stages of development, such as intrauterine nutrition and fetal development. Existing knowledge of the importance of intrauterine nutrition provided the basis for the current study.

1.2 Intrauterine Nutrition

Dietary factors can directly impact the health, fertility, growth and production of beef cattle (2015, Fiske and Adams, 1985, Formigoni et al.,

2006, He et al., 2015, Kane et al., 2002, Krishnamoorthy et al., 1995, Marongiu et al., 2002, Oliveira Filho et al., 2010, Robinson et al., 2006, Sullivan et al., 2009e, Waterman et al., 2012, Wettemann et al., 1986, Yaakub et al., 1999, Perry et al., 1999). In the developing fetus, different tissues develop at certain time points, or 'critical periods' (Widdowson and McCance 1975). Widdowson and McCance (1975) reported that even a short period of undernutrition during a critical period of development can affect fetal programming (Chapter 1.3) and has the potential to subsequently affect adult life. Furthermore, discrete periods such as oocyte maturation and fetal development were identified as critical periods of development (Funston et al., 2010, Wu et al., 2006). This supports the study of maternal diet in the periconception period. There is evidence in many species to suggest that preconception maternal nutritional status influenced fetal growth and development throughout gestation (Bloomfield et al. 2013). Multiple studies have investigated the effects of periconception maternal undernutrition in lambs (Fleming et al. 2012), although less research has been conducted in cattle to date (Mossa et al., 2013). Bovine gestation ranges between 280 and 289 days in beef cows (Amer et al., 2001, Walsh et al., 2011). It can be considered as trimesters of gestation. Chronologically, the time-points of investigation in previous research were initially late-gestation, as fetal growth rate is higher in late gestation compared with early gestation. Following this, the research focus shifted towards studying the first trimester and periconception.

1.2.1 Third Trimester of Gestation

Previous maternal dietary intervention studies focused on maternal nutrition throughout gestation, with nutritional intervention continuing into the third trimester of gestation. Rasby and colleagues (1990) studied the effect of moderate and low planes of maternal nutrition from 145 days postconception (dpc) until 259±1 dpc on bovine fetal development. Cows fed a low plane of nutrition from 145 dpc had lower body weight (419kg) compared with those fed a moderate plane of nutrition (511kg) (Rasby et al., 1990). Placental cotyledonary weights of cows fed low nutrition were greater (1.87kg) than those exposed to moderate maternal nutrition (1.44kg) (Rasby et al., 1990). Rasby and colleagues noted that amniotic fluid fructose concentration was decreased in placentas exposed to low maternal nutrition versus moderate

maternal nutrition. Fructose is the main fetal blood carbohydrate, and has been reported to decrease in fetal blood with decreasing ovine maternal nutrition (Battaglia and Meschia, 1978). Results reviewed by Battaglia and Meschia (1978) agree with Rasby and colleagues (1990). The focus shifted to earlier stages of gestation, as this coincides with developmental events sensitive to maternal nutrition (Rhind, 2004).

1.2.2 Second Trimester of Gestation

During the second trimester (93 to 180 dpc) of bovine gestation, studies of the effects of maternal dietary protein on fetal development were previously conducted (Micke et al., 2015, Sullivan et al., 2009b, Micke et al., 2011, Sullivan et al., 2010, Micke et al., 2010b, Sullivan et al., 2009c). Differing effects of maternal dietary protein were reported, such as greater free levels of the thyroid hormone triiodothyronine (T3) than total T3 in calves (Micke et al., 2015), increased placental bovine pregnancy associated glycoprotein (bPAG) levels, fewer cotyledons (Sullivan et al., 2009c, Sullivan et al., 2009b), increased muscle IGF1R mRNA in male and female offspring, and lower male plasma IGF1 at 29 to 379 days old (Micke et al., 2011, Micke et al., 2010b).

The average ewe gestation length is around 150-152 days (Susin et al., 1995) and can be shorter, reaching term at 147 days (Brameld et al., 2000). In ewes subjected to acute nutrient restriction during mid-gestation (83-90 dpc), fetal nutrient status was unaffected (McMullen et al., 2005). This was explained by alterations in maternal insulin-like growth factor 1 (IGF1) and insulin levels to maintain maternal glucose, and subsequently maintain fetal glucose supply (McMullen et al., 2005). Total placentome weight was 24.7% lighter in nutrient restricted ewes versus controls at 90 dpc, however following realimentation, placentome weights were not significantly different at 135 dpc (McMullen et al., 2005). Fetal growth was unaffected by short-term nutrient restriction (McMullen et al., 2005). This study is evidence that the effects of nutrient restriction are partly dependant on duration of nutrient restriction. Conversely to nutrient restriction, ewes fed *ad libitum* during 20 to 140 dpc produced 12.9% lighter fetal (100 dpc) mammary glands than maintenance diets (3.98 vs 4.53g, $p<0.05$) (Van Der Linden et al., 2009). This study demonstrated that dam body weight is also a factor affecting fetal development and future milk production (Van Der Linden et al., 2009). In

cows, male and female offspring exposed to low maternal nutrition during the second trimester resulted in greater *insulin-like growth factor I receptor* (*IGF1R*) mRNA expression in *semitendinosus* muscle (680 days old), and *longissimus dorsi* muscle area on carcass, compared to those exposed to high maternal nutrition (Micke et al., 2011). This suggests increased potential for muscle growth in offspring (Micke et al., 2011). Previous studies have elucidated that maternal nutrition during the first trimester is a more critical period in terms of the effects on fetal development, compared to late gestation (Brameld et al., 2000). This is thought to be due to the critical windows of oocyte, embryo and fetal development during preconception and periconception (Wu et al., 2006).

1.2.3 First Trimester of Gestation

Following previous studies of intrauterine nutrition in late gestation and the second trimester, research focus progressed to the first trimester of gestation. Maternal dietary restriction during the first trimester was shown to affect fetal growth (Copping et al., 2014, Hernandez-Medrano et al., 2015, Micke et al., 2010b). Low maternal dietary protein during the first trimester of bovine gestation increased dry term cotyledon weight by 59.8g compared to high protein (176.0 g vs 116.2g) (Perry et al., 1999). This suggests greater cotyledonary growth in heifers fed low protein during the first trimester compared to those fed high protein. Placentomes exposed to a change in dietary protein level at 99 dpc had decreased blood vessel volume, regardless of how dietary protein level changed (Perry et al., 1999). Males exposed to low maternal dietary protein during the first trimester had greater *semitendinosus* muscle cross-sectional area (mid-belly) compared to high protein counterparts, from 65 to 462 days old (Micke et al., 2011). In males, exposure to first trimester low maternal dietary protein increased *longissimus dorsi* area from 462 to 657 days of age, compared to high protein counterparts (Micke et al., 2011). In females, maternal dietary protein during the first or second trimester of gestation had no effect on postnatal *longissimus dorsi* area (Micke et al., 2011). This indicates a sex-specific response to maternal diet on postnatal muscle growth. First trimester maternal protein restriction (7% CP) was also shown to reduce fetal weight compared with high protein (14% CP) (Copping et al., 2014). Periconception protein restriction was shown to have a greater effect on growth than restriction in the first trimester (Copping et al., 2014).

1.2.4 Periconception

The period prior to and around conception is termed periconception. The periconception period in this study refers to day 60 preconception to 23 dpc. Other studies may have defined this period differently, based on differing experimental designs and specific sampling days, or day of dietary treatment change (Hernandez-Medrano et al., 2015, Sinclair and Watkins, 2014). The maternal environment comprises the metabolism, gastrointestinal, renal, cardiopulmonary and endocrine systems, which are thought to influence the uterine environment (Holland and Odde, 1992). The periconception maternal environment constitutes the conditions in which oocyte maturation and conception occurs, and has the potential to impact embryo and fetal development (Holland and Odde, 1992, Sinclair and Watkins, 2014). The effects of maternal periconception diet on embryo development has been well studied in sheep, cows and rodents (Sinclair and Watkins, 2014). However, studies investigating fetal mammary development are lacking. Rat and mouse studies have reported periconceptional maternal dietary low protein results in altered gene expression and elevated blood pressure in offspring (Kwong et al., 2006, Watkins et al., 2008). Maternal low protein during oocyte maturation had no effects on gestation length, birth weight, litter size, birth weight or postnatal growth (up to 28 weeks) of male and female offspring in mice (Watkins et al., 2008). This study concluded that despite no changes to birth or organ weights, gene expression was altered. In bovine, ovine and human offspring, sexually dimorphic effects have been reported in maternal nutrition studies (Sinclair et al., 2007, Tobi et al., 2009, Micke et al., 2011). Female but not male beef cow offspring exposed to low protein during the first trimester had lower expression of IGF2 mRNA in muscle (Micke et al., 2011). While Sinclair and colleagues (2007) reported differences in increased blood pressure in male but not female offspring of ewes fed a methyl-restricted diet (low vitamin B_{12}, methionine and folate). In heifers fed either 120% (high) or 60% (restricted) of maintenance energy requirements, from 11 days preconception to 110 dpc, offspring birth weights and postnatal growth rates were unaffected by maternal diet (Mossa et al., 2013). However, further analysis of female offspring at 7, 18, 56, 86 weeks-old indicated reduced fertility (lower secondary follicle counts) in females exposed to restricted maternal nutrition versus high nutrition (Mossa et al., 2013). These studies provide evidence for further investigation of the

molecular effects of maternal diet on placental development and highlight a lack of research into bovine fetal mammary development, which is of economic importance to industry and therefore warrants further investigation.

1.2.5 Postconception

The postconception period in the current study refers to day 23 postconception to day 98 postconception (dpc), around the end of the first trimester of gestation. It is again notable that other studies may define this period differently, based on differing experimental designs and specific sampling days, or day of dietary treatment change. Studies have highlighted the impact of producing beef cattle exposed to intrauterine growth restriction. Yield from finished beef cattle was reduced by 18kg (7%) in intrauterine growth restricted (low birth weight) animals compared with cattle well-grown (high birth weight) during gestation (Greenwood et al., 2006, Cafe et al., 2006). Growth restriction and subsequent low birth weight was caused by maternal nutrient restriction during gestation (Cafe et al., 2006). The feeding management for the low nutrition group was pasture based year round, with unfertilised, un-irrigated pasture (Cafe et al., 2006). While the high nutrition group was ensured by grazing on fertilised (urea), irrigated pasture, and further transfer to high quality grass species over winter-feeding (Cafe et al., 2006). This highlights the economic investments in fertilizer and irrigation required to achieve "high" nutrition pasture. During gestation, maternal protein levels were also shown to affect beef cow offspring growth (Micke et al., 2010a, Micke et al., 2011). Micke and colleagues (2010a) demonstrated temporal effects of maternal dietary protein restriction during the first trimester on male offspring growth. From 191 to 657 days post-partum, male offspring exposed to low maternal dietary protein during the first trimester were heavier than males of cows fed high protein (Micke et al., 2010a). Whereas in female offspring, carcass weight from 552 to 657 days post-partum was unaffected by maternal dietary protein level (Micke et al., 2010a). This demonstrates a sexually dimorphic response in male and female offspring to maternal dietary low protein, where a growth response was seen earlier in males than females (Micke et al., 2010a). Micke and colleagues (2011) reported similar findings. Male offspring of cows fed low protein during the first trimester had greater

muscle growth compared with males subjected to high maternal protein (Micke et al., 2011). In female offspring, there was no effect of maternal dietary protein intake during the first trimester on growth (Micke et al., 2011). These studies identify the effects of maternal dietary protein on offspring growth, which consequently affects the economic yield and sustainability of beef herds (Chapter 1.1). These studies are consistent with The Developmental Origins of Health and Disease (DOHaD) hypothesis.

1.3 Fetal Programming

The DOHaD, also known as fetal programming or the Barker hypothesis, is defined as the concept that a maternal stimulus during a critical period in fetal development has permanent effects on the offspring or subsequent generations (Barker et al., 1993, Godfrey and Barker, 2000). A critical period can be further defined for example, to periconception, or gestation and lactation, and permanent effects on the offspring may be, for example, a predisposition to adult-onset diseases in response to a maternal stimulus (Barker and Osmond, 1988, Barker, 1990, Barker, 1995, Godfrey and Barker, 1995, Armitage et al., 2005).

The effects of maternal nutrition on human offspring were demonstrated during the food shortage during the Dutch Famine of 1944-1945 (Banning, 1946), where a period of maternal malnutrition affected birth weight and placental weight (Lumey and Stein, 1997, Stein and Susser, 1975). This is evidence for human *in utero* growth being dependent upon maternal nutrition (Barker and Thornburg, 2013, Barker, 1995, Barker, 1997, Godfrey and Barker, 1995). In cattle, the effects of maternal nutrition on *in utero* growth have been investigated, and findings indicate lasting effects on offspring birth weight, growth and gene expression (Duarte et al., 2013, Micke et al., 2015, Micke et al., 2010b, Mohrhauser et al., 2015, Mulliniks et al., 2013, Perry et al., 2002, Perry et al., 1999, Sullivan et al., 2009b, Underwood et al., 2010).

Cattle studies also have observed that the *in utero* environment can have differential effects on male and female fetuses, termed sexually-dimorphic effects (Tobi et al., 2009, Sinclair et al., 2007, Micke et al., 2011). Furthermore, dietary interventions during the first and second trimesters of gestation affected fetal and placental development (Sullivan et al., 2009a,

Sullivan et al., 2009b, Sullivan et al., 2009c, Sullivan et al., 2009d, Sullivan et al., 2009e, Micke et al., 2010a, Micke et al., 2010b, Perry et al., 1999). Mouse studies have shown that adverse nutrition during gestation induces permanent modifications to the phenotype of the fetus and placenta (Armitage et al., 2005, Burdge et al., 2007, Rutland et al., 2007). Further understanding of the effect of maternal nutrition is important for the future health, fertility and production of beef cattle offspring. Nutrition is therefore an important factor in the production of beef cattle, as it can influence the consequent profitability of beef herds (Greenwood et al., 2006).

1.4 The Placenta

The placenta is critical to fetal development, as it facilitates the exchange of nutrients, respiratory gases and waste substances between the maternal and fetal circulatory systems, and is formed of both maternal and fetal tissues (Barcroft, 1946, Bloomfield et al., 2013, Ramsey and Crosby, 1982). The placenta is also critical to the survival of the semi-allogeneic fetus (Petty et al., 2006, Trowsdale and Betz, 2006). The function of the placenta thereby makes it a critical determinant of fetal development. In mammals, although the function of the placenta remains the same, there are physiological differences (Enders and Blankenship, 1999). Normal placental development is partially dependent on proliferation and differentiation of trophoblast cells, which are fetal cells derived from the trophectoderm (Forbes et al., 2008). The bovine placenta is considered intermediary in terms of invasiveness (Figure 1.2), compared with the invasive haemochorial human and mouse placentae and non-invasive diffuse porcine placenta (Figure 1.3) (Enders and Blankenship, 1999).

1.4.1 The Bovine Placenta

The bovine placenta is cotyledonary and synepitheliochorial (Peter, 2013, Lee et al., 1997). Bovine synepitheliochorial placentation is an intermediary trophoblast invasion (Wooding and Wathes, 1980). The word 'cotyledonary' refers to the fetal trophectodermal tissue of the placenta, collectively termed cotyledons (Wooding et al., 1996). The ectodermal epithelium that covers the blastocyst surface is termed trophoblast or trophectoderm (Barcroft et al., 1998). Endometrial caruncles are the maternal portion of placental tissue

(Reynolds et al., 1990). The caruncle is a highly vascularised basal membrane covered by branching crypts, consisting of cuboidal epithelium that interdigitates with the chorionic microvilli (Figure 1.2) (McNaughton and Murray, 2009). Together, the caruncles and cotyledons form the functional units of the bovine placenta, the placentomes (Figure 1.2) (Beagley et al., 2010). Microvillus interdigitation forms between the caruncles and cotyledons within the placentome which are also termed feto-maternal junctions (Figure 1.2. Figure 1.4) (Atkinson et al., 1984). Placentomes are smooth semi-circular elevations which are visible from 37 dpc and increase in number until 70 dpc (Table 1.2) (Assis Neto et al., 2009; Peter, 2013; Schlafer et al., 2000).

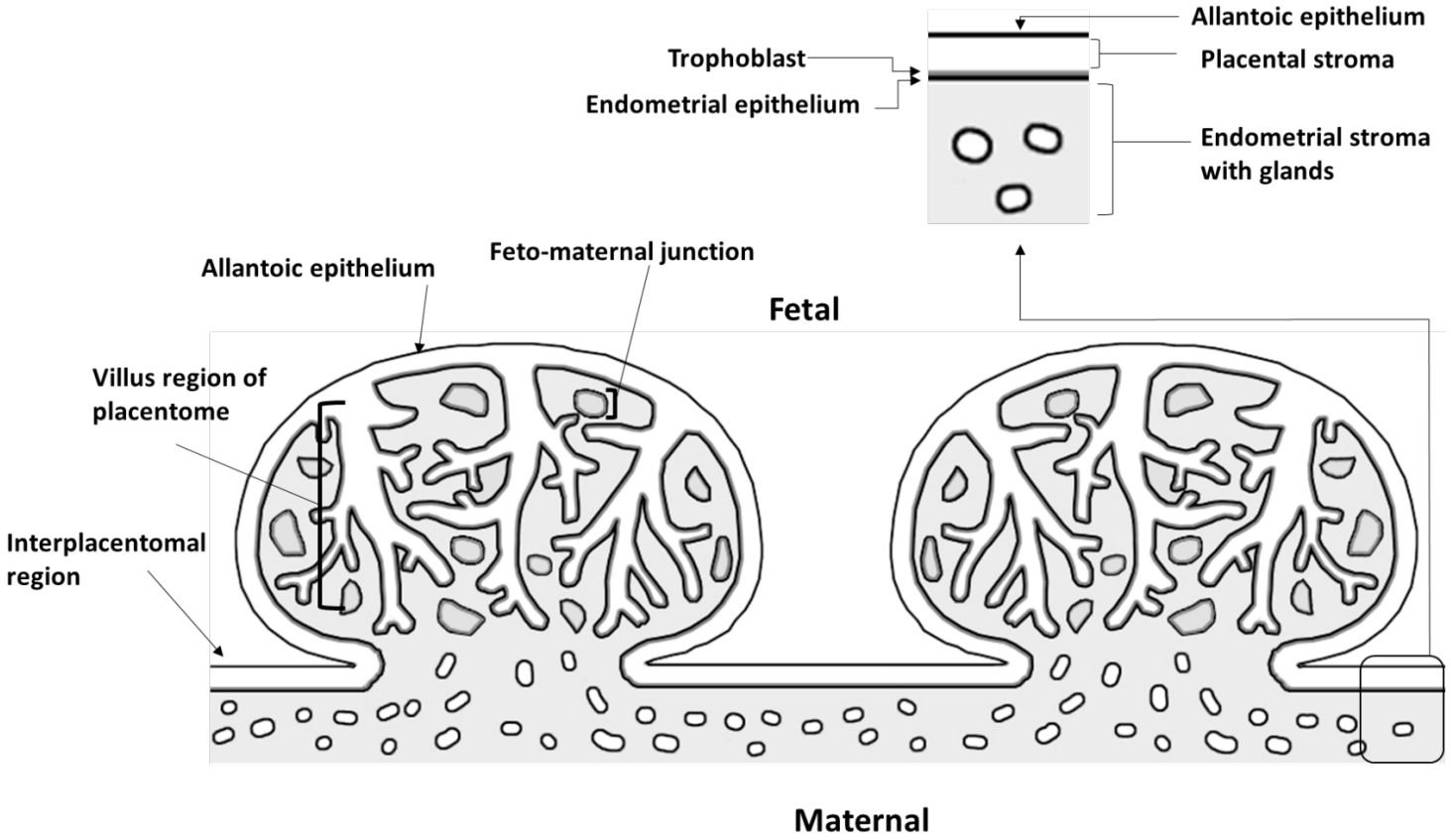


Figure 1.2 Schematic of the bovine placentome. Adapted from (Davies et al., 2000). For a full orientation of the bovine placentome, see Appendix 18.

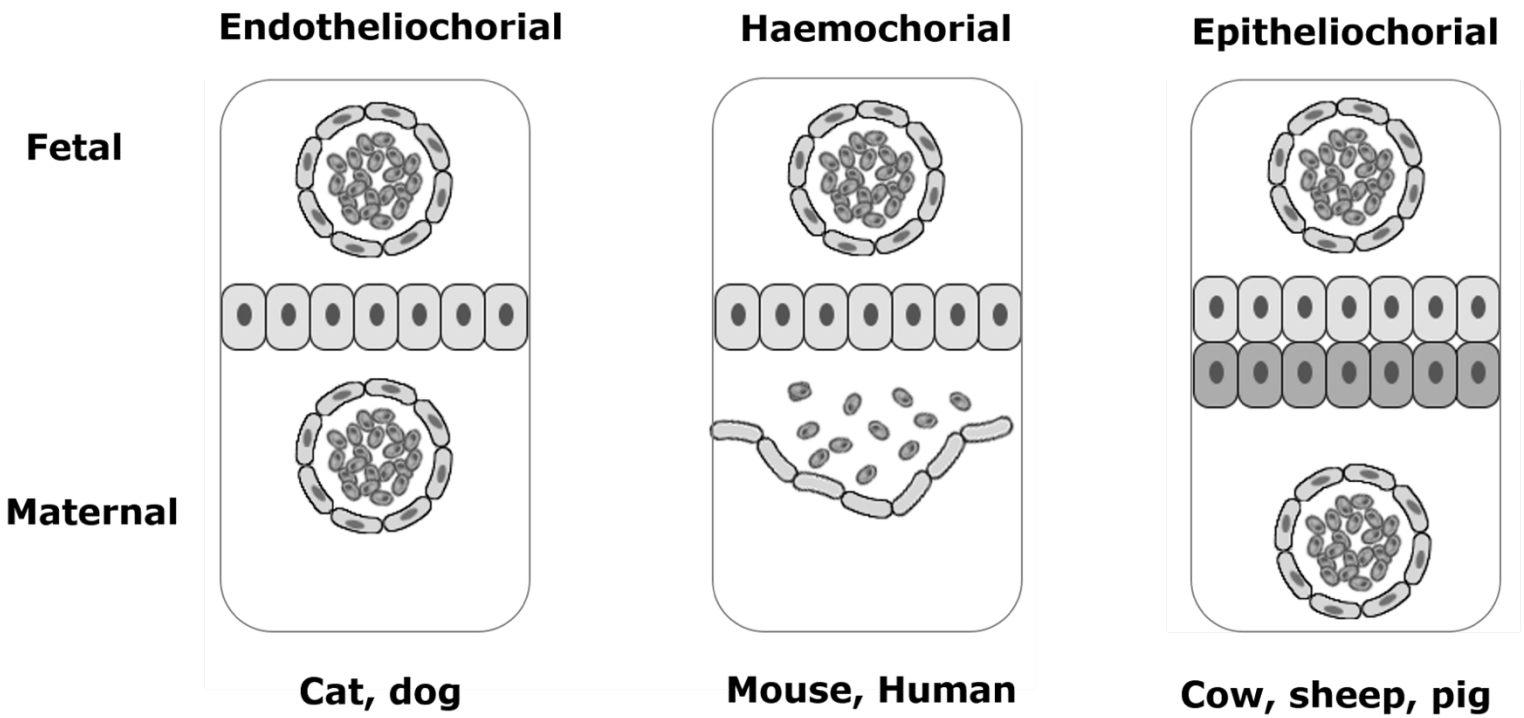


Figure 1.3 Schematic of the Endothelial, Haemochorial and Epitheliochorial placentae. Adapted from PrabhuDas et al. (2015). In the endotheliochorial placenta, the maternal vascular endothelium borders the chorion. In the haemochorial placenta, the chorionic villi are in direct contact with maternal blood. In the epitheliochorial placenta, the uterine epithelium (dark grey cells), and maternal vascular endothelium are the barrier between maternal blood and the chorion (light grey cells).

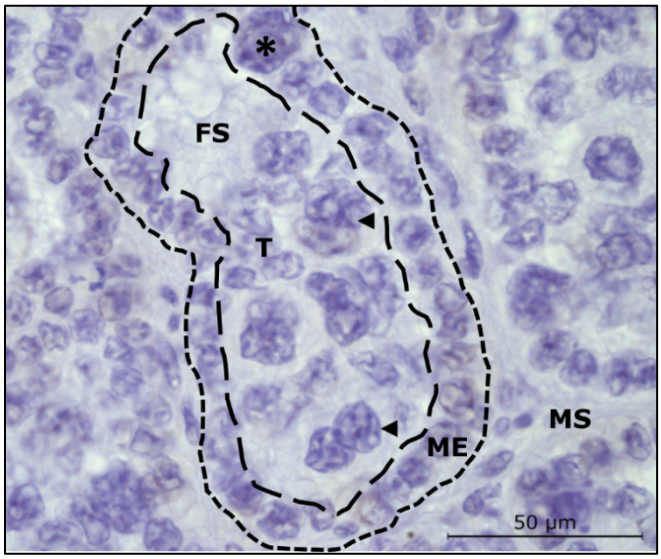


Figure 1.4 Example of a feto-maternal junction, displaying trophoblast binucleate cells at different stages of migration in the 98 days postconception bovine placentome. A maternal epithelial cell layer (ME) surrounds the trophoblast (T) and fetal stroma (FS). The basal lamina is located between the maternal stroma (MS) and epithelium (ME) (short dashed lines). Micro-villi connect the cotyledon and caruncle (long dashed line). Trophoblast binucleate cells (BNCs) are present (arrowhead) migrating towards the ME. A BNC is fused with an epithelial cell (*). For a full orientation of the bovine placentome, see Appendix 18.

Table 1.2 Key time points of placentome number and development in bovine gestation

Days postconception	Placentome Development
30	Specialised areas called cotyledons start to develop on the chorioallantois surface.
33	Visible umbilical cord extending from the fetus to chorioallantois, include vessels. Chorioallantois attaches to maternal endometrium. Close connection occurs at maternal caruncles, the specialised areas of the endometrium.
37	Placentomes are macroscopically visible.
37-40	Up to 20 placentomes can be present on the chorionic surface.
40-50	Placentome number increases three-fold.
60-70	Large cotyledons are located on the chorionic surface near the fetus. Vascularisation of the membranes continues.
70	Placentome number reaches a maximum. The average placentome number is 80 to 90. Placentome number ranges from 20 to 120.

(Assis Neto et al., 2009; Peter, 2013; Schlafer et al., 2000).

Two distinct bovine trophoblast cell populations exist; uninucleate trophoblasts (UTCs) and trophoblast binucleate cells (BNCs), which have specialised functions and molecular activity (Wathes and Wooding, 1980, Wooding et al., 2005, Wooding and Wathes, 1980, Red-Horse et al., 2004). BNCs and UTCs constitute approximately 20% and 80% of the trophectodermal layer respectively throughout gestation (Wooding et al., 1996). The distinguishing feature of the bovine placenta is the presence of BNCs (Wooding and Wathes, 1980). UTCs start to differentiate to either mature UTCs or BNCs from around 14-16 dpc (Wooding and Wathes, 1980, Spencer et al., 2006). UTCs become polyploid BNCs by the absence of cytokinesis (acytokinesis) during mitosis (Klisch et al., 1999, Ullah et al., 2009). UTCs express the pregnancy recognition hormone interferon tau, during conceptus elongation until implantation at around 19-19.5 dpc (Kusama et al., 2016, Kusama et al., 2018, Roberts et al., 1992). BNCs synthesise hormones and growth factors such as bovine pregnancy associated glycoprotein, (bPAG), and placental lactogen (bPL), which play a role in fetal growth (Wooding et al., 1996). bPAG and bPL are packaged in granulated vesicles within a BNC (Wooding et al., 1996, Wooding et al., 1986). Upon blastocyst implantation, BNCs migrate towards the maternal endometrial epithelium until term (Wooding and Wathes, 1980). Feto-maternal junctions (or interdigitations) are separated by microvilli, which BNCs migrate across in order to fuse with a maternal epithelial cell (Klisch et al., 2006, Lee et al., 1997). Granule contents such as bPAG are transferred to the maternal cell and subsequently the maternal blood circulation (Wooding et al., 1996, Wooding et al., 1986). It is therefore hypothesised that a BNC has stages of migration towards the epithelium in the order: stromal, stromal-villi, and stromal-epithelial. Bovine BNCs do not pass the basal lamina between the epithelium and maternal stroma (Spencer et al., 2006). The function of BNC migration and fusion is transfer of BNC glycoproteins to the epithelial cell (Hashizume, 2007, Klisch et al., 1999, Patel et al., 2004). From around 16-17 days post conception and throughout gestation (Wooding and Wathes, 1980), BNCs each fuse with a single epithelial cell, forming a trinucleate cell, and then is degraded to a cell remnant and phagocytosed by the epithelial cell (Klisch et al., 1999).

The close association between the caruncle and cotyledon allows communication of BNC glycoproteins from the placenta to the maternal circulation (Butler et al., 1982, Garth Sasser et al., 1986, Zoli et al., 1992, Mialon et al., 1993). Nearing the end of the first trimester, placental blood

flow and oxygenation increase to support increased mitochondrial oxidative metabolism in the placenta and fetus (Murray, 2012). The placenta channels oxygen to the fetal circulatory system, and utilises oxygen for placental energy demands, such as nutrient transport, protein synthesis, growth and hormone production (Murray, 2012). It is therefore important to consider how proteins expressed in the placental vasculature may alter placental function, and subsequent fetal growth.

The relationship between placental development, and subsequent placental weight has been shown to be positively correlated with fetal birth weight (Echternkamp, 1993, Zhang et al., 1999, Perry et al., 1999, Sullivan et al., 2009c). This is evidence for the importance of the placenta in beef production. Investigation of specific proteins expressed in the placenta, and their putative functions may further current understanding of the importance of placental changes in response to the maternal diet during gestation. The bovine placenta is nondeciduate, meaning the maternal placental tissues are retained and resorbed by the dam after parturition, while the fetal membranes are expelled with minimal trauma at parturition (Carter and Enders, 2013).

Studies have reported overcompensation of the placenta when under maternal malnutrition, where the placenta will grow larger to increase its capacity for maternal-fetal exchange (Han et al., 2014, Lumey, 1998). Remodelling is required for this compensatory growth, which involves breakdown of the extracellular matrix (ECM) (Macias and Hinck, 2012). Neutrophil elastase is an enzyme which degrades the ECM component elastin (Woessner, 1991), thereby allowing remodelling of the placenta. In placentomes, remodelling has also been observed when under maternal undernutrition (Han et al., 2004, Kawamura et al., 2007, Xu et al., 2006).

Both 98 dpc and term cotyledons were investigated in the current study. The study of these two time-points allows investigation of the cellular effects of maternal diet during periconception and postconception at the end of the first trimester (98 dpc) and term. Considering that after 98 dpc, maternal dietary treatments all returned to maintenance requirements, studying the term placenta may reveal whether the return to maintenance requirement diets (from 98 to 290 dpc, Chapter 2.1.6) allowed for compensation of earlier

protein restriction, or if cellular differences in structure and protein expression persisted to term.

1.4.2 Maternal Dietary Influences on Placental Function

Maternal dietary protein can affect placental development and function, which subsequently affects fetal growth (Echternkamp, 1993, Rasby et al., 1990). Perry and colleagues (1999) demonstrated that low maternal dietary protein (7% CP) during the first trimester followed by high (14% CP) maternal dietary protein in the second trimester of gestation increased placental volume. This was found to be due to a change in maternal dietary protein concentration between trimesters, as opposed to constant levels of maternal dietary protein (Perry et al., 1999). In placentomes exposed to altered dietary CP levels (7% to 14%, and 14% to 7%) between the first and second trimester, trophectoderm volume density and blood vessel volume density were increased and decreased respectively, compared with groups where protein levels remained the same (Perry et al., 1999). This is evidence for maternal dietary protein level affecting trophoblast and vasculature development in the bovine placenta.

In beef cows, the influence of maternal dietary protein during the first and second trimester on the placenta was investigated by Sullivan and colleagues (2009b, 2009c). Bovine pregnancy associated glycoprotein (bPAG) is a glycoprotein produced by BNCs, and Maternal plasma bPAG at 82 dpc was greater in heifers fed low protein (0.41 kg/heifer/day) during the first trimester of gestation compared with high protein (1.37 kg/heifer/day) counterparts (Sullivan et al., 2009b). bPAG was not affected by maternal diet at 28, 179 and 271 dpc (Sullivan et al., 2009b). The same study reported that term cotyledon number was greater in heifers exposed to high protein in both the first and second trimesters compared with those exposed to (1) low protein in both the first and second trimester, (2) high protein in the first trimester followed by low protein in the second trimester, or (3) low protein in the first trimester followed by high protein in the second trimester (Sullivan et al., 2009c). Although term cotyledon number was different between groups, cotyledonary weight and surface area were unaffected by maternal diet (Sullivan et al., 2009c). This suggests that trophoblast production of bPAG was altered in the first trimester (82 pdc), but potential placental adaptations unrelated to cotyledonary weight and surface area

occurred to support trophoblast function to term. Study of histological structural and protein expression differences around 82 dpc relating to placentome function may further elucidate the effects of first trimester maternal protein restriction.

Vonnahme and colleagues (2007) studied the effects of maternal nutrient restriction from 30 to 125 dpc, followed by realimentation (125 to 250 dpc), on placental vascularity in beef cows. Vonnahme and colleagues (2007) reported that nutrient restriction had little effect on vascularity at 125 dpc. However, cotyledonary vascularity was significantly lower at 250 dpc, which suggests that nutrient restriction combined with realimentation resulted in decreased cotyledonary vascularity. Caruncular capillary surface density was greater in nutrient restricted cows compared with control cows at 250 dpc, but there was no significant difference at 125 dpc. This suggests that a nutrient restricted diet leads to adaptation of the caruncular vasculature for increased capillary surface density upon realimentation. However, In the caruncles, no significant differences were reported for the density of blood capillaries within a certain area, or the number of capillaries, at either 125 or 250 dpc (Vonnahme, 2007). This suggests that caruncular vascularity was largely unaffected by nutrient restriction followed by realimentation.

This is contrary to the study by Perry and colleagues (1999), although there are differences in dietary treatment and time of diet during gestation. Vonnahme and colleagues (2007) commenced dietary treatment at 1 dpc, whereas Perry and colleagues (1999) commenced at 42 dpc. In order to understand the effect of the level of maternal dietary protein and the time of dietary implementation in relation to gestation on fetal development, it is necessary to identify proteins involved in placentome function or development which were affected by the dietary interventions in the current study.

1.5 The Mammary Gland

The mammary gland is the characteristic feature of mammals (Akers, 2002). The mammary gland is a unique structure originating from apocrine sweat glands that produces and secretes nourishment in the form of milk (Macias and Hinck, 2012, Oftedal, 2002). Milk is composed of emulsified fat, proteins,

vitamins, minerals, and sugars (Montalbetti et al., 2014). Fat enters the milk through vesicles which form from the apical membrane of the alveolar epithelium (Irmak et al., 2012). The milk produced in the first 24 to 36 hours post-partum is termed colostrum (Pakkanen and Aalto, 1997). Colostrum provides additional immunological defence to the neonate (Clark, 2005, Akers, 2002). The mammary gland is an exocrine organ, under hormonal regulation (Alkafafy et al., 2012, Musumeci et al., 2015, Ohtani et al., 2011, Tucker, 2000, Makarem et al., 2013). In primates and humans, two mammary glands are located within the breasts, on the upper ventral side in females (Clark, 2005). In many mammals such as mice, rats and pigs, the mammary glands are located along the abdomen, and teat number varies (Veltmaat et al., 2003, Hirooka et al., 2001). In cows, goats and sheep, the mammary gland is located in the udder, inguinal, between the hind limbs (Frandson et al., 2009, Hovey et al., 2002).

The mammary gland is unique as it undergoes cycles of growth and involution (Knight and Wilde, 1993, Smith et al., 2012). These stages are classified into five distinct stages: pre-puberty, puberty, pregnancy, lactation, and involution (Smith et al., 2012, Macias and Hinck, 2012, Brisken and O'Malley, 2010). Pre-pubertal development is considered hormone-independent, while mammary development during puberty, pregnancy, lactation and involution are hormone-dependent (Smith et al., 2012, Brisken, 2002).

Bovine fetal mammary development commences during embryogenesis (Capuco and Akers, 2010, Turner, 1930) and a number of developmental stages occur between 30 and 100 dpc (Rowson et al., 2012). The key stages are depicted (Figure 1.5). The postconception diet (23 to 98 dpc) in the current study falls within this 'critical window' in fetal mammary development. Fetal mammary development and the effects of maternal diet is therefore of critical interest. Further review of the bovine mammary gland and fetal mammary development is undertaken (Chapter 3.1).

Future research into the effect of maternal dietary protein on fetal gene expression is required to investigate the long-term effects on offspring. Areas such as offspring mammary development during postpartum, puberty, and pregnancy and lactation are of interest, as investigating these stages of development will provide information on the future productivity of offspring subjected to different maternal dietary protein diets. In terms of health,

future research into the effect of maternal dietary protein on offspring mastitis and other mammary diseases is of interest as it will identify the future health of the offspring mammary gland, which is linked to productivity and subsequent profitability. This research aims to further the understanding of maternal dietary protein during the periconception and postconception periods on the fetal mammary development, which may impact the future health and productivity of beef cattle offspring.

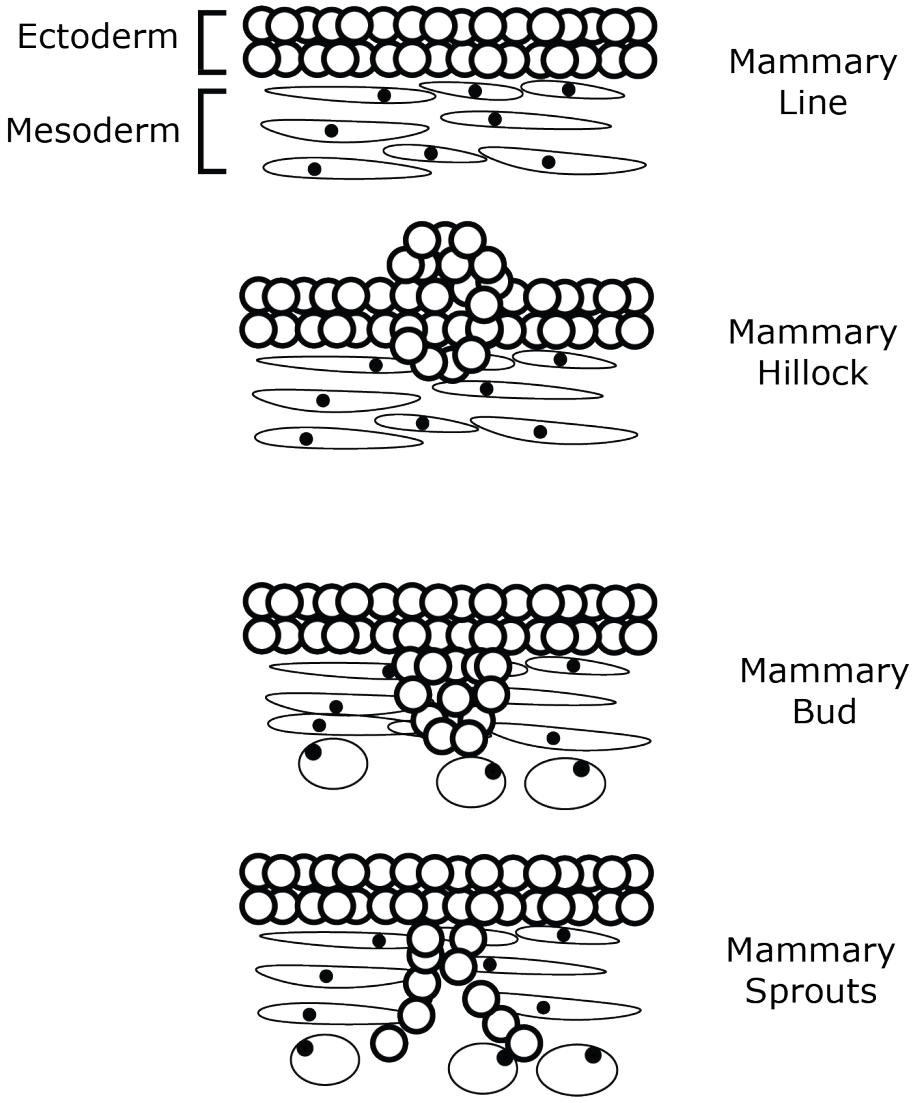


Figure 1.5 Key stages of fetal mammary gland development. Adapted from (Capuco and Akers, 2010, Turner, 1930).

1.6 Vascularisation

In cows, the vasculature is derived from the splanchnic mesoderm which is visible by day 21 postconception in bovine embryos (Maddox-Hyttel et al., 2003). Formation of the vasculature involves two distinct processes, vasculogenesis and angiogenesis. Vasculogenesis is the *de-novo* formation of blood vessels from undifferentiated precursor cells, whereas angiogenesis is the formation of new blood vessels from existing blood vessels (Andres and Djonov, 2010).

The cells which form the vasculature originate from the mesoderm (Table 1.2). Blood vessels start as primitive blood islands, which are formed of hemangioblasts (Table 1.2) (Lamalice et al., 2007). Primitive blood islands form more developed vascular structures in the presence of vascular endothelial like growth factor (VEGF) and a matrix of predominantly hyaluronan and collagen. VEGF stimulates cell migration while the matrix acts as a scaffold to allow cells to migrate and form a tube-like structure (Lamalice et al., 2007). The function of both the placenta and mammary gland relies on vascularisation. Functions such as nutrient transfer and gas exchange are vital parts of organ function.

Endothelial cells are derived from the mesoderm, specifically hemangioblasts (Ferkowicz and Yoder, 2005, Lamalice et al., 2007). Endothelial cell migration is an important part of angiogenesis (Matsumoto et al., 2005). Two cell types constitute blood vessels, endothelial cells and perivascular cells, also termed pericytes (Bergers and Song, 2005). Endothelial cells line the lumen of a blood vessel wall, while pericytes line the outer surface (Bergers & Song 2005). Pericytes constitute a cell body and multiple long projections anchored to the abluminal endothelium wall with the aid of adhesion plaques, providing scaffolding (Bergers and Song, 2005). Orlidge & D'Amore (1987) demonstrated that by direct contact with the endothelium, pericytes contribute towards vascular development by inhibition of endothelial cell growth and proliferation. Pericytes communicate with endothelial cells via paracrine signalling and direct cell contact (Bergers and Song, 2005). Direct connections between pericytes and endothelial cell cytoplasm are enabled by gap junctions (Bergers and Song, 2005). These connections allow the exchange of small molecules and ionic currents (Bergers and Song, 2005). Furthermore, pericytes produce and release basic fibroblast growth factor (bFGF) (Watanabe et al., 1997) and VEGF

(Greenberg et al., 2008) which are known to be essential in angiogenesis, directly stimulating the proliferation and migration of endothelial cells (Beck and D'Amore, 1997, Greenberg et al., 2008).

1.7 Conclusions

In conclusion, evidence supports further study of the effect of maternal dietary protein on beef cattle offspring. The effect of maternal dietary protein on the development and gene expression in the bovine placenta is of interest due to its' critical role in fetal growth and development. The effect on the placenta is also of economic interest. Optimal growth and development of healthy offspring is vital to herd productivity and subsequently affects output and income of beef farming. Improved productivity may lead to improved revenue and beef farming sustainability, thereby improving the future of the beef industry. Furthermore, feed costs are a large proportion of costs to cattle farming. Therefore, further understanding into dietary effects on the future herd health, growth and development would provide important information for diet formulation. Further understanding may ensure efficient diet formulation in terms of minimising excess protein, which is the most expensive macronutrient in a ruminant diet. Secondly, efficient formulation for return on investment such as increased calf live weight, reduced veterinary costs, lifetime milk production and longevity may all be affected by diet. This subsequently could improve the sustainability of beef farming. Some effects of maternal dietary protein on the placenta have been identified. Further investigation of the relationship between maternal diet and cellular effects on the bovine placenta is required. The potential effects of maternal dietary protein on mammary gland development have been discussed, which may affect the ability of future mature beef cows to produce weaned marketable calves.

Previous evidence supports fetal programming and how malnutrition during gestation alters offspring gene expression, subsequent protein expression, growth, development and future health, often with negative lasting effects.

The overarching hypothesis of the current study is that low maternal dietary protein during periconception and postconception, negatively affects tissue development ((a) fetal mammary tissue, (c) placentome) and gene expression ((b) fetal mammary tissue) in the fetus.

To test this hypothesis:

a) Fetal mammary tissue - Immunohistochemical, histological, stereological analysis was conducted (Chapter 3) to assess the structural and cellular differences in 98 dpc fetal mammary tissues exposed to the four maternal dietary interventions (HH, HL, LH and LL).

b) Fetal mammary tissue - RNA-Sequencing, and validation by RT-qPCR was conducted (Chapter 4) to assess differential gene expression in 98 dpc mammary tissues exposed to the four maternal dietary interventions.
    i. Further immunohistochemical analysis of the proteins of the differentially expressed genes was performed, to investigate the presence and localisation of the proteins of interest (IL33 and FGFR2).

c) Placentome - Immunohistochemistry was performed to assess the presence and localisation of proteins of interest within 98 dpc placentomes (Chapter 5) and term cotyledons (Chapter 6) exposed to the four maternal dietary interventions.

The current study aims to further understand the cellular and molecular effects of low maternal dietary protein on bovine fetal mammary development and the bovine placenta. This may provide insights into the future mammary health and production of beef cow offspring exposed to low maternal dietary protein during gestation.

2 General Methods

2.1 Animals, Dietary Treatment and Management

2.1.1 Animals

Twelve-month-old primiparous Santa Gertrudis (*Bos Taurus x Bos indicus*) heifers (n=360) were selected based on body weight and age. All procedures were under ethical agreement between the University of Adelaide and the University of Nottingham (ethics code 1010 131112). Heifers were transported to Sedan, South Australia where they were housed in shaded feedlot pens in November 2011. Heifers were acclimatised to the housing for 60 days before dietary intervention commenced and fed an acclimatisation diet (Table 2.1). During acclimatisation, heifers learned to eat the ration portion of their partial mixed ration in individual stalls as was performed during the treatment periods. Heifers were fed a pellet ration daily in individual stalls, and straw (5% crude protein,(CP)) was offered in pens on an *ad libitum* basis prior to the diet treatment (Chapter 2.1.5) (Copping et al., 2014). Dietary treatments were a 2x2 factorial design resulting in four maternal dietary interventions (High High (HH)/ High Low (HL) / Low High (LH) / Low Low (LL)), as described previously (Copping et al., 2014). Heifers were randomly assigned, stratified by body weight, to one of four dietary treatments (Figure 2.1), 60 days preconception. Heifers were fed either low (L; 9% crude protein (CP)) or high (H; 14.6% CP) protein during periconception (60 days preconception to 23 days postconception (dpc) (Figure 2.1). At 23 dpc, heifers were split equally and either entered the low (7.6% CP) or high (12.4% CP) treatment group during postconception (23 dpc until 98 dpc) (Figure 2.1). The diet formulations are detailed in Table 2.1.

A subset (n=48) of heifers and fetuses (of which n=20 were female) were culled at 98 days postconception (Figure 2.1). Fetal mammary tissue and placentomes were collected and either fixed in 10% formalin and processed into paraffin wax blocks for histology, or frozen at -80°C for RNA isolation. The following sections in detail each stage of the processes (Chapter 2.2, Chapter 2.5).

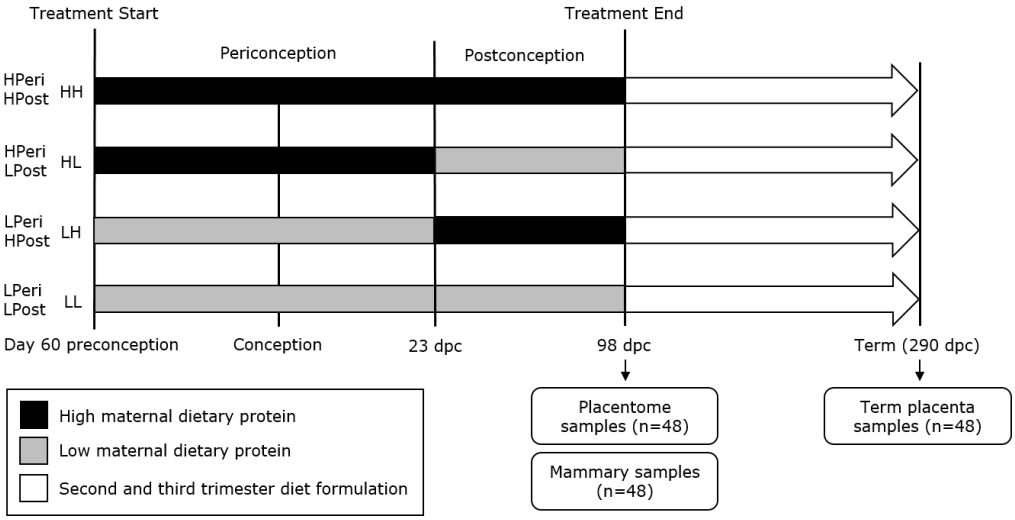


Figure 2.1 Timeline of maternal dietary interventions. Schematic of the four maternal dietary interventions (treatment). Schematic illustrates periconception, from 60 days preconception, until 23 dpc (days postconception), and postconception, from 23 dpc to 98 dpc. Key indicates the level of dietary protein in each treatment. At 98 dpc, placentome and fetal mammary samples were taken. Term cotyledons were sampled at calving.

2.1.2 Ovulation Synchronisation

Synchronisation of oestrous cycles was achieved 50 days after initiation of diet treatment using intravaginal devices which release progesterone (progesterone 1.56g, CUE-MATE, Bioniche Animal Health, Bayer Australia Ltd, Pymble, Australia). These devices were inserted day -10 with an intramuscular injection of 2mg oestradiol benzoate (Bomerol, Bayer Australia Ltd, Pymble, Australia). On day -2, heifers received intramuscular injections of prostaglandin (PG)F2 alpha (2ml; Ovuprost) and equine chorionic gonadotrophin (300 IU; eCG; Pregnecol), the intravaginal devices were also removed. Heifers were injected with 1mg oestradiol benzoate intramuscularly within 24 hours after the devices removal and inseminated 24 hours after the injection (day 0 = conception day) using frozen semen from a single Santa Gertudis bull (*Bos indicus x Bos taurus*).

2.1.3 Pregnancy

Pregnancy diagnosis via trans-rectal ultrasound (Sonosite, M-Turbo; Sonosite Inc., Bothell, USA) was performed 36 dpc. Of the original 360 heifers, 124 were positively diagnosed as pregnant (HH – 33, HL – 37, LH – 30, LL – 28). Non-pregnant heifers were immediately removed from the trial. Fetuses were sexed at 60 dpc using ultrasonography which allowed equal numbers of each sex to be selected and excised at slaughter.

2.1.4 Diet Formulation

The crude protein content of dietary treatments varied, however inclusion of a commercial vitamin and mineral premix met dietary requirements (Personal communication, Dr Viv Perry). Biofos® is a feed grade monocalcium phosphate (MDCP) (Mosaic®, Plymouth, Minnesota, USA). At 98 days post conception, the end of the first trimester, the remaining pregnant heifers were individually fed the National Research Council (NRC) recommended ration for body weight, containing 12% CP until parturition.

Table 2.1 Diet formulations of heifer rations

Diet ingredients	Acclimatisation	Peri		Post		Second and third trimester
		High	Low	High	Low	
Wheat (kg)	0.66	0.48	1.81	0.56	2.12	0.6
Canola Meal (kg)	2.23	-	-	-	-	0.89
Soybean Meal (kg)	-	1.83	0.48	2.14	0.56	0.44
Barley Straw (kg)	7[a]	6.7	5.5	10.7	10.2	8.6
Molasses (g)	90	72	72	84	84	60
Biofos® MDCP (g)	-	-	19	-	22	-
Salt (g)	15	12	12	14	14	10
Vitamin/trace Mineral Premix (g)	3	2	2	3	3	2
Dry Matter	9.1[b]					

[a]Assumed value. [b]Estimated value, diet previously published (Copping et al., 2014). Peri, Periconception (60 days before artificial insemination to 23 days post conception). Post, Postconception (23 days post conception to 98 days post conception). Second and third trimester (98 dpc to 290 dpc). MDCP, mono-dicalcium phosphate.

Table 2.2 Nutrient analysis of heifer rations

Diet ingredients	Peri-conception (60 days before AI to 23 days post conception)		Postconception (23 days post conception to 98 days post conception)		Second and third trimester (98 dpc to 290 dpc
	High	Low	High	Low	
Dry Matter (kg)	8.3	7.2	12.3	11.8	9.6
Dry Matter (%)[b]	88.0	87.7	88.0	87.8	
Forage (% DM)[b]	73.7	69.9	79.3	78.6	
Concentrate (% DM)[b]	26.3	30.1	20.7	21.4	
Total Energy ME (MJ)	71	63	102	98	79
M/D[b]	8.47	8.67	8.07	8.07	
% of Energy Requirements[a]	96	85	142	136	125
Total Crude Protein (kg)	1.18	0.62	1.49	0.88	0.92
Crude protein (% DM)[b]	14.6	9.0	12.4	7.6	
ERDP[b]	714	405	901	562	
UDP[b]	303	120	355	141	
% of Protein Requirements[a]	127	67	123	72	88
% CP (total Diet)	14.2	8.6	12.1	7.4	9.6
Total Calcium (g)	26	22	38	37	33
% of Calcium Requirements[a]	130	110	190	185	132
Total Phosphorous (g)	17	17	21	21	20
% of Phosphorous Requirements[a]	130	130	160	160	125

[a]Dietary requirements were calculated using Nutrient Requirements of Domesticated Ruminants (2007, CSIRO). Input values were based on nutrient analysis of component ingredients in the total diet, live weight and age of heifers at each diet change, mature cow weight of 550kg and the desired growth target. [b]Estimated value using Ultramix, (Ultramix, AGM Systems Ltd, Romsey, England), run by David Wilde. DM, Dry Matter; ME, Metabolisable Energy; MJ, Mega Joule; ERDP, Effective Rumen Degradable Protein; UDP, Undegradable Protein.

2.2 Tissue Collection, Sampling and Sectioning

2.2.1 Tissue Collection 98 days Post Conception

A subgroup of heifers (n=48) were slaughtered 98 dpc in a commercial abattoir. The fetuses were excised from the uterus, sexed, weighed, measured and cord blood was collected. Placentomes from 48 heifers (n=48) and female fetal mammary tissues (n=20) were among the tissues collected from the fetuses. Whole placentas were weighed, and their volumes recorded, before individual dissection of 5 placentomes per placenta (5 x n=48 samples). Individual placentomes were labelled accordingly then frozen at -80°C. The four quarters of the mammary fat pad were dissected separately. Per fetus, quarters were individually weighed, and two quarters were randomly assigned either molecular analysis and frozen at -80°C, and two quarters were randomly assigned for fixing in 4% paraformaldehyde (Chapter 2.2.3). This process was completed by Katrina Copping under the supervision of Dr Viv Perry in Australia.

2.2.2 Term Cotyledon Tissue Collection

Term fetal placental membranes were collected at the time of each calving under the supervision of Dr Viv Perry in Australia. Total fetal membranes were weighed, and the expulsion times for each heifer were recorded. Retained fetal membranes (n=2) were excluded from analysis.

2.2.3 Tissue Fixation for Histological Analysis

Tissue fixing was carried out by Katja Hummitzsch. Placental tissues (d98 and term), and mammary tissues (98 dpc) were processed and fixed as described below in Australia. Individual tissues were fixed in 4% paraformaldehyde in 0.1M PBS for 12 hours (1:5 tissue fixative ratio). Tissues were then washed in 0.1M PBS for 1 hour. Following this, samples were dehydrated through a series of 70%, 90%, 95%, and 100% ethanol for 2 hours, followed by 2x xylene washes for 1 hour each. Following sample transfer to molten paraffin (Paraffin, Leica TP1020, Automatic Tissue Processor, Nussloch, Germany) for 1 hour, samples were orientated and embedded in paraffin blocks and left to cool before sectioning. Samples were individually labelled with ID numbers only. Fetal sex and maternal diet were not included in tissue labelling. This allowed for blinded investigation and

analysis of all tissues, whether for histological or molecular analysis. Samples in paraffin blocks were transported to The University of Nottingham for further processing.

2.2.4 Tissue Sectioning

Tissue sectioning of mammary tissues, placentomes, cotyledons, and murine placentae was carried out by Aziza Alibhai. In order to create tissue slices for immunohistochemistry, a RM2255 Microtome (Leica, Wetzlar, Germany) at 20µm thickness setting, was used to trim the paraffin and expose the tissue. Each sample was then cut into sections using the RM2255 Microtome, at a thickness of 10µm for all tissues. Sections were floated on ethanol in order to remove potential tissue folds, floated on a water bath at 45ºC, and then transferred onto positively charged polysine microscope slides (Gerhard Menzel GmbH, Germany).

2.3 Immunohistochemistry

Slide mounted tissue sections were deparaffinised in xylene for 5 minutes. Following rehydration in a series of graded alcohol baths (100%, 90%, 70% ethanol respectively), slides were then washed in deionised water (dH_20) for 5 minutes. A microwaved sodium citrate buffer bath (1mM concentration) was used to achieve antigen retrieval for an optimised amount of time for each antibody. Thereafter the sections remained in citrate buffer for 20 minutes to cool down, ensuring full antigen retrieval. Sections were then washed in dH_20 for 5 minutes. Endogenous peroxidase was neutralised using 3 drops of Novocastra peroxidase block (Novocastra RE7101, Leica, Wetzlar, Germany) on each slide for 5 minutes. Parafilm M® (Bemis Company, Inc., California, USA) coverslips were used during incubation steps to prevent evaporation of reagents. The samples were then washed twice in 1x phosphate buffered saline (PBS; GIBCO, USA) for 5 minutes. Protein blocking was achieved by incubating with 150µl protein blocking solution (RE70102, Leica, Wetzlar, Germany) per slide for 30 minutes. The sections were then incubated in primary antibody (at a predetermined concentration which was optimised for each antibody) diluted in 5% normal goat serum for 60 minutes. Following a wash step, sections were then incubated in approximately 150µl per slide of Novocastra post-primary block (Leica, Wetzlar, Germany) for 30 minutes. Slides were incubated in approximately

150µl per slide of Novolink polymer (Leica, Wetzlar, Germany) for 30 minutes, followed by a wash step. The peroxidase was activated using diaminobenzidine reagent (DAB) working solution. 50µl Novocastra DAB chromogen (Leica, Wetzlar, Germany) was added to 2000µl of Novolink DAB substrate buffer (Leica, Wetzlar, Germany), vortexed briefly and 200µl was applied per slide (at a predetermined length of time optimised for each antibody). Slides were placed in deionised water bath for 10 minutes to deactivate DAB.

Haematoxylin (Richard-Allan Scientific, USA) stain was used to counterstain the tissue for 2 seconds and immediately washed in a bath of running tap water for 10 minutes. Sections were washed in deionised water for 5 minutes and then rehydrated in a series of alcohol baths (70%, 90%, and 100%) for 5 minutes each. Slides were placed in a xylene bath for 2x3 minutes before mounting with Distrene, Plasticiser, Xylene (DPX) (Sigma-Aldrich, Missouri, USA) and glass coverslips. Slides were left to dry overnight at room temperature.

2.4 Microscopy and Sampling

All photomicrographs of tissue sections were taken using a DM5000B microscope (Leica DM5000B microscope, Leica, Wetzlar, Germany). Systematic random sampling was used to acquire photomicrographs for image analysis (Gundersen and Jensen, 1987, Mayhew, 2008) throughout the studies.

2.5 RNA Isolation and Quality Control

Samples were removed from the -80°C freezer and placed on ice. Approximately 200 mg per tissue was added to gentleMACS™ M tubes (Miltenyi Biotec, Bergisch Gladbach, Germany) with 1000ml Trizol, and homogenised. The gentleMACS™ M tubes were flushed with the resulting homogenate using a Gilson pipette. Homogenates were transferred into 1.5ml microcentrifuge tubes and incubated at room temperature for 5 minutes. Chloroform (200µl) was added to the homogenates, shaken vigorously for 15 seconds, then incubated at room temperature for 3 minutes. Microcentrifuge tubes containing homogenate and chloroform were

centrifuged at 12,000xg for 15 minutes at 4°C. Following centrifugation, the upper aqueous phase containing RNA was transferred to new 1.5ml microcentrifuge tubes. Care was taken to not disturb the layer of protein. 70% ethanol (600µl) was added and the samples vortexed. A RNeasy micro kit was used according to the manual for animal tissue (Qiagen, Hilden, Germany). At room temperature, the samples (700µl/sample) were transferred to RNeasy Mini Spin columns (Qiagen, Hilden, Germany) placed in 2ml collection tubes (Qiagen, Hilden, Germany) and centrifuged at ≥ 8000xg for 15 seconds. The flow-through were discarded and the remainder of the samples were added to the RNeasy Mini Spin columns in the collection tubes, and centrifuged at ≥ 8000xg for 15 seconds. Subsequently, flow-through was discarded from the collection tubes. Buffer RW1 (350µl/sample) was added to the RNeasy Mini Spin columns and centrifuged at ≥ 8000xg for 15 seconds. DNase I (80µl/sample: Qiagen, Hilden, Germany) was added to the spin columns and incubated for 15 minutes. Care was taken to ensure that DNase I was pipetted directly onto the spin column tube membranes. Buffer RW1 (350µl /sample) was again added to the RNeasy Mini Spin columns, and centrifuged at ≥ 8000xg for 15 seconds. The flow-through of each collection tube was discarded. Buffer RPE (500µl /sample) was added to the RNeasy Mini Spin columns, and centrifuged at ≥ 8000xg for 15 seconds. Flow-through was discarded, then the RNeasy Mini Spin columns were placed in new 2ml collection tubes and centrifuged at full speed, for 1 minute. The RNeasy Mini Spin columns were then placed in new 1.5ml microcentrifuge tubes for RNA collection and RNase-free water (33µl) (Qiagen, Hilden, Germany) was added directly to the RNeasy Mini Spin column membranes, and centrifuged at ≥ 8000 x g for 1 minute to elute the samples. The elution step was repeated using the eluate in the microcentrifuge tube after centrifugation, pipetted back onto the RNeasy Mini Spin column membrane and centrifuged at ≥ 8000 x g for 1 minute. The RNeasy Mini Spin columns were discarded, leaving RNA samples in the microcentrifuge tubes. An aliquot of each sample (1µl /sample) was used to quantify RNA yield using a NanoDrop 8000 (Thermo Fisher Scientific, Waltham, Massachusetts, United States). The samples were then immediately stored at -80°C.

2.6 cDNA Synthesis

Isolated RNA samples were diluted to 500ng/µl. qScript™ cDNA SuperMix (Quanta Biosciences, Beverley, Massachusetts, USA) was used for cDNA synthesis. A 10µl volume of components (2µl qScript™ cDNA SuperMix, 8µl RNA at 500 ng/µl) was pipetted into each microcentrifuge tube, sealed securely, gently vortexed and centrifuged briefly to force the components to the bottom of the microcentrifuge tube. Microcentrifuge tubes were then incubated in a water bath (42.00°C) for 1 hour. Following incubation, samples were removed from the water bath and RNase free water (190µl) was added. The samples were then immediately stored at -80°C.

2.7 Statistical Analysis

Statistical analysis was carried out using SPSS® (IBM, Armonk, New York, United States). Graphs were created using Prism software (Prism 7, GraphPad Software, Inc, United States).

Levene's Test for Equality of Variances was used to test for equality of variances, to determine whether data was normally distributed. For the four diet groups (HH, HL, LH, LL), when data was normally distributed, a one-way analysis of variance (ANOVA) was used to compare means. The Post-Hoc test, LSD, was used to test for equal variance. When performing statistical analysis on periconception and postconception dietary interventions, an independent samples t-test was used to compare means. The individual statistics methods for each experiment are outlined in detail for each results chapter where applicable.

3 The Cellular Effects of Maternal Dietary Intervention on Fetal Mammary Gland Development

3.1 Introduction

The mammary gland originates from an apocrine sweat gland, and produces and secretes milk (Macias and Hinck, 2012, Oftedal, 2002). The functional units of the mammary gland are the epithelial alveoli (Yart et al., 2014). The alveoli are the mature milk producing units which contain a lumen, where milk is secreted and stored. Milk filters down into the tertiary, interlobular ducts connecting to the gland cistern, which stores milk (Yart et al., 2014). Depicted below is a schematic of one of the four quarters of a bovine mammary gland, which is contained within the udder (Figure 3.1).

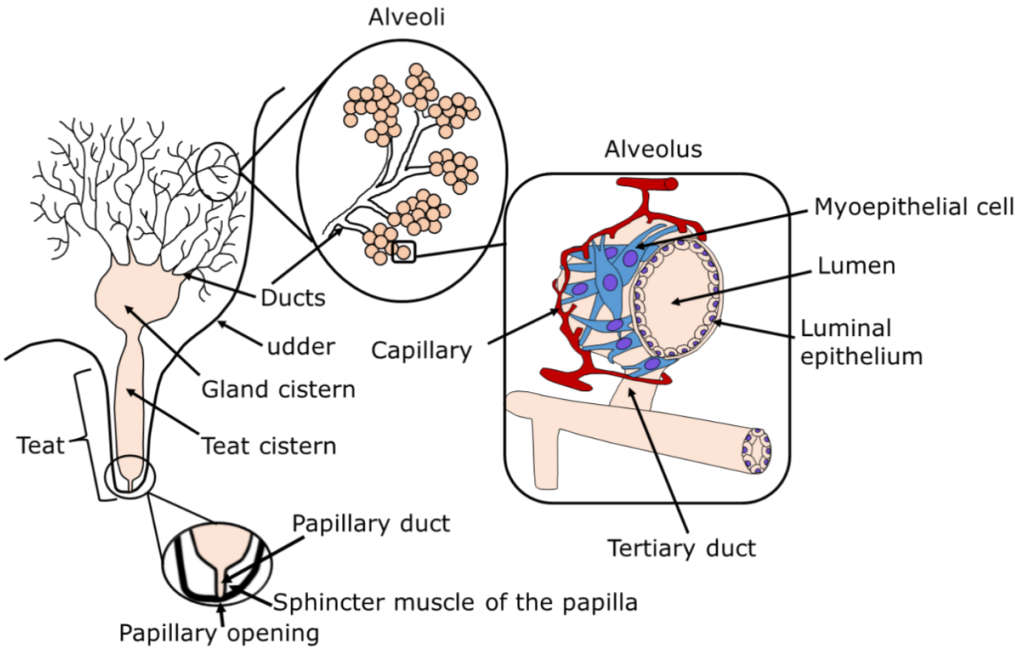


Figure 3.1 The structure of the adult bovine mammary gland. Adapted from (Sharma and Jeong, 2013, W. Lee Wilke, 2009). Schematic of one of the four bovine mammary glands contained within an udder.

The glandular ductal tissue is situated within the mammary stroma (Yart et al., 2014). The mammary gland consists of numerous cell types (Table 3.1). The basement membrane is important for duct morphogenesis as it provides a structural barrier and attachment to the surrounding stroma (Berry et al., 2003b, Fata et al., 2003, Lochter, 2011). The stroma contains cells including

fibroblasts, adipocytes, lymphocytes, and blood vessels (Yart et al., 2014). The extracellular matrix (ECM) also forms part of the stroma, and consists of laminin, collagens I and IV, fibronectin and proteoglycans (Yart et al., 2014). Milk yield is a genetic trait, and environmental factors such as nutrition can result in epigenetic modifications which influence milk production (Connor et al., 2008, Li et al., 2016). During development, gene promoter regions are under epigenetic regulation, which are then retained in the organism, long-term (Burdge et al., 2007). Fetal programming can result in permanent alterations to gene expression and tissue function (Tobi et al., 2009, Fleming et al., 2015). For these reasons, research into the effects of maternal diet on fetal mammary tissue was performed in this thesis.

The current study investigated fetal mammary gland tissue at 98 days postconception (dpc), which is around the end of the first trimester. This research will enable further understanding of the impact of maternal diet on fetal mammary gland development. A review of fetal mammary gland development was conducted and presented below in order to comprehend the current knowledge and the factors involved in development.

3.2 Mammary Gland Development

The mammary gland presents first as a thickening of the ectodermal epithelium surface, which descends into the mesodermally derived stroma, or mesenchyme (Brownfield et al., 2013, Watson and Khaled, 2008, Jeong et al., 2017a, Gjorevski and Nelson, 2011). Ectoderm-mesoderm signalling is required for mammary development to occur (Jeong et al., 2017a). Previous research has predominantly been conducted in mice (Cowin and Wysolmerski, 2010), with differences in terminology between murine and bovine developmental stages. In cows, the first visible stage of development, the parallel thickening of the ectoderm, is termed the mammary line (Figure 1.5), which is also known as a streak, or band (Capuco and Akers, 2010). Rowson and colleagues (2012) define the terminology by gestational day (Table 3.2).

Table 3.1 Cell type and structures originating from the ectoderm in the adult mammary fat pad

Cell Type	Structure formed
Epithelial	Ductal network
Vascular endothelial	Blood vessel
Perivascular	Blood vessel
Luminal /alveolar epithelial	The duct lumen lining
Myoepithelial	The basal epithelium surrounding the luminal epithelium. Cells are in close contact with the duct's basement membrane.

(Fata et al., 2003, Watson et al., 2011)

At 30 dpc in the cow, mammary bands are established, and development begins (Table 3.2). At around day 35 postconception, the mammary bands develop into mammary lines (Figure 1.5, Table 3.2). Mammary morphogenesis commences during the embryonic stage in gestation, pauses at parturition, and resumes at puberty (Sternlicht et al., 2006). At puberty, the ductal tree further develops within the mammary fat pad, providing the structure for alveoli (Sternlicht et al., 2006). The alveoli of the mammary gland further develop during pregnancy to prepare for lactation (Figure 3.1) (Fridriksdottir et al., 2011). To allow for each of these developmental stages, stromal tissue remodelling is essential for mammary epithelial duct development as it allows outgrowth and branching morphogenesis (Berry et al., 2003b, Fata et al., 2003). During pregnancy, histological and stromal gene expression indicates that the proportional area of stroma decreases (Rudolph et al., 2003).

Table 3.2 Bovine fetal mammary gland development

Gestational day	Development
30	Mammary bands are established via parallel thickenings of the ectoderm, along the ventral side of the inguinal area.
35	Mammary bands develop into mammary lines. Mammary anlagen are first visible.
37	Cell proliferation along the paired mammary lines forms the mammary crests, while neighbouring cells do not proliferate along the mammary line.
40	Mammary hillock stage, proliferative epithelial zones in each crest become rounded and sink simultaneously into the mesenchyme.
43	The bud stage marks the final stage of embryonic mammary development. Sexual dimorphism of bud size, shape and position is apparent.
65	Each mammary bud is forced to obtrude above the existing plane of the epithelium and skin by the proliferation of mesenchymal cells surrounding each bud.
80	Mammary buds start proliferating ventrally and dorsally lengthwise. A solid critical mass of epithelial cells, known as the primary sprouts form, which are visible within the rudimentary teats.
90	Further epithelial cell branching occurs, secondary sprouts branch off primary sprouts. The secondary sprouts develop into the milk-collecting ducts that empty into the gland cistern.
100	Canalization occurs in the primary sprouts, producing the gland and teat cisterns.
110	Further tissue remodelling and apoptosis of gland occurs.
130	Further tissue remodelling and apoptosis of teat cisterns occurs.

Adapted from Rowson and colleagues (2012).

Each mammary line (Table 3.2) gives rise to two mammary crests, which eventually develop into the four quarters of the bovine mammary gland (Rowson et al., 2012). At parturition, the neonatal mammary gland comprises of rudimentary ducts which are supported by the mammary fat pad (Berry et al., 2003b). Mammary epithelial cell numbers and activity directly influence milk yield (Capuco et al., 2003, Knight and Wilde, 1993, Purup et al., 2000, Boutinaud and Guinard-Flament, 2004). Therefore, *in utero* effects on fetal mammary epithelial cell proliferation may affect future

milk production. Further to cell number, the structure of the ductal network is important for function.

Desmosomes and adherens junctions are cell-cell adhesion structures, comprised of cadherins (Knudsen and Wheelock, 2005). They are vital for maintaining epithelial structure and forming an organised multicellular population such as the stratified epithelia of a duct (Runswick et al., 2001, O'Brien et al., 2002). Desmosomes and E-cadherin are particularly important for luminal epithelial cell morphogenesis (Runswick et al., 2001). E-cadherin and desmosomes are also associated with formation of complex epithelial structures such as the mammary acini (alveoli) and tubules (ducts, secondary ducts) (O'Brien et al., 2002). The formation of these structures requires branching morphogenesis, which in turn requires cell adhesion and orientation by collagens (O'Brien et al., 2002, Brownfield et al., 2013). Extensive branching coincided with elongation into the collagenous intralobular stroma in calves (Capuco et al., 2002). 3-month-old calves were treated with oestradiol-17β, a mammary epithelial mitogen, to observe its effect on epithelial cell proliferation. However, extensive proliferation was also observed in control calves (Capuco et al., 2002). This provides evidence for prepubertal allometric mammary growth, as puberty commences around 9.5-months-old in Holstein calves (Capuco et al., 2002). Proliferation was analysed in all locations of the gland; cisternal, medial, outer, and was greatest at the interface between advancing stroma and fat pad (Capuco et al., 2002). At 3-months-old, terminal ductal units exist, the distal branching ductal structures and were 300 μm in diameter. Proliferating epithelial cells were observed throughout the terminal ductal units, and distribution was unaffected by oestradiol treatment (Capuco et al., 2002). Growth was predominantly dorsal, away from the teat and adjacent mammary glands, towards the fat pad. Proliferation between solid epithelial cords and more mature patent lumena was equal (Capuco et al., 2002). This suggests that epithelial proliferation is not a distinguishing characteristic of more mature ducts, and that presence of patent lumena (one lumen) or microlumina (multiple developing lumina not yet at patent lumena stage) are no indication of epithelial proliferation.

3.2.1 Mammary Gland Vasculature

Blood vessel development and subsequent blood supply to the mammary gland is vital to its function (Andres and Djonov, 2010, Cai et al., 2018). Milk is the predominant source of nutrients to pre-weaned beef calves, and nutrient availability to the mammary gland is partly dependent on blood-flow to the gland (Cant et al., 2002, Madsen et al., 2015). Further to nutrients and oxygen supply, blood supplies important hormones to the adult mammary gland which are vital for normal function (Prosser et al., 1996). Therefore, mammary vascular development is important for future milk production in cows, and subsequent growth and development of pre-weaned calves.

The external pudendal artery supplies blood to the mammary gland and branches into the caudal and cranial mammary arteries (Gorden and Timms, 2015). The caudal and cranial mammary veins remove blood and diverge into the external pudendal vein (Götze et al., 2010, Gow et al., 2003). The prominent milk veins of a cow are the superficial cranial and caudal epigastric veins (Piotrowska-Tomala et al., 2012). Smaller blood capillaries associate within the ductal network (Gjorevski and Nelson, 2011).

Mammary vascular development involves two distinct processes, vasculogenesis and angiogenesis (Woodward et al., 1998, Wang and Laurie, 2004). Vasculogenesis is the *de-novo* formation of blood vessels from differentiating endothelial cells (Risau and Lemmon, 1988, Risau et al., 1988). Angiogenesis describes the formation of new blood vessels from existing ones (Gupta and Zhang, 2005, Ribatti, 2008). Briefly, arteries and veins are composed of three layers: the *tunica intima*, *tunica media*, and *tunica adventitia*, whilst capillaries comprise of a single layer of endothelial cells and connective tissue (Barros et al., 2003, Cleaver and Melton, 2003, MacAlpin, 1980, Masani, 1986). Cell types and fibres which constitute arteries include vascular smooth muscle cells, endothelial cells, pericytes, elastic lamina, and collagen (Bergers and Song, 2005, Hellstrom et al., 1999, Masuda et al., 1999, Neary et al., 2017, Biasato et al., 2018). Endothelial cells line the blood vessel lumen wall, while pericytes line the abluminal surface (Bergers and Song, 2005). Pericytes constitute a cell body and multiple projections anchored to the basement membrane surrounding the endothelium (Bergers and Song, 2005). Adhesion plaques aid pericyte

attachment which provides scaffolding (Bergers and Song, 2005). Orlidge and D'Amore (1987) demonstrated *in vitro* that pericytes (of the bovine retina) directly inhibit endothelial cell growth and proliferation. Pericytes communicate with endothelial cells via paracrine signalling, and direct cell contact via gap junctions (Bergers & Song 2005). Gap junctions allow exchange of small molecules and ionic currents (Bergers and Song, 2005). Furthermore, pericytes produce and release basic fibroblast growth factor (Watanabe et al., 1997) and vascular endothelial growth factor (Greenberg et al., 2008) which are essential in angiogenesis (Beck and D'Amore, 1997, Greenberg et al., 2008).

Neovascularization and subsequent blood-flow to the mammary gland supports milk production. Expression of genes involved in angiogenesis were altered by increased milking frequency, when stimulators and inhibitors of vascular development were increased and decreased respectively (Connor et al., 2008). Therefore, there is potential for the periconception uterine environment to affect fetal mammary vasculature in the current study.

3.2.2 Adipocytes in the Mammary Fat Pad

The fetal mammary fat pad is thought to be present in mammary parenchyma at 80 dpc (Hovey et al., 1999). Triacylglycerol is stored in adipocyte cytoplasm, and can constitute the majority of the cell volume, resulting in the nuclei being visibly forced to the periphery of the cell (Tang et al., 2008). Adipocytes within the mammary fat pad can signal to mammary epithelial cells, regulating cell proliferation, development and activity (Hovey et al., 1999, Jeong et al., 2017b, Suzuki et al., 2015). This is relevant considering the epithelial cell roles in blood vessels, vascularisation, duct formation and maintenance (Section 2.1.1). Adipose tissue also has an endocrine function, producing adipokines such as leptin and adiponectin (Kershaw and Flier, 2004, Chilliard et al., 2001, Lecchi et al., 2015). Adipocytes also express inflammatory and immune related genes, and expression was altered by body condition and nutrition (Vailati-Riboni et al., 2016, Depreester et al., 2018, Contreras et al., 2016).

3.2.3 Collagen in Fetal Mammary Tissue

Collagens are heterogeneous, fibrous glycoproteins which form supramolecular networks such as fibrils (Crowley et al., 2000). A collagen

molecule is synthesised from procollagen (Bellamy and Bornstein, 1971, Robinson et al., 1998), which collectively form a collagen protein, following cleavage (Kadler et al., 1987). The basement membrane consists of proteins such as collagen IV, laminin 5, laminin 1, fibronectin and heparin sulphate proteoglycans (Lochter, 2011). The importance of collagen and other ECM components in mammary gland development has been demonstrated by *in vitro* cell cultures where human or rodent mammary epithelial cells or cell lines were cultured with and without ECM components (Berdichevsky et al., 1992, Li et al., 1987). Cultured mammary epithelial cells proliferate poorly in a monolayer, however when cultured on collagen I gels, cells formed organised branching tubular structures, or spheres (Lochter, 2011). This demonstrates the effect collagen I has on mammary epithelial cell development and indicates the importance of the ECM and its components in bovine mammary gland development. It has also been shown that type VII collagen is important in binding epithelial cells and stroma together (Van der Rest and Garrone, 1991). Therefore, it is possible that type VII collagen is present in 98 days post conception bovine mammary fat pad in the current study. However, there is no published evidence of collagen in fetal bovine mammary fat pads. Berry and colleagues (2003b) investigated collagen protein abundance and localisation in 6-months-old prepubertal Holstein heifers (n=14). Collagen VI was localised in the basal lamina (basement membrane) of mammary ducts, and fibronectin and laminin in the parenchymal stroma (Berry et al., 2003b). Overall, connective tissue, including collagen, is an essential component in the mammary gland of cows, for development and structural support (Nickerson and Akers, 2011). Within the adult bovine mammary gland, the four quarters are separated by the median suspensory ligament, and the connective tissue septum, supporting the weight of tissue and stored milk, which can amount to ~70kg (~25kg milk) in dairy cows (Nickerson and Akers, 2011, Franz et al., 2009). Furthermore, degradation of the suspensory ligament fibres occurs with age, leading to pendulous teats and udder, consequently increasing risk of mastitis (Nickerson and Akers, 2011, Abebe et al., 2016, Islam et al., 2010). Alterations in collagen development or structure in early development may lead to life-long increased risk of infection.

3.2.4 The Effects of Maternal Diet on Fetal Mammary Development

Few studies have previously investigated the effects of maternal diet on fetal mammary development, and most studies were in mice and sheep (Hilakivi-Clarke et al., 1998, Van Der Linden et al., 2009, Blair et al., 2010). Studies previously investigated calf mammary development, and the effects of early nutrition on development (Brown et al., 2005; Sejrsen et al., 1982). Pre- (7-month-old) and post-pubertal (13-month-old) heifer nutrition altered mammary growth (Sejrsen et al., 1982). *Ad libitum* or restricted (60%) feed was offered (length of dietary treatment not stated), resulting in 1218g and 613g daily bodyweight gain (Sejrsen et al., 1982). Pre-pubertal heifers fed *ad libitum* had 23% and 32% less mammary ductal tissue and DNA respectively, compared to restricted counterparts (Sejrsen et al., 1982). Whereas no differences were seen in post-pubertal heifers (Sejrsen et al., 1982). This indicates a critical period of mammary epithelial development susceptible to altered growth by nutrition. Furthermore, the effects of crude protein on heifer mammary development were studied (Brown et al., 2005; Dobos et al., 2000).

Dobos and colleagues (2000) studied the effects of CP level and proportion of rumen-undegradable-protein (UDP) on pre-pubertal mammary development in Holstein heifers (Dobos et al., 2000). The dietary intervention period was from 5 to 10 months-old, followed by culling of a subset of each treatment group at 16-months-old for mammary tissue collection and analysis (Dobos et al., 2000). Udder composition of fat free tissue (protein, DNA) was unaffected by CP and UDP (Dobos et al., 2000). Similarly, the percentage of collagen and secretory tissue area were unaffected by CP and UDP level (Dobos et al., 2000). Adipose tissue area was unaffected by UDP but was greater in heifers fed low CP versus high CP (Dobos et al., 2000). The ratio of fat: secretory tissue was greater in low CP versus high CP fed heifers, but was unaffected by UDP (Dobos et al., 2000). Overall, it appears CP was more critical than UDP level for pre-pubertal mammary development. Although this may also apply to fetal mammary development, it is currently unknown. Age and live weight at calving, milk volume, protein and fat yields were unaffected by CP (Dobos et al., 2000). This suggests that dietary intervention in 5 to 10 months-old heifers had no permanent effects on milk production or udder size. The feed substrates, heifer age and breed varied considerably compared with the current study,

however the findings of Dobos and colleagues (Dobos et al., 2000) support early development as a critical period for fetal programming and nutritional influence on mammary development (Dobos et al., 2000).

Brown and colleagues (2005) investigated even earlier dietary protein and energy interventions between 2 and 14 weeks of age, in milk replacer (Moderate or High 2-8 weeks of age) and concentrate (Low or High) throughout. Increased rate of parenchymal development and extra-parenchymal adipose tissue mass was observed in heifers fed high protein and energy compared to moderate levels (Brown et al., 2005). High CP during 8-14 weeks of age increased parenchymal and extra-parenchymal adipose, compared with low CP although parenchymal mass was unaffected (Brown et al., 2005). This indicates that pre-weaning diet influenced parenchymal development, whereas post-weaning diet affected level of fat deposition. These findings suggest parameter-specific critical periods of development.

3.2.5 Chapter Aims:

1) To understand and demonstrate the gross histology (including vasculature, duct development and adipocyte presence) of the bovine mammary fat pad at 98 dpc.
2) To quantify the vasculature within the 98 dpc fetal mammary fat pad under the four dietary interventions.
3) To quantify duct presence and describe developmental stages observed within the duct system, including associated vasculature, within fetuses exposed to the four dietary interventions.
4) To quantify and qualitatively describe collagen presence within the 98 dpc bovine mammary fat pad and compare between fetuses from the four dietary interventions.

Hypothesis:
Maternal dietary intervention will negatively affect duct and blood vessel development, and collagen presence in 98 days postconception mammary fat pad.

3.3 Methodology

In this chapter, immunohistochemistry and other histological stains alongside microscopy and systematic random sampling was used to investigate fetal mammary duct, duct micro lumina, patent lumen, duct associated blood vessels, and stromal blood vessels (Chapter 3.3.1), total collagen (Chapter 3.3.3) and adipocytes (Chapter 3.3.4). Statistical analysis was performed for quantitative data (Chapter 3.3.1, Chapter 3.3.3). Van-Gieson's stain (Chapter 3.3.2) was investigated in preliminary work to identify collagen and connective tissue. It is described in the methodology solely due to the use of this stain in confirming presence of duct associated blood vessels, which was displayed in results, to highlight duct associated blood vessels (Figure 3.7). Van-Gieson's stain was not selected for further qualitative or quantitative analysis.

3.3.1 Mammary Duct, Lumen, Associated Blood Vessel and Stromal Blood Vessel Analysis

To positively identify mammary epithelial cells which constitute the mammary ducts, immunohistochemistry for keratin (KRT) 18 in 98 days postconception bovine fetal mammary tissues was performed, by Henry Johnson (Undergraduate student, supervised by Dr Catrin Rutland). The general immunohistochemistry protocol (Chapter 2.3) was performed with some modifications in protocol detailed below. Prior to deparaffinisation in xylene, tissue sections were heated on a hot plate at 60ºC to soften the paraffin wax, then deparaffinised in xylene for 3 minutes. A microwaved sodium citrate buffer was optimised to 1 minute on medium power and 4 minutes on medium-low power microwave settings. Protein blocking was achieved by incubating tissue sections in 100µl of fetal calf serum for 1 hour. Sections were then incubated in the primary antibody, mouse monoclonal Anti-human KRT18 (Catalogue number SAB4700411, Sigma-Aldrich, Missouri, USA) diluted 1:100 in 5% fetal calf serum. Sections were incubated in the primary antibody for 1 hour and 15 minutes at 37ºC.

Photomicrographs of Anti-KRT18 stained tissue sections were taken using a DM5000B microscope (Leica DM5000B microscope, Leica, Wetzlar, Germany) using the 20x magnification objective, for optimal tissue coverage and precision of image analysis. Two slides of sectioned tissue per sample

(n=20 total, HH n=6, HL n=5, LH=4, LL n=5) were selected for microscopy. Systematic random sampling was used to select photomicrograph location on tissue sections, ensuring no overlapping of tissue photography within a section. One section per slide was utilised for systematic random sampling. Ten images per section (n=2x10) were taken to give an overall observation of tissue conformation throughout the sample. Photomicrographs for mammary duct, lumen and blood vessel analysis were taken by Henry Johnson (Undergraduate student, supervised by Dr Catrin Rutland and laboratory supervised by the author of this thesis), then photomicrographs (n=420 total photomicrographs, n=20 photomicrographs per sample) were analysed by the thesis author.

Image analysis was carried out using computerised image analysis software, Image-Pro 6.3 (Image-Pro 6.3, Media Cybernetics, Washington, USA). The area of individual mammary ducts, mammary duct lumina, duct-associated (within duct) blood vessels, and stromal blood vessels and blood vessel lumina were measured. The number of total ducts, ducts with (micro lumina, patent lumen) and without a lumen, per field of view (photomicrograph) were counted. Furthermore, the number of duct-associated blood vessels, and stromal blood vessels were counted. Proportions were further calculated in Excel 2013. (Excel 2013, Microsoft, Washington, USA). An example of duct and duct associated blood vessel image analysis is presented (Figure 3.2).

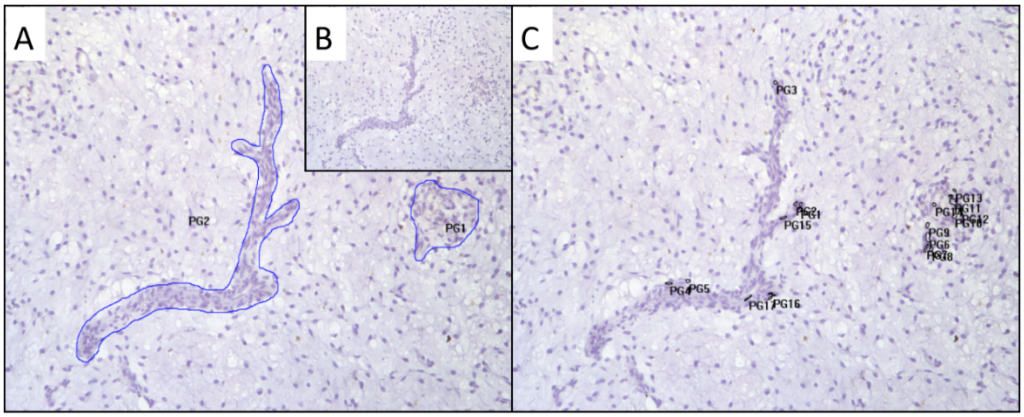


Figure 3.2 Example of duct image analysis performed in bovine fetal mammary tissues. Duct (A) and duct-associated blood vessel areas (C) in 98 days postconception bovine fetal mammary tissue. The original image (B) analysed is displayed. Photomicrographs obtained using the 20x magnification objective. Data output e.g. area of PG1, was exported to Microsoft Excel for further data analysis such as total and proportional areas.

3.3.2 Van-Gieson's Stain

Van-Gieson's stain was used in preliminary work to determine the optimum histological technique to stain for collagen and other connective tissues in 98 dpc mammary tissues. It was not used in the final analysis for collagen, therefore the n represents this preliminary stage of investigation.

Tissue sections (n=2x3 total preliminary work) were deparaffinised in xylene and a series of graded alcohols (100, 90 and 70% ethanol) and hydrated in distilled water for 5 minutes each. Weigert's Working Haematoxylin was prepared by combining Weigert's Iron Haematoxylin A, and Weigert's Iron Haematoxylin B at a 1:1 ratio. Sections were incubated in Weigert's Working Haematoxylin (200µl per slide) for 10 minutes at room temperature. Following a wash step in running tap water and distilled water ($_dH_2O$) for 5 minutes each, sections were incubated in Van Gieson's solution (200µl per slide) for 3 minutes. Sections were then dehydrated in 95% ethanol followed by 100% alcohol, for 2x 5 minutes each. Following clearing in Xylene for 2x 5 minutes, sections were mounted with DPX (Sigma-Aldrich, Missouri, USA) and a coverslip was applied, then left overnight to dry.

3.3.3 Collagen Histological Staining via Masson's Trichrome and Colourimetrics Analysis

One slide of sectioned tissues per sample (HH n=6, HL n=5, LH n=4, LL n=5) was used to investigate collagen in the fetal mammary gland. Tissue sections were deparaffinised in xylene for 4 minutes. Tissue sections were rehydrated in a series of graded alcohols (100%, 90%, 70% ethanol respectively), then washed twice in 1x phosphate buffered saline (PBS; GIBCO, USA) for 5 minutes. Sections were incubated in Bouin's solution at 60°C for 60 minutes. Sections were then washed in running tap water for 5 minutes, followed by a rinse step in $_dH_2O$. Sections were incubated in 100% haematoxylin (200µl per slide) for 2 minutes, followed by a wash step, and rinse step. Sections were incubated in Biechrich Scarlet acid-Fuchsin (200µl per slide for 30 seconds per slide, followed by a rinse step. Sections were incubated in 5% Phophotungstic/phosphomolybdic acid (diluted in $_dH_2O$; 200µl per slide) for 10 minutes. Sections were incubated in 200µl per slide of light green for 30 minutes. Following a rinse step, sections were transferred to 1% Acetic acid (200µl per slide) for 1 minute. Following a rinse step, sections were dehydrated in a series of alcohol (70%, 90%, 2x 100% ethanol respectively)

followed by 2x xylene baths for 4 minutes each. Sections were mounted with DPX (Sigma-Aldrich, Missouri, USA), cover slips were added, and gentle pressure was applied on the coverslip to remove any bubbles. Sections were left over night to dry at room temperature.

Photomicrographs of Masson's Trichrome stained tissue sections were taken using the 20x ocular lens (Leica DM5000B microscope, Leica, Wetzlar, Germany). Systematic random sampling was used to select photomicrograph location on one tissue section per sample (n=20 total, HH n=6, HL n=5, LH=4, LL n=5), ensuring no crossover of tissue photography within a section. Ten images per tissue section (n=1x10) were taken to give an overall observation of tissue composition throughout each sample (n=210 photomicrographs total, n=10 per sample). Image analysis was carried out using computerised image analysis software, Image-Pro 6.3 (Media Cybernetics, Washington, USA). Image-Pro 6.3 (Media Cybernetics, Washington, USA) was used to measure the area of collagen using the colour metrics feature, Masson's Trichrome stained blue-green collagen was selected manually until all blue stained objects were measured (Figure 3.3). The data output from Image-Pro 6.3 included the mean, mode, and median collagen area. The sum of the total collagen area was further calculated in Excel 2013 (Microsoft, Washington, USA).

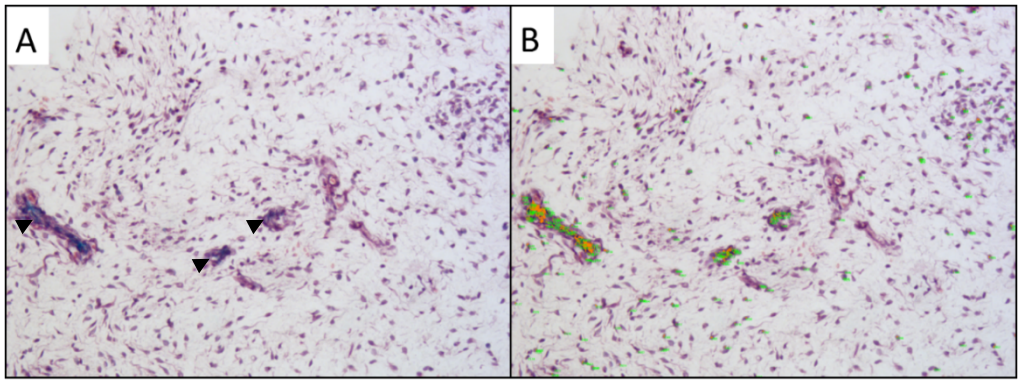


Figure 3.3 Example of colourimetrics image analysis performed in bovine fetal mammary tissues. The original photomicrograph (A) of 98 days post conception mammary tissue and a snapshot of the analysed photomicrograph (B) are displayed. Masson's Trichrome stained collagen (blue-green) is visible (arrowheads) in ducts with dense epithelial cells (A).

3.3.4 Adipocyte Histology

Immunohistochemistry was performed according to the general method described (Chapter 2.3). Uncoupling protein (UCP)-1 and UCP2 were used to positively identify adipocytes (Ricquier and Bouillaud, 2000). The antibodies used were: UCP1 (Thermo Fisher, Waltham, Massachusetts, United States) UCP2, (Abcam, Cambridge, UK) (Table 3.3). Antigen retrieval was optimised to 20 minutes medium low. Antibody concentration was optimised to 1:100 for each antibody. No statistical analysis was performed for qualitative analysis. One slide per sample (n=20 total, HH n=6, HL n=5, LH=4, LL n=5) was used to perform immunohistochemistry for each antibody.

Table 3.3 Commercial uncoupling protein antibodies used in mammary tissue immunohistochemistry

Anti-body	Catalogue number	Supplier	Details
UCP1	PA1-24894	Thermo Fisher (Waltham, Massachusetts, USA)	Rabbit anti-human polyclonal
UCP2	ab203244	Abcam (Cambridge, UK)	Rabbit anti-mouse polyclonal

3.3.5 Statistical Analysis

For maternal dietary intervention diets, firstly a Levene's test for equality of variance was performed (Chapter 2.7). When data was normally distributed, a One-Way ANOVA was performed (Chapter 2.7). For non-parametric data, Kruskell-Wallis test was performed. For periconception and postconception diets, statistical analysis was as previously described (Chapter 2.7).

Total collagen data was not normally distributed. For analysing the four diet groups, Kruskal-Wallis non-parametric test was used to compare means. Following this, pairwise Mann Whitney U tests were completed for all diets. For analysing periconception and postconception diets, Mann Whitney U test was used.

3.4 Results

At the time of conducting this study, there were no published works on the histology of the bovine 98 dpc mammary gland. Therefore, initially the tissue, cell types, and structures were identified and classified. This was necessary prior to making decisions on structures to be measured. At 98 dpc, mammary ducts were observed histologically within all tissue samples and were classified based on lumen development and categorised into one of three categories: no lumen, micro lumina, or patent lumen. Ducts either had no lumen, or multiple micro lumina, meaning some lumen development but no single central lumen (Figure 2.3.1). These ducts were classified as 'immature' and were analysed together as 'immature ducts'. More mature ducts were characterised as having a single central lumen, termed a patent lumen, and compact epithelial cells (Figure 2.3.1). In light of these structural findings, multiple analyses were performed. Measurements of all ducts (no lumen, micro lumina, and patent lumen) were undertaken and presented as total duct analysis (Appendix 1, Appendix 2). Further distinct analysis of ducts with patent lumena (Appendix 3, Appendix 4) was performed to investigate this sub-group of ducts. Additionally, duct associated blood vessels were measured (Figure 3.7). Results were partly presented as structural findings, which included qualitative presentation of structural findings observed across multiple areas of work (3.4.1). Further quantitative analysis of bovine fetal mammary ducts (Chapter 3.4.2), stromal blood vessels (Chapter 3.5.3), and total collagen area (Chapter 3.4.4) was presented, and the effects of maternal dietary protein was investigated for each structure. Finally, adipocytes were positively identified in bovine fetal mammary glands and their presence in each sample was reported (Chapter 3.4.5).

3.4.1 Structural Findings Within the 98 Days Postconception Bovine Mammary Gland

Initially, structures and cell types were identified within the 98 days postconception (dpc) fetal bovine mammary gland. Ducts, the teat structure, blood vessels, epidermis, fibroblasts, immune cells, adipocytes and collagens were observed at 98 dpc. The epidermis layer was observed in the majority of samples (Figure 3.4), however due to the nature of tissue sectioning this structure was not present in all sections. Structures thought to be a fetal

hair follicle or glandular structures were observed in the majority of samples (Figure 3.4, B). Fibroblasts and putative lymphocytes were identified within the mammary stroma, based on cell morphology alone (Figure 3.5). Rudimentary teat and teat cisterns were observed in a number of samples (Figure 3.6). The teat was characterised, by the presence of a thick epidermis at the base of the teat (Figure 3.6). The base of the teat was proximal to a developing papillary duct (also termed teat canal), with multiple layers of keratinocytes (Figure 3.6). The basal cell layer at the base of the teat was thicker and was observed protruding up into the mammary mesenchymal stroma (Figure 3.6). The epithelial lining of a teat cistern was thick and adjacent with the rudimentary papillary duct (Figure 3.6). The cells constituting the papillary duct were present, although presence of a lumen within the papillary duct was observed, the presence of a papillary opening (also termed streak canal, or *ostium papillare*) was not evident (Figure 3.6). Ducts and duct associated blood capillaries were also observed, where endothelial cells were identified as having a flattened nucleus. (Figure 3.7).

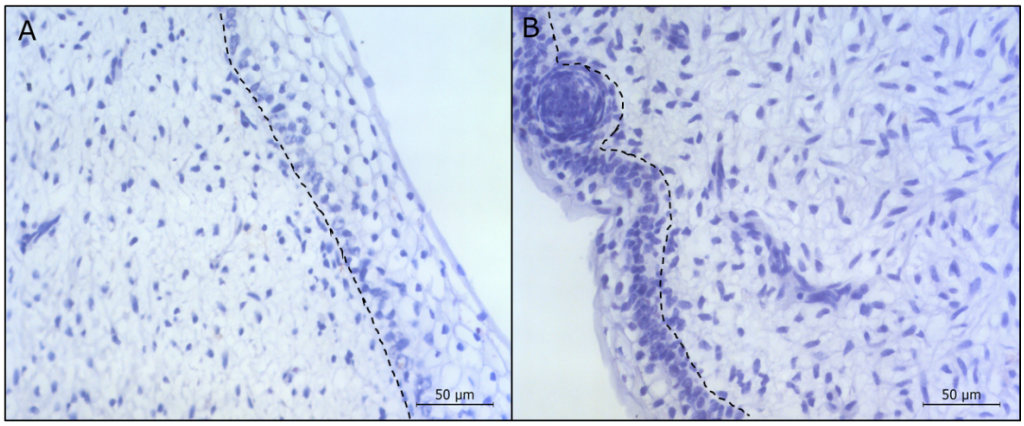


Figure 3.4 Examples of the epidermis in the 98 days postconception bovine mammary fat pad. Dashed line indicates the basement membrane bordering the stratum basale and the epidermis where keratinocytes are present, and dermis. (A) Epidermis is present (right of the dashed line) although typical dermis tissue was absent in the majority of tissue sections and only mammary stroma (left of dashed line) was observed. (B) Epidermis (left of the dashed line) and dermis (right of the dashed line observed by presence of elongated slightly flattened nuclei of fibroblasts. Scale bar represents 50 µm.

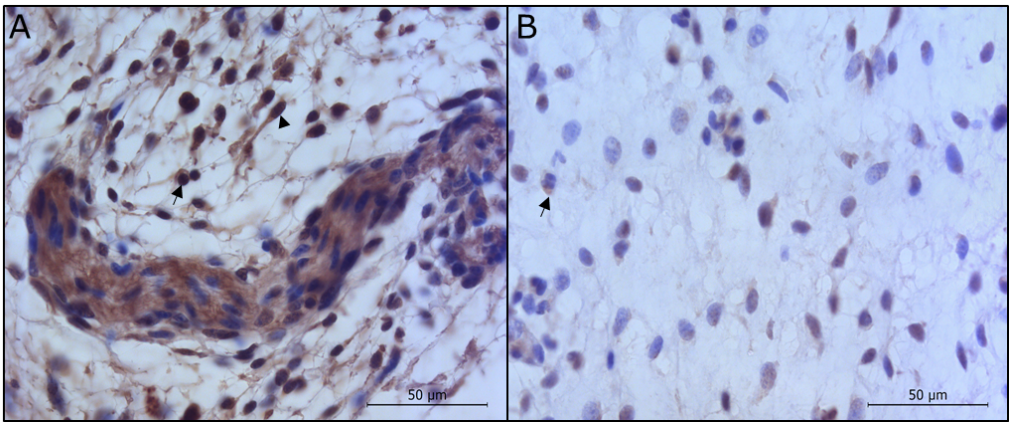


Figure 3.5 Examples of lymphocytes, fibroblasts, and neutrophils in the 98 days postconception mammary fat pad. Photomicrograph (A) displaying a lymphocyte (arrow) and fibroblast (arrowhead) in loose extra-cellular matrix of the fetal mammary stroma. Photomicrograph (B) displaying a neutrophil (arrow) stained positive for IL33 (For methods, see Chapter 4.2.5), localised within the mammary stroma. Scale bar represents 50 µm.

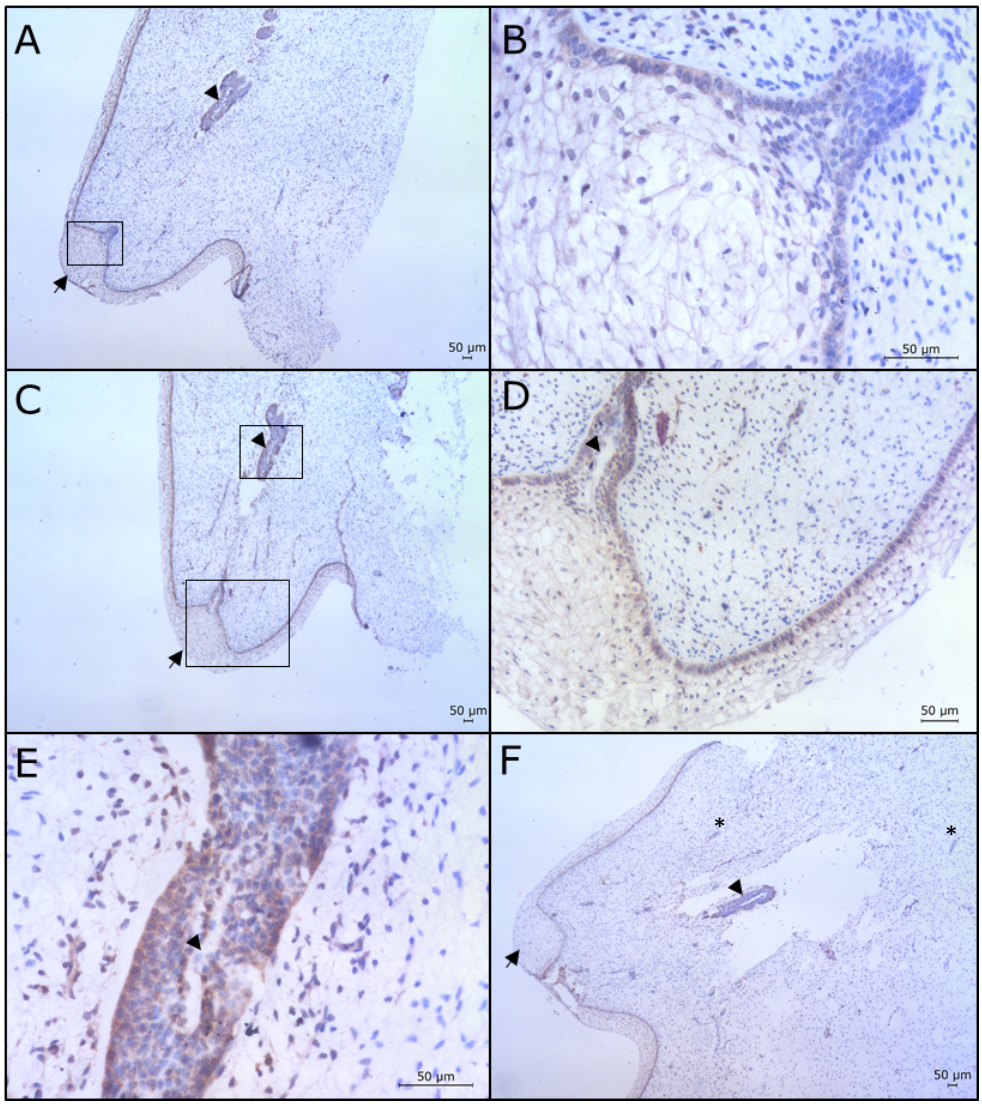


Figure 3.6 Examples of developing teats in the 98 days postconception bovine mammary fat pad. Tissue sections of observed fetal teats where the residue of mammary bud is indicated (arrow). Photomicrographs (A, C) are neighbouring sections of the same sample. (B) Higher magnification of a developing papillary duct, where the tissue section does not cross a lumen. Higher-magnification photomicrographs (D, E) of tissue section (C), showing the developing papillary duct (D) and canalisation of the primary sprout (E) each with a lumen (arrowhead). Second example of a developing teat (F) with residue of mammary bud (arrowhead). Epithelial ductal cords (*) are observed (A, C, and F), distal to the teat. Sections show FGFR2 staining. Scale bar represents 50 µm.

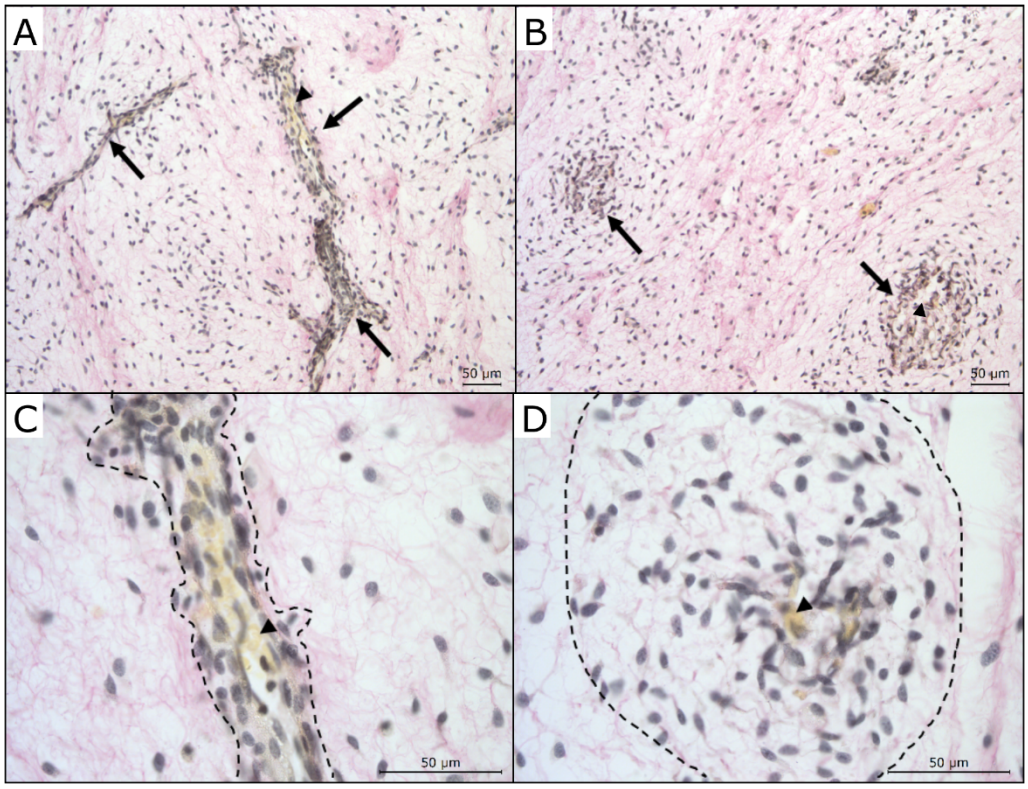


Figure 3.7 Observed duct-associated blood vessels within ducts of differing developmental stages in the 98 days postconception bovine mammary fat pad. Photomicrographs of 98 dpc mammary fat pads stained with Van-Gieson's stain. Photomicrograph displaying A) more 'mature' ducts (arrow) and associated blood vessels, visible by non-specific yellow staining for red blood cells (arrowhead), and B) 'immature' ducts (arrow) with microlumina and associated blood vessels (arrowhead). Higher magnification of C) more 'mature' duct and D) 'immature' duct (dotted line) and associated blood vessels (arrowhead). Scale bars represent 50µm.

3.4.2 The Effect of Maternal Dietary Intervention on Total Mammary Duct Tissue

Developing ducts were observed in fetal mammary tissues at 98 dpc in the current study. Ducts were qualitatively observed to differ in epithelial density and presence of a lumen (Table 3.4). This is notable as duct size was not indicative of duct developmental stage. Generally, three variables differed in ducts; size, epithelial density and lumen presence (Table 3.4). In epithelial ductal cords (epithelial ducts with no lumen), all types of epithelial density and duct size were observed. However, ducts with a patent lumena (mature lumen) tended to have high epithelial density. In descriptive terms only, ducts with low epithelial density generally had a larger duct size (Figure 3.7, B, D), although there were exceptions. Epithelial density was not quantified or part of total duct analysis and was strictly an observation.

Table 3.4. Descriptive variables in observed ducts

Duct size	Epithelial Density	Lumen presence
Small	Low	None, epithelial cord
Medium	Medium	Partial expansion, microlumina
Large	High	Patent lumena

Descriptive variables are arbitrary.

Total duct tissue and duct associated blood vessel parameters were unaffected by maternal dietary intervention (Appendix 1; $p>0.05$). Mammary duct tissue area was unaffected by periconception diet (Appendix 2; $p>0.05$). Results suggest that mammary tissues exposed to HPost exhibited 45.9% larger duct lumen (micro lumina, patent lumen) sizes compared with LPost counterparts (High protein, 1107.32±209.06μm^2 versus low protein, 599.10±111.47μm^2, $p<0.05$; Figure 3.8).

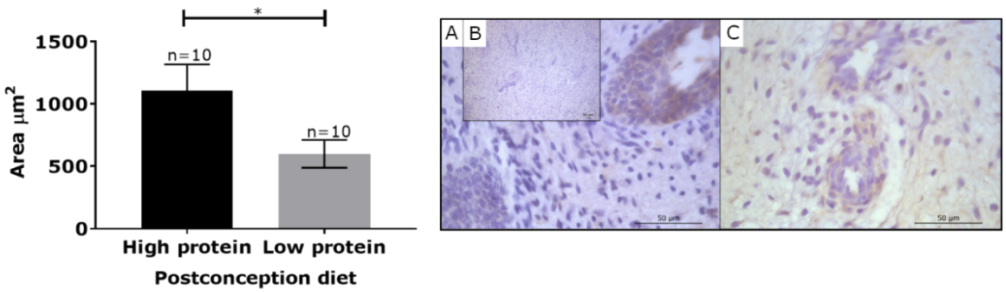


Figure 3.8 Increased duct lumen size in 98 days postconception mammary tissues exposed to high versus low maternal dietary protein. Graph displays the mean ± SEM duct lumen size in 98 dpc fetal mammary fat pads exposed to postconception high maternal dietary protein or low maternal dietary protein (Independent samples t-test; *$p<0.05$). Photomicrographs display HPost (A) or LPost (C) exposed bovine fetal mammary glands and a negative control (B). KRT18 staining is displayed in epithelial cells throughout ducts (A, C). Scale bar represents 50 μm.

3.4.3 The Effects of Maternal Dietary Intervention on Blood Vessels in the Mammary Stroma

Vasculature was present within the 98 dpc mammary stroma (Figure 3.9). Overall, stromal blood vessels were unaffected by maternal dietary intervention (HH, HL, LH, LL) and postconception diet (Appendix 5, Appendix 6). Mammary tissues exposed to LPeri had a 59% greater mean number of stromal blood vessels compared to HPeri counterparts (LPeri, 0.22±0.05 versus HPeri, 0.09±0.03, $p<0.05$; Figure 3.9 A). Tissues exposed to LPeri had a 65.6% greater proportion of blood vessel lumen area to total blood

vessel area compared with HPeri counterparts (LPeri, 0.032±0.009 versus HPeri, 0.011±0.004 proportional number, $p<0.05$; Figure 3.9 B). This equates to 3.2% and 1.1% of blood vessel area comprising of lumen area, in LPeri and HPeri exposed mammary tissues respectively. Tissues exposed to LPeri had a 68.75% greater ratio of stromal blood vessels: ducts compared to HPeri counterparts (LPeri, 0.08±0.02 versus HPeri, 0.02±0.01 proportional number, $p<0.05$; Figure 3.9 C). Further distinction between mature and immature ducts revealed a 77% greater proportion of stromal blood vessels to immature ducts in mammary tissues exposed to LPeri compared with HPeri (LPeri, 0.11±0.04 versus HPeri, 0.03±0.01 proportional number, $p<0.05$; Figure 3.9 D).

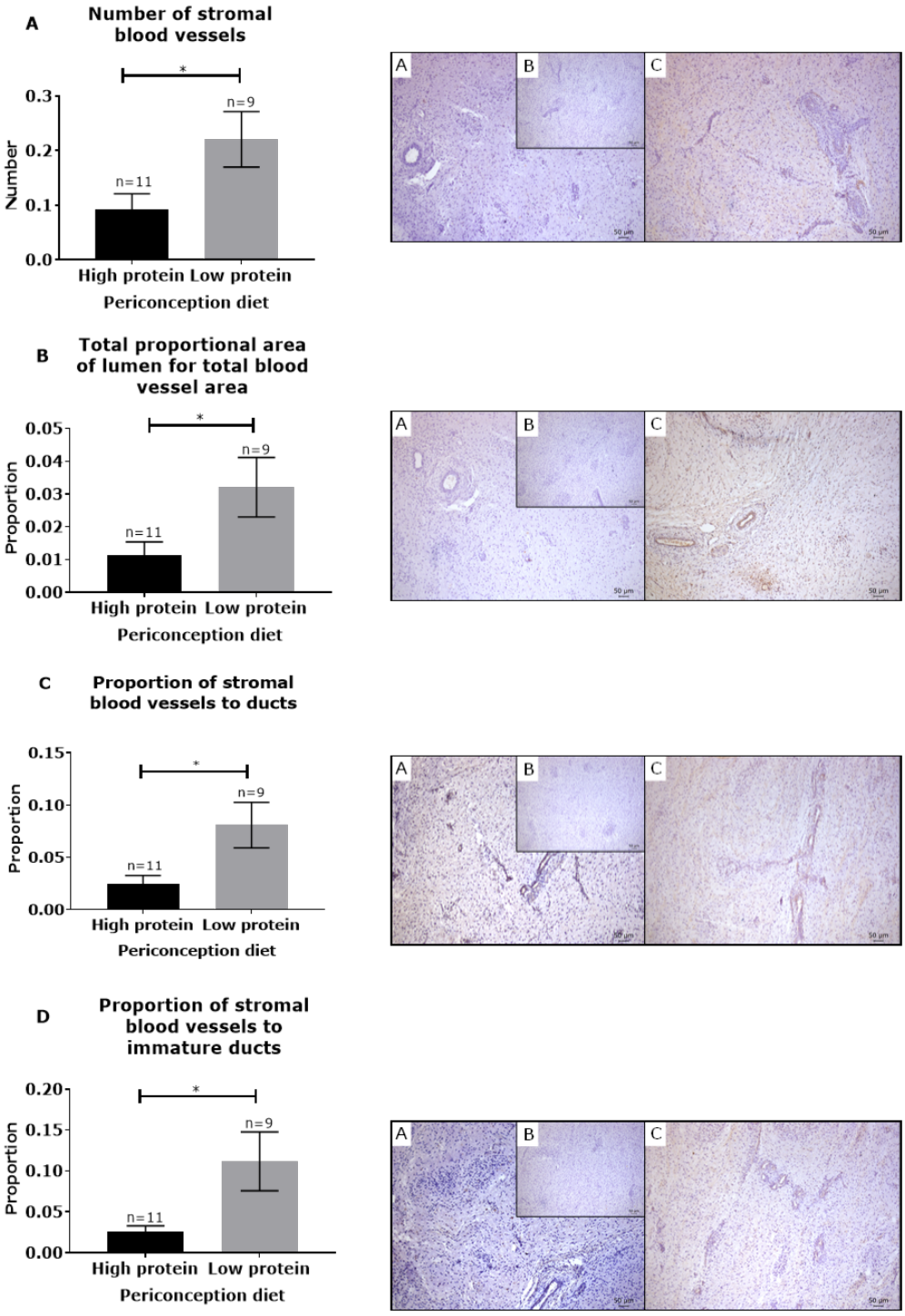


Figure 3.9 The effects of periconception diet on stromal blood vessels in the 98 days postconception bovine mammary gland. Graphs and complimentary photomicrographs (B=negative control, A=High protein, C=Low protein) display mean ± SEM of the number of stromal blood vessels in mammary fat pad (A), total proportional area of lumen to total duct area (B), the proportion of stromal blood vessels to ducts (C), and the proportion of stromal blood vessels to immature ducts (D), in 98 dpc mammary glands exposed to high or low maternal dietary protein during periconception (-60 to 98 dpc). Independent samples t-test, *p<0.05. Scale bar represents 50 μm.

3.4.4 The Effect of Maternal Dietary Intervention on Total Collagen Area

Total collagen was histologically localised within the 98 dpc bovine mammary fat pad (Figure 3.10). Collagen staining was stromal, periductal (around a duct), intraductal, and intravascular (Figure 3.10). Mean collagen area, which was the mean area of discrete collagen staining within each field of view, was unaffected by maternal dietary intervention (Figure 3.11, A; $p>0.05$). The mean discrete collagen areas were 0.28μm^2, 0.20μm^2, 0.21μm^2, and 0.33μm^2 in HH, HL, LH and LL exposed 98 dpc mammary tissues respectively (Figure 3.11, A; $p>0.05$). Discrete collagen area was unaffected ($p>0.05$) by periconception diet which was 0.25μm^2 and 0.28μm^2 in HPeri and LPeri respectively (Figure 3.11 B). Discrete collagen area was unaffected by postconception diet which was 0.26μm^2 and 0.27μm^2 in HPost and LPost respectively (Figure 3.11, B; $p>0.05$).

Total collagen area in fetal mammary tissues was unaffected ($p>0.05$) by maternal dietary intervention (Figure 3.11 C). The mean total collagen areas were 197.5μm^2, 88.9μm^2, 131.0μm^2, and 101.7μm^2 in HH, HL, LH and LL respectively (Figure 3.11 C). Total collagen area was unaffected ($p>0.05$) by periconception diet which was 148.2μm^2 and 114.7μm^2 in HPeri and LPeri respectively (Figure 3.11 D). Total collagen area was unaffected ($p>0.05$) by postconception diet which was 170.9μm^2 and 95.3μm^2 in HPost and LPost respectively (Figure 3.11 D).

However, qualitative differences in collagen presence were observed between ducts, which was strictly descriptive, and no further analysis was performed. Ducts with a high density of epithelial cells (compact) had qualitatively greater levels of collagen staining (Figure 3.10) than ducts with low epithelial cell density (dispersed), as displayed in photomicrographs in Figure 3.11 A, photomicrographs A and B. In stroma, collagen was fibrous or filamentous in structure (Figure 3.11 A, B). Immature ducts with low density or sparsely compacted epithelial cells tended not to contain collagen (Figure 3.11 A-C). Collagen was observed in ducts with a high density of epithelial cells regardless of lumen presence (Figure 3.11 D).

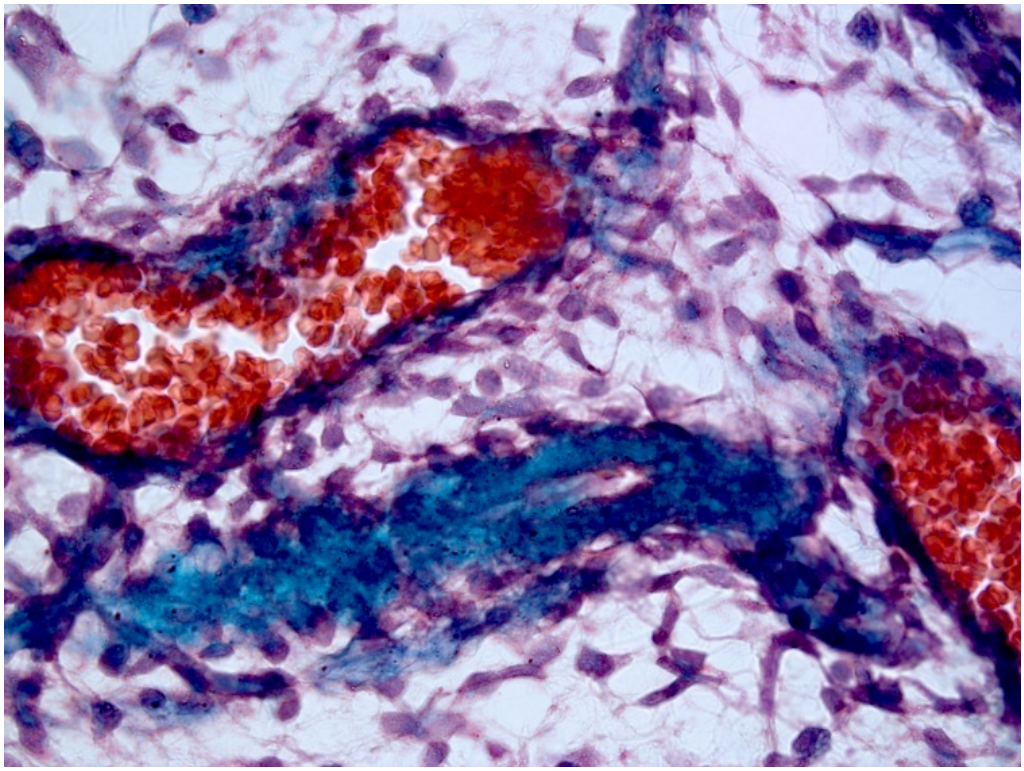


Figure 3.10 Collagen in the 98 days postconception mammary fat pad. Photomicrograph displaying Masson's trichrome staining of collagen (blue-green). Two blood vessels with intravascular collagen staining, and a duct with periductal and intraductal staining were observed. Stromal collagen staining was observed**.** Example of a duct with compact epithelial cells and collagen staining is displayed.

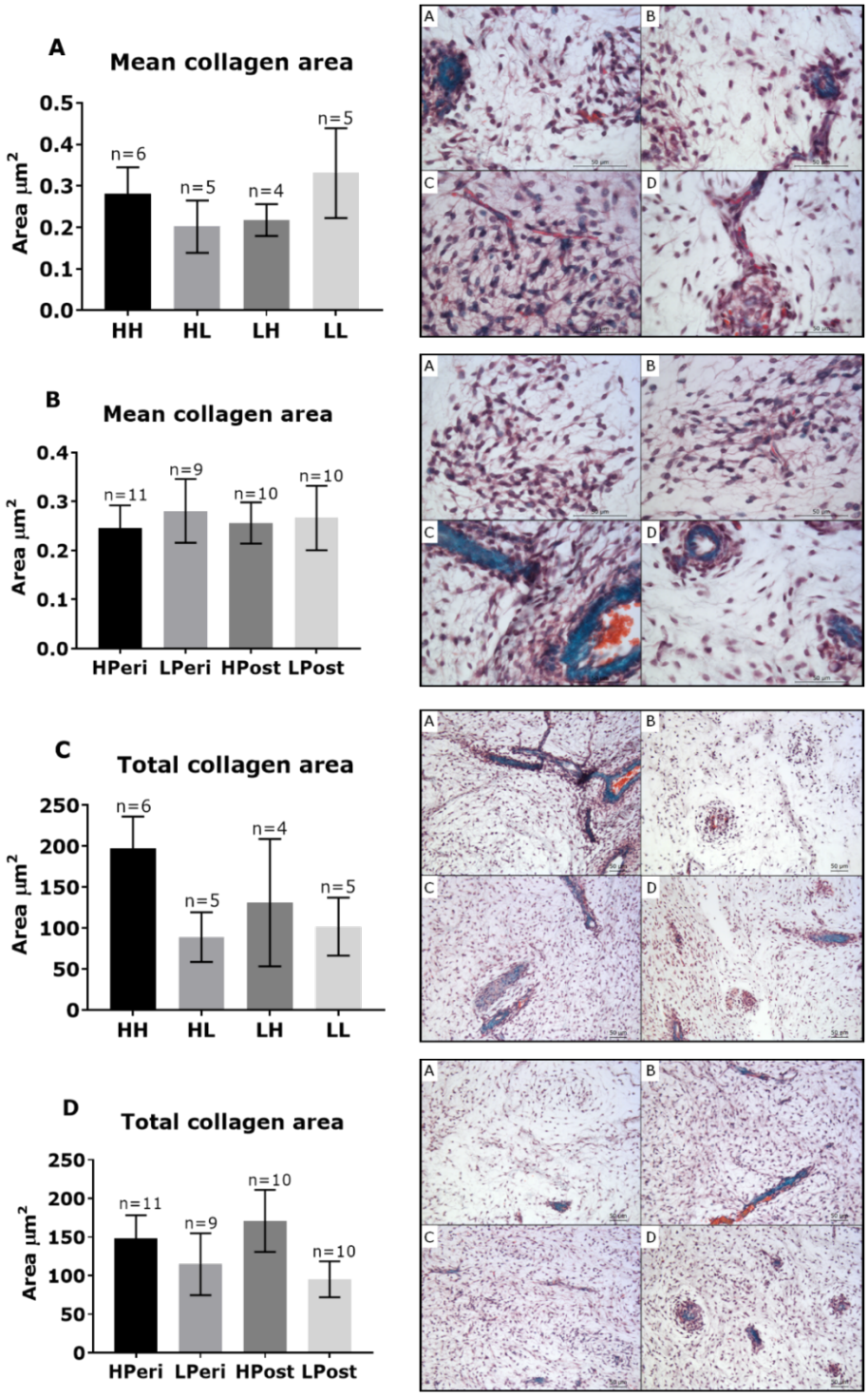


Figure 3.11 Mean ±SEM of discrete and total area of collagen, and the cellular context of localisation in 98 days postconception bovine mammary glands. Graphs and complimentary photomicrographs (photomicrographs A-D represent each graph key e.g. A=HPeri, B=LPeri, C=HPost, D=LPost) display the mean discrete areas (A, B) and total collagen areas (C, D) between maternal dietary interventions (A, C), periconception (Peri) and postconception (Post) diets (B, D). H=high protein, L=low protein. Letters denote periconception (Peri) and postconception (Post) diets, e.g. HL= high protein periconception, low protein postconception. ANOVA, $p>0.05$ (A, C), t-test, $p>0.05$. Scale bar represents 50 µm.

3.4.5 Adipocytes in the 98 Days Postconception Mammary Fat Pad

Adipocytes were immunohistochemically localised via UCP1 and UCP2 within 98 dpc mammary fat pads to positively identify adipocytes (Figure 3.12, Figure 3.13). Adipocytes were characterised as having a circular cell membrane, with a distally localised nucleus of varying flatness depending on cell size. Adipocytes were observed within and surrounding developing ducts, and infrequently in the mammary stroma (Figure 3.12). UCP1 immunopositivity was observed within adipocyte cytoplasm, while immunopositivity was absent in nuclei regardless of cell-type (Figure 3.12, A, B). The number of samples with observed adipocytes was not equal to the total n per group for both UCP1 and UCP2 qualitative analysis.

In UCP1 stained samples, the number of samples which presented adipocytes were n=3/6 (50%), n=4/5 (80%), n=1/4 (25%), and n=4/5 (80%) for HH, HL, LH and LL respectively (Figure 3.12, A). In UCP1 stained samples, the number of samples which presented adipocytes were n=7/11 (63.63%), n=5/9 (55.55%), n=4/10 (40.00%), and n=8/10 (80.00%) for HPeri, LPeri, HPost and LPost respectively (Figure 3.12). In samples with observed adipocytes, 100% of those samples presented adipocyte immunopositivity for UCP1 and UCP2. Qualitative immunopositivity in adipocytes was therefore dependant on the presence of adipocytes. Therefore, the results for adipocyte immunopositivity reflect whether adipocytes were observed within samples. As described above, samples without adipocytes were not excluded from results (Figure 3.12).

In UCP2 stained samples, the number of samples which presented adipocytes were n=3/6 (50%), n=2/5 (40%), n=1/4 (25%), and n=0/5 (0%) for HH, HL, LH and LL respectively (Figure 3.13). In UCP2 stained samples, the number of samples which presented adipocytes were n=5/11 (45.45%), n=1/9 (11.11%), n=4/10 (40.00%), and n=2/10 (20.00%) for HPeri, LPeri, HPost and LPost respectively (Figure 3.13). UCP2 immunopositivity was cytoplasmic and nuclear, and was observed in adipocyte cell, epithelial cells, vasculature, and stromal cells (Figure 3.13). Other cells presented immunopositivity for UCP1 and UCP2, although this was not the aim of this part of investigation (Appendix 7).

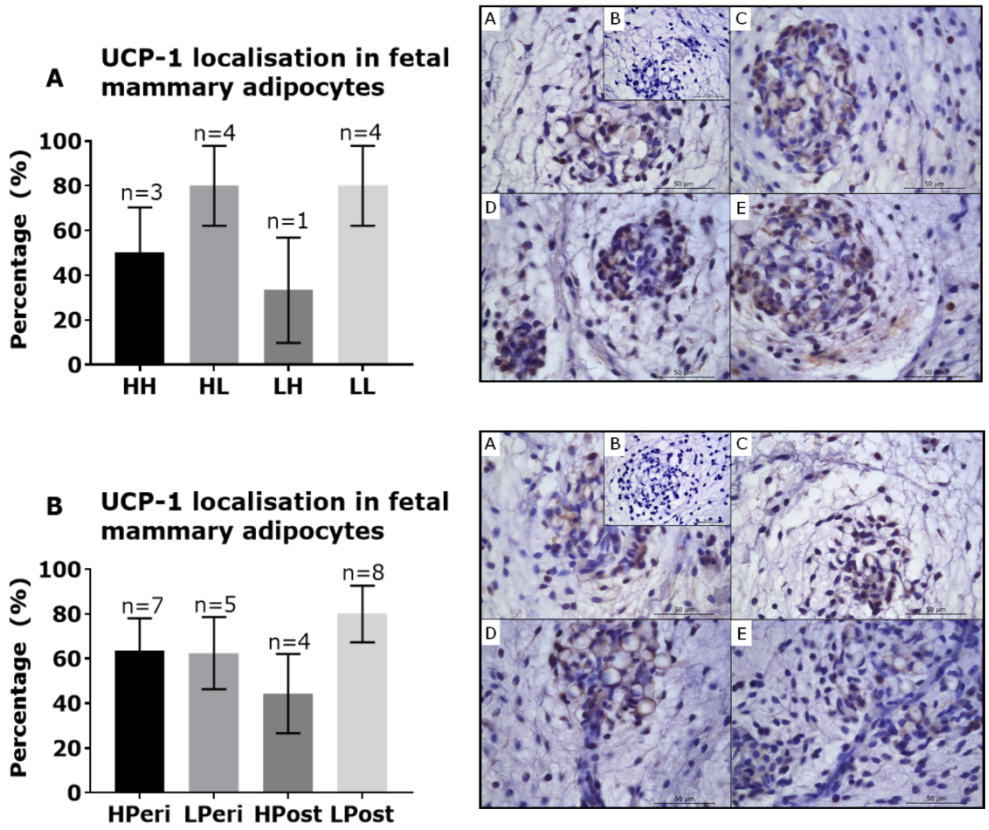


Figure 3.12 The percentage ±SEM of samples displaying adipocyte UCP1 immunopositivity and adipocyte localisation in 98 days postconception bovine mammary glands. Graphs and complimentary photomicrographs (B=negative control, A, C-E represent each graph key e.g. A=HH, C=HL, D=LH, E=LL) display results between maternal dietary interventions (A), and periconception and postconception diets (B). A putative terminal ductal unit (TDU) (arrowhead) is visible with a subtending or secondary duct (*), terminating with a circular unit of epithelial cells and adipocytes (3.12, B, Photomicrograph D, E) H=high protein, L=low protein Letters denote periconception (Peri) and postconception (Post) diets, e.g. HL= high protein periconception, low protein postconception. Total samples per group: HH n=6, HL n=5, LH n=4, LL n=5. HPeri n=11, LPeri n=9, HPost n=10, LPost n=10. Samples with observed adipocytes (UCP1): HH n=3/6, HL n=4/5, LH n=1/4, LL n=4/5. HPeri n=7/11, LPeri n=5/9, HPost n=4/10, LPost n=8/10. Scale bar represents 50 μm.

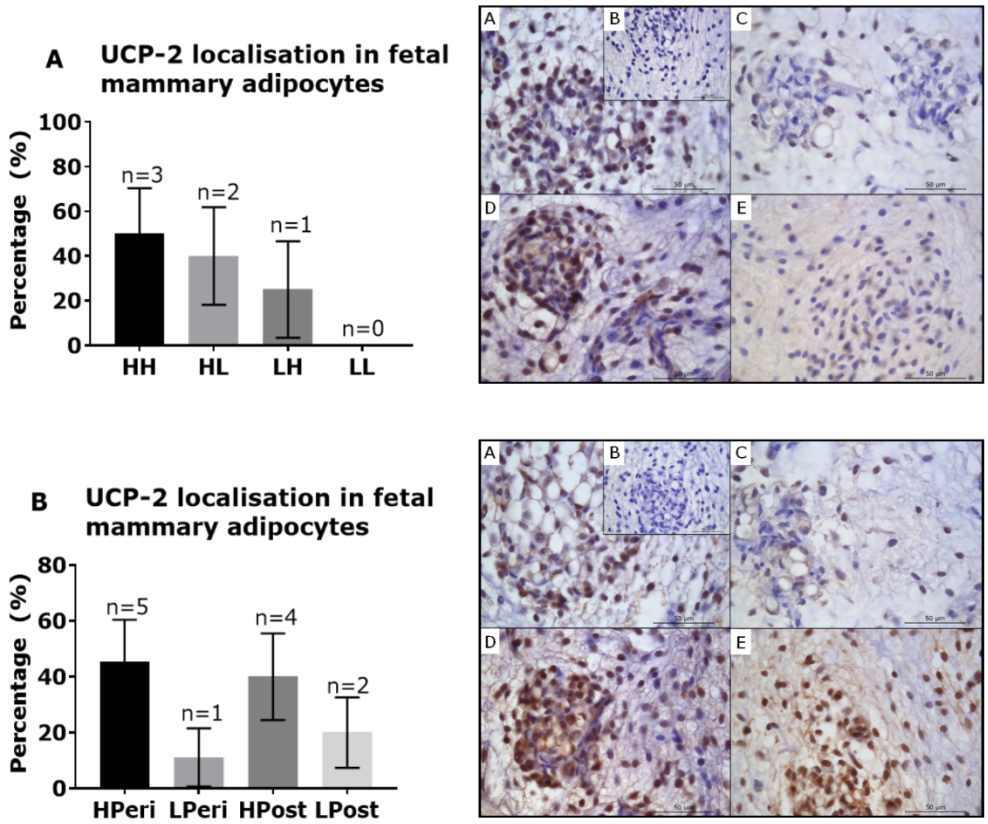


Figure 3.13 The percentage ±SEM of samples displaying adipocyte UCP2 immunopositivity and adipocyte localisation in 98 days postconception bovine mammary glands. Graphs and complimentary photomicrographs (B=negative control, A, C-E represent each graph key e.g. A=HH, C=HL, D=LH, E=LL) display results between maternal dietary interventions (A), and periconception and postconception diets (B). H=high protein, L=low protein Letters denote periconception (Peri) and postconception (Post) diets, e.g. HL= high protein periconception, low protein postconception. Total samples per group: HH n=6, HL n=5, LH n=4, LL n=5. HPeri n=11, LPeri n=9, HPost n=10, LPost n=10. Samples with observed adipocytes (UCP2): HH n=3/6, HL n=2/5, LH n=1/4, LL n=0/5. HPeri n=5/11, LPeri n=1/9, HPost n=4/10, LPost n=2/10. Scale bar represents 50 μm.

3.5 Discussion

The works presented in this chapter represented a histological analysis of the bovine fetal mammary gland at 98 days postconception (dpc). The majority of structural findings discussed relates to immunohistochemical staining of KRT18 (Chapter 3.4.1). In stereology, the study of a 2-dimentional image from a 3-dimentional structure has its limitations. It is therefore important to note that some smaller ducts with a lumen may be part of the secondary or tertiary duct and a terminal ductal unit as previously

described in calves (Capuco et al., 2002). This may explain the differences in areas observed within a tissue section, rather than the size difference being due to stage of duct development (Montalbetti et al., 2014). However, as this applied to all samples, this did not impose a bias or discrepancy between samples, the histological analysis, or results. Further to structural findings, quantitative image analysis of ducts, associated blood capillaries, stromal blood vessels were discussed (Chapter 3.5.2, Chapter 3.5.3). Total collagen area was quantified and the observed variations in collagen presence between ducts were described (Chapter 3.5.4). Finally, the presence of adipocytes in the 98 dpc bovine mammary gland were discussed (Chapter 3.5.5).

3.5.1 Structural Findings Within the 98 Days Postconception Bovine Mammary Gland

Ducts, blood vessels, epidermis, fibroblasts, lymphocytes, adipocytes and collagens were observed in 98 dpc bovine mammary tissue.

Firstly, bovine ducts were previously defined as either terminal ductal units (TDUs) (distal branching ductal structures) or subtending ducts (mature, patent lumen) (Capuco et al., 2002). TDUs were not expected to be present in the fetal 98 dpc bovine mammary gland, as they are the distal structures of the ductal network which will further develop into milk synthesising alveoli. Therefore, TDUs can be considered the most advanced structures in mammary ductal development. However branched ductal structures were observed with a subtending duct, which suggests presence of tertiary ducts expanding to a rudimentary TDU (Figure 3.12, B, photomicrograph D). This was unexpected as the majority of branching morphogenesis occurs during pubertal development (Sternlicht et al. 2006).

In prepubertal heifers, TDUs with a subtending duct were previously observed at 8 and 12 weeks-of-age (Brown et al., 2005; Capuco et al., 2002). In the present study, evidence for terminal ductal units were observed at 98 dpc (Figure 3.12 B, photomicrograph D). In comparison to the TDU of 8 weeks old heifers published by Brown and colleagues (2005), the rudimentary TDU at 98 dpc in the current study was less organised, lacking a clear organised basal cell layer, with a lower density of epithelial cells, indicating immaturity. The difference in age between 98 dpc and 8-weeks-old is approximately 34 weeks, thus the differences in TDU

development at these ages are understandable. Regarding the subtending duct (mature, patent lumen), the epithelial cell density at 98 dpc was also less dense compared to the 8 weeks old subtending duct published by Brown and colleagues (2005). These differences described between 98 dpc in the current study and 8-weeks-old were similar to the structural differences between 98 dpc in the current study and previous findings in 12-weeks-old ducts published by Capuco and colleagues (2002).

Furthermore, within a single tissue section, ducts were observed and were distinct from neighbouring ducts. This is consistent with a previous mouse study which demonstrated ductal spacing and outgrowth away from neighbouring ducts (Hinck and Silberstein, 2005). Furthermore, teat cisterns (primary ducts) were observed proximal to a teat (Figure 3.6). Within a single tissue section, ducts were observed longitudinally as epithelial cords, and at higher magnification, as ducts with lumen if developed, in the transverse plane (Figure 3.6).

Additionally, ranges in duct size, cell density, and lumen development were observed (Appendix 1). These observations may warrant further analysis of the effect of dietary intervention on fetal mammary duct development. It is unclear whether epithelial tissue measured was ductal (milk collecting) or rudimentary spherical alveoli (milk synthesising) structures. 3-dimensional image analysis of sections of a whole tissue sample would further elucidate the fetal mammary tissue structure (Capuco et al., 2002).

In the current study, anti-KRT18 immunohistochemistry positively stained mammary epithelial cells (Figure 3.8). KRT18 was previously identified as an epithelial cell marker in mouse mammary, a human mammary epithelial cell line, and human breast tumours (Wilson and Dering, 2004, Charafe-Jauffret et al., 2006, Bièche et al., 2002, Sun et al., 2010). This allowed immunopositive identification and further analysis of ductal epithelial tissue. Recently, Holstein fetal mammary tissues were reported to display primary ducts at ~90 dpc (n=3), and primary and secondary ducts at ~100 dpc (n=2) (Hara et al., 2018). Tissues were analysed via immunohistochemistry for keratin-18 (KRT18) and α-smooth muscle actin (α-SMA), reportedly for specifically staining differentiated luminal and myoepithelial cells respectively (Hara et al., 2018). This partly agrees with the current study, which also used anti-KRT18 to immunopositively identify epithelial cells.

Future investigation in the current study may include immunohistochemistry of α-SMA to determine presence of myoepithelial cells in the 98 dpc bovine mammary gland. However, the report of myoepithelial cell identification in fetal mammary glands is converse to earlier results by Capuco and colleagues (2002), who stated that in 3-month-old heifers, although terminal ductal units were present, no cells were characterised as myoepithelial cells. This contradicts the reports by Hara and colleagues (2018), who claimed to have observed myoepithelial cells in bovine fetal mammary glands. In the current study, the presence of myoepithelial cells was not investigated. Additional investigation into this cell-type at 98 dpc would benefit the current understanding of myoepithelial presence in the bovine mammary gland. This is of interest to dairy production given the function of myoepithelial cells in lactating cows.

It was revealed that at ~80 dpc, KRT18 immunopositivity was not present in bovine mammary glands, however by ~90 dpc, KRT18 was localised to central cells of primary ducts only (Hara et al., 2018). In the current study, KRT18 immunopositivity was observed throughout ducts, and staining was not restricted to central cells (Figure 3.8). Therefore the findings of the current study partly agree with the findings by Hara and colleagues (2018), although there are discrepancies between the distribution of KRT18 staining within ducts at a similar age. Furthermore, in 90 dpc mammary samples, α-SMA was localised in the peripheral cells in the primary mammary duct, which was displayed as over ten cells in thickness (Hara et al., 2018). In 100 dpc mammary samples, α-SMA immunopositivity was localised in the peripheral cells of primary and secondary ducts, and KRT18 was localised in primary and secondary ducts (Hara et al., 2018).

As previously discussed, immunohistochemical identification of fetal bovine and ovine fetal mammary epithelial cells with KRT18 was recently published (Hara et al., 2018). Photomicrographs of the bovine teat at ~80, ~90 and ~95 dpc were displayed, and it was reported that primary ducts were absent at 80 dpc, and present at ~90 and ~100 dpc, while secondary ducts were reported at 100 dpc (Hara et al., 2018). This recent work was not published at the time of 98 dpc bovine mammary gland image analysis presented in this chapter, and structures such as the teat and ductal tissue were analysed without reference material as no work had ever been reported previously on this age and species. However, Hara and colleagues (2018) obtained Holstein

fetuses from an abattoir, from unknown cows with unknown diets, and age was estimated by crown-rump length (CRL) alone, based on previous work in the Jersey cow breed (Richardson et al., 1990). Marked differences between these two breeds were previously documented (Swett et al., 1948). Similarly, Rasby and colleagues (1990) reported crown-rump lengths (32.1-33.5 cm) in 260 dpc Hereford fetuses which were unaffected by dam body condition. Maternal nutrition was shown to alter 98 dpc CRL, where periconception low protein (7% CP) reduced CRL compared with high protein (14% CP) counterparts (Copping et al., 2014). Although the breed differs from Holstein and Jersey, there is a marked difference in the crown-rump lengths (30.0cm = ~130 dpc, 75.0cm = ~240 dpc) stated by Hara and colleagues (2018) used to estimate gestational age. This raises concern of the accuracy in crown-rump length and the estimated age reported by Hara and colleagues. Furthermore, the sample size of fetal samples ranges from n=1-3, where a maximum of n=3 were ~90 dpc (14.5 to 16cm CRL).

3.5.2 The Effect of Maternal Dietary Intervention on Total Mammary Duct Tissue

Total ductal area was unaffected by maternal dietary intervention (Appendix 1). Maternal dietary intervention during periconception and postconception did not affect total duct area, size, or number (Appendix 2). This suggests that maternal diet did not influence ductal epithelial cell proliferation to the point where growth was affected. Results of the current study agree with findings of a previous study which reported that Friesian heifer mammary secretory tissue area was unaffected by dietary CP during five to ten months of age (Dobos et al., 2000). Although there are breed, age and dietary intervention differences between the study by Dobos and colleagues, and the current study, overall findings were consistent. Further to mammary epithelial cell proliferation, previous studies have investigated bovine mammary cell proliferation via immunohistochemistry using *Marker of proliferation Ki-67* (*MKI67*), which is not cell-type specific (Capuco et al., 2001, Hara et al., 2018). Further immunohistochemistry and qualitative image analysis of a proliferation marker such as *MKI67* in tissue sections would localise cell proliferation within ducts, and determine whether proliferation differed between mammary tissues exposed to the four maternal dietary interventions. Recently, immunohistochemical localisation of *MKI67* was presented in bovine mammary epithelial cells, and was

distributed throughout bovine mammary ducts (n=3) at ~90 dpc (Hara et al., 2018). This is evidence for this *MKI67* as an appropriate marker of proliferation in bovine fetal epithelial cells. Equally, future investigation via RT-qPCR of *MKI67* in all mammary cDNA samples would further elucidate whether maternal diet affected fetal mammary epithelial cell proliferation.

Postconceptional high protein resulted in increased lumen size in ducts with lumen (Figure 3.8, $p<0.05$). Loss of cellular junctions and adhesion between central cells in epithelial cords was reportedly involved in ductal canalisation (Capuco et al., 2002). This would allow for remodelling and formation of a lumen. Since mammary duct development commences at 30 days postconception, postconceptional maternal diet may be more critical than periconception. Evidence for the effect of maternal dietary protein on fetal mammary development in literature is currently limited. Previous studies focused on the following postnatal mammary development stages: prepubertal, pubertal, postpubertal, pregnancy, and lactation. Literature reviews have indicated energy intake to be a critical factor in mammary development (Sejrsen, 1994). In first-parity ewes fed restricted (60%), control (100%) or high (140%) plane of nutrition from 50 dpc to term, gestational diet affected ewe mammary development (Swanson et al., 2008). Mammary gland weight (g) was lower in restricted versus control and high diets (Swanson et al., 2008). However, this was not an indication of composition.

LPeri exposed mammary tissues presented a 65% greater mean number of blood vessels per field of view than HPeri counterparts at 98 dpc (Figure 3.9 A). This suggests that in LPeri exposed tissues, a greater number of blood vessels were present during duct development at 98 dpc compared to HPeri exposed mammary glands. Mammary duct development is thought to commence at around 30 dpc in the cow (Rowson et al., 2012). Considering this, mammary vasculature in combination with a change from LPeri to HPost (LH) may have influenced duct development (Figure 3.9 C D). However, total duct analysis indicates that overall, maternal dietary intervention did not affect total duct area (Appendix 1). Alternatively, the periconception environment had potential to affect mammary progenitor cells. The protein *LGR5* was identified as a mammary stem cell marker in the murine fetal mammary gland (Trejo et al., 2017). Further investigation of gene expression and immunohistochemical localisation of stem cell specific genes

and proteins may reveal whether stem cell gene expression or numbers were altered by maternal dietary intervention.

In addition to fetal development, it is vital for animal health and production that maternal diet has no detrimental effects on the gestating heifer. Sullivan and colleagues (2009e) reported negative effects to beef heifer milk production following a high protein diet in the first trimester, which is comparable to the HH diet in the current study.

Feeding level studies have previously focused on the birth to first calving stage of bovine growth and development (Sejrsen et al., 2000). Although mammary growth during puberty is less evident than mammary growth during pregnancy, growth and development during this stage can impact future milk production potential (Sejrsen, 1994). This is likely dependent on the stage of development during which dietary intervention occurs (Dobos et al., 2000, Sejrsen, 1994). Previous studies of the effects of dietary intervention on mammary development were postnatal (Akers, 1985, Capuco et al., 1995, Sejrsen et al., 2000, Silva et al., 2002, Sejrsen et al., 1982). Growth hormone and IGF1 were reduced and increased respectively in heifers fed a high energy diet (950g/day daily weight gain) versus control (725g/day daily weight gain) (Capuco et al., 1995). In this study, total mammary parenchymal DNA and RNA were reduced, and mammary glands contained a greater volume of adipocytes than epithelial cells compared to those fed lower energy diets. High energy diet during prepubertal allometric growth reduced mammary parenchyma during heifer prepubertal growth (Capuco et al., 1995, Silva et al., 2002). During postpubertal growth, these effects of high energy diet were not observed (Capuco et al., 1995). Both studies suggested body fat as a key influence on mammary growth (Capuco et al., 1995, Silva et al., 2002). Although these studies varied greatly from the present study in breed, dietary intervention time and diets, their findings identified critical period of prepubertal mammary development on mammary growth.

Dietary CP levels in the trial by Capuco and colleagues (1995) were 22% and 15% CP for lucerne and maize silage respectively. The control diet protein level (15% CP) was comparable with the high protein diet in the current study (14% CP). However, considerations should be made to the energy contents, protein digestibility and age of heifers at dietary intervention.

Results of the current study suggest no effect of maternal diet on total area of ductal epithelial tissue. Ductal tissue size at 98 dpc was expectedly smaller than prepubertal ductal tissue previously reported (Brown et al., 2005; Capuco et al., 2002). Cell proliferation was previously observed throughout the terminal ductal unit (Capuco et al., 2002). Peripheral epithelial cells were more receptive to epithelial mitogens IGF1 and TGF-β than medial cells (Ellis et al., 2000). The peripheral zone formed a multi-cell layer, resembling parenchymal tissue (Ellis et al., 2000). This suggests that proliferation was greater in peripheral cells of ductal tissue.

In the current study, specific staining of mammary stem cell, myoepithelial and luminal cell populations were not performed. Previous work reported an absence of myoepithelial cells in 3-month-old heifers (Capuco et al., 2002). Studies using bovine myoepithelial cell markers such as α-smooth muscle actin, vimentin, and membrane metalloendopeptidase, also confirmed this (Hellmen and Isaksson, 1997, Pechoux et al., 1999, Smith and Chepko, 2001, Hu et al., 2009). Furthermore, the proportion of mammary epithelial cell types may be investigated based on previous bovine work (Capuco et al., 2002; Ellis et al., 2000). The reasons supporting this crucial further work are threefold. Firstly, there is potential for on-farm economic gain in further understanding the effect of maternal dietary intervention on fetal mammary stem cell populations. Understanding would aim to identify beneficial maternal diet for optimal improvements of fetal mammary development and future milk production in both beef and dairy cattle. Secondly, similarities between human and bovine mammary development appear greater than mice, making the cow a better model for human research (Capuco et al., 2002). Thirdly, insights into bovine mammary epithelial populations may benefit human cancer research, as cows essentially never develop mammary cancer (Povey and Osborne, 1969). The human and cow genome was compared, although the mammary gland was not discussed (Band et al., 2000). In addition, a recent multi-species report on cancer resistance did not include cows, or the mammary gland (Ferris et al., 2018).

Previous research emphasised the association between nutrition for increased heifer growth rate, and the consequences for mammary development (Gabler and Heinrichs, 2003, Harrison et al., 1983, Hoffman, 1997, Lammers et al., 1999, Sejrsen and Purup, 1997, Valentine et al., 1987). Particularly the negative effects of high energy on mammary growth

(Brown et al., 2005; Sejrsen et al., 1982; Sejrsen et al., 2000). Critical periods in heifer mammary development were previously identified (Sejrsen et al., 1982). However, the current study also identified critical periods where cellular effects were observed or unaffected in peri- or postconception. Current understanding suggests that multiple critical periods exist, where mammary development is susceptible to changes. Previous research also suggests that critical periods are parameter-specific, such as mammary parenchymal composition.

Previously, mammary parenchyma was unaffected by diet in pre- and post-pubertal heifers (Sejrsen et al., 1982). Sejrsen and colleagues (1982) analysed the percentage of epithelial cells, connective tissue, adipose, and ductal lumen at different distances from the teat (base of outgrowth). Variation in tissue composition reportedly differed with distance from teat, and between front and rear quarters (Brown et al., 2005; Nickerson and Akers, 2011; Sejrsen et al., 1982). This may explain within-group variation of cellular parameters qualitatively observed in the present study. This is because the current study did not categorise tissue sections by distance from teat, as it was not possible. Results of the current study are not invalid because the parenchymal zone was not distinguished. Results represent the total composition throughout the fetal parenchyma. Work in 3-month-old heifers by Capuco and colleagues (2002) support the overall analysis of epithelial tissue as proliferation in their study was seen throughout the mammary tissue. Although proliferation was reportedly greatest at the interface between parenchymal stroma and the fat pad (Capuco et al., 2002). Systematic random sampling of mammary sections in the current study presented overall tissue analysis. Tissue parameters relative to distance from the teat were not analysed, as observed teat structures were rare in tissue sections. Future work may involve identifying precise quarter analysis. Future systematic sampling within proximal, mid and distal tissue sections, relative to the teat within the mammary tissue would allow more comprehensive analysis.

3.5.3 The Effects of Periconception Diet on Blood Vessels in the Stroma

Mammary tissues (98 dpc) exposed to periconception low protein had 65% more stromal blood vessels than high protein counterparts (Figure 3.9, A). No significant differences in stromal vasculature were observed between

maternal dietary interventions (HH, HL, LH, and LL) (Appendix 5) or postconception diets (Appendix 6). This suggests that postconception diet did not alter the effects of periconception low protein by 98 dpc. This indicates a persistent change to fetal mammary vasculature in the first trimester of gestation, and that periconception (60 days preconception to 23 dpc) maternal diet altered fetal mammary vasculature. However, it is unclear whether these differences would persist to pre-pubertal, post-pubertal or adult mammary development. Previously in mice, maternal malnutrition during oocyte maturation was demonstrated to have effects on the cardiovascular system of offspring (Watkins et al., 2008). In cows which were feed-restricted during the first trimester of gestation, offspring at 94 dpc, and 94 weeks old had 1.19 and 1.1-fold enlarged aorta diameters and circumferences respectively (circumference of aorta at base)(Mossa et al., 2013). This indicates that developmental differences in vasculature established during gestation can be permanent. In the current study, if differences in vasculature number persist to puberty, periconception development may influence pubertal mammary development and later production, particularly in terms of milk solids (Davis and Collier, 1985). At puberty, mammary growth is hormonally influenced (Chapter 1.5). Hormones are delivered to peripheral tissues via the blood circulation (Blouin et al., 2002, Jacobsen et al., 2000). Therefore, distribution of hormonal-delivery in mammary tissues with greater numbers of stromal blood vessels may increase in later development. Subsequently, the rate of hormone-dependent growth may increase during puberty. However, blood-flow requires investigation, as blood vessel diameter alone does not wholly determine adult bovine mammary blood-flow (Götze et al., 2010).

Luminal expansion (total proportional area of lumen to total blood vessel area) in stromal blood vessels was greater in tissues exposed to periconception low protein compared with high protein counterparts (Figure 3.9, B). As blood vessel size was unaffected by periconception diet (Appendix 6), this suggests a greater proportion of blood vessel area consisted of lumen in low protein exposed tissues. This is similar to the increased aortic diameters of calves of under-fed dams compared with controls (Mossa et al., 2013). Further analysis of blood vessel diameter would further elucidate the effect of maternal diet on blood vessel structure, as vascular lumen size was unaffected by maternal dietary intervention (Appendix 5). The overall implication of this is unclear, although adult bovine mammary blood flow

volume is thought to be dependent on cross-sectional area and the time-averaged maximum velocity of blood (Götze et al., 2010).

The proportional number of stromal blood vessel to ducts was increased 3.3-fold in tissues exposed to LPeri compared with HPeri (Figure 3.9 C). 98 dpc mammary tissues exposed to HPeri or LPeri contained 1:50 and 1:25 stromal blood vessel: ducts, respectively. Furthermore, the number of immature (4.4-fold increase) but not mature ducts were affected by periconception diet and stromal blood vessel number (High, 1:40 vs. Low 1:9 stromal blood vessels: immature ducts) (Figure 3.9 D). This suggests that tissues exposed to LPeri had a greater proportion of blood vessels to 'immature' ducts than HPeri counterparts. Although stereology is useful in determining tissue structure, it does not provide a complete picture of vasculature or ductal branching. What was initially considered an 'immature' duct may be early developing terminal ductal units, which would later mature to alveoli by puberty. Presence of developing terminal ductal units suggests that the fetal mammary ductal network at 98 dpc is more advanced than originally expected. If these 'immature' epithelial structures are confirmed to be TDUs, for example, by three-dimensional analysis of serial sections the results of the current study suggest that periconception maternal diet may alter ductal network development via fetal mammary vasculature. However, further research is required to test this hypothesis. A limitation of the duct image analysis was that ducts which did not contain a lumen, but which presented compact epithelial cells were grouped with ducts with micro lumina. The latter were predominantly circular with sparsely structured epithelial cells. Further categorisation of ducts may improve understanding of the proportion of ductal structures present. However, more advanced methods may prove more valuable. Capuco and colleagues (Capuco et al., 2002) created a 3-dimensional image using serial sections of mammary glands from 3-month-old Holstein heifers. Validation of the present study's results are possible with multiple *in vitro* techniques such as 3D analysis of serial sections, or 3D cell cultures (Simons et al., 2015, Huh et al., 2011). Existing research in fetal programming on vasculature is limited in the bovine mammary fat pad, although the placenta and general fetal vascularisation have been studied (Hernandez-Medrano et al., 2015, Vonnahme, 2007, Vonnahme et al., 2007, Farin et al., 2006, Vonnahme and Lemley, 2011). Mammary blood flow in lactating dairy cows varied and moderately correlated with milk yield (Götze et al., 2010). Blood vessel area was greater in cows with three or more

lactations compared with cows in their second lactation, and blood vessel area also increased in late lactation compared to early lactation (Götze et al., 2010). This demonstrates continuous vascular remodelling in the adult lactating cow, which is supported by other studies (Safayi et al., 2010, VanKlompenberg et al., 2016).

3.5.4 Total Collagen in Fetal Bovine Mammary Glands was Unaffected by Maternal Dietary Intervention

Both vasculature and parenchyma require structural support from the stroma which contains fibres such as collagens (Chapter 3.2.3). Total collagen analysis suggested no effect of maternal dietary intervention on fetal mammary collagen (Figure 3.11). This compliments previous work in mammary development by Dobos and colleagues, who concluded that the percentage of collagen was unaffected by pre-pubertal calf dietary CP levels (Dobos et al., 2000). Although there are discrepancies between the current study and that of Dobos and colleagues (2000), such as the stage of mammary development and maternal or offspring dietary intervention, both studies agree that total collagen was unaffected by diet. Furthermore, results do not allow inferences of individual collagen types. To determine whether individual specific collagens were affected by dietary intervention, collagen mRNA expression from mammary tissues of fetuses subjected to the maternal dietary interventions could be compared (Appendix 13). Nonetheless, differences in localisation were observed between ducts (Figure 3.10, Figure 3.11).

In mammary stroma, collagen was fibrous or filamentous in structure (Figure 3.11, A-B). Immature ducts with low density or sparsely compacted epithelial cells tended not to contain collagen (Figure 3.11, A-C). Collagen was observed in ducts with a high density of epithelial cells regardless of lumen presence (Figure 3.11, D). This suggests a difference in structural development unrelated to lumen presence. Alternatively, as sections were randomly selected for staining, it is possible that a lumen existed but was absent in the observed section. Ducts with more compact epithelial cells were thought to be more mature and therefore more likely to contain collagen compared to immature ducts. Gon (1965) reported moderate green-blue and red staining of stroma in Masson's Trichrome stained human epithelial cell tumour tissue sections. This agrees with the current study's results, where

stroma was stained green-blue. Evidence from Gon (1965) supports the results of Masson's Trichrome stained mammary fat pad as positively staining collagen.

Considering duct analysis and total collagen analysis results, no further analysis was performed. However, further work could involve categorisation of collagen could into discrete contexts within the mammary gland: ECM (stromal), perivascular (around blood vessel), ductal epithelial, and periductal (around a duct). Total collagen analysis with Masson's Trichrome (Chapter 3.3.3) in the current study was insufficient to determine specific collagen type. Collagen I is known to direct ductal branching, as extracellular periductal collagen I thickening combined with increased tissue pressure by cell proliferation directs branching or epithelial expansion towards ECM areas of low collagen I (Hinck and Silberstein, 2005). Presence of periductal collagen I may suggest branching morphogenesis of observed ducts (Hinck and Silberstein, 2005). Furthermore, connective tissue was suggested to influence mammary development (Hovey et al., 1999). The current study's results of total collagen analysis are not sufficient to interpret an influence of stromal connective tissue on parenchymal development.

Connective tissue is vital for adult mammary gland structural support (Chapter 3.2.4), as the weight of tissue and stored milk can amount to ~70 kg (Nickerson and Akers, 2011). It is unclear whether collagen gene expression affected future development of medial or lateral suspensory ligaments. As dietary intervention altered lumen size in ducts with patent lumena, there is potential for this effect to persist in future ducts not yet at patent lumena stage. The biological implication of lumen size is unclear. Previous research suggests that lumen size can affect the volume available to store milk. However, myoepithelial cell contraction is thought to increase lumen size. Therefore, it is unclear whether lumen size at 98 dpc has future implications for milk storage. Ductal tissue weight requires structural support in the form of the medial and lateral suspensory ligaments (Nickerson and Akers, 2011). Relative to collagen, future ductal or parenchymal weight may correlate with collagen expression and the proportion of connective tissue. However, further investigation is required to determine this.

3.5.5 Adipocytes in the Bovine Fetal Mammary Gland

The analysis of adipocytes was limited to immunohistochemical staining of UCP1 and UCP2, which were immunopositively localised in adipocytes (Figure 3.12). However, both UCP1 and UCP2 immunopositivity were not specific to adipocytes (Appendix 7). Therefore, although immunopositivity was observed in adipocytes, neither antibody was a suitable adipocyte-specific marker in the 98 dpc mammary fat pad. Nonetheless, adipocytes were immunopositively identified. Results are displayed as the percentage of all samples within each group, indicating the variability of adipocyte observations between fetal mammary tissues exposed to different maternal diets (Figure 3.12, Figure 3.13). However, results between UCP1 and UCP2 also indicate the variability of adipocyte observations in tissue sections of the same sample (Figure 3.12, Figure 3.13).

Capuco and colleagues (1995) reported an increased adipocyte volume in Holstein heifer mammary stroma in cows fed high energy maize silage, compared to lower energy, higher protein Lucerne silage. These detrimental effects on mammary gland development are thought to be due to the high energy diet leading to excess body fat deposition and rapid body growth rate (Capuco et al., 1995). Similarly, Brown and colleagues (2005) reported greater mammary fat mass in dairy heifers fed high energy and protein during 2 to 14 weeks of age. These results support quantification of adipocytes in the present study, as they suggested that prepubertal bovine mammary adiposity was affected by diet. The mammary fat pad is dorsally localised relative to the teat, and is gradually invaded by mammary parenchyma (Nickerson and Akers, 2011). The localisation of adipocytes relative to the teat was not investigated, as previously explained in this chapter. Mammary adipocyte localisation relative to distance from teat would be of interest in the current study as localisation is known to be distal relative to the teat. Furthermore, parenchymal and extra-parenchymal adipocytes were observed but not quantified. Previous studies reported these adipocyte populations to be differentially affected by dietary intervention (Brown et al., 2005). Mammary fat was suggested to influence mammary development (Hovey et al., 1999). Overall, presence of adipocytes at 98 dpc suggests susceptibility to fetal programming which may affect future mammary function and development. UCP1 and UCP2 were localised in bovine epithelial cells in the current study, therefore confirming that these are unsuitable

adipocyte specific markers in bovine mammary glands, as both cell types are present.

In summary, adipocytes were identified in multiple samples across all four maternal diets. The structural identification of adipocytes is in agreement with Hara and colleagues, who identified adipocytes in n=2 ~100 dpc bovine mammary glands (2018). Cells were identified as adipocytes via Oil-Red-O fat globule stain in frozen tissues (7 μm thick), embedded in OCT compound, and immunohistochemically with anti-ERα in paraffin embedded tissue (5μm thick) (Hara et al., 2018). Oil-Red-O histological stain was performed in the current study, however, it was unsuccessful due to all mammary tissues being embedded in paraffin wax (Appendix 15). Briefly, Oil-Red-O stains fat globules red, however deparaffinisation also removed fat globules contained within adipocyte cytoplasmic vesicles, thereby meaning the technique was unsuitable for tissue sections in the current study. However, future analysis of immunopositive identification of adipocytes using anti-ERα is possible.

3.6 Conclusion

Developing teats, adipocytes and ducts were observed in tissue samples, which were previously not known to be present at 98 dpc. In the cow, results suggest no effect of dietary intervention during -60 to 98 dpc on overall mammary epithelial area. However, differences were observed in response to periconception and postconception diets. This analysis does not infer structural differences such as cell-cell adhesion within ducts and blood vessels. The practical implications are evident as optimal development of the mammary parenchyma and associated stroma are vital for optimal function of the mammary gland. Fetal mammary stromal vasculature number was increased in tissues exposed to LPeri and may influence duct development. Secondly, lumen size was greater in tissues exposed to postconception high protein than low protein. Although adipocytes were identified, further quantitative analysis of cell number, area and localisation within the mammary gland relative to distance from teat would further understanding of dietary protein influence on the fetal mammary adipocyte population. Further research is required to fully elucidate the lifetime effect of *in utero* dietary protein on mammary morphogenesis and future function.

4 The Molecular Effects of Maternal Dietary Protein on the Fetal Mammary Gland

4.1 Introduction

As briefly discussed (Chapter 1.2), maternal diet can affect fetal gene expression during gestation. The mammary gland is of critical importance in beef and dairy production. It is therefore paramount that the effects of maternal diet during early mammary development are better understood. With advances in understanding, recommendations to nutritional and reproductive management practices may be modified to ensure optimal fetal development which promotes sustainability in beef farming. The aim of this chapter was to identify the effects of maternal dietary protein during periconception on fetal mammary gene expression.

This chapter focuses on the molecular effects of maternal diet on the fetal mammary fat pad. Ribonucleic acid (RNA) sequencing (RNA-Seq), also referred to as transcriptomics, was developed to assess messenger RNA (mRNA) expression (Nagalakshmi et al. 2006; Marioni et al. 2008; Wang et al. 2009; Wilhelm & Landry 2009). RNA-Seq identifies the presence and amount of each mRNA (Mortazavi et al. 2008). The presence and quantity of each mRNA can be subsequently compared with sequences of other samples (Nagalakshmi et al. 2006; Mortazavi et al. 2008; Wang et al. 2009). RNA-Seq is therefore able to identify differences in gene expression between samples. RNA-seq was used to identify the effects of dietary treatments on gene expression, for example, whether gene expression was up or down regulated in response to a treatment (Wang et al. 2009; Mortazavi et al. 2008). RNA-Seq identifies and quantifies the transcriptome, all transcripts within a cell (Wang et al. 2009). RNA-Seq data is in the form of counts and expression of a specific gene can be quantified as such (Li et al. 2012).

The genes of interest, or candidate genes studied in the current study are biologically relevant to the fetal mammary gland, and also met selection criteria (Section 4.3.1). For example, genes with a role in mammary morphogenesis, adipocyte function, immune function, milk production and angiogenesis. Candidate genes and their roles in mammary development were investigated. Identified candidate genes were validated in all four diet groups with Real Time quantitative Polymerase Chain Reaction (RT-qPCR). This chapter introduction highlights the genes of interest investigated: *CDH1*

(Chapter 4.1.1), *ITGB2* (Chapter 4.1.2), *KRT80* (Chapter 4.1.3), *IL33* (4.1.4), *TWIST1* (Chapter 4.1.5), *FGFR2* (Chapter 4.1.6), and *TFAP2* (Chapter 4.1.7).

4.1.1 The Functions of *E-cadherin* in the Mammary Gland

The cadherin family are transmembrane cell surface proteins which have a role in cell-cell adhesion, controlling the interactions between cells during tissue development (Takeichi, 1995, Shapiro and Weis, 2009, Gumbiner, 2005, Gumbiner, 1996). Numerous subclasses of cadherins exist, such as E-(epithelial), P-(placental) and N-(neuronal) cadherins (Knudsen and Wheelock, 2005, Takeichi, 1988). Subclasses are grouped depending on their localisation and immune specificity, although all cadherins are Ca^{2+} sensitive and have similar molecular weights (Takeichi, 1988, Patel et al., 2003). Previous observations indicate *E-cadherin* (*CDH1*) is vital for epithelial tissue structural development, epithelial homeostasis, and adult tissue maintenance (Larue et al., 1994, Cano et al., 2000, Gumbiner, 1996). E-cadherin adhesion receptors are homophilic, meaning E-cadherin binds to other E-cadherins on the extracellular domain of adjacent cells (Takeichi, 1988, Gumbiner, 1996). In the mammary gland, *E-cadherin* is expressed in luminal epithelial cells, and is required for normal structure and function of the mammary gland (Chapter 3.2) (Daniel et al., 1995, Knudsen and Wheelock, 2005). *E-cadherin* expression is spatiotemporal, grouping cells of an identical cell-type together, such as luminal epithelial cells (Takeichi, 1988, Cano et al., 2000). Cadherins can form adherens junctions (Gumbiner, 1996).

The classical cadherin is localised in the transmembrane domain and forms adherens junctions with catenins, which are cytoplasmic plaque proteins (Sobolik-Delmaire et al., 2006). Developmental processes such as cell migration, branching morphogenesis, and cell compaction are adhesion-dependant (Sobolik-Delmaire et al., 2006). Cell migration, and condensation and compaction occurs during the mesenchymal to epithelial transition, which occurs during embryonic development (Sobolik-Delmaire et al., 2006). E-cadherin was reported to be involved in binding within mouse epithelial cells and T-lymphocytes (Cepek et al., 1994). This suggests a role of E-cadherin in mammary immune function. Bovine embryonic *CDH1* expression was increased with interferon-tau treatment (Bao et al., 2014). Since

interferon-tau is produced by trophoblast cells, there may be a link between trophoblast cell number, molecular activity and fetal *E-cadherin* expression (Bao et al., 2014). Further to this cell surface protein, members of another family of cell surface proteins, integrins, were identified as genes of interest in the current study.

4.1.2 The Integrin Family and Their Functions

The integrins are a superfamily of ubiquitously expressed cell surface adhesion receptors (Takada et al., 2007, Raymond et al., 2012). Integrins bind to ligands which can be extracellular, soluble or cell-surface ligands (Takada et al., 2007, Song et al., 2015, Raymond et al., 2012, Nasiri Kalmarzi, 2015). Integrin α (α-1 to –11, 11b, E, D, L, M X) and β (β-1 to -8) subunits exist, which exclusively form αβ heterodimers (Takada et al., 2007, Song et al., 2015, Barczyk et al., 2010, Raymond et al., 2012). Integrins facilitate cell attachment to the extracellular matrix (ECM) (Tan, 2012, Raymond et al., 2012, Barczyk et al., 2010). *Integrin subunit beta 2* (*ITGB2*) encodes the integrin subunit β2 (Nasiri Kalmarzi, 2015). Integrin subunit β2 forms a heterodimer with αL (*ITGAL*), αM, αX, and αD subunits to form leukocyte specific receptors (Tan, 2012, Takada et al., 2007, Bilsland et al., 1994, Barczyk et al., 2010, Raymond et al., 2012) (Table 4.1).

Table 4.1 Integrins containing integrin subunit β2, their cellular expression, and ligands

Integrin	Other name	Expression	Ligands	Reference
αMβ2	MAC-1, CD11b/CD18	Neutrophils, monocytes, macrophages, NK cells	Fibrinogen, fibronectin, laminin, collagen –I, –II, -IV, neutrophil inhibitory factor ICAM - 1, -2, -3, -4.	(Wang et al., 2014b, Han et al., 2010, Muller, 2003)
αXβ2	CD11c/CD18, CR4	Dendritic cells, Macrophages, monocytes, NK cells	Fibronectin, Collagen I, ICAM-1	(Urlaub et al., 2017)
αLβ2	CD11a/CD18, LFA-1	All leukocytes	ICAM-1, -2, -3, -4, -5 and JAM-1	(Marlin and Springer, 1987)
αDβ2	CD11d/CD18,	Eosinophils, macrophages	VCAM-1, ICAM-3	(Han et al., 2010)

Integrins are transmembrane spanning receptors, with an small intercellular and large extracellular domain (Raymond et al., 2012). The extracellular domain binds to ECM ligands (Raymond et al., 2012). In the mammary gland, integrins are located at the contact site between myoepithelial cells and the basement membrane in mammary ducts (Taddei et al., 2003). Integrin subunits localised in luminal epithelial and myoepithelial cells do not include β2 containing integrins (Table 4.1) (Taddei et al., 2003). A broader role of integrins is in the immune system (Urlaub et al., 2017, Abram and Lowell, 2009). Ligand-integrin binding initiates recruitment of signalling and adaptor proteins to the intercellular domain. This recruitment leads to intracellular changes in cell activity, such as proliferation, differentiation, apoptosis, migration, and polarity (Taddei et al., 2003).

4.1.3 The Functions of Keratins in the Mammary Gland

Keratins are intermediate filament proteins which function as structural support to epithelial cells (Langbein et al., 2010). Keratins are either keratin I or II family members, and type I keratins will form a heteropolymer with specific type II keratins (Langbein et al., 2010, Moll et al., 2008). Keratins

are an established epithelial cell marker. Keratin 80 (KRT80) is a type II keratin, and heteropolymerises with over 20 type I keratins (Langbein et al., 2010). Consequently, KRT80 forms intermediate filaments in various epithelia including the mammary gland and skin, although *KRT80* is widely expressed in stratified and non-stratified epithelia (Langbein et al., 2010). Human KRT80 is known to heteropolymerise with Keratin 10 (KRT10) in luminal duct and interfollicular epidermis (Langbein et al., 2010). *KRT10* is considered a keratinisation marker of keratinocytes (Moll et al., 2008). *KRT10* down-regulation was implicated in tissue stretching in a human cell culture study (Yano et al., 2004). The human and cow *KRT80* are homologues, having highly conserved amino acid sequences (Langbein et al., 2010).

KRT80 is involved in the cytoskeleton (Ulbrich et al., 2011). *KRT80* gene expression was increased in human leukocytes, in humans treated with glucose-based fluid, compared to macromolecule based fluid (icodextrin) (Wilflingseder et al., 2009). This demonstrates the ability of *KRT80* to be affected by nutritional factors. *KRT80* was associated with gene ontology (GO) terms: cell structure and motility, ectoderm development, developmental processes (Wilflingseder et al., 2009).

4.1.4 The Role of *IL33* in the Mammary Gland Immune System

Interleukins (IL) are cytokines with roles in the immune system (Liew et al., 2016, Netea et al., 2015). Numerous interleukin families exist. The interleukin-1 family contains 11 members of which *interleukin 33* (*IL33*) (also termed *IL-1F11*) is the 11th, newest member (Boraschi et al., 2018, Dinarello, 2009, Wood et al., 2009). *IL33* was identified by Schmitz and colleagues (2005). *IL33* functions in a complex with its receptor interleukin 1 receptor like 1 (IL1RL1/ST2) (encoded by *IL1RL1*), and co-receptor IL1RAcP (Schmitz et al., 2005, Peine et al., 2016, Lamkanfi and Dixit, 2009). *IL33* is part of the IL-1 receptor/TLR family (Haraldsen et al., 2009, Schmitz et al., 2005). *ST2* is expressed on T helper (T_H) -1 cells, T_H -2 cells, natural killer cells, $CD4^+$ and $CD8^+$ T cells, mast cells and regulatory T cells (Treg) (Liew et al., 2016, Boraschi et al., 2018). This gives *IL33* multiple potential target cells within the immune system. *IL33* plays a role in inflammation, apoptosis and cell necrosis (Miller, 2011). *IL33* can act in a pro- or anti-inflammatory manner depending on the inflammatory disease (Miller, 2011).

Although predominantly cytoplasmic in action, *IL33* can also act directly in the nucleus (Moussion et al., 2008, Haraldsen et al., 2009).

Mastitis is a major inflammatory disease affecting dairy cows and incurs huge economic losses as a consequence of sub-clinical and clinical mastitis, and remains a costly disease in the dairy industry globally (Jamali et al., 2018, Sharma et al., 2017). Mastitis also affects beef cows (Murrieta et al., 2006, Paape et al., 2000). For the dairy industry, mastitis economic losses are estimated at £63931.24 /farm/year, or around £310.05 per case in the first month of infection (Guimarães et al., 2017, Rollin et al., 2015). Reduced milk production accounts for the largest percentage of economic losses (Rollin et al., 2015, Hadrich et al., 2018, Goncalves et al., 2018). Mastitis reduces future milk production by causing lasting damage to the mammary gland (Schukken et al., 2009, Seegers et al., 2003). Mastitis is associated with apoptosis and necrosis of epithelial cells, which subsequently reduces cell number and activity (Zhao and Lacasse, 2008). The effect of maternal diet on fetal immune function is therefore a highly relevant area of research both economically and for future animal health. Mammary epithelial cells are of interest as they are exposed to the environment and express and store *IL33* constitutively (De la Fuente et al., 2015). Constitutive expression of *IL33* suggests it is important in the healthy state, potentially as a quick-response, protective function. In healthy adult cows, *IL2*, *IL6*, *IL8*, and *IL12* mRNA were expressed in milk derived cells (Leutenegger et al., 2000). This suggests that these interleukins are not necessarily a marker of infection.

4.1.5 The Putative Functions of *TWIST1* in the Fetal Mammary Gland

Twist family bHLH transcription factor 1 (*TWIST1*) encodes *TWIST1*, a basic-helix-loop-helix (bHLH) protein (Way et al., 2014). There are three subclasses of the bHLH family, A, B and C, of which *TWIST1* is in subclass B (Castanon and Baylies, 2002). *TWIST1* has been localised in the mesoderm and mesodermal nuclei of embryos, and is vital for mesodermal development (Thisse et al., 1988). *TWIST1* was identified in Drosophila as a positive regulator of mesoderm formation, and is said to be tissue specific (Castanon and Baylies, 2002, Leptin, 1991). This relates to the mesodermally derived stroma in the fetal mammary gland. *TWIST1* functions by regulating downstream expression of genes expressed in the mesoderm (Leptin, 1991).

This was demonstrated to include suppression of cytokine and *SNAIL* genes (Šošić et al., 2003, Forghanifard et al., 2017). The implications of this regulation may be associated with aberrant development. Additionally *TWIST1* was expressed in white (WAT) and brown (BAT) adipose tissue (Pan et al., 2009, Qin et al., 2012, Pettersson et al., 2009, Šošić et al., 2003). In these locations *TWIST1* regulates mesenchymal stem cell differentiation (Pan et al., 2009, Qin et al., 2012). This suggests localisation of *TWIST1* in the mammary stroma and adipocytes within the bovine fetal mammary gland. Furthermore, abnormal *TWIST1* expression was associated with loss of *CDH1* related cell-cell adhesion (Yang et al., 2004). *TWIST1* therefore has a critical role in embryonic and fetal morphogenesis and was therefore selected as a candidate gene.

4.1.6 The Role of *FGFR2* in Mammary Development

Another gene related to growth is *Fibroblast growth factor receptor 2* (*FGFR2*) (Lu et al., 2008). *FGFR2* is a regulator of mammary morphogenesis, and has been located in the epithelium in young mouse and adult cows (Sternlicht et al., 2006, Spencer-Dene et al., 2001, Pond et al., 2013, Parsa et al., 2008, Mailleux et al., 2002, Luqmani et al., 1992, Casey et al., 2011). Fibroblast growth factor signalling pathways are involved in regulating cell fate determination in ectodermal organ development, such as the mammary gland (Jimenez-Rojo et al., 2012). FGFR2 like all mammalian FGF receptors, contains an extracellular domain, a transmembrane domain and an intracellular domain (Lu et al., 2008). The transmembrane domain allows interaction with extracellular growth factors such as FGFs (Lu et al., 2008). This interaction between extracellular growth factors and the FGFR2 signals to the cell, and allows a cellular response to the environment (Parsa et al., 2008). FGFR2 is vital to mammary development, playing a key role in establishing mammary epithelial placodes (Hens and Wysolmerski, 2005). The ligands of FGFRs are fibroblast growth factors (FGFs), of which multiple FGFR2 ligands were located in mouse mammary mesenchyme (FGF1, FGF3, FGF7, and FGF10) (Hens and Wysolmerski, 2005, Mailleux et al., 2002). FGFR2 is critical for epithelial-mesenchymal communication during early development (Mailleux et al., 2002). An FGFR2 ligand *FGF10* was expressed in mouse mammary adipocytes (Parsa et al., 2008). This suggests *FGFR2* functions in signalling between epithelia and adipocytes. Reduced *FGFR2* expression decreased mammary size by 40% in neonatal mice, compared

with controls (Parsa et al., 2008). Additionally, branching morphogenesis and terminal end bud development was negatively affected by decreased *FGFR2* expression (Parsa et al., 2008, Pond et al., 2013, Lu et al., 2008). These studies provide evidence for the function of *FGFR2* in mice.

In prepartum multiparous cows, *FGFR2* was localised in epithelial cells, which agrees with mouse studies of FGFR2 mammary localisation (Casey et al., 2011, Mailleux et al., 2002, Hens and Wysolmerski, 2005). In lactating cows, *FGFR2* expression was up-regulated in mammary glands milked four times daily versus twice daily (Connor et al., 2008). Evidence in mouse and cow supports *FGFR2* function as a positive promotor of mammary epithelial development. In bovine mammary epithelial cell culture, *FGF2-FGFR2* was shown to stimulate cell proliferation, and cell cycle progression to S and G2/M phase (Jeong et al., 2017a). *FGFR2* mRNA expression was confirmed whereas there was little to no *FGFR1*, *FGFR3* and *FGFR4* expression in mammary epithelial cells (Jeong et al., 2017a). This suggests that *FGFR2* is vital in bovine mammary development and remodelling in response to increased milking (Connor et al., 2008). Importance of *FGFR2* was confirmed in mice, as mammary placode development failed with reduced *FGFR2* expression (Mailleux et al., 2002, Kim et al., 2013).

4.1.7 The Role of *TFAP2* Family Members in Mammary Development

The family of AP-2 transcription factors (TFAP2) were identified as genes of interest in the fetal mammary gland in part for their functions in development, and also due to differential expression in transcriptomic analysis. The AP-2 family of transcription factors during fetal development are involved in cell differentiation (Eckert et al., 2005). There are five known members in the human and mouse, *TFAP2A*, *TFAP2B*, *TFAP2C*, *TFAP2D*, and *TFAP2E* (Eckert et al., 2005). *TFAP2C* promotes normal development of the luminal epithelial cell type (Cyr et al., 2015). It is unclear whether under expression of *TFAP2C* leads to physiological effects. However, complete loss of *TFAP2C* reduced branching morphogenesis (Jäger et al., 2010). In pubertal mice mammary glands, TFAP2C was localised in myoepithelial and luminal epithelial cells (Jäger et al., 2010). Loss of *TFAP2C* did not detrimentally affect myoepithelial differentiation, however the *TFAP2C* $^{-/-}$ mice presented quantitatively reduced ductal elongation and branching

compared to controls (Jäger et al., 2010). Furthermore, a deficiency in TFAP2C reduced prepubertal branching morphogenesis (Jäger et al., 2010). This suggests that a reduced expression of *TFAP2C* may lead to reduced ductal development in the bovine fetal mammary fat pad. This warrants further research into *TFAP2* expression in all four diet treatments, and immunohistochemical localisation in the bovine mammary gland in fetal, prepubertal, pregnant and lactating mammary glands.

4.1.8 The Mammary Immune System and Related Genes

The mammary gland is an exocrine gland, and is thereby in direct contact with the environment and open to bacterial infection such as *Escherichia coli* (*E. coli*) and *Staphylococcus aureus* (*S. aureus*) (Gilbert et al., 2013). Hernandez and colleagues (2002) analysed blood lymphocyte RNA expression, and reported over 200 differentially expressed genes between cattle with a high or low immune response.

The lymphatic system is a one-way transport of plasma fluid, immune cells and proteins from interstitial spaces to the circulatory system (Swartz, 2001). The lymphatic system comprises of capillaries, collecting vessels, lymph nodes, trunks and ducts (Swartz, 2001, Gasbarre et al., 2001). Lymph nodes are reservoirs for leukocytes such as lymphocytes, which often increase in numbers in response to bacterial or parasitic infection (Swartz, 2001, Gasbarre et al., 2001). Further understanding of the impact of maternal diet on fetal mammary gland immune function, is vital for enabling informed decision-making of nutrition during gestation, to reduce disease incidence in future cattle.

Integrin subunit alpha L (ITGAL) heterodimerises with *ITGB2* to form lymphocyte function-associated antigen 1 (LFA-1, CD11a/CD18; Table 4.1) (Taddei et al., 2003). *ITGAL* is expressed in all leukocytes and has a role in leukocyte intercellular adhesion, and lymphocyte costimulatory signalling (Marlin and Springer, 1987).

4.2 Methodology

4.2.1 RNA Isolation and Quality Control

Total RNA was isolated from fetal mammary tissue samples (total n=20; HH n=6, HL n=5, LH n=4, LL n=5) using Qiagen RNeasy with on-column DNase digestion, according to the method described in Chapter 2.4. Further to the general protocol, aliquots of samples (1µl /sample) were used to further validate RNA yield and quality (RNA integrity value, RIN) using an Agilent Bioanalyser 2100 (Agilent Bioanalyser 2100, Agilent Technologies, Santa Clara, California, USA).

4.2.2 RNA-seq

RNA samples with a RIN of >7, (Table 4.2) were submitted to the Bart's and the London Genome Centre, Queen Mary, University of London, for cDNA library preparation and sequencing on an Illumina NextSeq/HiSeq. Samples with a RIN of <7 were deemed to not be of sufficient quality, and thus were not analysed. Thus, only samples which passed quality control were selected for analysis, and which led to only the comparison between the HH and LH samples being studied (Table 4.2). A minimum of 20 million 150bp-paired end reads per sample were generated. The resultant fastq files were quality processed (phred score >30 retained) by Professor Nigel Mongan. Adapters were trimmed using the Trimgalore wrapper for fastqc and cutadapt. Following this, the QC-processed reads were aligned to the reference genome (bovine UMD3.1) using the Tophat aligner using the Ensembl gene annotation set. Differential gene expression was calculated using Cufflinks and Cuffdiff, the final step in analysis performed by Prof. Nigel Mongan. Quality control (QC) criteria were applied to the Cuffdiff gene list (>2 Log^2 fold-change, $p<0.05$), to identify differentially expressed genes. Differentially expressed genes were analysed using ConsensusPathDB and Cytoscape (Genemania). Genes which met QC criteria and were of biological relevance were considered genes of interest/candidate genes.

Table 4.2 Mammary fat pad RNA samples used in RNA-seq.

Lab ID	Ear tag animal number	Diet group	RIN
12/R61	22	HH	7.1
12/R62	23	HH	7.7
12/R57	18	LH	7.4
12/R63	24	LH	7.1

RIN, RNA integrity value. HH, high protein periconception, high protein postconception. LH, low protein periconception, high protein postconception.

4.2.3 Reverse transcription of cDNA

RNA (total n=20; HH n=6, HL n=5, LH n=4, LL n=5) of 98 dpc mammary fat pad samples were reverse transcribed to synthesise cDNA. The method is described (Chapter 2.5).

4.2.4 RT-qPCR

To validate the RNA-Seq data, a total of n=19 mammary fat pad cDNA samples (HH n=5, HL n=5, LH n=4, LL n=5) (Table 4.3) were used to validate candidate genes (Table 4.4) identified after quality control of RNA-seq data. One HH sample was removed due to low quality RNA and subsequent cDNA.

Table 4.3 List of mammary fat pad cDNA samples used for RT-qPCR validation of RNA-seq.

Lab ID	Ear tag animal number	Diet group
12/R61	22	HH
12/R62	23	HH
12/R75	36	HH
12/R78	39	HH
12/R79	40	HH
12/R42	3	HL
12/R56	17	HL
12/R60	21	HL
12/R69	30	HL
12/R70	31	HL
12/R51	12	LH
12/R52	13	LH
12/R57	18	LH
12/R63	24	LH
12/R43	4	LL
12/R46	7	LL
12/R68	29	LL
12/R82	43	LL
12/R88	49	LL

n=19. (HH n=5, HL n=5, LH n=4, LL n=5).

Table 4.4 List of Taqman® assays, and reagent volumes used in RT-qPCR reaction mixes.

Gene	Assay number
CDH1	Bt03210089_m1
ITGB2	Bt03216637_m1
KRT80	Bt03252931_m1
IL33	Bt03245533_m1
TWIST1	Bt04302308_g1
FGFR2	Bt04318017_m1
TFAP2A	Bt03253726_m1
UXT	Bt03229278_m1

Reagent	Volume per well (µl)
Roche LightCycler 480 Probe Master[1]	7.5
Taqman® Assay	0.65
RNase-free water	5.35
cDNA template	1.5

[1]Roche Life Science, Penzberg, Germany). (Taqman®, Thermo Fisher Scientific, Massachusetts, USA). *UXT* was used as an internal control.

Real-time-quantitative PCR (RT-qPCR) was used to measure relative mRNA expression of candidate genes using TaqMan® Gene Expression Assays (Thermo Fisher Scientific, Waltham, Massachusetts, USA). A reaction mix was formulated for each assay (Table 2.3). Assays were specific to candidate gene and species. Reagents (Table 4.4) as a master mix were pipetted into a LightCycler 480 Multiwell Plate 96 (Roche, Basel, Switzerland) and a LightCycler 480 (Roche, Basel, Switzerland) was used to run RT-qPCRs using the settings: 95°C for 10 minutes, 95°C for 10 seconds, followed by 45 cycles of 60°C for 30 seconds, 72°C for 1 second, followed by cooling at 40°C for 30 seconds. Samples were analysed in triplicates, with one triplicate non-template control per probe, per plate. Bonferroni's multiple comparison test was used for statistical analysis. Relative gene expression was calculated following the Pfaffl method (Pfaffl, 2001), where E is equal to primer efficiency and Ct is cycle threshold, using the formula:

$$\text{Ratio} = \frac{(E_{\text{target}})^{\Delta Ct \text{ target (Control–treated)}}}{(E_{\text{reference}})^{\Delta Ct \text{ reference (Control–treated)}}}$$

4.2.5 Immunohistochemistry

Immunohistochemistry was performed as described (Chapter 2.3) with the specific antibodies and concentrations as detailed (Table 4.5). Antigen retrieval was optimised to 20 minutes at medium-low microwave settings. In each diet group, n=3 sample slides (each slide containing multiple consecutive tissue sections) were stained and qualitatively analysed for each antibody (HH n=3, HL n=3, LH n=3, LL n=3), equalling n=12 per antibody.

Table 4.5 Commercial antibodies used in mammary tissue immunohistochemistry

Anti-body	Catalogue number	Supplier	Details	Dilution
IL33	NBP-1-76394	Novus Biologicals	Rabbit anti-Human polyclonal	1:200
FGFR2	ab227202	Abcam	Rabbit anti-Human polyclonal	1:400

Novus Biologicals, Littleton, Colorado, USA. Abcam, Cambridge, UK.

4.3 Results

4.3.1 RNA-Seq Data Overview

Comparisons of HH and LH samples allowed investigations of the effects of low or high protein during periconception, followed by high protein during the postconception period (LH vs. HH). Samples were mapped to the *Bos taurus* genome, and 24616 genes in total were in the data set (Figure 4.1, Figure 4.2). 7994 genes were not present in 98 dpc mammary tissues (Figure 4.1). While 731 and 914 genes were only expressed in HH or LH respectively (Figure 4.1). These genes were discarded from further analysis as the Log-fold change was subsequently infinite. The remaining 14977 genes was subjected to quality control (QC). The gene list was analysed for characterised and novel genes, and filtered using QC selection criteria (alpha level = $p<0.05$, defined fold-change = Log FC >2) to yield post QC genes (Figure 4.2).

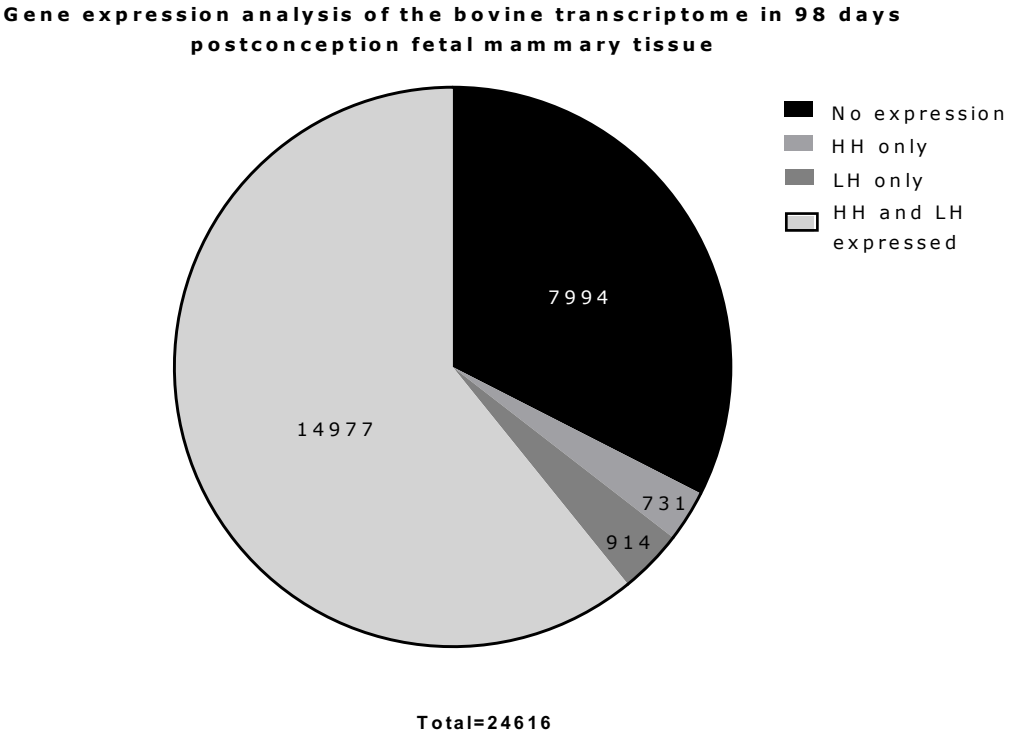


Figure 4.1 Pie chart showing the total number of genes mapped to the *Bos taurus* genome. Unexpressed genes, or genes only expressed in one treatment group were discarded. The remaining 14977 genes expressed in both HH and LH tissues were subjected to quality control criteria.

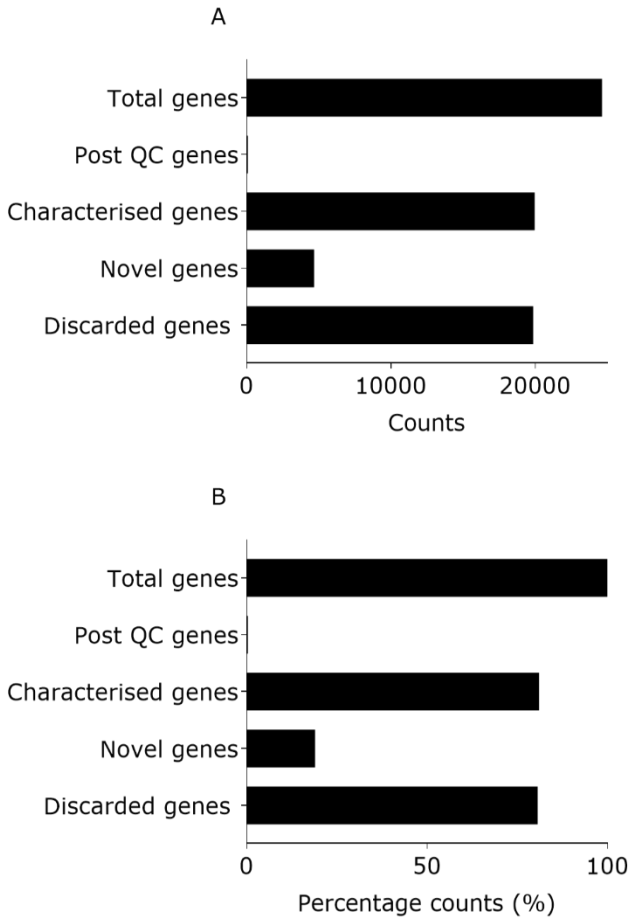


Figure 4.2 Counts of total, post QC, characterised and novel genes mapped to the *Bos taurus* genome Post quality control (QC) genes represents the number (A) and percentage (B) of genes remaining after QC criteria (Log Fold Change >2, $p<0.05$), these were also termed differentially expressed genes (DEGs). Characterised genes represented genes well characterised in literature. Novel genes represented genes that were characterised but little or nothing was known. Discarded genes were genes that did not pass QC. Total genes (100%).

4.3.2 Differentially Expressed Genes in the 98 Days Postconception Fetal Mammary Gland

Quality control of differentially expressed genes (DEGs) to a cut off criteria of Log Fold Change >2, $q<0.05$ identified 11 (one novel) DEGs (Table 4.6). These DEGs were further investigated in the literature. The cut-off value of $q<0.05$ appeared too strict for a whole animal study, where biological variability is greater than in cell culture research. 103 DEGs (Log Fold-Change >2, $p<0.05$) were identified as candidate genes (Table 4.7).

Table 4.6 Down- and up- regulated genes in LH versus HH exposed mammary tissues which met q value significance

Ensembl ID	Gene symbol	Log2 FC	p value	q value
Down-regulated				
ENSBTAG00000015106	DSP	-4.44	5.00E-05	9.22E-03
ENSBTAG00000020824	KRT10	-4.27	5.00E-05	9.22E-03
ENSBTAG00000046295	CCER2	-3.59	5.00E-05	9.22E-03
ENSBTAG00000017188	C20ORF82	-3.29	1.50E-04	2.59E-02
ENSBTAG00000012992	CDH6	-3.13	5.00E-05	9.22E-03
ENSBTAG00000005154	APCDD1	-2.71	5.00E-05	9.22E-03
ENSBTAG00000002129	KLF5	-2.57	5.00E-05	9.22E-03
ENSBTAG00000006694	CXCL14	-2.54	5.00E-05	9.22E-03
ENSBTAG00000043568	MT-ND3	-2.52	5.00E-05	9.22E-03
ENSBTAG00000047547	ENSBTAG00000047547	-2.39	5.00E-05	9.22E-03
ENSBTAG00000046256	TMEM132C	-2.31	5.00E-05	9.22E-03
Up-regulated				
ENSBTAG00000010645	BOLA-DRA	2.07538	5.00E-05	9.22E-03
ENSBTAG00000011766	C7	2.57789	5.00E-05	9.22E-03
ENSBTAG00000008631	CORO1A	2.12612	1.00E-04	1.75E-02

Table 4.7 Summary of the 103 DEGs, down- and up-regulated in LH versus HH exposed bovine fetal mammary tissues

Down-regulated DEG		Up-regulated DEG	
Characterised	Novel	Characterised	Novel
46	3	45	9

(p<0.05, logFC >2). LH= periconception low maternal dietary protein, postconception high maternal dietary protein. HH= periconception high maternal dietary protein, postconception high maternal dietary protein. Novel = no official gene symbol exists.

4.3.3 Up-regulated Genes in LH versus HH Exposed Fetal Mammary Tissues

The top ten up-regulated genes in the periconception low protein group versus high protein with the greatest fold-changes were *CD48 molecule (CD48)*, *complement C3d receptor 2* (*CR2), MHC Class I JSP.1 (JSP.1), major histocompatibility complex, class II, DQ beta (BOLA-DQB), CD53 molecule (CD53), Integrin subunit alpha L* (*ITGAL), T cell immunoglobulin and mucin domain containing 4 (TIMD4), C-C motif chemokine ligand 19 (CCL19), CD27 molecule (CD27)* and *protein tyrosine phosphatase, receptor type* C (*PTPRC*, *CD45*) (Table 4.8). The full up-regulated genes list is displayed (Appendix 8). *ITGAL* was up-regulated in LH versus HH (Table 4.8).

Enriched pathway analysis revealed T cell lymphocyte activation, T cell receptor activation, and signalling (Appendix 9) (Kamburov et al., 2009). Additionally, 190 enriched gene ontology (GO) terms were identified for the up-regulated gene set (Appendix 10), of which the top ten GO terms are displayed (Table 4.9). *ITGAL* but not *ITGB2* was associated with GO term GO: 0042110 T cell activation (Appendix 10). The GO term integrin alphaL-beta2 was the greatest enriched GO term, with 100% enrichment of the gene set (Table 4.9). *ITGB2* and *ITGAL* physically interact with each other, share a protein domain, and are in the same pathway (Warde-Farley et al., 2010). These two genes encode the proteins which form the complex integrin alphaLbeta2 which was subsequently enriched (Table 4.10). Another lymphocyte related gene was T-cell surface glycoprotein CD8 alpha chain (*CD8A*) which was up-regulated 3.02-fold in LH samples compared with HH sample, along with multiple other family members (Appendix 8). The immune system, adaptive immune system and innate immune system were among the enriched GO terms observed (Appendix 10).

Table 4.8 The top ten[a] up-regulated genes in LH versus HH exposed bovine fetal mammary tissues

Ensembl ID	Gene symbol	Log^2FC	*p* value
ENSBTAG00000011238	CD48	4.40	7.60E-03
ENSBTAG00000038496	CR2	4.33	5.05E-03
ENSBTAG00000020116	JSP.1	4.30	1.46E-02
ENSBTAG00000019588	BOLA-DQB	4.19	9.55E-03
ENSBTAG00000006466	CD53	3.77	1.09E-02
ENSBTAG00000007103	ITGAL	3.72	6.15E-03
ENSBTAG00000013185	TIMD4	3.71	1.37E-02
ENSBTAG00000012684	CCL19	3.68	1.79E-02
ENSBTAG00000014725	CD27	3.65	4.82E-02
ENSBTAG00000023144	PTPRC	3.54	1.65E-03

[a]See Appendix 8 for the full list of up-regulated genes. FC, Fold Change.

Table 4.9 The top ten[a] gene ontology terms for up-regulated genes in LH versus HH exposed bovine fetal mammary tissues

Gene ontology term	Category	Set size	Candidates contained (%)	*p*-value	*q*-value
GO:0034687 integrin alphaL-beta2 complex	CC	2	2 (100.0%)	4.40 E-06	6.15E-05
GO:0030369 ICAM-3 receptor activity	MF	3	2 (66.7%)	1.32 E-05	0.00029
GO:0001771 immunological synapse formation	BP	13	3 (23.1%)	2.50 E-06	3.82E-05
GO:0001767 establishment of lymphocyte polarity	BP	9	2 (22.2%)	0.00 0157	0.0014
GO:0032395 MHC class II receptor activity	MF	11	2 (18.2%)	0.00 0239	0.00263
GO:0001911 negative regulation of leukocyte mediated cytotoxicity	BP	16	2 (12.5%)	0.00 0518	0.00326
GO:0042605 peptide antigen binding	MF	31	3 (9.7%)	3.82 E-05	0.00107
GO:0001772 immunological synapse	CC	34	3 (8.8%)	5.07 E-05	0.000693
GO:0002577 regulation of antigen processing and presentation	BP	23	2 (8.7%)	0.00 108	0.00571
GO:0042611 MHC protein complex	CC	27	2 (7.4%)	0.00 149	0.00835

[a]See Appendix 10 for the full list of GO terms.
BP, biological process. CC, cellular component. MF, molecular function.

Table 4.10 Enriched complexes of up-regulated genes in LH versus HH exposed bovine fetal mammary tissues

Complex name	Set size	Candidates contained (%)	*p*-value	*q*-value
CD8A-LCK complex	2	2 (100.0%)	7.07E-06	8.21E-05
Integrin alphaLbeta2	2	2 (100.0%)	7.07E-06	8.21E-05
alphaL/beta2 Integrin/ICAM1	3	2 (66.7%)	2.12E-05	8.21E-05
alphaL/beta2 Integrin/ICAM2	3	2 (66.7%)	2.12E-05	8.21E-05
alphaL/beta2 Integrin/ICAM3	3	2 (66.7%)	2.12E-05	8.21E-05
alphaL/beta2 Integrin/ICAM4	3	2 (66.7%)	2.12E-05	8.21E-05
GNB2L1 forms a complex with ITGB2 :: ITGAL	3	2 (66.7%)	2.12E-05	8.21E-05
Integrin alphaLbeta2:F11R	3	2 (66.7%)	2.12E-05	8.21E-05
IL2/IL2R beta/gamma/JAK1/LCK/JAK3	6	2 (33.3%)	0.000105	0.000363
IL2/IL2R alpha/beta/gamma/JAK1/LCK /JAK3	7	2 (28.6%)	0.000147	0.000434

4.3.4 Down-regulated Genes in LH versus HH Exposed Fetal Mammary Tissues

The top ten greatest down-regulated genes in LH mammary tissue versus HH counterparts were *Myosin XVI (MYO16)*, *Plakophilin 1 (PKP1)*, *G protein-coupled receptor 37* (*GPR37), Desmoplakin (DSP), Keratin 10 (KRT10), transcription factor AP2 -A and -C (TFAP2A, TFAP2C), B cell CLL/lymphoma 11B (BCL11B), coiled-coil glutamate rich protein 2 (CCER2), and family with sequence similarity 83 member H (FAM83H)* (Table 4.11). The full down-regulated genes list is displayed (Appendix 11). The top ten gene ontology terms, and enriched pathways are listed (Table 4.12, Table 4.13). Furthermore*, E-cadherin (CDH1)*, *Keratin 80 (KRT80), and Twist family bHLH transcription factor 1 (TWIST1)* were also down-regulated genes in LH versus HH exposed mammary tissues (Table 4.14). These genes were identified in the enriched pathway, Developmental Biology (Appendix 12). Their biological relevance (Chapter 4.1.1, Chapter 4.1.2, and Chapter 4.1.4) warranted further investigation.

E-cadherin, *PKP1* and *DSP* were collectively identified in three enriched gene ontology terms: apoptotic cleavage of cell adhesion proteins, programmed cell death, and apoptosis (Appendix 12). *CDH1 and FGFR3* were identified as

candidate genes in the FGF signalling pathway (Appendix 11). *CDH6*, *TFAP2A*, and *TFAP2C* were identified in the enriched ectoderm differentiation pathway (Appendix 13). GO term GO:0007155 cell adhesion was enriched by 0.8% in the down-regulated gene set (Appendix 12). *CDH1* and *DSP* were identified candidate genes in the post-translational regulation of adherens junction stability and disassembly pathway (Appendix 13).

Keratinisation was also identified as an enriched pathway with *KRT80*, *KRT10*, *DSP* and *PKP1* identified in this pathway (Appendix 13). Enriched pathway analysis identified both activation and inhibition of the TFAP2 family pathway (Table 4.13). *TFAP2C* and *TFAP2A* expressions were 3.8-fold and 4.3-fold lower in LH mammary tissue compared to HH respectively (Table 4.11). *FGFR2* and *GATM* were 1.0-fold and 0.92-fold down-regulated in LH versus HH 98 dpc bovine mammary tissues respectively (Appendix 14). Enriched pathway analysis suggested enrichment of pathways involved in cell-cell communication, collagen chain trimerisation, apoptosis, cell adhesion, and structural elements of the cytoskeleton (Table 4.13, Appendix 13).

Table 4.11 The top ten[a] down-regulated genes in LH versus HH exposed bovine fetal mammary tissues

Ensembl ID	Gene symbol	Log^2FC	p value
ENSBTAG00000018237	MYO16	-5.40	0.0039
ENSBTAG00000046350	PKP1	-4.77	0.00215
ENSBTAG00000013732	GPR37	-4.59	0.005
ENSBTAG00000015106	DSP	-4.44	5.00E-05
ENSBTAG00000020824	KRT10	-4.27	5.00E-05
ENSBTAG00000001250	TFAP2A	-4.26	0.00835
ENSBTAG00000018019	BCL11B	-4.17	0.00815
ENSBTAG00000002065	TFAP2C	-3.79	0.0137
ENSBTAG00000046295	CCER2	-3.59	5.00E-05
ENSBTAG00000038682	FAM83H	-3.58	0.01445

[a]See Appendix 11 for the full list of down-regulated genes.

Table 4.12 The top ten[a] gene ontology terms of the down-regulated genes in LH versus HH exposed bovine fetal mammary tissues

Gene ontology term	Category	Set size	Candidates contained (%)	p-value	q-value
GO:0030280 structural constituent of epidermis	MF	8	2 (25.0%)	1.41 E-04	2.83 E-03
GO:0019215 intermediate filament binding	MF	13	2 (15.4%)	3.91 E-04	2.99 E-03
GO:0021756 striatum development	BP	16	2 (12.5%)	5.98 E-04	1.09 E-02
GO:0030057 desmosome	CC	26	3 (11.5%)	2.78 E-05	9.16 E-04
GO:0007379 segment specification	BP	18	2 (11.1%)	7.61 E-04	1.35 E-02
GO:0045109 intermediate filament organization	BP	20	2 (10.0%)	9.42 E-04	1.41 E-02
GO:0021544 subpallium development	BP	22	2 (9.1%)	1.14 E-03	1.13 E-02
GO:0017134 fibroblast growth factor binding	MF	25	2 (8.0%)	1.48 E-03	6.79 E-03
GO:0036075 replacement ossification	BP	26	2 (7.7%)	1.60 E-03	1.72 E-02
GO:0001958 endochondral ossification	BP	26	2 (7.7%)	1.60 E-03	4.31 E-02

[a]See Appendix 12 for the full list of GO terms.
BP, biological process. CC, cellular component. MF, molecular function.

Table 4.13 The top ten[a] enriched pathways of down-regulated genes in LH versus HH exposed bovine fetal mammary tissues

Pathway name	Set size	Candidates contained (%)	p-value	q-value
TFAP2 (AP-2) family regulates transcription of other transcription factors	4	3 (75.0%)	6.13 E-08	7.17 E-06
TFAP2 (AP-2) family regulates transcription of cell cycle factors	5	2 (40.0%)	6.34 E-05	1.05 E-03
Apoptotic cleavage of cell adhesion proteins	11	3 (27.3%)	2.50 E-06	1.46 E-04
Negative regulation of activity of TFAP2 (AP-2) family transcription factors	10	2 (20.0%)	2.83 E-04	2.60 E-03
Activation of the TFAP2 (AP-2) family of transcription factors	12	2 (16.7%)	4.14 E-04	3.23 E-03
TFAP2 (AP-2) family regulates transcription of growth factors and their receptors	15	2 (13.3%)	6.55 E-04	4.79 E-03
SUMOylation of transcription factors	20	2 (10.0%)	1.18 E-03	7.49 E-03
Adherens junctions interactions	31	3 (9.7%)	6.57 E-05	1.05 E-03
Apoptotic cleavage of cellular proteins	38	3 (7.9%)	1.22 E-04	1.58 E-03
Syndecan-3-mediated signalling events	26	2 (7.7%)	1.99 E-03	7.49 E-03

[a]See Appendix 13 for the full list of enriched pathways.

4.3.5 Candidate Genes for Validation

The candidate genes (Chapter 4.1) differentially expressed in 98 dpc mammary tissues (Table 4.14) were validated across all maternal dietary intervention groups (HH, HL, LH and LL) using RT-qPCR (Figure 4.3). No significant differences were seen between the groups for *E-cadherin*, *KRT80*, *ITGB2, TWIST1* and *TFAP2A* (Figure 4.3). *IL33* gene expression was initially observed to be 1.8-fold greater in LH versus HH diet using RNA-Seq. RT-qPCR analysis revealed an 8.3-fold and 11.6-fold greater *IL33* relative expression in LL versus HL, and LL versus HH respectively (ANOVA, $p<0.05$; Figure 4.3). *FGFR2* relative gene expression was 2.6-fold and 4.1-fold greater in HL versus LH (ANOVA, $p<0.01$) and HL versus LL (ANOVA, $p<0.001$) respectively (Figure 4.3). Between periconception diets, *TWIST1*,

KRT80, and *TFAP2A* relative expressions were unaffected (Figure 4.4). Relative expression of *IL33*, *ITGB2* and *E-cadherin* were 5.9-fold, 1.8-fold, and 3.5–fold greater in LPeri versus HPeri respectively. Relative expression of *FGFR2* was 0.5-fold down-regulated in LPeri versus HPeri exposed fetal mammary tissues (Figure 4.4). Between postconception diets, *TWIST1*, *FGFR2*, and *ITGB2* relative gene expressions were unaffected. *IL33*, *KRT80*, *E-cadherin*, and *TFAP2A* relative gene expressions were upregulated 6.4–fold, 2.0-fold, 6.3-fold and 6.5–fold in LPost versus HPost exposed bovine fetal mammary tissues respectively (Figure 4.4).

Table 4.14 List of candidate genes selected for RT-qPCR

Gene ID	Gene	Log^2 FC[a]	p value
ENSBTAG00000015991	*E-CADHERIN*	-3.49	0.01735
ENSBTAG00000017060	*ITGB2*	2.27	0.00425
ENSBTAG00000007204	*KRT80*	-2.26	0.0138
ENSBTAG00000018347	*IL33*	1.84	5.00E-05
ENSBTAG00000046922	*TWIST1*	-1.10	0.0065
ENSBTAG00000014064	*FGFR2*	-1.01	0.0015
ENSBTAG00000001250	*TFAP2A*	-4.26	0.00835

[a]Log^2 fold-change (FC) between HH and LH exposed 98 days postconception mammary tissues. Negative, and positive fold-change represents down- and up-regulation in LH versus HH exposed mammary tissues respectively.

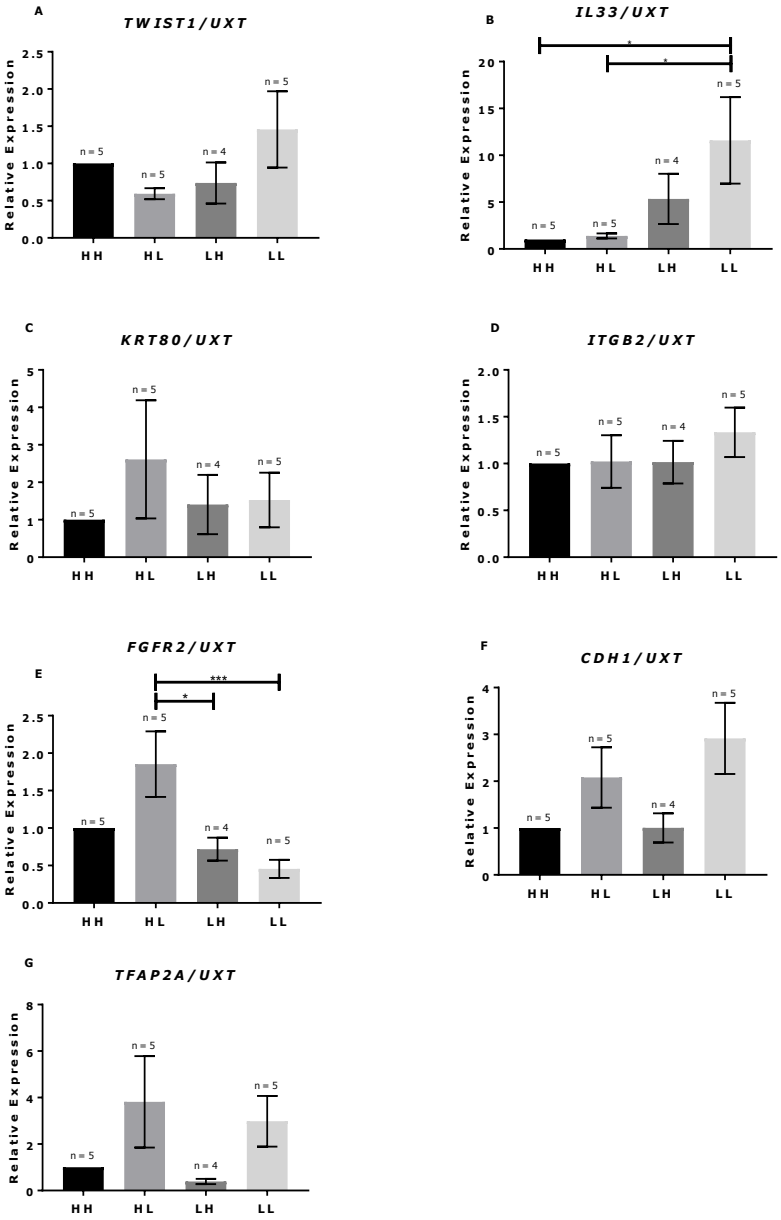


Figure 4.3 The effects of maternal dietary intervention on relative gene expression normalised to *UXT* expression in 98 days postconception mammary tissues. *TWIST1, Twist family bHLH transcription factor 1* (A), *IL33*, *Interleukin 33* (B), *KRT80*, *Keratin 80* (C), *ITGB2*, *integrin beta subunit 2* (D) *FGFR2, Fibroblast growth factor receptor 2* (E), *CDH1*, *E-cadherin, Cadherin 1* (F), *TFAP2A, Transcription factor-AP2A* (G). *UXT, Ubiquitously expressed prefoldin like chaperone,* HH=High/High, HL=High/Low, LH=Low/High, LL=Low/Low. (ANOVA, **p*<0.05, ***p*<0.01, ****p*<0.001).

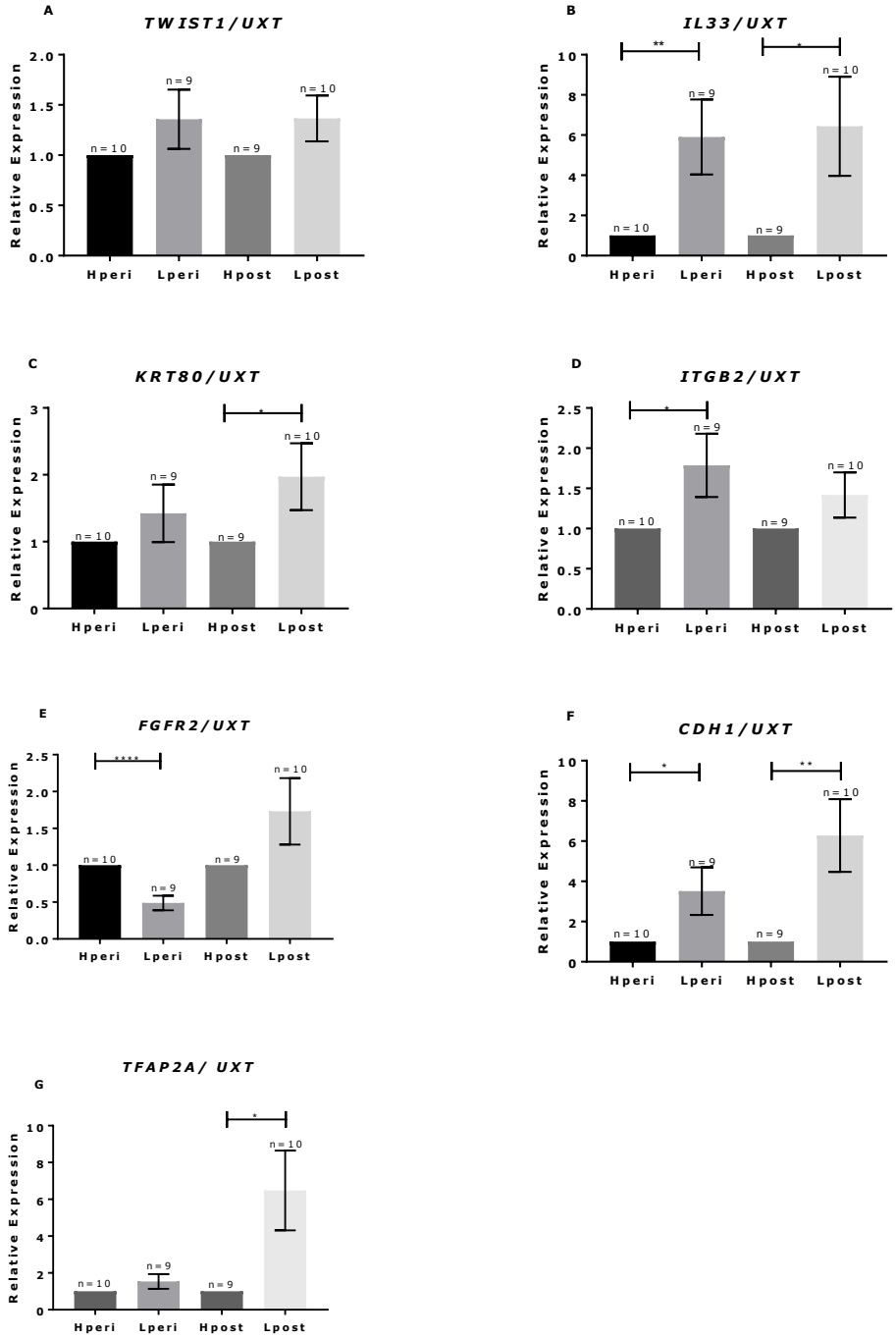


Figure 4.4 The effects of periconception and postconception maternal diet on relative gene expression normalised to *UXT* expression in 98 days postconception mammary tissues. *TWIST1, Twist family bHLH transcription factor 1* (A), *IL33, Interleukin 33* (B), *KRT80, Keratin 80* (C), *ITGB2, integrin beta subunit 2* (D) *FGFR2, fibroblast growth factor receptor 2* (E), *CDH1, E-cadherin, Cadherin 1* (F) and *TFAP2A, Transcription factor-AP2A* (G). *UXT, Ubiquitously expressed prefoldin like chaperone,* H=high L=low, Peri=periconception, Post=postconception, HPeri= (HH, HL), LPeri (LH, LL), HPost (HH, LH), LPost (HL, LL), (Independent samples t-test, *p<0.05, **p<0.01, ****p<0.0001).

4.3.6 Immunohistochemistry

IL33 and *FGFR2* genes identified as DEG's and validated by RT-qPCR were further investigated by immunohistochemistry (Chapter 2.3, 4.2.5) to localise the encoded proteins.

4.3.7 IL33 Immunohistochemical Localisation in the 98 Days Postconception Mammary Gland

IL33 protein was immunopositively localised in 98 days postconception bovine mammary tissues (Figure 4.5). IL33 immunopositivity was predominantly cytoplasmic, and was observed in epithelial, epidermal, adipocyte, endothelial, and immune cells (Figure 4.5). Within cell cytoplasm, no variation in the percentage of samples with immunopositive cell cytoplasm was observed between mammary glands exposed to different diets (Figure 4.6). Fetal mammary tissue exposed to HH, HL and LH maternal diets appeared to positively stain for *IL33* in endothelial nuclei (Figure 4.6). Stromal cells include B-lymphocytes and T-lymphocytes, which are collectively termed immune cells.

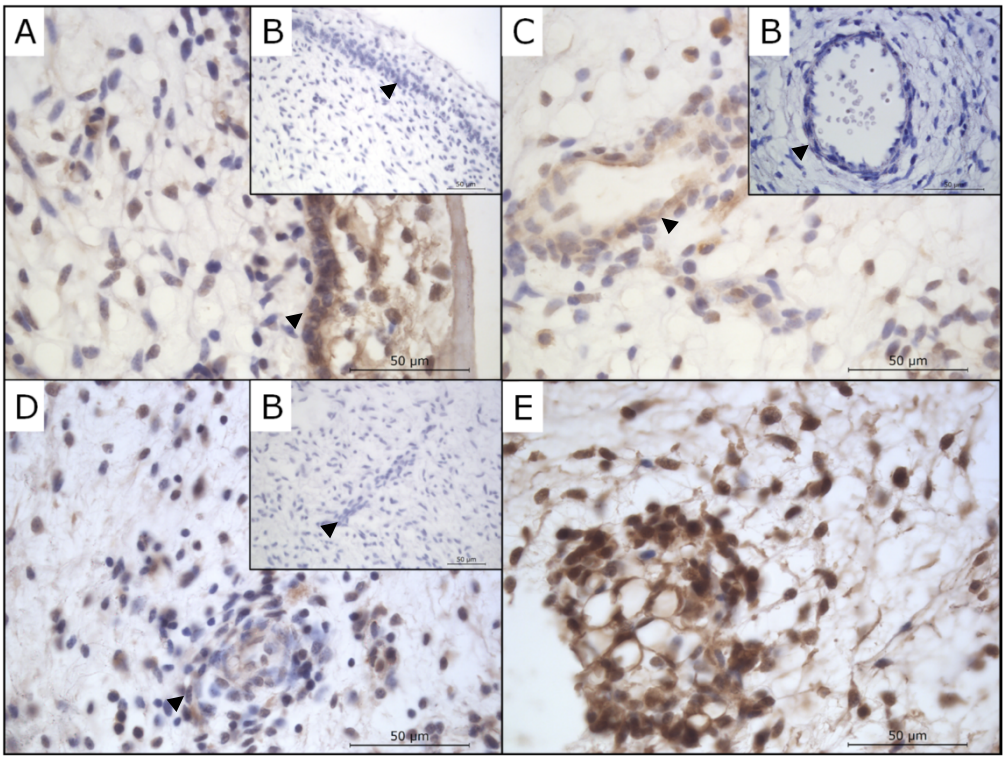


Figure 4.5 Immunohistochemical localisation of IL33 in 98 days postconception bovine mammary tissues. Photomicrographs display (A) mammary epidermis (arrowheads), (C) blood vessels (arrowheads), (D) duct (arrowheads), and (E) adipocytes (*), where photomicrographs labelled B are negative controls of respective structures. Negative controls (B) are displayed. A negative control is not displayed for adipocytes as the cells were not observed in the negative control tissue sections. Scale bar represents 50µm.

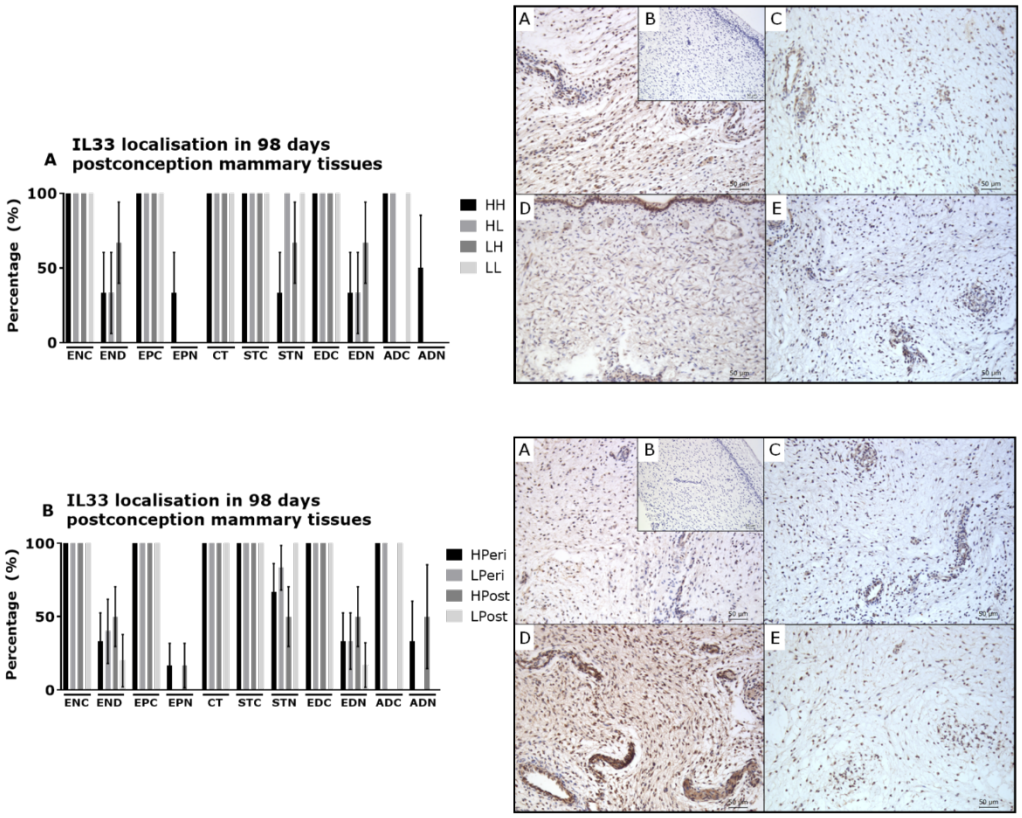


Figure 4.6. The percentage of samples displaying *IL33* immunopositivity and the cellular localisations in the 98 days postconception bovine mammary gland. The percentage of samples displaying IL33 immunopositivity and the cellular localisation in 98 dpc mammary tissues exposed to maternal dietary interventions (A), periconception, and postconception diets (B). Graphs and complimentary photomicrographs (B=negative control, A, C,-E represent each graph key e.g. photomicrograph A=HH, C=HL, D=LH, E=LL). H=high protein, L=low protein. Key: Letters denote periconception (Peri) and postconception (Post) diet e.g. HL=high protein periconception, low protein postconception. ENC=endothelial cell cytoplasm, END=endothelial cell nucleus, EPC=epithelial cell cytoplasm, EPN=epithelial cell nucleus, CT=duct adjacent connective tissue/stroma, STC=stromal cytoplasmic, STN=stromal nuclear, EDC=epidermal cytoplasm, EDN=epidermal nucleus, ADC=adipocyte cytoplasm, ADN=adipocyte nucleus. n=3 per maternal dietary intervention. Scale bar represents 50μm.

4.3.8 FGFR2 Immunohistochemical Localization in the 98 Days Postconception Mammary Gland

FGFR2 protein was immunopositively identified within 98 days postconception (dpc) fetal mammary tissues (Figure 4.7). Overall, FGFR2 protein was localised in the cell cytoplasm of all observed cell types (Figure 4.7). Epithelial cells constituting developing ducts stained positively for FGFR2 in their cytoplasm (Figure 4.7). FGFR2 was also present in surrounding connective tissue proximal to ducts, however it was also observed at a weaker density throughout the ECM (Figure 4.7). FGFR2 staining in stromal cells was cell-specific, however staining was predominantly cytoplasmic. Stromal adipocytes were either surrounded by the ECM or proximal to a duct, although not observed in all samples. This was likely due to the early stage of mammary development investigated, as adipocytes were uncommonly observed at 98 dpc. The n number stated for each maternal dietary intervention reflects only samples (n=2, 1, 0 and 1 for HH, HL, LH and LL respectively) with observed stromal adipocytes (Figure 4.8). Therefore, the percentage of samples positively stained for FGFR2 represents only observations where adipocytes were present (Figure 4.8).

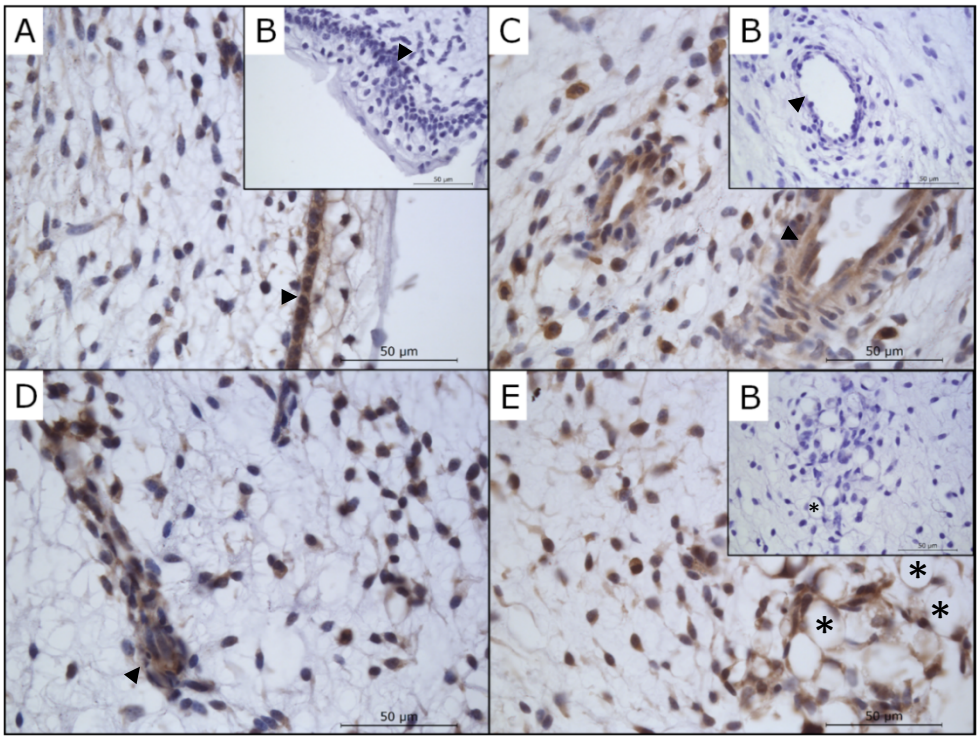


Figure 4.7 Immunohistochemical localisation of FGFR2 in 98 days postconception bovine mammary tissues. Photomicrographs display 63x magnification of mammary epidermis (A), blood vessels (C), duct (D), and adipocytes (E), where microphotographs labelled B are negative controls of respective structures. Scale bar represents 50µm.

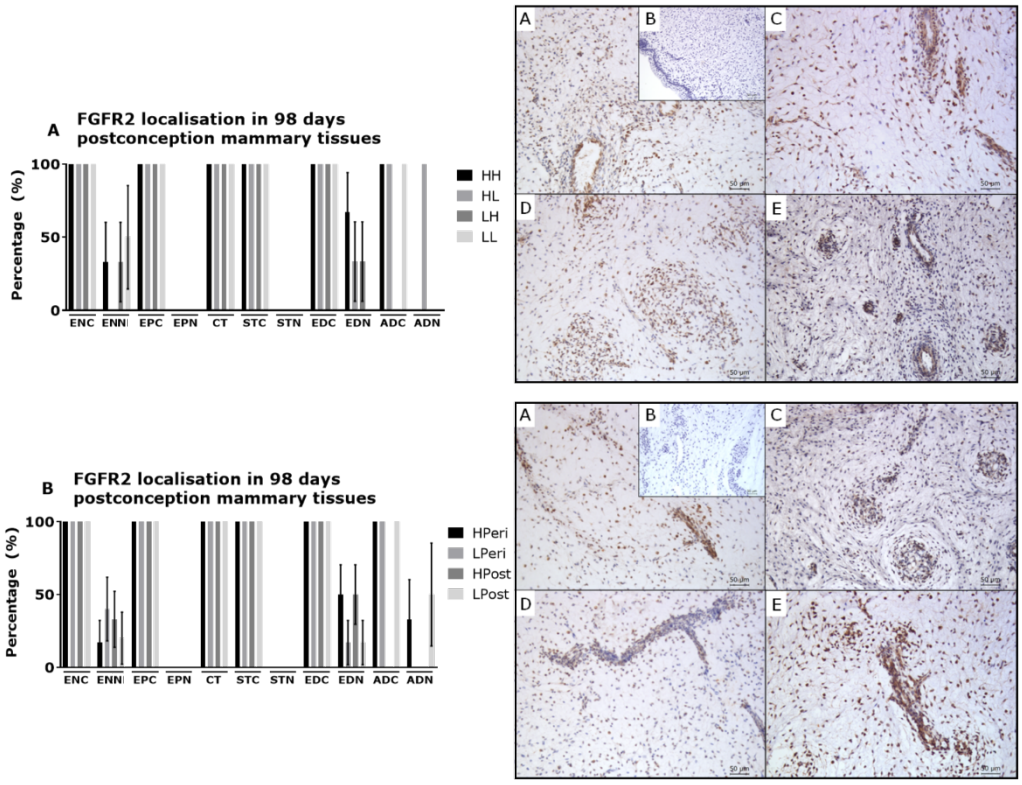


Figure 4.8 The percentage of samples displaying FGFR2 immunopositivity and the cellular localisations in the 98 days postconception bovine mammary gland. The percentage of samples displaying FGFR2 immunopositivity and the cellular localisation in 98 dpc mammary tissues exposed to maternal dietary interventions (A), periconception, and postconception diets (B). Graphs and complimentary photomicrographs (B=negative control, A, C, D, and E represent each graph key e.g. photomicrograph A=HH, C=HL, D=LH, E=LL) display examples of localisation. H=high protein, L=low protein. Key: Letters denote periconception (Peri) and postconception (Post) diet e.g. HL=high protein periconception, low protein postconception. ENC=endothelial cell cytoplasm, ENN=endothelial cell nucleus, EPC=epithelial cell cytoplasm, EPN=epithelial cell nucleus, CT=duct adjacent connective tissue/stroma, STC=stromal cytoplasmic, STN=stromal nuclear, EDC=epidermal cytoplasm, EDN=epidermal nucleus, ADC=adipocyte cytoplasm, ADN=adipocyte nucleus. n=3 per maternal dietary intervention. Adipocytes were observed in n=2, 1, 0 and 1 for HH, HL, LH and LL respectively. Scale bar represents 50μm.

4.4 Discussion

The bovine mammary gland is of critical importance to cattle production, for milk production, and raising a weaned marketable beef calf (Summers and Funston, 2013). The present study investigated the molecular effects of maternal dietary intervention on fetal 98 dpc mammary gene expression. The present study investigated low protein diet during periconception, compared to high protein diet periconception via RNA-sequencing (LH versus HH). This chapter compared gene expression in mammary fat pad samples exposed to LH compared with HH. In the current study, the majority of up-regulated genes had a role in immune function.

4.4.1 Genes Up-regulated in LH versus HH Exposed 98 Days Postconception Fetal Mammary Tissues

Maternal dietary intervention resulted in up-regulation of 45 genes in the fetal mammary fat pad (Table 4.7). Up-regulated genes were generally immune related (Table 4.8). Enriched pathways were supported by GO terms: GO:0006955 immune response, GO:0046649 lymphocyte activation, and GO:0045321 leukocyte activation (Table 4.9). Results indicated that LH exposed mammary tissues may have increased immune cell activation (Table 4.9). Other GO terms included GO:0046651 lymphocyte proliferation, and GO:0070661 leukocyte proliferation (Table 4.9, Appendix 10). This may suggest that LH exposed tissues had an increased adaptive immune response to pathogens in comparison to HH counterparts (Heinzel et al., 2018). However, the maternal diet (LH) may have signalled as a stress factor, signalling downstream effects on the developing immune system. In the maternal system, nutrient restriction such as the LH diet, may have induced a stress response in the hypothalamic-pituitary-adrenal (HPA) axis. Maternal hormones synthesised and released in response to nutrient restriction may affect fetal immune system development. Cortisol is a stress hormone produced in the adrenal gland (Racz et al., 1985).

BOLA-DQA1, *BOLA-DRA*, *ITGAL*, and *ITGB2* were genes up-regulated in the low protein periconception diet, which overlapped with pathway name *Staphylococcus aureus* infection (Table 4.8, Appendix 10). *S. aureus* is an environmentally derived mastitis-causing bacteria which evokes an inflammatory response (Lutzow et al., 2008). This is supported by enriched GO terms: GO:0006955 immune response, GO:0046649 lymphocyte

activation, and GO:0045321 leukocyte activation (Table 4.9). Results indicate that low protein during periconception is related to increased immune activation. These GO terms in combination with GO:0046651 lymphocyte proliferation, and GO:0070661 leukocyte proliferation suggest adaptive immune response to pathogens (Heinzel et al., 2018). Cytokine (C-C motif), and interleukin family members were up-regulated in adult bovine mammary tissue, following *S. aureus* challenge (Lutzow et al., 2008).

Overall, previous evidence in adult mammary glands challenged with bacteria highlight similar genes to those up-regulated in the fetal mammary gland in the current study. However, results are no indication of whether an infection was present, or whether gene expression was adaptive to prepare for future immune challenge. Further study is required to elucidate whether the mammary gland immune system and resistance to mammary infection differs in adult offspring exposed to different maternal dietary interventions during gestation.

4.4.2 Hematopoietic Cell Surface Receptors

Three CD family members *CD48*, *CD53*, and *CD27,* and *PTPRC* were up-regulated in 98 days postconception mammary tissue exposed to low protein during periconception compared with high protein (Table 4.8, Appendix 8). Co-expression occurs between all genes (Warde-Farley et al., 2010). Furthermore, Genemania network analysis (2010) revealed *LCK*, *PTPRC*, and *LTB* as related genes, which were all up-regulated in tissues exposed to low protein periconception (LH) compared with high protein counterparts (HH) (Table 4.8, Appendix 8).

CD48 is an immunoglobulin-like receptor, widely expressed on the surface of ($CD4^+$, $CD8^+$) T- and B-lymphocytes, natural killer cells, mast cells, eosinophils, dentritic cells, and monocytes in human and mouse cells (Elishmereni and Levi-Schaffer, 2011, McArdel et al., 2016). *CD48* is a ligand for *CD2* and *CD244* (McArdel et al., 2016). However, in the current study *CD244* was not expressed in tissues, and only LH exposed tissues expressed *CD2* (Appendix 14). This suggests development of the immune system for future infection, rather than activation in response to current infection. *CD48* has a role in response to bacterial infection, including but not limited to mastitis-causing *E. coli* and *S. aureus* (Minai-Fleminger et al., 2014, Rocha-de-Souza et al., 2008, Shin et al., 2000, Malaviya et al., 1999). In dairy

cattle mammary tissue, *CD48* and *CD53* were previously up-regulated in response to *E. coli* infection (Rinaldi et al., 2010). This may suggest an adaptive response to enhance defence against bacterial infection in mammary tissues exposed to low protein during periconception in the present study. *CD48* appears to be pro-inflammatory (McArdel et al., 2016). IL-2 pathways were suggested to be enriched in relation to up-regulated gene set analysis, specifically *CD48* (Table 4.10). This is supported by literature, such as CD48 having a role in T cell activation(Patel et al., 2001b). *CD48* stimulates LCK-kinase activity (Elishmereni and Levi-Schaffer, 2011). Literature suggests that fetal mammary tissues exposed to the LH diet, had up-regulated genes which provide the molecular framework for T cell activation.

CD53 is a leukocyte cell surface antigen, and expression has been identified on human B-lymphocyte plasma membranes (Zuidscherwoude et al., 2015). *CD53* expression was identified on human B-lymphocyte cell membranes, and dendrites (Katikaneni and Jin, 2018, Zuidscherwoude et al., 2015). This suggests either an increase in these cells in LH exposed fetal mammary tissues, or alternatively an increase in *CD53* gene expression in the 98 dpc mammary tissue, compared to HH counterparts.

Furthermore, the 3.5-fold up-regulation of *PTPRC* in LH exposed bovine fetal mammary tissue versus HH counterparts was similar to the above genes (Table 4.8). *PTPRC* is also a leukocyte cell surface molecule, which is expressed on all nucleated hematopoietic cells including pre-cursors (Penninger et al., 2001). *PTPRC* was previously increased in milk-derived cells extracted from cows milk with *S. aureus* infection compared to healthy controls (Riollet et al., 2001). It was not discussed whether the increase in *PTPRC* was due to increased immune cell number, or increased gene expression of milk-derived cells between healthy and infected milk (Riollet et al., 2001). A potential role of *PTPRC* in mammary tissue is regulation of apoptosis during T-lymphocyte development and maturation, which is dependent on *PTPRC* expression, where *Galectin-1* associates with *PTPRC* (Penninger et al., 2001, Demetriou et al., 2001). In the current study *Galectin-12* (*LGALS12*) was expressed in all mammary tissues and was unaffected by diet (Appendix 14). The expression of *LGALS1* highlights a potential role of *PTPRC* in the current study, and suggests enhanced potential for T-lymphocyte development and maturation in mammary tissues exposed

to low protein during periconception compared with high protein. Further research is required to identify the specific function of *PTPRC* in response to maternal dietary protein level. Further to lymphocytes, genes related to a wider range of leukocytes were identified as DEGs, such as *integrin-β2* (*ITGB2*) and *integrin- αL* (*ITGAL*) (Table 4.8, Appendix 8).

4.4.3 *ITGB2* Up-regulation in the LH Exposed 98 Days Postconception Mammary Tissues

In the mammary gland, integrins are vital for epithelial cell response to changes in the ECM and fat pad components, and act as receptors of collagen, fibronectin and laminin (Kass et al., 2007). Integrin-β2 was previously immunohistochemically localised on human neutrophils and bound fibrinogen (pulmonary artery samples) (Wang et al., 2014b). *ITGAL* was up-regulated in fetal mammary tissues exposed to LH compared with HH samples (Table 4.8). *ITGAL* heterodimerises with *ITGB2* to form lymphocyte function-associated antigen 1 (LFA-1) (Taddei et al., 2003). The expression of *junction adhesion molecule 3* (*JAM-3*) (Appendix 14) may suggest that ITGB2 forms integrin αMβ2 with the alpha integrin ITGAM (Muller, 2003). However, JAM-3 is known to be present on resting and inflamed endothelium, and therefore is not indicative of inflammation in the fetal mammary tissues (Muller, 2003). Enriched pathway analysis revealed T cell lymphocyte activation, T cell receptor activation, and signalling (Kamburov et al., 2009). *ITGAL* but not *ITGB2* was associated with GO term GO: 0042110 T cell activation. *ITGAL* physically interacts with *PTPRC* (Warde-Farley et al., 2010). Increased integrin subunit β2 expression was associated with acute infection in humans ($p<0.001$) where a median of 92% of human samples with acute infection expressed integrin subunit β2, compared to no-infection controls (90% integrin subunit β2 expression) (Song et al., 2015). Although there was a differential expression between infected and non-infected groups, β2 expression is not associated specifically with infection (Song et al., 2015). It is therefore not clear whether *ITGB2* expression in the current study was associated with infection. Integrin subunit β2 is also a core protein of venous thrombus, a blood clot (Wang et al., 2014b, Song et al., 2015).

4.4.4 *IL33* Up-regulation in the LH Exposed 98 Days Postconception Mammary Tissues

IL33 expression was 1.8-fold up-regulated in LH versus HH exposed fetal mammary tissues (Table 4.14) and was selected as a candidate gene (Chapter 4.1.5). RT-qPCR was performed to validate differential expression of *IL33* between diet groups (Figure 4.3, B). Additionally, immunohistochemistry confirmed *IL33* protein localisation in the 98 dpc mammary fat pads (Figure 4.5, Figure 4.6). Although the fold-change criterion (>2) was not met, this gene was selected for further investigation due to its biological relevance in the mammary gland. RT-qPCR did not statistically support the differential expression between HH and LH (Figure 4.3, B). However, RT-qPCR identified differential expression between LL exposed tissues and HH ($p<0.05$), and HL ($p<0.05$) (Figure 4.3, B). No significant difference between LH and LL exposed tissues suggest that dietary protein during postconception did not alter *IL33* expression (Figure 4.3, B). Although the error-bars between LH and LL demonstrate biological variability in whole organisms which would not be expected in cell culture (Figure 4.3 B). Although further investigation using cell culture of specific mammary cells, such as epithelial, adipocyte or endothelial cells may be of interest to further elucidate *IL33* expression in the bovine mammary gland, and the influence of maternal dietary protein exposure, ultimately whole-organism research is valid and more applicable to industry. In the present study, expression of *IL33* in specific immune cells is unknown, however non-immune cell-types, such as endothelial and epithelial cells are known to express *IL33* and were observed in the current study. Therefore, the focus will be on potential functions of *IL33* in these cell-types.

IL33 up-regulation is consistent with other up-regulated immune-related genes in the current study (Table 4.8, Appendix 8). Specifically, *IL33* is suggested to increase differentiation of various T-lymphocytes, as its receptor *ST2* (*IL1RL1*) is located on multiple cell types. *IL1RL1* was lowly expressed and unaffected by diet in the current study (Appendix 14). *IL33* was associated with skin *S. aureus* infection in adult mouse studies (Yin et al., 2013). There are many differences between the study by Yin and colleagues and the current study. In adult male skin, *IL33* potentially functioned to increase *collagen 3A* and *fibronectin* (*FN1*) to aid tissue healing (Yin et al., 2013). *IL33* potentially plays a developmental role in fetal mammary ECM development. However, *COL3A1* expression was not

identified in mammary tissues in the current study. *FN1* was highly expressed but unaffected by diet (Appendix 14). This suggests no biological response to differential *IL33* expression regarding *FN1* and *COL3A*, and related ECM development. Nevertheless, *IL33* was shown to enhance immune defence against various bacteria in mucosal tissues (Yin et al., 2013).

Caspase-3 (*CASP3*) was 0.62-fold down-regulated in LH exposed mammary tissues compared with HH (Appendix 14). *Caspase-7* (*CASP7*) was expressed and unaffected by maternal diet (Appendix 14). In apoptosis, caspase-3 and -7 cleave and inactivate *IL33*, preventing *IL33-ST2/IL-1RAcP* binding and subsequent downstream expression of interleukins by immune cells (Lamkanfi and Dixit, 2009, Liew et al., 2016). In necrotic endothelial or epithelial cells, cleavage does not occur, and *IL33* is exocytosed and interacts with its receptor complex, eliciting downstream upregulation of interleukins -4, -5, and -13 (Liew et al., 2016). Data in the current study suggests no necrosis and supports apoptosis as *IL4*, *IL5* and *IL13* were not expressed in mammary tissue (Appendix 14). *IL33* upregulation and *CASP3* downregulation in LH suggests potential reduced regulation of *IL33* stimulation of *ST2* membrane-bound cells, and enhanced immune activity.

Immunohistochemical localisation identified cytoplasmic and nuclear immunopositive staining of IL33 protein in the 98 days postconception bovine mammary gland (Figure 4.6). Qualitative analysis suggested no overall differences in localisation between diet groups (Figure 4.6). Localisation was predominantly cytoplasmic (Figure 4.5). In addition to immune cells, *IL33* was also expressed in endothelial and epithelial cells. This is in agreement with previous reports where *IL33* was constitutively expressed by endothelial and epithelial cells in humans and mice, in steady-state conditions (Boraschi et al., 2018). This is a feature of these barrier cells as they are in contact with the environment, e.g. the gut (Boraschi et al., 2018). Therefore, the reason for *IL33* up-regulation in LH exposed tissues is unknown, as it could be due to differences in endothelial and or epithelial cells, or cell molecular activity.

IL33 was expressed in human adipocytes (Wood et al., 2009, Saidi et al., 2011). This was supported by localisation in the current study (Figure 4.5). In 50% of HH exposed tissue samples, nuclear localisation of IL33 protein

was observed, while HL, LH and LL tissues displayed no nuclear localisation (Figure 4.5). Further to *IL33*, the gene *C7* was also up-regulated in LH exposed fetal mammary tissues versus HH, and is part of the innate immune system.

4.4.5 *C7* Up-regulation in the LH Exposed 98 days Postconception Mammary Tissues

Complement component 7 (C7) gene expression was up-regulated in LH versus HH (Appendix 8) and encodes the protein Complement component 7 (C7). The complement system is complex, regulated by three different pathways, and comprised of over 30 proteins (Carroll, 2004). Complement component genes form part of the innate immune system and coordinate inflammatory and immunological processes, including T and B cell regulation (Ricklin et al., 2010, Carroll, 2004). The complement system identifies and distinguishes between healthy host cells, apoptotic host cells, cellular debris and microbial pathogens (Ricklin et al., 2010, Maye et al., 2017). The complement is a highly conserved part of the innate immune system (Carroll, 2004). Complement components can be cleaved to form a and b fragments, such as C5b (Nauta et al., 2002, Würzner, 2000). C7 is one of multiple complement components (C5b, C6, C7, C8, and C9) which form the C5b-C9 membrane attack complex (MAC) on the phospholipid membrane of target cells (Würzner, 2000, Nauta et al., 2002). The C5b-C9 MAC has a role in activation of apoptosis, or opsonisation of target cells (Nauta et al., 2002). *C7* has a hydrophobic domain, allowing association with target cell membranes (Bossi et al., 2009, Yorulmaz et al., 2015). This is the specific function of *C7* within the C5b-C9 MAC. Up-regulation of *C7* in mammary fat pad tissues exposed to the LH diet versus HH diet suggests an increased immune modulation in host defence, particularly against pathogens in the circulatory system (Bossi et al., 2009). Alternatively *C7* may be involved in transport of cholesterol, high density lipoprotein (HDL), and lipids, as identified previously in the disease-state human circulatory system (Schwarz et al., 2008, Vasil and Magro, 2007). Expression of other complement components in the C5b-C9 MAC were observed in the current study (Appendix 14). Of the *C5b-C9* genes, not all were identified as expressed in RNA-Seq data. This suggests that *C7* up-regulation was potentially a preparatory rather than active biological process in the 98 dpc (Appendix 14), however this remains unclear.

4.4.6 Chemokine Up-regulation in the LH Exposed 98 Days Postconception Mammary Tissues

Briefly, chemokines are a family of small molecules which regulate leukocyte trafficking (Zlotnik and Yoshie, 2000). Chemokines are subdivided into CC or CXC classes based on cysteine residue locations (Moore et al., 2018, Zlotnik and Yoshie, 2000). *CCL19* encodes *chemokine C-C motif ligand 19*, which was 3.70-fold up-regulated in LH exposed tissues compared with HH (Table 4.8). Human *CCL19* has a homeostatic function, and its receptor is *CCR7* (Widdison and Coffey, 2011). *CCR7* was lowly expressed in the current study and was unaffected by diet (Appendix 14). Nonetheless, *CCL19* may play an important role in mammary gland immune function in later life (Moore et al., 2018). Previously, *CCL19* was up-regulated 24-fold in adult sheep mammary tissues challenged with infection compared to controls (Chopra-Dewasthaly et al., 2017). The fold-change between LH and HH exposed was not as great as the infected sheep study. This may indicate an adaptive up-regulation in LH exposed fetal mammary tissues rather than infection at 98 dpc.

The immune system may affect structural development. Immune activation may reduce *IGF1* expression and lead to altered body composition, adipokine, and HPA-axis signalling (Bourke et al., 2016). In the present study, genes involved in immune activation, particularly leukocytes and the lymphatic system were up-regulated (Table 4.8, Appendix 6). *IGF1* expression was increased 0.99-fold in LH compared to HH (Appendix 14; $p<0.05$). This does not support the suggestion of Bourke and colleagues (2016). IGF1 may increase mammary epithelial protein synthesis via the phosphatidylinositol 3-kinase (PI3K) - serine/threonine kinase 1 (Akt) pathway (Burgos and Cant, 2010).

In healthy beef cows, *IGF1* was associated with endometrial growth and proliferation, and postpartum (Foley et al., 2012). In cows, *IGF1* was expressed at various stages in mammogenesis, and was highest at involution (Plath-Gabler et al., 2001). These studies are evidence for the role of IGF1 in growth and proliferation in cows, and specifically the mammary gland. In adult human mammary tissue, a low protein diet reduced *IGF1* expression (Levine et al., 2014). The switch from low protein to high protein (LH) during postconception may have altered the *IGF1* pathway towards increased expression compared to fetuses exposed to a maintained maternal high

protein diet (HH). This suggests that the potential effect of low protein during periconception on *IGF1* expression was not alleviated by a switch to high protein during the postconception period. RT-qPCR analysis of *IGF1* would further elucidate the effects of maternal dietary intervention on *IGF1* expression in the fetal mammary gland.

Apoptosis was identified as an enriched pathway in analysis of down-regulated genes in LH versus HH exposed fetal mammary tissues (Appendix 11). Apoptosis occurs during bovine lactation and involution (Suchyta et al., 2003). However, apoptosis is unlikely to be involved in bovine fetal mammary development, as it was previously reported not to be involved in postnatal bovine mammary development (Capuco et al., 2002). In human breast cancer cell lines, *IGF1* was identified as having a role in apoptosis (Suchyta et al., 2003). Aside from *IGF1*, *C7* may have played a role in apoptosis associated genes. It was previously reported that the membrane attack complex (*C5b-C9*) was involved in induction of apoptosis in *in vitro* rat mesangial cells isolated from kidney (Nauta et al., 2002).

4.4.7 Genes Down-regulated in LH versus HH Exposed 98 Days Postconception Fetal Mammary Tissues

Further to up-regulation of the discussed genes, maternal dietary intervention during periconception and postconception also resulted in down-regulation of 49 genes in 98 dpc bovine mammary tissues (Appendix 11). The functions of down-regulated genes were more diverse (Table 4.12, Appendix 12) compared with the up-regulated gene set, which were predominantly immune related. Although there was crossover between gene function and interactions, down-regulated genes were grouped into the following themes: milk production, structural development, immune function, and adipocyte related genes.

4.4.8 Future Milk Production

In adult cattle, gene expression of milk constituent or production related genes have been reported (Connor et al., 2009, Li et al., 2016). Li and colleagues (2016) focused on candidate genes influencing milk proteins, and investigated expression levels at peak lactation and dry-period. *SLC1A1*, *TFAP2A*, *TFAP2C, AQP3,* and *GATM* were down-regulated in 98 dpc mammary

tissues exposed to LH compared with HH (Appendix 11, Appendix 14). Their functions indicate a putative role in future milk production and were therefore biologically relevant.

Solute carrier family 1 member 1 (SLC1A1) is a glutamate and aspartate amino acid transporter, previously temporally expressed in the bovine endometrium, fetus and adult mammary gland (Forde et al., 2014, Mitko et al., 2008, Wang et al., 2012, Manjarin et al., 2014). *SLC1A1* was previously localised at the membrane of mammary epithelial cells (Manjarin et al., 2014, Millar et al., 1996). *SLC1A1* expression was shown to be influenced by progesterone (Forde et al., 2014). In dairy cows, plasma progesterone concentrations were lower in *ad libitum* versus cows with restricted grazing access and no ration, suggesting a quicker progesterone clearance rate in *ad libitum* (Rabiee et al., 2001). In the present study LH dietary exposure reduced *SLC1A1* expression 3.1-fold, compared with HH exposure (Appendix 11). In adult mammary glands at 30 days postpartum, *SLC1A1* expression was up-regulated compared with 30 days prepartum (Wang et al., 2012). This is evidence for up-regulation of *SLC1A1* during lactation versus the dry-period. Adult endometrial *SLC1A1* was affected by progesterone concentration during the bovine oestrus cycle (Forde et al., 2014). On oestrus day 7, heifers with high progesterone levels had higher *SLC1A1* expression compared with control and low progesterone groups, but decreased by day 13 and was unaffected by progesterone level (Forde et al., 2014). Progesterone was affected by dietary energy and protein levels in dairy cows (Butler, 2000). Differential expression of *SLC1A1* may be a consequence of differential maternal serum progesterone concentrations in heifers of the current study.

Serum or blood analysis, or body condition scoring, or body weight changes of the heifers were not performed in the current study. Additionally, decreasing body condition and negative energy balance have been associated with lower progesterone (Butler, 2003). Fetal mammary tissues exposed to the LH diet may have been exposed to altered progesterone levels and subsequent altered *SLC1A1* expression. In summary, altered *SLC1A1* expression may be directly influenced by maternal diet or indirectly through maternal progesterone. However, further investigation is required to confirm this. Reduced *SLC1A1* expression in LH versus HH exposed fetal mammary tissues may lead to reduced amino acid transport into milk in

future milk production, as a conservative mechanism of maintaining maternal body reserves during an assumed low protein environment. Unfortunately, analysis of adult offspring mammary development and milk production was beyond the scope of the current study.

TFAP2A relative gene expression was not significantly affected by maternal dietary intervention in qPCR validation (Figure 4.3, G). However, LPost exposed tissues had greater relative expression than Hpost counterparts (Figure 4.4, G). This may suggest that in LPost exposed 98 dpc mammary tissues, cell proliferation may have reduced. How maternal diet may have directly influenced this change is unclear, although previous studies observed a correlation between liver *TFAP2A* mRNA expression and indicators of negative energy balance (Loor et al., 2005). This may be an adaptive response to the *in utero* environment, and adaptation of the fetal mammary gland for future development. This is a novel insight into postconception dietary effects on *TFAP2A* gene expression as LPost was not investigated by RNA-seq (LH and HH both being HPost). This novel finding reveals an effect of postconception diet on 98 dpc mammary tissue development.

A third DEG associated with milk production is *Aquaporin-3* (*AQP3) (*Appendix 11*). AQP3* is a water, urea and glycerol transporter (Ishibashi et al., 1994), and was previously immunohistochemically localised in adult bovine basolateral membrane of epithelial cells localised in the teat, cistern, and tubuloalveoli (Mobasheri et al., 2011). *AQP3* was also localised in some apical membranes in cistern and teat duct epithelial cells (Mobasheri et al., 2011). It is not known whether *AQP3* down-regulation in fetal mammary tissues exposed to LH versus HH would result in a permanent alteration in AQP3 protein expression in the bovine mammary gland. Equally, it is unknown whether this differential expression was as a result of differences in cell numbers expressing this gene, or cell gene expression.

Furthermore, *GATM* may also affect future milk protein yield of offspring (Appendix 14). In lactating dairy cows, *GATM* was previously 1.04-fold up-regulated in mammary samples with high milk protein percentage compared to low protein percentage at peak lactation (Li et al., 2016). This suggests either increased creatine production due to increased *GATM* expression, meaning more energy available for milk protein synthesis so the amount of

milk protein increases. Alternatively, milk volume may be reduced, meaning constituents may be more concentrated and thus may leads to a higher milk protein percentage. However, this is not as logical an argument. In LH exposed mammary samples versus HH tissues, it is hypothesised that the -0.9 down-regulated *GATM* expression (Appendix 14) may lead to reduced milk protein, as a conservative mechanism of fetal programming in a low dietary protein environment. Although the fold-change of *GATM* may not be sufficient for physiological implications. Nonetheless, the overall implication of reduced *GATM* expression may be a potentially reduced future milk protein production. Mammary *GATM* gene expression was up-regulated in lactating versus non-lactating dairy cows (Dai et al., 2018).

4.4.9 Mammary Epithelial Development and Branching Morphogenesis

Genes specific to mammary epithelial development and branching morphogenesis were identified. This may influence future structural development and subsequent future milk production of offspring in the current study. Down-regulated genes (LH vs HH) included *CDH6*, *KRT80*, *KRT10*, *TFAP2A*, *TFAP2C*, *E-cadherin*, *FGFR2,* and *S100A4, and* are involved in embryonic mammary development (Table 4.11, Appendix 11).

CDH6, also termed K-cadherin, is a type II member of the cadherin superfamily of cell adhesion proteins (Yagi and Takeichi, 2000, Yang et al., 2017, Gugnoni et al., 2017). Current literature is lacking in mammary or bovine knowledge regarding *CDH6*. A previous mouse mammary study identified *CDH6* expression in basal epithelial cells during lactation (Acosta et al., 2016). This indicated a role of CDH6 in cell adhesion during lactation, potentially relating to remodelling during this stage. In the current study, *CDH6* expression was 3.14-fold down-regulated in LH versus HH exposed mammary tissues *(q<0.01;* Table 4.6). This may suggest a difference in cell-adhesion in bovine fetal mammary epithelial cells. Alternatively, it may indicate a difference in epithelial cell populations, although the presence of myoepithelial (basal) and luminal epithelial cells remains unknown at 98 dpc. The current study would benefit from further study of firstly whether these sub-types exist at 98 dpc, and the transcriptomic analysis of these cell-types to determine the specific effects of maternal dietary intervention on the bovine fetal mammary transcriptome. Previously, loss of *CDH6* expression

altered cell architecture, morphology and inhibited cell proliferation in human thyroid carcinomas (Gugnoni et al., 2017). Existing work may suggest a change in mammary structure and morphology. Although there are many discrepancies between previous studies to construct a hypothesis for the present study. The general role of cadherins would support the suggestion that down-regulation of *CDH6* suggests a reduction in cell adhesion and potential and tissue structure. Results in the current study contribute to the knowledge that *CDH6* expression was reduced in fetal mammary tissues exposed to LH compared with HH, however as discussed above, further investigation is required. Genes better understood in mammary tissue, and which also function in tissue structure and development are the keratins.

Keratinisation was identified as an enriched pathway in the down-regulated gene set, with *KRT80*, *KRT10*, *DSP* and *PKP1* identified in the pathway (Appendix 13). Squamous epithelial keratinisation occurs in the bovine mammary gland, in order to form a physical barrier (Franz et al., 2001, Paulrud, 2005). Keratinisation in the bovine mammary gland is vital for function, particularly within the papillary duct, which is responsible for retaining milk in between milkings. Keratin in the papillary duct is a physical barrier against environmental pathogens (Paulrud, 2005). There is potential for differential rates of development of the papillary duct in the current study. Thus, potential for differential gene expression of related genes exists. Keratinocytes line the papillary duct lumen and express *KRT10* (Paulrud, 2005, Smolenski et al., 2015). This is notable as *KRT10* was q-value significant (Table 4.6), although *KRT80* initially held more scope for further investigation due to its affinity with type I keratins (Langbein et al., 2010). Other keratinisation associated genes were *DSP* and *PKP1*, which were also down-regulated in LH exposed tissues versus HH counterparts (Table 4.11). *DSP* was suggested to be in the same pathway as *junction plakoglobin* (*JUP*) (Warde-Farley et al., 2010). However, *JUP* expression was unaffected by diet (Appendix 14). *Desmoplakin* (*DSP*) encodes the cell-membrane localised protein Desmoplakin, which is involved in desmosome formation between cells (Sobolik-Delmaire et al., 2006).

Desmosomes are a form of intercellular junction and densely localised on epidermal cell surfaces, particularly during keratinisation (Gumbiner, 1996). As *DSP* was down-regulated in LH exposed mammary tissues compared to HH exposed mammary tissues, this may suggest that desmosome formation

may be impaired in mammary fat pads exposed to LH, compared with HH diet. Desmosome formation is dependent on the presence of an adherens junction between cells (Sobolik-Delmaire et al., 2006). *DSP* and *JUP* are integral to desmosome formation, while either of the plakophilin family, *PKP1*, *PKP2* or *PKP3* may be present (Neuber et al., 2010). This suggests that *PKP1* may not be crucial to desmosome formation as alternative proteins exist. However, desmosome function may differ with varying PKP protein, and or availability may differ depending on tissue. *DSP* and *PKP1* were down-regulated by low protein during periconception versus high protein counterparts. *JUP* has a role in gene regulation (Dusek and Attardi, 2011), therefore its expression is potentially more tightly regulated than *DSP* or *PKP1*. The function of the papillary duct relies on tightly adherent cells (Paulrud, 2005). Therefore, any reduction in adherens or desmosome number or formation may impact papillary duct function. Any permanent change in adherens or desmosome formation may lead to future susceptibility to infection, such as mastitis. This would be detrimental to health and milk production (Seegers et al., 2003, Rollin et al., 2015, Guimarães et al., 2017).

The AP-2 family of transcription factors (*TFAP2*) are involved in stimulating cell proliferation and supressing differentiation during embryonic development (Eckert et al., 2005). *TFAP2A* expression was decreased 4.3-fold in LH compared with HH (Table 4.11). *TFAP2A* is involved in embryonic mammary development. *TFAP2A* and *TFPA2C* were expressed in basal cells and ducts of human fetal mammary tissue at 98 dpc (Friedrichs et al., 2007). This is comparable to 98 dpc in the present study. *TFAP2A* and *TFAP2C* are thought to control cell proliferation, apoptosis and differentiation in mammary epithelial development (Zhang et al., 2003). *TFAP2C* promoted normal development of the luminal epithelial cell type (Cyr et al., 2015). It is unclear whether down-regulation of *TFAP2C* led to physiological effects in the current study. However, previously a complete loss of *TFAP2C* reduced branching morphogenesis and ductal elongation, but myoepithelial differentiation was unaffected compared with controls (Jäger et al., 2010). In line with this, the number and activity of mammary epithelial cells has been previously correlated with milk production (Boutinaud and Guinard-Flament, 2004). Both *TFAP2A*, and *TFAP2C* were down-regulated in LH samples versus HH, as determined by RNA-Seq (Table 4.11). These genes are both thought to be involved in promoting branching morphogenesis

(Jäger et al., 2010). A morphological consequence potentially is reduced branching morphogenesis (Jäger et al., 2010). RT-qPCR validation of *TFAP2A* indicated that relative gene expression was 6.5–fold greater in LPost versus HPost exposed 98 dpc mammary tissues (Figure 4.4). In developing pubertal mice mammary glands, TFAP2C was localised in myoepithelial and luminal epithelial cells (Jäger et al., 2010). This suggests that a reduced expression of *TFAP2C* may lead to reduced terminal ductal unit (TDU) development in the bovine fetal mammary fat pad. Although it is uncertain whether a biological response exists and is linked to a 3.8-fold decrease in expression in LH compared to HH counterparts (Table 4.11). These studies suggest a biological importance of *TFAP2C*, and thus warrants future validation of *TFAP2C* expression in all four diet treatments via RT-qPCR. Further study may also involve investigation of *TFAP2C* in adult bovine lactating offspring. For instance, RT-qPCR analysis of gene expression in luminal epithelial cells of bovine lactating mammary glands, which can be extracted from the cytoplasmic crescent within milk fat globules (Lemay et al., 2013). Simultaneously, immunohistochemical localisation and Western Blot of protein in the bovine mammary gland in fetal, prepubertal, pregnant and lactating mammary glands may be valuable to determine whether the potential effects are permanent. The potential implication of *TFAP2A* and *TFAP2C* down-regulation, although unclear, may be future reduction in milk yield potential.

4.4.10 *E-cadherin* Down-regulation in LH versus HH Exposed Fetal Mammary Tissues

Another gene linked with branching morphogenesis is *E-cadherin* (*CDH1*) as it is vital for mammary epithelial cell remodelling, and subsequent fetal duct development (Yang et al., 2017). *E-cadherin* is also thought to be important for alveoli and tubule (ducts, secondary ducts) formation (O'Brien et al., 2002). *CDH1* was 3.4-fold down-regulated in mammary fat pads exposed to LH compared with HH (Table 4.14). This may indicate differences in the formation of complex epithelial structures, or level of development of alveoli and or tubules between LH and HH exposed fetal mammary tissues. In the adult bovine mammary gland, down-regulated genes with gene ontology terms related to adhesion were also reported during the onset of lactation (Gao et al., 2013). This may support evidence for epithelial remodelling in the fetal mammary gland. *CDH1* is co-expressed with *KLF5* (Warde-Farley et

al., 2010), which was 2.6-fold down-regulated in tissue exposed to low protein in the current study (Appendix 11). It is unclear whether *CDH1* expression affected desmosome formation, as *CDH1* RT-qPCR results of *CDH1* expression between maternal dietary interventions did not validate the RNA-seq data (Figure 4.3). However, analysis of peri- and post-conception diets displayed greater *CDH1* expression in mammary tissues exposed to LPeri and LPost, compared with HPeri and HPost counterparts respectively (Figure 4.4). LPost was not a diet investigated with RNA-seq (Chapter 4.2.2). Overall, based on the discussed literature for *CDH1*, results may suggest increased remodelling and structural changes in fetal mammary epithelial development. However, the cellular effects of maternal diet on ductal epithelial tissue does not support this (Appendix 1, Appendix 2). Alternatively, it may indicate an increased rate of remodelling at the 98 dpc time-point. This novel finding reveals an effect of postconception diet on 98 dpc mammary tissue development which would previously be unknown based on RNA-seq analysis alone. Previously in adult lactating dairy cows, four-times-daily milking altered the expression of 50 ECM and cell adhesion related genes, however *E-cadherin* was unaffected (Connor et al., 2008). Previous studies inferred that down-regulated expression of cell-adhesion genes indicated remodelling (Connor et al., 2008, Gao et al., 2013). Differential *E-cadherin* gene expression in the current study may indicate differential remodelling in the fetal mammary gland. Although further analysis of the tissue structure and appropriate markers is required to elucidate this.

4.4.11 *FGFR2* Down-regulation in LH versus HH Exposed Fetal Mammary Tissues

FGFR2 was identified as a candidate gene in the current study by RNA-Seq, based on its biological functions in the bovine mammary gland. In 98 dpc mammary tissues, *FGFR2* down-regulation in LH versus HH (Table 4.14) was validated via RT-qPCR in all samples (n=20) (Figure 4.3, E). Although statistical differences were initially observed between LH and HH in RNA-seq, in RT-qPCR, *FGFR2* relative expression in LL and LH samples was significantly lower than HL (Figure 4.3, E). Based on these results and previous evidence, exposure to LH and especially LL were detrimental in terms of molecular effects on *FGFR2*. Analysis of peri- and post-conception diets revealed down-regulated *FGFR2* expression in LPeri exposed mammary tissues compared

with HPeri, while *FGFR2* expression was unaffected by postconception. This is a novel result as LPost was not a diet investigated with RNA-seq (Chapter 4.2.2).

Previously, *FGF2* and *FGFBP2* expression was up-regulated in lactating dairy cows versus non-lactating cows, and were postulated to promote mammary cell growth and differentiation (Dai et al., 2018). *FGFR2* was previously not a DEG in lactating versus non-lactating dairy cows (Dai et al., 2018). It is possible that *FGF2* is not yet a major mechanism of fetal mammary growth. *FGFBP2* was not present in RNA-seq data of the current study. While *FGF2* was lowly expressed in all samples (13 FPKM in LH and HH, $p>0.05$). As *FGFR2* was down-regulated in LH exposed samples (Appendix 14), this may suggest reduced receptivity for *FGF2* in the future, if a permanent down-regulation of *FGFR2* exists. This was beyond the scope of the current study. Expression persisted to 98 dpc, suggesting postconception diet did not alter gene expression response to periconception low protein exposure. FGFR2 protein was immunohistochemically localised in stromal adipocytes (Figure 4.6, C, D). This compliments pre-existing knowledge that *FGF10* is expressed in adipocytes (Parsa et al., 2008). In summary, the implications of reduced *FGFR2* expression in bovine mammary tissue exposed to LH are vast. The link between milk production and epithelial cell number is well established, and evidence suggests that reduced *FGFR2* expression reduced bovine mammary epithelial proliferation (Jeong et al., 2017a). The findings of the current study build on previous bovine *in vitro* work and are novel findings adding to the effects of maternal diet on bovine fetal mammary development.

4.4.12 *TWIST1* Down-regulation in LH versus HH Exposed Fetal Mammary Tissues

The 1.10-fold downregulation of *TWIST1* in LH exposed 98 dpc bovine mammary tissues versus HH counterparts (Table 4.14) may indicate a differential rate of adipocyte differentiation in either white or brown adipocytes. There is evidence for *TWIST1* expression in white and brown adipose tissue (Pan et al., 2009, Qin et al., 2012). Additionally, *TWIST1* was highly expressed in human adipocytes (Pettersson et al., 2010). In human and mice, *TWIST1* expression was shown to increase during preadipocyte differentiation (Pettersson et al., 2010, Ma et al., 2014, Ren et al., 2016). A

possible implication of *TWIST1* down-regulation in LH exposed mammary tissues may be a reduced rate or number of preadipocytes undergoing adipocyte differentiation. *TWIST1* was previously reported to regulate inflammatory factors *tumour necrosis factor-α* (*TNFα)*, interleukin-6 (*IL-6)*, and *monocyte chemoattractant protein 1* expression (Pettersson et al., 2010). However, overall RT-qPCR did not validate the down-regulated gene expression of *TWIST1* between maternal dietary interventions, periconception, or postconception diets (Figure 4.3, Figure 4.4). Results of the current study do not support further investigation of *TWIST1* as a differentially expressed gene involved in fetal bovine mammary development. If *TWIST1* expression was restricted to adipocytes, this may explain the -1.1-fold-change originally identified via RNA-seq (Table 4.14), and highlights a requirement for further quantification of adipocytes in the fetal mammary gland.

4.4.13 Mammary Gland Immune Function

A number of down-regulated genes were related to immune function including *GPR37*, *BCL11B*, *BOLA*-DRA, and *BCL11A* (Table 4.11, Appendix 11). *GPR37* was the third most down-regulated gene in LH versus HH exposed mammary tissues (Table 4.11).

G protein-coupled receptor 37 (*GPR37*) is a signal transduction gene (Gao et al., 2013). G protein-coupled receptors (GPRs) transmit extracellular signals and trigger intracellular responses to the extracellular environment (Agrawal et al., 2017). In dairy cows, GPRs are of interest due to their potential role in the immune system and fatty acid metabolism (Agrawal et al., 2017). GPR expression is reportedly tissue specific, and specific GPRs were previously expressed in adipose tissue with a potential role in adipose inflammation (Agrawal et al., 2017). *GPR37* is associated with non-alcoholic fatty liver disease, and was down-regulated in adult dairy cows with severe negative energy balance (Fatima et al., 2014). This supports the finding that *GPR37* was 4.6-fold down-regulated in LH mammary glands exposed to maternal low protein during periconception (Table 4.11). The role of *GPR37* in the fetal mammary gland remains unclear, although previous research suggests a link between metabolism and adipose inflammation. Another gene related to immune function was *BCL11A*.

B cell CLL/lymphoma 11B, a paralog of *BCL11A*, encodes a zinc finger transcription factor (Wakabayashi et al., 2003, Li et al., 2010). *BCL11B* was associated with lymphocytes (Wakabayashi et al., 2003, Li et al., 2010, Cismasiu et al., 2006) and human mammary stem cells (Miller et al., 2018, Cai et al., 2017). Mouse *BCL11B* is required for T-cell commitment, is expressed in T-lymphocyte precursors and is then unique to differentiated T-cells amongst other hematopoietic cells (Li et al., 2010). *HLA-DRA*, or *BOLA-DRA* (*Bos taurus*) expression was increased in the LH mammary fat pad compared with HH counterparts (Appendix 11). Antigens of B cells *CD19*, *CD20* (*MS4A1*), and *CD22* were not significantly affected by diet. While *CD21* (*CR2*) were both expressed (Appendix 14). *BOLA-DRA* is the first antigen to be expressed in the early stage of development, whereas *CD20* expression occurs later (Loken et al., 1987). Results may indicate that B cells present in the 98 dpc mammary fat pad were in the early development stage.

The pattern of down-regulated genes is comparable to mouse gene expression profiles of mammary glands during involution (Clarkson et al., 2004). *CXCL14* was down-regulated in mammary fat pads exposed to LH diet compared with HH counterparts (Appendix 11). Human *CXCL14* has a role in inflammation, with an unknown receptor (Widdison and Coffey, 2011). *CXCL14* is a chemoattractant for lymphocytes, macrophages and monocytes (Clarkson et al., 2004). Overall, previous evidence suggests that down regulation of *CXCL14* may reduce the inflammatory response of the fetal mammary gland. However, multiple components are involved in the inflammatory response, therefore it is unclear whether a biological response will occur as a result of down-regulated *CXCL14* specifically.

4.4.14 *KRT80* Down-regulation in LH versus HH Exposed Fetal Mammary Tissues

Further to immune function, *KRT80* was identified as a gene of interest for its structural role in mammary ducts. *KRT80* gene expression was 2.5-fold down-regulated in mammary tissues exposed to LH compared with HH (Table 4.14). However, this was not supported by RT-qPCR results (Figure 4.3, Figure 4.4). *KRT80* is expressed in epithelial cells, therefore the 2.5 fold down-regulation of *KRT80* may be a result of a difference in cell number expressing the gene, which is also postulated (Nuutila et al., 2012). Alternatively, it is possible that a differential proportion of epidermal cells

were included between individual samples used for RNA extraction. Human *KRT80* and *KRT10* together form an intermediary filament located in luminal ducts and in the interfollicular epidermis (Langbein et al., 2010). *KRT10* also was a DEG in the current study (Table 4.11). As keratins are expressed by epithelial cells, it is possible that a differential cell number exists between mammary tissues. Alternatively, it is possible that cell number was unaffected by maternal diet while *KRT10* and *KRT80* gene expressions were altered. Furthermore, another possibility is that a combination of cellular and molecular effects may have occurred. As *KRT80* gene expression was not validated by RT-qPCR between maternal dietary interventions, no further investigation was performed (Figure 4.3, Figure 4.4). However, immunohistochemical analysis would elucidate which cells expressed KRT80 protein, and immunofluorescence may reveal co-localisation of KRT80 and KRT10. Immunohistochemical investigation of KRT80 and KRT10 is of interest in terms of defining their localisation within individual ductal structures, and overall in the fetal ductal network at 98 dpc. For example, whether protein localisation of KRT80 and KRT10 differ between the originally defined 'immature' ducts, and whether localisation is consistently observed in patent ducts. Currently, evidence in literature is lacking, therefore there is little evidence to support this future work.

4.4.15 *MYO16* Down-regulation in LH versus HH Exposed Fetal Mammary Tissues

Myosin 16 (*MYO16*) was 5.4-fold down-regulated in LH versus HH exposed 98 dpc bovine mammary glands (Table 4.11). *MYO16* was the most down-regulated gene (Table 4.11). *MYO16* is a more recently evolved myosin superfamily member compared to other family members (Thompson and Langford, 2002). *MYO16* is expressed in few organisms and cell types, and performs specific functions (Thompson and Langford, 2002). *MYO16* appeared around mammalian evolution (Thompson and Langford, 2002, Lee et al., 2017, Patel et al., 2001a). This may suggest a specialist function in the mammary gland, an organ unique to mammals. However, current knowledge is predominantly in human central nervous system and muscle (Thompson and Langford, 2002, Patel et al., 2001a, Kengyel et al., 2017, Basson et al., 2014). Cytoskeletal cell cycle regulation requires *MYO16*, and expression levels vary depending on stage of cell cycle (Cameron et al., 2013). Although *MYO16* function in the mammary gland is poorly

understood, it was not disregarded, being the most down-regulated gene by 5.4-fold-change (Table 4.11). In human patients, mesenchymal stem cells were isolated from abdominal and facial adipose tissue, *MYO16* mRNA was lowly and highly expressed, respectively (Wang et al., 2010). This may suggest *MYO16* expression in bovine mammary fat pad adipocytes. In beef calves, *MYO16* was reported to be expressed in DNA extracts from blood samples (Marley et al., 2018). Smooth muscle cells are present within the connective tissue of the adult bovine teat (Koyama et al., 2013). *MYO16* down-regulation in LH exposed mammary tissues may suggest altered cell number of molecular activity of adipocytes, or smooth muscle cells. Further research is required to identify the cellular expression and localisation of *MYO16*, and its role in the mammary gland.

4.4.16 *CCER2* Down-regulation in the LH Exposed 98 Days Postconception Mammary Tissues

Coiled-coil glutamate rich protein 2 (*CCER2)* was 3.6-fold down-regulated in LH exposed fetal bovine mammary tissues versus HH counterparts (Table 4.11). *CCER2* encodes a 226 amino acid sequence, which has an unknown biochemical function (Mukawa et al., 2017). *CCER2* protein has a C-terminus coiled-coil domain, and an N-terminal secretory signal peptide (Mukawa et al., 2017). *CCER2* was lowly expressed in a range of human tissues, including skin, skeletal muscle and thymus, though mammary tissue was not investigated (Fagerberg et al., 2013, Mukawa et al., 2017). It is possible that *CCER2* was expressed in the skin surrounding the mammary fat pad in the current study. However, further research is required to elucidate the possible function of *CCER2* in the developing mammary gland. Human *CCER2* neighbours *SIRT2* on chromosome 19, a gene involved in metabolic homeostasis (Zhang et al., 2017). Contrastingly, *Bos taurus CCER2* is located on chromosome 18 (locus 18:48891578-48894035). Further investigation of *CCER2* function by gene knock-down in cell culture may reveal why *CCER2* was down-regulated in tissues exposed to LH, compared to HH counterparts. Finally, genes known to be expressed in adipocytes were identified as differentially expressed genes (DEGs).

4.4.17 Adipocyte Related Genes

UCP2 was 1.3-fold up-regulated in LH versus HH ($p<0.01$; Appendix 14). However, there is no indication as to whether UCP2 protein expression differed. UCP2 immunohistochemistry indicated that UCP2 protein was present in 98 dpc mammary fat pad (Figure 3.13), although quantitative analysis between mammary tissues was not performed for UCP1 or UCP2. Further quantitative analysis such as RT-qPCR, is required to elucidate whether *UCP2* gene expression was differentially affected by maternal diet. Simultaneous investigation following the method of Hara and colleagues (2018) in adipocyte immunohistochemistry may of interest, as this study used anti-oestrogen receptor (ER)α to localise fetal adipocytes. These future studies combined may provide more robust evidence of characterised fetal adipocytes in the bovine fetal mammary gland. Further RT-qPCR of ERα would elucidate and validate mRNA expression in all fetal mammary samples further to evidence in RNA-sequenced tissues.

Another adipocyte related gene reported to be differentially expressed is *Adenomatosis polyposis coli down-regulated 1* (*APCDD1)* (-2.7-LogFC; Appendix 11*). APCDD1* is a Wnt inhibitor expressed in skin and fat, and positively regulates adipogenic differentiation (Yiew et al., 2017). The 2.7-fold decrease in *APCDD1* expression in LH mammary samples versus HH counterparts suggests an effect on adipogenesis (Appendix 11). However, GO terms of the down-regulated gene set did not indicate enrichment relating to adipogenesis (Appendix 12). Yiew and colleagues (2017) report that down-regulation of *APCDD1* may result in reduced adipogenic differentiation. The Wnt signalling pathway is one of the pathways involved in mesenchymal stem cell commitment to adipogenic differentiation (Moseti et al., 2016). Yiew and colleagues (2017) reported that *APCDD1* expression was greater in mature adipocytes compared with undifferentiated preadipocytes. This finding may suggest that, in the current study, mammary tissues exposed to low protein during periconception (LH) may have reduced or delayed adipogenic differentiation compared with high (HH) protein periconception counterparts. Further histological analysis is required to elucidate the cell number and size of adipogenic cells, and further understand the potential effects of differentially expressed genes relating to adipogenesis.

The final DEG related to adipocytes was *Krüppel-like factor 5 (KLF5)* (-2.6-LogFC; Appendix 11). *KLF5* was down-regulated in mammary tissues exposed to LH compared with HH (Appendix 11). *KLF5* is a transcription factor involved in regulation of adipogenesis (Oishi et al., 2005). In mouse (cell line 3T3-L1) *KLF5* is one of four (*KLF4*, *KLF5*, *KLF9*, and *KLF15*) *KLF* genes involved in regulation of adipogenesis, and activates PPARγ (Moseti et al., 2016). Adipocyte size was reduced in $KLF5^{+/-}$ mouse cell line compared to wild-type control, while cell number was unaffected (Oishi et al., 2005). Previous knowledge suggests reduced adipogenesis and cell size in mammary tissues exposed to LH versus HH counterparts. Further analysis of adipocyte cell size and number is required to elucidate a cellular response to *KLF5* expression levels.

4.5 Conclusions

In conclusion, low protein (9% CP) during periconception appeared to be detrimental to mammary development compared with high protein (14.6% CP) during periconception. Furthermore, a switch from low to high dietary protein at 23 dpc did not significantly restore expression to that of the expression seen in HH samples. Thus, indicating that maternal dietary intervention during periconception evoked potentially permanent changes in fetal gene expression into the first trimester of gestation. Further research is required to determine whether transcriptional differences in the fetus translate into morphological differences in the term, pre-pubertal, pubertal and lactating adult offspring.

The fetal mammary immune related genes were up-regulated when exposed to low maternal dietary protein during periconception (LH diet). Findings from IHC of IL33 confirmed protein localisation in the tissue, while there were no overall differences in protein localisation between dietary treatments. As the mammary gland is an exocrine gland, this may explain the transcriptional response of the gland compared with other organs which are not directly exposed to the environment.

Results suggest that low maternal dietary protein during periconception had negative consequences for mammary structural development, particularly in rudimentary duct development and cell-cell adhesion. The down-regulated genes indicate negative or reduced developmental responses, such as cell-

cell adhesion and tissue remodelling. This may be an adaptive or protective response, to reduce output of future milk production in a low-nutrition environment, and potentially reduce the metabolic demands on the adult female during lactation. However, if structural integrity is impaired, this may be detrimental to the structural ability of the mammary gland to protect against infection. This potential loss of structural barrier in the keratinised epithelium may be linked to the up-regulation of immune related genes.

This evidence supports further investigation of the role of candidate genes affected by maternal diet and their biological significance in the developing mammary gland. From an industry perspective, knowledge of the molecular effects of maternal dietary intervention in the developing mammary gland is of multifactorial importance. Permanent effects on fetal gene expression may influence future herd mammary health and production. Decision-making of nutritional and or reproductive management practices which influence mammary genes are of interest. This study supports further in-depth research into potential consequences of the molecular effects to the future adult mammary gland following fetal exposure to differing protein levels, which may impact the sustainability, efficiency and economics of beef production.

From a practical perspective, there are a number of points the current study would support. In cattle production where reproduction is controlled, the current study does not support artificial insemination during low quality pasture or mixed ration (7% CP) throughout the periconception and postconception periods. Additionally, increasing crude protein at postconception following periconceptional low protein is not sufficient to compensate for the initial maternal low protein, in terms of gene transcription in the mammary fat pad. Further research is required to identify whether 14% CP is optimal for fetal programming or an alternative level is more beneficial to fetal programming. Ultimately, a meta-analysis of the overall effects of maternal diet on multiple fetal organs and fetal-sex specific effects, with a greater sample size, and additional time-points in neonatal development, would further understanding and improve recommendation for best management practices in beef farming.

5 The Cellular Effects of Maternal Dietary Intervention on the 98 Days Postconception Bovine Placentome

5.1 Introduction

As described in Chapter 1.4, the bovine placentome is a complex structure of maternal and fetal tissue, essential to fetal growth. The structural composition of 98 dpc placentomes included in the current study was previously analysed by Edwards (2016). Relevant results are summarised for discussion and context of protein localisation. The proportion of uninucleate trophoblast cells (UTCs) and binucleate trophoblast cells (BNCs) was unaffected by dietary intervention (Edwards, 2016). The estimated weight of BNCs was around 0.4 and 0.6g greater in HH versus LL and HL respectively (Edwards, 2016). Collagen proportion was reduced in placentomes exposed to periconception high protein compared with low protein counterparts (Edwards, 2016). Further to cellular findings, RNA-sequencing analysis in the 98 pays postconception bovine placentome was previously investigated (Edwards, 2016).

Gene expression of *SerpinB1, GATM, STRA6, GJA1, FOXO4, and Notch1* were differentially expressed between maternal dietary interventions (Edwards, 2016). The genes encoding these proteins were differentially expressed in the 98 days postconception bovine placentome and met stringent selection criteria (Edwards, 2016). The aims of the current chapter were to 1) confirm localisation of each protein encoded by these genes via immunohistochemistry, and 2) to investigate the effect of maternal dietary intervention and/or fetal sex on protein localisation.

5.1.1 The Putative Roles of SerpinB1 in the Bovine Placentome

The serpin superfamily has many regulatory functions, including extracellular matrix remodelling, cell migration, proliferation, apoptosis, intracellular proteolysis, and inflammatory response modulation (Potempa et al., 1994, El Ouaamari et al., 2016, Arecco et al., 2016). Serpins are single stranded proteins and activation requires a structural change (Potempa et al., 1994). *Serine peptidase inhibitor, clade B, member 1 (SerpinB1*), also known as

leukocyte elastase inhibitor (LEI), is traditionally a neutrophil serine protease inhibitor (Farley et al., 2012, Heit et al., 2013, Law et al., 2006). The three target proteases of *SerpinB1* are neutrophil elastase, proteinase-3 (PR3) and cathepsin G (Gettins, 2002, Benarafa et al., 2011). The formation of a serpin-protease complex was confirmed by Huntington and colleagues (2000). Neutrophil serine proteases are stored within neutrophil granulocytes, specifically cytoplasmic azurophilic granules (Korkmaz et al., 2013). Serine proteases have multiple unique or shared targets, allowing numerous functions (Figure 5.1) (Heutinck et al., 2010a). In the placenta, serine proteases and matrix metalloprotease members were involved in embryo implantation in multiple species, however they were not known SerpinB1 targets (Salamonsen, 1999).

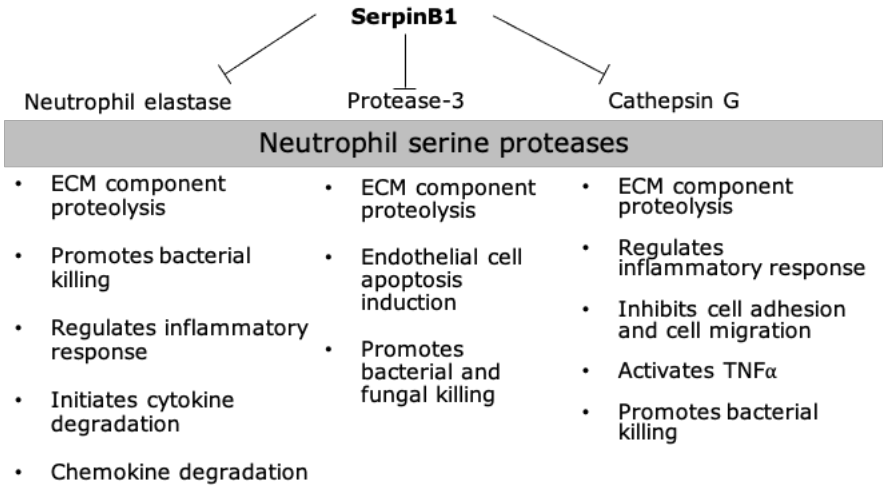


Figure 5.1 SerpinB1 - A serine protease inhibitor. Adapted from Heutinck and colleagues (2010a). Lines from SerpinB1 to neutrophil serine proteases indicate inhibition of the proteases by SerpinB1. Functions of each neutrophil serine protease are listed below respectively.

Neutrophil elastase degrades extracellular matrix (ECM) components such as fibrin and elastin (Figure 5.1) (Woessner, 1991, Bach-Gansmo et al., 1995). Elastin was a component of the placental ECM in first trimester and term human placentomes (Chen and Aplin, 2003). The established functions of neutrophil serine protease, and neutrophil cell characteristics are of interest in the placenta. The fetal cotyledons are considered semi-allogeneic and are therefore susceptible to maternal immunological attack, similar to pathogen killing by neutrophil serine proteases (Trowsdale and Betz, 2006, Benarafa et al., 2011, Reeves et al., 2002). However, this is not the case. Maternal immune tolerance to the fetus and placenta occurs (Trowsdale and

Betz, 2006, Petty et al., 2006, Aluvihare et al., 2004). There are several known mechanisms for protection against maternal immune attack in mammals (Xu et al., 2000, Rouas-Freiss et al., 1997, Makrigiannakis et al., 2001, Aluvihare et al., 2004). Arguably the most aggressive aspect of bovine placentation is BNC fusion to the maternal endometrial epithelium (Wooding and Wathes, 1980, Wooding et al., 1986). Although ECM component degradation is required for cell migration, it must be regulated (Chen and Aplin, 2003). As previously described (Chapter 1.4), BNCs migrate towards the epithelium, and this is thought to be in the order: stromal, stromal villi, stromal epithelial.

SerpinB1 may have a novel function of regulating proteases at implantation and gestation (Salamonsen, 1999). To date, *SerpinB1* expression is yet to be reported in 98 dpc bovine placentomes. In cattle, Riding and colleagues (2008) reported SerpinB1 protein expression in bovine conceptus allantoic fluid, but not fetal tissue or amniotic fluid, which were also sampled, at 45 dpc. *SerpinB1* was also expressed in bovine follicles, adult uterus, and sheep (Hayashi et al., 2011, Subramaniam et al., 2010, Ledgard et al., 2015, Wang et al., 2016). As described above, SerpinB1 has many roles in various tissues and cell-types, some of which are controversial.

One hypothesis for the role of *SerpinB1* in the current study is spatio-temporal downregulation to allow for tissue remodelling and BNC migration. Down-regulation of *SerpinB1* would reduce inhibition of target proteases, such as neutrophil elastase (Figure 5.1). This would allow degradation of elastin in the ECM and consequently enable cell migration. However, regulation would also involve up-regulation of *SerpinB1* following cell migration and remodelling.

Exposure to protein restriction during gestation may lead to placental remodelling for compensatory growth of the placenta. Therefore, at 98 dpc, individual cell protein expression may differ depending on cell activity. The effect of maternal dietary intervention on BNC migration is unknown. Furthermore, SerpinB1 protein expression in endothelial cells was reported in humans, and was up-regulated in virally infected cells (Carroll et al., 2004). The role of SerpinB1 in bovine placental vasculature is unknown, although it may be involved in immune response, and tissue remodelling.

The effect of maternal dietary intervention on SerpinB1 protein expression and localisation in the 98 dpc bovine placentome is unknown.

5.1.2 An Overview of GATM in the Placenta

The current study also investigated *GATM* localisation in the bovine placenta. Previously, *GATM* was down-regulated in FLH (female LH) versus FHL (female HL) bovine 98 dpc placentomes (Edwards, 2016). The mitochondrial enzyme L-arginine: glycine amidinotransferase (AGAT) is encoded by the *GATM* gene (Humm et al., 1997, Miyamoto et al., 2005, Sandell et al., 2003). L-arginine: glycine amidinotransferase catalyses the first and rate-limiting step in creatine synthesis (Miyamoto et al., 2005, Sandell et al., 2003). Creatine synthesis is a two-step pathway, requiring the two enzymes GATM and guanidinoacetate methyltransferase (GAMT). GATM catalyses the production of guanidinoacetic acid and ornithine from amino acids arginine and glycine (Humm et al., 1997, Ellery et al., 2017). Creatine synthesis occurs mainly in the pancreas, liver and kidneys, and is transported via the circulatory system to organs by creatine transporters (Guimbal and Kilimann, 1993, Sora et al., 1994). Intracellularly, creatine is involved in energy homeostasis, especially in cells with changing energy requirements (Ireland et al., 2009). *GATM* is known to be maternally imprinted in the mouse placenta (Sandell et al., 2003, Miyamoto et al., 2005), although *GATM* is not a known bovine imprinted gene.

Placental tissue remodelling occurs throughout gestation (Cartwright et al., 2010, Schlafer et al., 2000). For example, the ovine placenta undergoes remodelling at mid-gestation, to meet increasing fetal nutrient requirements (Heasman et al., 1999). In pregnant ewes, intravenous creatine supplementation at 122 to 131 dpc increased maternal blood creatine concentration but did not affect fetal blood creatine concentration, tissue or amniotic fluid concentrations (Baharom et al., 2017). This indicates that creatine is not transferred from maternal to fetal circulation, suggesting that the fetus relies on *de novo* synthesis via the two-step pathway. In cows, the level of dietary energy and protein and urinary creatine was analysed in cows at prepartum and postpartum (Vandehaar et al., 1999). This study found no differences in urinary creatine concentration between high (16.2% CP), medium (14.2% CP) or low (12.2% CP) dietary protein and energy at 2 weeks prepartum or postpartum (Vandehaar et al., 1999). This suggests that

CP between 12.2-16.2% did not affect maternal urinary creatine during peripartum. It is not known whether 7% CP affected maternal creatine.

In human term placentae, GATM protein was immunohistochemically localised in fetal endothelial and stromal cells (Ellery et al., 2017). This indicates that *de novo* creatine synthesis is possible in human placentae. As GATM was expressed in the 98 dpc bovine placentome (Edwards, 2016), this suggests capacity for creatine synthesis. Crude protein and energy supply differed between diets in the current study (Table 2.1). Therefore, differential expression of *GATM* between maternal diets suggests an adaptive mechanism, potentially to meet placental energy demands. This may relate to the findings by Vandehaar and colleagues (1999). In rats, GATM was immunohistochemically localised in discrete areas within the kidney, specifically tubular epithelial cells in the cortex (McGuire et al., 1986). This indicates that GATM is located in cells involved in ion transfer via active transport. This is also evidence for GATM protein expression in epithelial cells, which may include trophoblast cells, a specialised epithelial cell-type. The fetal villus in the process of remodelling, may require ATP and creatine phosphate, and therefore is likely to express GATM to support energy requirements.

Endogenous fetal and placental creatine production and subsequent GATM expression may change in response to maternal nutrient supply (McMinn et al., 2006). Particularly as arginine is the substrate of GATM, altering crude protein likely alters the amino acid content of maternal diet (Ellery et al., 2017). Creatine is an important energy source to the placenta, and dam, as the placenta and fetus have creatine requirements (Wyss and Kaddurah-Daouk, 2000). *GATM* expression was decreased in intrauterine growth restriction human placentae versus controls (McMinn et al., 2006). *GATM* was down-regulated in FHL (F, Female) versus FLH, MLL (M, Male), MLH, and MHL by -1.69, -2.61, -1.86, and -2.18 LogFC respectively (Edwards, 2016). This indicates that placental sex responded differently to maternal dietary intervention. It was therefore hypothesised that GATM protein localisation may vary in placentomes exposed to low maternal dietary protein to compensate and ensure adequate creatine supply to the placenta and potentially the fetus.

5.1.3 The Function of STRA6 in the Placenta

Vitamin A metabolites (e.g. retinoic acid, retinol) are required for vision, normal growth, and development (Harris et al., 2018, Nozière et al., 2006, Kawaguchi et al., 2007). In ruminant diets, β-carotene is a major carotenoid, and is a retinol precursor (Nozière et al., 2006). *Stimulated by retinoic acid 6* (*STRA6*) is a multi-transmembrane cell-surface receptor for plasma retinol-binding protein (RBP) (Kawaguchi et al., 2007, Kawaguchi et al., 2008). STRA6 contributes to retinol homeostasis through the influx and efflux depending on how retinol is bound (Kawaguchi et al., 2012, Isken et al., 2008). RBPs facilitate retinol transport from hepatic storage to target tissue via the circulation (Kawaguchi et al., 2012, Blaner, 2007). *STRA6* has a high affinity for RBP and retinol uptake into the cell (Wu et al., 2014, Kawaguchi et al., 2007, Kawaguchi et al., 2012). Once RBP is bound to *STRA6*, retinol bound RBP is removed by enzymes and transported into the cell cytoplasm by STRA6 (Kawaguchi et al., 2011). Fetal vitamin A supply is thought to originate from maternal blood and requirements are vital for proper organogenesis (Costabile et al., 2016). It would therefore be likely that the bovine placenta contained retinol transport machinery such as STRA6.

STRA6 expression enhanced retinoic acid uptake by soluble retinol-binding protein (RBP) which may explain the importance of *STRA6* expression in the placenta for efficient transfer (Kawaguchi et al., 2007, Blaner, 2007). In the ovine placenta, both maternal-fetal and fetal-maternal transport of retinol was demonstrated (Donoghue et al., 1982).

If fetal demands for retinol increase, *STRA6* expression and protein localisation may occur. Alternatively, if maternal availability of retinol is deficient, placental STRA6 expression and protein localisation may increase to facilitate transport to the fetus. It is therefore hypothesised that *STRA6* will be localised in the maternal blood vessels and fetal trophoblast cells of the bovine placenta.

5.1.4 The Roles of GJA1 in Fertility and the Placenta

Additionally, the current study also investigated *GJA1* localisation in the 98 dpc bovine placentome. *GJA1* is a 43 kDa gene encoding *gap junction protein, alpha 1 protein* (*GJA1*) (Kumar and Gilula, 1996). GJA1 protein is one of the most prevalent connexin proteins which form gap-junctions and

hemichannels in mammals (Elfgang et al., 1995, Cronier et al., 2002, Ho et al., 2013, Kerr et al., 2011, Smyth and Shaw, 2013, Kar et al., 2012). Connexins are ubiquitously expressed in mammals, although their study in the placenta is poorly documented (Kar et al., 2012). A hexamer of connexins constitutes one gap-junction termed a connexon (Al-Lamki et al., 1999, Fernandes et al., 2004). Gap junctions are specialised transmembrane channels connecting the cytoplasm of neighbouring cells (Goldberg et al., 2002). Gap-junctional connections are clusters of intercellular transmembrane channels that transfer small (<1000 Da) molecules and ions between adjacent cells via passive diffusion (Kumar and Gilula, 1996, Crespin et al., 2010). Molecules transported through gap junctions include ATP, glucose, and glutathione (Goldberg et al., 2002). Connexin proteins also form hemichannels, which do not connect adjacent cells but allow paracrine communication (Iyyathurai et al., 2013, Kar et al., 2012).

Expression of GJA1 protein forms gap-junctions, allowing communication of molecules between cells, which are required for cell activity such as proliferation and differentiation (Cronier et al., 2002, Moorby and Patel, 2001). Conversely, GJA1 down-regulation resulted in reduced gap-junction formation and subsequent suppression of cell proliferation and differentiation (Cronier et al., 2002, Moorby and Patel, 2001). In humans and mice, GJA1 has been identified in many tissue types, the third highest protein expression being in the placenta (Churko and Laird, 2013, Cronier et al., 2002, Errede et al., 2002, Guo et al., 1992, Kibschull et al., 2004). GJA1 expression in reproductive tissues was subject to hormonal regulation (Salilew-Wondim et al., 2010, Bernal, 2007, Bauersachs et al., 2005, Dong et al., 2009). Although it was not possible to comment on serum hormones levels in heifers in the current study, this may have been a factor involved in placental GJA1 expression.

Previously, connexins were present in preconception bovine endometria, and expression varied throughout the oestrus cycle, as it is up- and down-regulated by oestradiol and progesterone respectively (Salilew-Wondim et al., 2010, Bernal, 2007). GJA1 was up-regulated in the bovine endometrium at oestrus (Bauersachs et al., 2005). In mouse maternal stromal cells *GJA1* was up-regulated in oestrogen treated mice (Laws et al., 2008). Grummer and colleagues (2004) reported that during preimplantation in rats, oestrogen increased endometrial *GJA1* expression via oestrogen receptor.

Progesterone receptor decreased GJA1 expression (Dong et al., 2009). This agrees with in vivo expression of GJA1. At day 0 of oestrus, GJA1 expression was greater than day 12 (Bauersachs et al., 2005). Oestrogen is greater at day 0 and low at day 12, while progesterone levels are inverse. Salilew-Wondium and colleagues (2010) compared the endometrial transcripts of receptive and non-receptive bovine endometria at day 7 and 14 of the oestrus cycle. In both days, *GJA1* expression was lower in receptive versus non-receptive endometria, i.e. successful and non-successful pregnancy and calvings (Salilew-Wondim et al., 2010). Lower GJA1 expression may be explained by greater progesterone and or progesterone receptor in receptive endometria, thereby favouring reproductive success. GJA1 has previously been reported in bovine placentomes (Pfarrer et al., 2006).

GJA1 facilitated the establishment of a communication network for post-implantation development stages during pre-implantation development (Pfarrer et al., 2006). *GJA1* expression was greater at 220 dpc compared with 120 dpc (Pfarrer et al., 2006). *GJA1* expression was increased in the maternal stroma in response to trophoblast invasion (Pfarrer et al., 2006). In bovine 240 and 270 dpc placentomes, *GJA1* mRNA was present in caruncular connective tissue and to a lesser amount in the fetal mesenchyme (Pfarrer et al., 2006). This supports evidence for potential localisation in caruncles and cotyledons of 98 dpc placentomes.

Bovine retinal endothelial cells exposed to high glucose concentration (25mM vs. 5.5mM control) had 50% reduced *GJA1* expression levels, and 40% decreased gap-junctions, compared with controls (Fernandes et al., 2004). High glucose decreased *GJA1* half-life from 2.3 to 1.9 hours (Fernandes et al., 2004). Proteasome inhibitors (MG132 and lactacystin) prevented high glucose induced reduction in *GJA1* expression and increased GJA1 half-life, effectively reversing the degradation of *GJA1* triggered by high glucose (Fernandes et al., 2004). This demonstrated the ability of a nutrient to affect *GJA1* expression and gap-junction numbers, resulting in altered cell-cell communication, but also inhibitors exist to stabilise GJA1. Expression of *GJA1* was reported to vary temporally during pregnancy and was reduced before rat implantation (Grummer et al., 2004). Connexon uncoupling between cells was reported to be important in epithelial cell migration (Grummer et al., 2004). This may relate to trophoblast migration as trophoblasts are a specialised epithelial cell-type (Prossler et al., 2014).

As the placenta has been shown to overcompensate for maternal malnutrition, the current study hypothesises that maternal low protein may result in increased *GJA1* protein expression, to increase gap-junctional communication and support increased trophoblast cell migration in the placenta. Moreover, another protein of interest involved in transport is STRA6.

5.1.5 The Functions of FOXO4 in the Placenta

FOXO4 was also a protein of interest. *Forkhead box O (FOXO)* transcription factors modulate gene expression, such as genes involved in oxidative stress dependent apoptosis, DNA damage repair, glucose metabolism, cell cycle arrest and other cellular functions (Huang and Tindall, 2007). FOXO4 expression was enriched in the placenta compared with other tissues (Saben et al., 2014b). *FOXO4* expression was regulated post-transcriptionally (Ho et al., 2010).

In the human placenta, *FOXO4* was localised in chorionic trophoblasts and syncytiotrophoblasts (Lappas et al., 2010). Low levels of oxidative stress induce *FOXO4* activation (Essers et al., 2004). *FOXO4* activation was shown to be protective against inflammation and oxidative stress, as demonstrated in FOXO4-null mice exposed to oxidative stress, and human FOXO4-dependent transcription of antioxidant enzyme SOD2 gene (Araujo et al., 2011, Zhou et al., 2009). Saben and colleagues (2014b) reported that *FOXO4* could be a marker for oxidative stress. The current study of FOXO4 localisation may identify an association between maternal diet and oxidative stress in the placenta.

Human *in utero* growth-restricted placentas were shown by Takagi and colleagues (2004) to have higher expression levels for oxidative stress-related molecules compared to placentas of normal gestations. This suggests a hypothesis that low maternal dietary protein in the current study would cause *in utero* growth restriction, which would have increased expression of molecules related to oxidative stress. The current study hypothesised that low maternal dietary protein diets may alter FOXO4 localisation in the bovine placentome. FOXO4 was expressed in elongated bovine embryos (Betsha et al., 2013). The FOXO pathway but not specifically FOXO4, was targeted by

microRNAs up-regulated in plasma samples of gestating dairy heifers (Ioannidis and Donadeu, 2017).

5.1.6 Notch1 Signalling in the Placenta

Additionally, the current study aimed to investigate the localisation of *Notch1* in the bovine placentome. The *Notch1* gene encodes a 300 kDa single-pass type-I transmembrane receptor (Artavanis-Tsakonas et al., 1999). *Notch* signalling is an evolutionarily conserved signalling pathway which regulates many stages of development and is therefore essential for normal embryonic development (Ge and Stanley, 2010, Gridley, 2007, Kopan and Ilagan, 2009). Notch1 was therefore a candidate for further investigation in the current study. Notch signalling is intercellular, where both the Notch receptor and its ligands are transmembrane bound (Bray, 2006). Notch signalling (Figure 5.2) therefore requires adjacent cells to possess a ligand for signal transduction to occur, which restricts signalling to between neighbouring cells (Krebs et al., 2000, Bray, 2006).

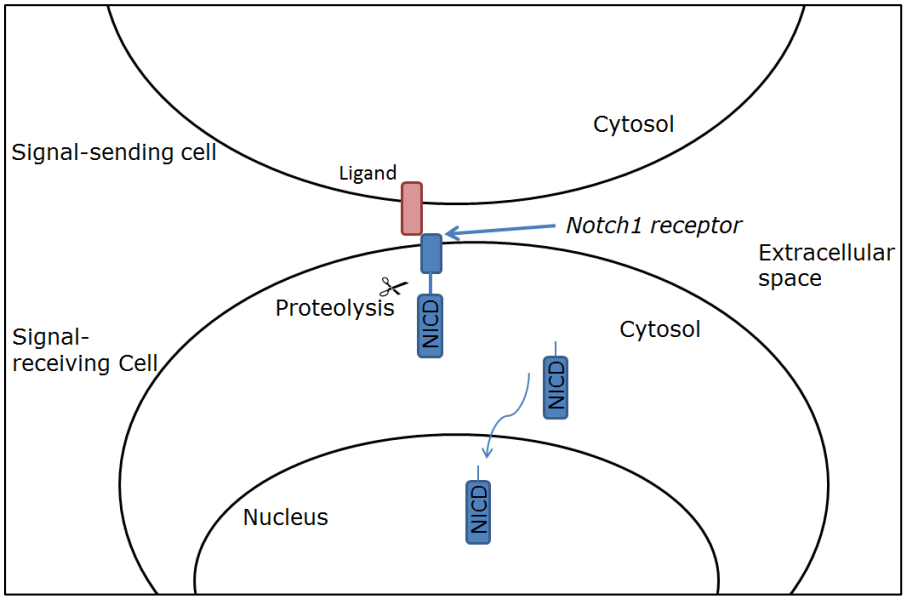


Figure 5.2 Notch1 receptor-ligand binding and proteolytic cleavage of the Notch intracellular domain (NICD) (Liu et al., 2017).

In mammals, there are four notch receptors (Notch1-4) and five Notch ligands: Jagged-1, -2, Delta-like-1, -3 and -4 (Gridley, 2007, Herr et al., 2011, Musse et al., 2012, Nickoloff et al., 2002, Roca and Adams, 2007, Shimanuki et al., 2014). The *Notch* intracellular domain (NICD) is bound to the cell membrane, and two proteolysis steps are required for NICD

activation, allowing translocation to the nucleus (Figure 5.2) and association with a DNA-binding protein (Iso et al., 2003, Katoh and Katoh, 2006, Kopan, 2002, Kopan and Ilagan, 2009, Musse et al., 2012, Bray, 2006). Once bound to a DNA binding protein, the NICD forms part of a transcription complex that activates *Notch*-dependent target genes (Iso et al., 2003, Katoh and Katoh, 2006, Kopan, 2002, Kopan and Ilagan, 2009, Musse et al., 2012). In humans and mice, notch signalling has been observed in various cell types, including epithelial cells, trophoblasts, and vascular endothelial cells (Ng et al., 2008, De Falco et al., 2007, Yu et al., 2014, Hellström et al., 2007).

In epithelial cells, *Notch* signalling influences growth and differentiation processes (Ng et al., 2008). In the bovine placentome, Notch1 may therefore play a role in growth and differentiation of maternal epithelial cells, and trophoblast cells. Contact between cells (Figure 5.2) is necessary for transmission of *Notch* signals, which can stimulate or suppress cell proliferation, activation of differentiation, or apoptosis (Kopan and Ilagan, 2009, Bray, 2006). Leptin-induced Notch signalling has been studied in human breast cancer (Battle et al., 2014, Saxena and Sharma, 2013). This indicates the ability of body fat reserves to influence Notch1 expression. This may relate to the current study and suggest a link between body condition and Notch1 expression during gestation, further supporting a genetic link between BCS and fertility in cows (Berry et al., 2003a).

The *Notch* family members play a role in vascular development during embryogenesis (Benedito and Hellström, 2013, Cao et al., 2009). *Notch1* expression decreased from the first to third trimester in human cytotrophoblast cytoplasm, but was maintained in syncytiotrophoblast (De Falco et al., 2007). In the human placenta Notch1 was expressed in epidermal keratinocytes (Ng et al., 2008). These findings suggest a temporal role of *Notch1* in epithelial differentiation, such as the trophoblast differentiation, which are specialised epithelial cells (Prossler et al., 2014). In humans, Notch1 was involved in cell fate determination and has varying roles in numerous cell types, including epithelial and endothelial cells (Baldi et al., 2004, De Falco et al., 2007, Gridley, 2007).

Notch1 inhibition decreased trophoblast migration and invasion (Yu et al., 2014). Therefore, placental *Notch1* expression may be important throughout

gestation, as trophoblast migration is continuous throughout gestation (Pfarrer et al., 2006). The current study hypothesises that *Notch1* is localised in both d98 and term placentomes, with expression decreasing in term versus 98 dpc placentomes. The current study hypothesises that placentomes from offspring subjected to a diet which causes decreased *Notch1* expression will have reduced areas of d98 fetal cotyledonary trophoblast migration into the caruncular epithelium, due to reduced trophoblast invasion and migration. Maternal malnutrition previously lead to compensatory placental growth (Lumey, 1998). Angiogenesis may also compensate for a restricted maternal diet, to increase surface area of placental vasculature (Perry et al., 1999). Human Notch1 receptor expression was enhanced by leptin in extra villus trophoblast *in vitro* (Wang et al., 2014a). Existing research into bovine Notch expression is limited, with the exception of beef cattle myogenesis, and bovine herpesvirus-1 (Sinani et al., 2012, Sinani and Jones, 2011, Workman et al., 2011, Liu et al., 2017). Notch1 was expressed in bovine blastocysts (Goossens et al., 2013). Notch1 was identified as a marker for rumen epidermal stem cells and progenitor cells (Yohe et al., 2016). Notch1 was stipulated to be involved in dairy cow mammary involution (Piantoni et al., 2010). Overall, Notch1 may have cell-type specific roles within the bovine placentome.

5.2 Methodology

5.2.1 Immunohistochemistry

Immunohistochemistry was performed for SerpinB1, GATM, STRA6, GJA1, FOXO4, and Notch1 proteins in 98 dpc tissues according to the general protocol (Chapter 2.3) with optimised modifications specific to each antibody (Table 5.1). Microscopy and qualitative image analysis were performed (Chapter 2.4). One slide (per sample) containing multiple tissue sections was used in each immunohistochemistry. n=3 samples per sex (Female (F) and Male (M)) and dietary intervention were analysed (FHH n=3, FHL n=3, FLH n=3, FLL n=3; MHH n=3, MHL n=3, MLH n=3, MLL n=3) equalling n=24 samples per target protein/antibody. Qualitative analysis was performed using approximately 40 fields per sample. Qualitative localisation was binary, defined as positive staining (+), or immunopositivity, while qualitative absence (-) was defined as no specific staining or immunopositivity present (Table 5.2).

Table 5 Commercial antibodies used in immunohistochemistry of 98 days postconception bovine placentome tissue sections

Anti-body	Catalogue number	Supplier	Details	Dilution	Optimised antigen-retrieval step
SerpinB1	ab181084	Abcam	Rabbit anti-Human Monoclonal	1:100	20 minutes medium-low
GATM	GTX114423	GeneTex	Rabbit anti-Human Polyclonal	1:100	1 minute high, 4 minutes medium-low
STRA6	SAB1401800-100ug	Sigma-Aldrich	Rabbit Anti-Human Polyclonal (full length protein)	1:100	20 minutes medium-low
GJA1	LS-A9453	LS Bio	Rabbit anti-Human Polyclonal (C-terminus)	1:10	20 minutes medium-low
FOXO4	LS-C12273	LS Bio	Rabbit anti-Human Polyclonal (aa108-157)	1:10	20 minutes medium-low
Notch1	ab52627	Abcam	Rabbit anti-Human Monoclonal	1:50	20 minutes medium-low

Abcam, Cambridge, England. GeneTex, San Antonio, Texas, USA. Sigma-Aldrich, St. Louis, Missouri, USA. LS Bio, Seattle, WA, USA.

5.2.2 Image Analysis of SerpinB1 Immunopositivity in BNCs

Quantitative analysis of BNCs irrespective of SerpinB1 staining was performed in Image-Pro and Microsoft Excel. Image-Pro was used to analyse each photomicrograph and provide cell IDs which were transferred to Microsoft excel, where each cell ID were classified as one of four cellular contexts, and positive or negative staining (Table 5.3). Twenty photomicrographs per sample (n=24), equalling a total of 480 photomicrographs), were analysed to achieve adequate observed BNCs. Photomicrographs were taken at 20x magnification, according to the general method for microscopy and sampling (Chapter 2.4). Pearson's chi-squared and Bonferroni post-hoc testing was performed.

5.2.3 Statistical Analysis

For quantitative data in Chapter 5.3.1, a Pearson's Chi-squared test was used followed by Bonferroni post-hoc analysis.

5.3 Results

This section presents immunohistochemical qualitative and quantitative (SerpinB1) results of proteins: SerpinB1, GATM, STRA6, GJA1, FOXO4 and Notch1 (Table 5.2).

Table 5.2 Protein localisation in the 98 days postconception bovine placentome

Localisation		Protein of interest					
		SerpinB1	GATM	STRA6	GJA1	FOXO4	Notch1
Cotyledonary	UTC nuclei	+	-	-	-	-	+
	UTC cytoplasm	-	-	+	+	+	+
	BNC nuclei	*	-	-	-	+	+
	BNC cytoplasm	+	-	+	+	-	+
	Stroma	-	+	+	+	+	+
	Vascular endothelial cell			+	+		+
	Villus	-	+	+	+	+	+
Caruncular	Epithelial cell nuclei	+	-	-	+	-	+
	Epithelial cell cytoplasm	+	+	+	+	-	+
	Stroma	+	+	-	-	-	+
	Vascular endothelial cell	+	-	+	+	+	+

Immunopositive localisation (+), and absence of immunopositivity (-) are displayed for each antibody. *Indicates further analysis of immunopositivity in this location in this antibody. UTC= unitrophoblast cells. BNC= binucleate trophoblast cells. Positive and negative localisation were determined by comparison with a negative control sample for tissue age for each protein of interest.

5.3.1 SerpinB1 Localisation in the 98 Days Postconception Bovine Placentome

In caruncular tissue, SerpinB1 caruncular stromal expression was seen in stroma proximal but not distal to epithelial cells. Overall, in caruncular stroma, SerpinB1 immunopositivity was observed within connective tissue, and was rarely nuclear (Figure 5.3). The majority of epithelial cells did not express SerpinB1. Fetal and maternal vasculature was consistently SerpinB1-immunopositive in 100% of samples (Figure 5.3). Positive staining was observed in all samples and when using a binary scale for staining as described (Chapter 5.2.1), no differences in localisation of SerpinB1 protein were seen between diet groups or sex of fetus (Figure 5.3).

In cotyledonary tissue, BNCs were observed in the fetal villus in a number of samples, and SerpinB1 positive staining was qualitatively observed as ~50%. This BNC location was categorised as 'fetal villus' (Table 5.3). Overall, cotyledonary stroma or connective tissue was absent of SerpinB1 expression. Fetal villi were observed to express SerpinB1 (Figure 5.4). Blood vessels were observed within fetal villi. Vascular endothelial cells expressed SerpinB1 consistently (Figure 5.4). Furthermore, connective tissue within the fetal villi presented SerpinB1 expression differentially, two samples are presented where there is little or no stain in villus connective tissue (Figure 5.4 A B) in one sample, whereas specific staining is present in another sample (Figure 5.4 C D).

A proportion of BNCs expressed SerpinB1 protein. The current study defined four discrete cellular contexts to sufficiently describe SerpinB1 localisation in the binucleate cell (Table 5.3). Initial descriptive analysis identified a discrete pattern of staining dependant on cellular localisation (Table 5.3). Quantitative analysis of binucleate cells was performed (Table 5.4) to determine statistical significance of the initial observation (Table 5.3). Firstly, the percentage of BNCs within each cellular context were analysed independent of SerpinB1 immunopositivity (Table 5.5)

The cellular context of BNCs within the feto-maternal junction, and fetal villus differed between treatment groups and fetal sex (Table 5.5). In stromal localised BNCs, FLL had the greatest (28.0%) percentage of BNCs compared to all other groups (14.67-20.8%, $p<0.05$; Table 5.5). In stromal villi localised BNCs, FHH had a 4.19% greater percentage of BNCs compared with MLL (Table 5.5; $p<0.05$) but a lower percentage compared to FHL, FLH, MHH, MHL and MLH (52.14-60.13%, $p<0.05$; Table 5.5). The percentage of stromal villi localised BNCs was greater in FHL (60.13%) compared to FHH, FLL, MHH, MHL and MLL (37.59-54.27%, $p<0.05$; Table 5.5). In stromal epithelial localised BNCs, FHH had the greatest (37.63%) percentage of cells compared to all other groups (18.19-25.81%, $p<0.05$; Table 5.5). The percentage of BNCs located in the fetal villus, was greater in MLL (28.29%) compared to all other groups (0.25-10.11%, $p<0.05$; Table 5.5). Following this analysis, SerpinB1 immunopositivity was quantified and the effects of maternal dietary intervention and sex were investigated (Table 5.6).

The percentage of SerpinB1-negative stromal localised BNCs was greater in FLL (24.43%) compared with all other sex-diet groups (12.56-20.10%, $p<0.05$; Table 5.6). SerpinB1 immunopositivity was absent in 12.56% of MLL, which was significantly lower than all other sex-diet groups (15.74-24.43%), except FLH (13.99%) ($p<0.05$; Table 5.6). In the stromal villi cellular context, the percentage of SerpinB1-negative BNCs was greater in FLH (41.69%) compared to all other sex-diet groups (16.94-37.61%, $p<0.05$; Table 5.6). In the stromal villi cellular context, MLL had the lowest percentage (16.94%) compared to all other diet groups (24.19-41.69%, $p<0.05$; Table 5.6). In stromal epithelial localised BNCs, the percentage of SerpinB1-negative BNCs was greater in MLL (3.77%) compared to all other diets (0.13-0.78%, $p<0.05$; Table 5.6). In the fetal villus, the percentage of SerpinB1-negative BNCs was greater in MLL (14.69%) compared with all other diet groups (0.07-4.37%, $p<0.05$; Table 5.6).

In positively stained BNCs, the percentage of stromal localised BNCs was greater in MLL (3.37%) compared to FHH, FHL, FLH, MHL and MLH (0.68-1.51%, $p<0.05$; Table 5.6). In stromal villi localised BNCs, the percentage of positively stained cells in FHH (16.95%) and FLH (17.18%) were lower compared with FHL, FLL, MHH, MHL, MLH and MLL (20.65-28.78%, $p<0.05$; Table 5.6). The percentage of immunopositive BNCs in the stromal villi was greater in MHH (27.95%) and MLH (28.78%), than FHH, FHL, FLH, FLL, MHL and MLL (16.95-23.41%, $p<0.05$; Table 5.6). The percentage of positively stained stromal epithelial localised BNCs was greater in FHH (36.84%) compared to all other groups (14.42-25.48%, $p<0.05$; Table 5.6). MLL displayed the lowest percentage (14.42%) of SerpinB1 immunopositive BNCs in the stromal epithelial cellular context compared to all other groups (18.13-36.84%, $p<0.05$; Table 5.6). In SerpinB1 immunopositive BNCs located in the fetal villus, the percentage of cells was greater in MLL (13.61%) compared to all other groups (0.08-5.43%, $p<0.05$; Table 5.6).

Further to placentome maternal dietary intervention (Table 5.6), the effects of periconception (Table 5.7) and postconception (Table 5.8) diet on SerpinB1 localisation within the four discrete cellular contexts were investigated. In stromal BNCs, the percentage of cells lacking SerpinB1 immunopositivity was greater in FHPeri (19.63%) compared to MHPeri and MLPeri, which were 17.62% and 14.32% respectively ($p<0.05$; Table 5.7). The percentage of SerpinB1-negative stromal localised BNCs in was 5.31%,

4.69%, and 3.3% lower in MLPeri, compared with FHPeri, FLPeri and MHPeri respectively (p<0.05; Table 5.7). In stromal villi localised BNCs, the percentage of SerpinB1-negative cells was greater in FHPeri (32.41%) and FLPeri (33.55%) compared to MHperi (27.37%) and MLPeri (24.72%, p<0.05; Table 5.7). In the stromal villi, MLPeri exposed placentomes displayed a 2.65%, 7.69% and 8.83% lower percentage of SerpinB1-negative BNCs compared with MHPeri FHPeri, and FLPeri respectively (p<0.05; Table 5.7). In stromal epithelial localised BNCs, the percentage of SerpinB1-negative cells was 1.45%, 1.43% and 1.23% greater in MLPeri than MHPeri, FHPeri, and FLPeri respectively (p<0.05; Table 5.7).

In stromal localised BNCs, SerpinB1 immunopositivity (positive stain) was 1.19%, 0.95% and 1.46% lower in FHPeri than FLPeri, MHPeri, and ML respectively (p<0.05; Table 5.7). In stromal villi localised BNCs, SerpinB1 immunopositivity was 5.53% and 7.12% greater in MHPeri than FHPeri and FLPeri respectively (p<0.05; Table 5.7). In stromal epithelial localised BNCs, SerpinB1 immunopositivity was 5.31%, 4.12% and 9.32% greater in FHPeri than FLPeri, MHPeri, and MLPeri respectively (p<0.05; Table 5.7). In fetal villus localised BNCs, SerpinB1 immunopositivity was 1.93%, 2.85% and 7.03% lower in FHPeri than FLPeri, MHPeri, and MLPeri respectively (p<0.05; Table 5.7).

The effects of sex and postconception diet on SerpinB1 localisation within the feto-maternal junction and fetal villus were analysed (Table 5.8). In stromal localised BNCs, the percentage of BNCs absent of SerpinB1 stain was 5.95%, 5.81%, and 6.68% greater in FLPost than FHPost, MHPost, and MLPost respectively (Table 5.8; p<0.05). In SerpinB1-negative stromal villi localised BNCs, the percentage of BNCs in FHPost (34.44%) was 2.71%, 6.51%, and 10.76% greater than FLPost, MHPost and MLPost respectively (p<0.05; Table 5.8). In stromal epithelial localised BNCs, the percentage of SerpinB1-negative cells was 1.46%, 1.87%, and 1.86% greater in MLPost than FHPost, FLPost and MHPost respectively (p<0.05; Table 5.8). In fetal villus localised BNCs, the percentage of SerpinB1-negative cells was 4.92%, 5.82%, and 3.96% greater in MLPost (7.61%) compared to FHPost, FLPost, and MHPost respectively (p<0.05; Table 5.8).

In stromal localised BNCs, the percentage of cells which displayed Serpinb1 immunopositivity was 1.17%, 1.03% and 1.48% lower in FHPost (0.86%)

than FLPost, MHPost and MLPost respectively (Table 5.8; $p<0.05$). In stromal villi localised BNCs, the percentage of SerpinB1-immunopositive cells was 4.40%, 11.32%, and 4.90% lower in FHPost than FLPost, MHPost and MLPost respectively ($p<0.05$; Table 5.8). In stromal epithelial localised BNCs, the percentage of SerpinB1-positive cells was 6.76%, 8.55%, and 6.92% greater in FHPost compared to FLPost, MHPost and MLPost respectively ($p<0.05$; Table 5.8). In fetal villus localised BNCs, the percentage of SerpinB1-immunopositive BNCs was 5.64%, 6.39%, and 2.63% greater in MLPost compared to FHPost, FLPost and MHPost respectively ($p<0.05$; Table 5.8).

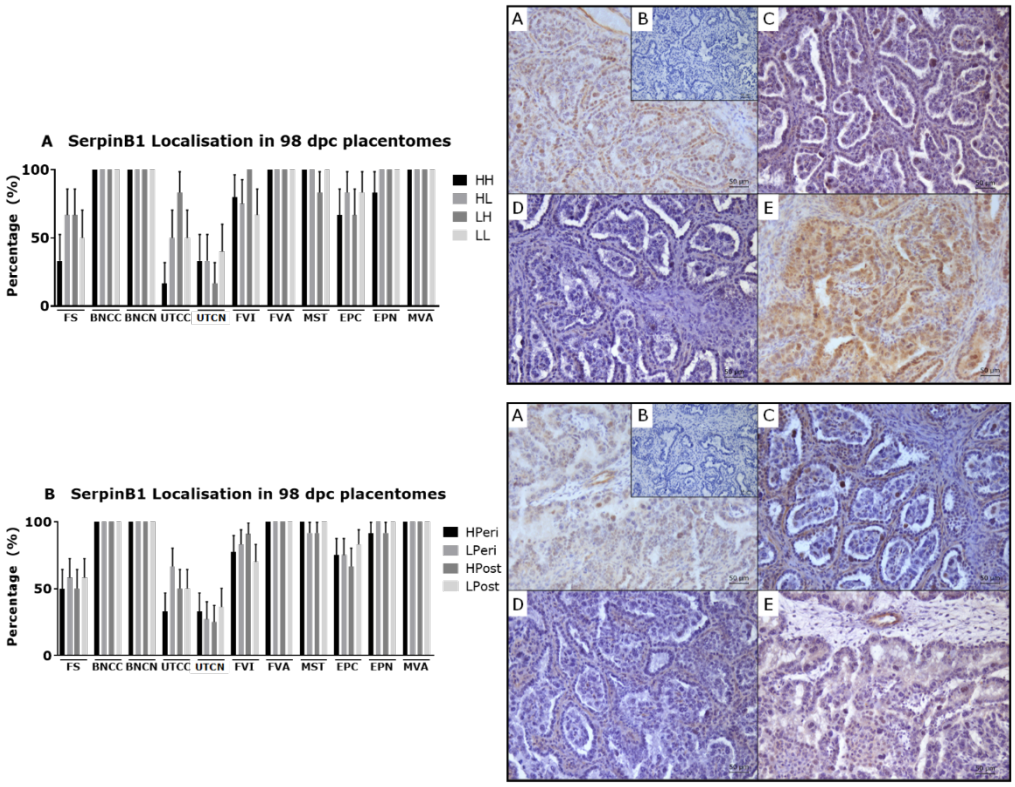


Figure 5.3 The percentage of samples displaying SerpinB1 immunopositivity and the cellular localisations in 98 days postconception placentomes. Graphs and complimentary photomicrographs (B=negative control, A, C-E represent each graph key, e.g. A=HH, E=LL) display results between maternal dietary interventions (A), periconception, and postconception diets (B). H=high protein, L=low protein. Letters denote periconception and postconception diet, e.g. HL= high protein periconception, low protein postconception. Peri=periconception, Post=postconception, FS=fetal stroma, BNC=binucleate cell, UTC=uninucleate trophoblast cell, FVI=fetal villus, FVA=fetal vasculature, MST=maternal stroma, EP=epithelial cell, MVA=maternal vasculature, C=cytoplasm, N=nucleus. Scale bars represent 50 µm.

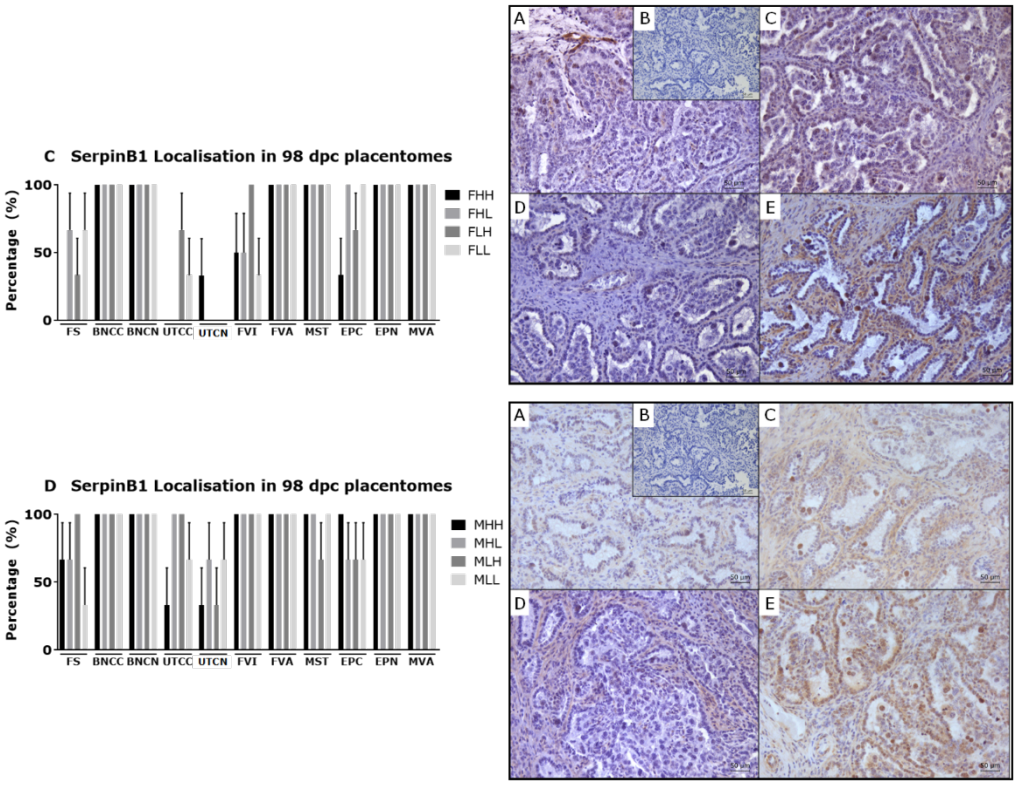


Figure 5.3 The percentage of samples displaying SerpinB1 immunopositivity and the cellular localisations in 98 days postconception placentomes continued. Graphs and complimentary photomicrographs (B=negative control, A, C-E represent each graph key, e.g. A=FHH, E=FLL) display results between female (C) and male (D) placentomes exposed to maternal dietary interventions. F=female, M=male, H=high protein, L=low protein. Letters denote periconception and postconception diet, e.g. HL= high protein periconception, low protein postconception. FS=fetal stroma, BNC=binucleate cell, UTC=uninucleate trophoblast cell, FVI=fetal villus, FVA=fetal vasculature, MST=maternal stroma, EP=epithelial cell, MVA=maternal vasculature, C=cytoplasm, N=nucleus. Scale bars represent 50 μm.

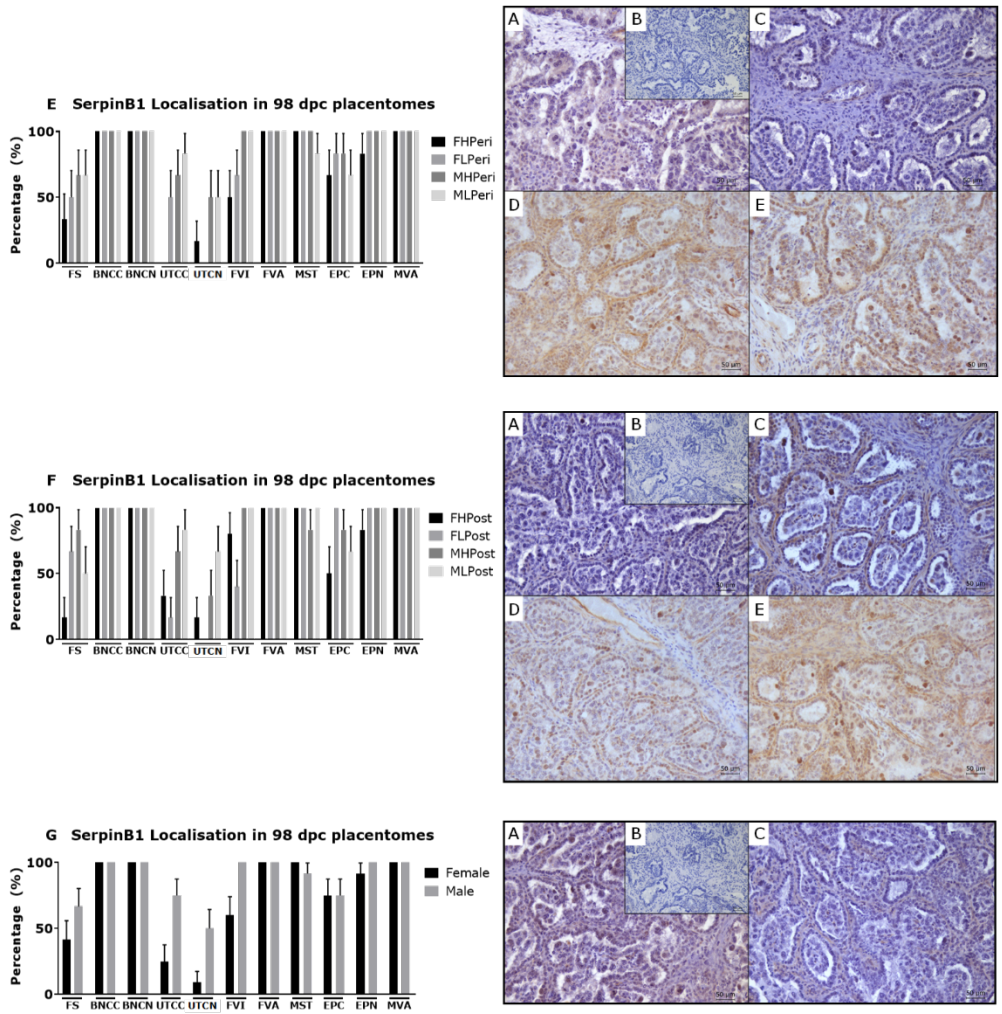


Figure 5.3 The percentage of samples displaying SerpinB1 immunopositivity and the cellular localisations in 98 days postconception placentomes continued. Graphs and complimentary photomicrographs (B=negative control, A, C-E represent each graph key, e.g. A=FHPeri, E=MLPeri) display results between female and male placentomes exposed to periconception (E) and postconception diets (F), and female and males (G) independent of dietary intervention. F=female, M=male, H=high protein, L=low protein, Peri=periconception, Post=postconception, FS=fetal stroma, BNC=binucleate cell, UTC=uninucleate trophoblast cell, FVI=fetal villus, FVA=fetal vasculature, MST=maternal stroma, EP=epithelial cell, MVA=maternal vasculature, C=cytoplasm, N=nucleus. Scale bars represent 50 μm.

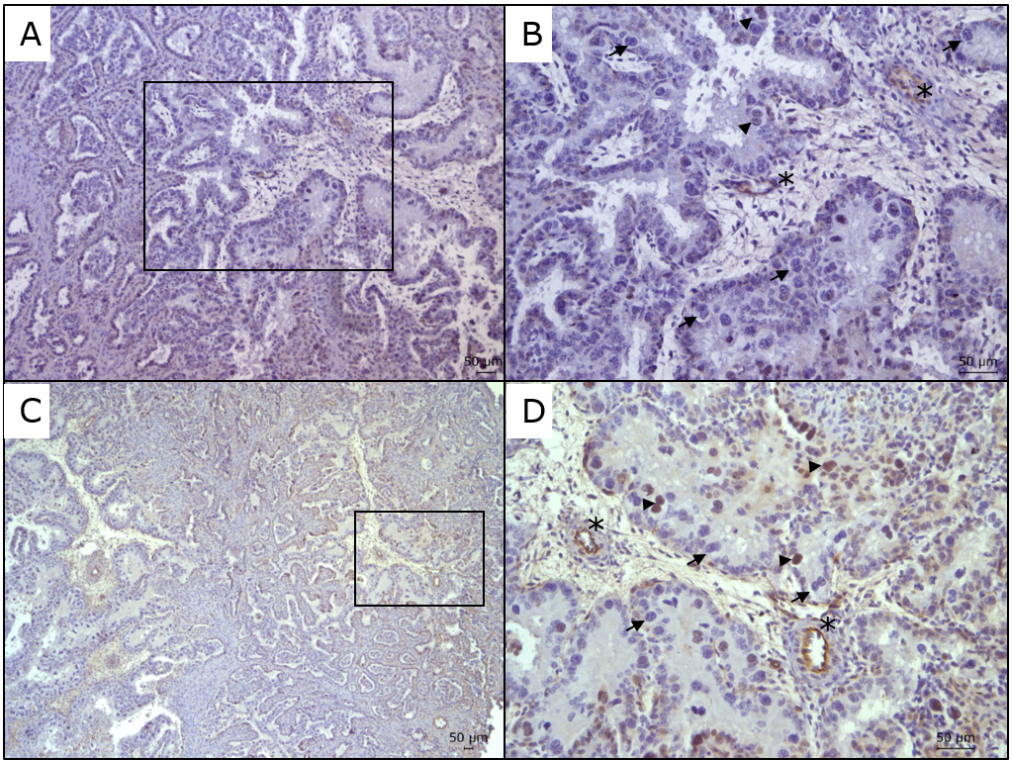


Figure 5.4 Immunohistochemical localisation of SerpinB1 in BNCs in villi of 98 days postconception placentomes. Photomicrographs display fetal villus tissue (A, C). Blood vessels (*) within the villus consistently expressed (brown) SerpinB1 in all samples (B, D). In this sample, the majority of trophoblast binucleate cells (BNCs) neighbouring the fetal villi did not express SerpinB1 (arrows) although expression is present in a minority of BNCs (arrowheads). Example of strong SerpinB1 immunopositivity in villus tissue (C). Example of a placentome with a greater proportion of SerpinB1 immunopositive BNCs neighbouring the fetal villus (arrowheads). Scale bars represent 50 µm.

Table 5.3 Descriptive analysis of trophoblast binucleate cellular context, and qualitative SerpinB1 staining percentage

Cellular context	Descriptive analysis	
	Location description	Stained (%)
Stromal	BNC surrounded by fetal stroma	~0
Stromal villi	BNC bordering fetal stroma and microvillar junction	~50
Stromal epithelial	BNC fusing with a maternal epithelial cell	~100
Fetal villus	BNC within the fetal villus, not within a feto-maternal junction	~50

BNC= trophoblast binucleate cell.
~=qualitative, estimated percentage of cells with SerpinB1-immunopositivity, following microscopic observation of n=24 samples based on cellular context.

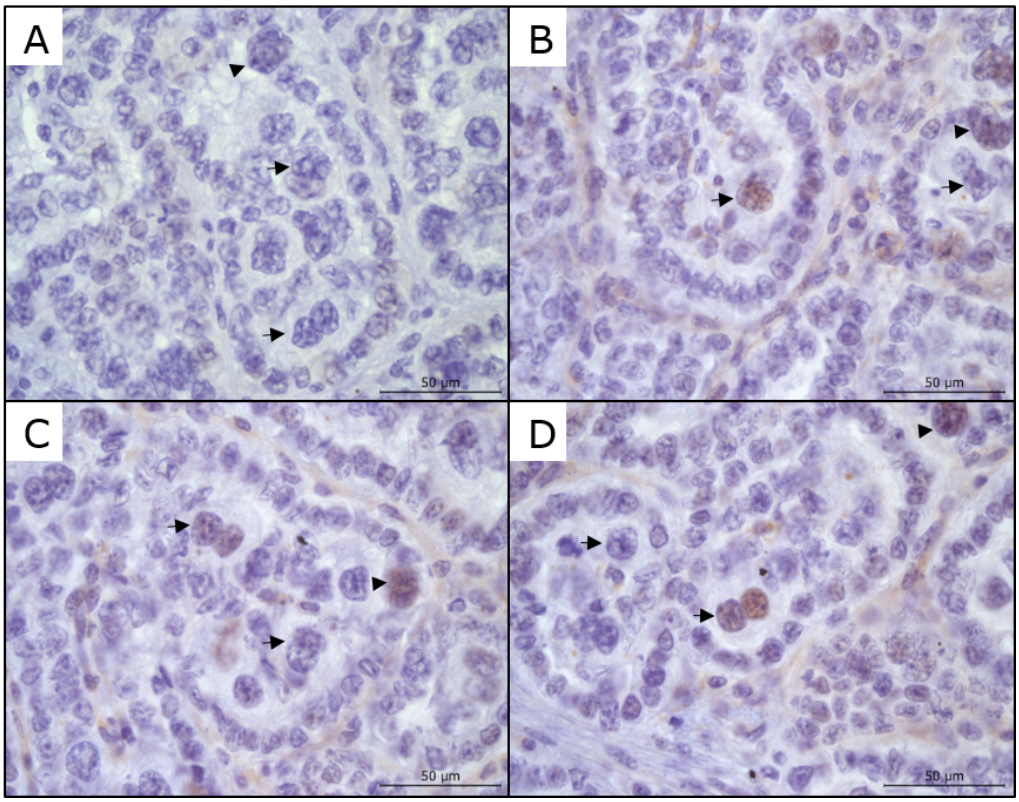


Figure 5.5 Immunohistochemical localisation of SerpinB1 in 98 days postconception placentome BNCs. Photomicrograph (A) is a negative control, showing the absence of staining in all structures and cell types. Photomicrographs (B-D) show examples of positive staining (brown) in different cellular contexts of trophoblast binucleate cells (BNCs), including fusing with a maternal epithelial cell (arrowheads). Stromal villi BNCs displayed variation in SerpinB1 expression (arrows). Scale bars represent 50 µm.

Table 5.4 The quantified percentage of SerpinB1 expression in BNCs according to cellular context

		SerpinB1 expression (%)	
Cellular context	Location description	Negative	Positive
Stromal	BNC surrounded by fetal tissue	90.8	9.2[a]
Stromal villi	BNC bordering fetal tissue	56.9	43.1[a]
Stromal epithelial	BNC fusing with a maternal epithelial cell	3.5	96.5[a]
Fetal villus	BNC within the fetal villus, not within a feto-maternal junction	55.4	44.6[a]
Expected %		51.70	48.30

Negative=no staining present. Positive=staining was present. Negative and positive stained percentages are significantly different to respective expected percentages ($p<0.001$). [a]Significant difference between negative and positively stained cells ($p<0.05$). Expected percentage (%) is the Pearson's chi-squared expected value for each cellular location if there were no significant differences between staining in each cellular context.

Table 5.5 The quantified percentage of BNCs in each cellular context, irrespective of SerpinB1 expression

	Sex and Dietary Intervention							
Cellular context	FHH	FHL	FLH	FLL	MHH	MHL	MLH	MLL
Stromal	$20.05^{b,c}$	20.8^{b}	14.67^{d}	28.01^{a}	$19.25^{b,c}$	$19.68^{b,c}$	$17.25^{c,d}$	15.93^{d}
Stromal villi	41.78^{c}	60.13^{a}	58.87^{a}	45.01^{c}	52.14^{b}	54.27^{b}	59.83^{a}	37.59^{d}
Stromal epithelial	37.63^{a}	18.51^{b}	$19.57^{b,c}$	22.14^{c}	18.49^{b}	25.81^{b}	18.29^{b}	18.19^{b}
Fetal villus	0.55^{e}	0.54^{e}	6.88^{c}	4.85^{d}	10.11^{b}	0.25^{e}	4.63^{d}	28.29^{a}

Pearson's chi-squared (p<0.001), Bonferroni (p<0.05). Superscripts that differ indicate significant difference between sex and dietary intervention (p<0.05).

Table 5.6 The quantified percentage of SerpinB1 immunopositive and immunonegative BNCs, according to cellular context, in sex and maternal dietary interventions

		Sex and Dietary Intervention							
SerpinB1 Stain	cellular context	FHH	FHL	FLH	FLL	MHH	MHL	MLH	MLL
Negative	Stromal	$18.95^{b,c}$	20.10^{b}	$13.99^{e,f}$	24.43^{a}	$16.89^{c,d}$	$18.42^{b,c,d}$	$15.74^{d,e}$	12.56^{f}
	Stromal villi	24.82^{d}	37.61^{b}	41.69^{a}	24.75^{d}	24.19^{d}	30.86^{c}	31.05^{c}	16.94^{e}
	Stromal epithelial	0.78^{b}	$0.32^{b,c}$	$0.53^{b,c}$	0.13^{c}	$0.33^{b,c}$	$0.32^{b,c}$	0.16^{c}	3.77^{a}
	Villus	0.47^{d}	0.46^{d}	4.37^{b}	$3.38^{b,c}$	4.68^{b}	0.07^{d}	2.79^{c}	14.69^{a}
Positive	Stromal	$1.10^{c,d}$	0.73^{d}	0.68^{d}	3.57^{a}	$2.36^{a,b}$	$1.25^{c,d}$	$1.51^{b,c}$	3.37^{a}
	Stromal villi	16.95^{c}	22.51^{b}	17.18^{c}	20.26^{b}	27.95^{a}	23.41^{b}	28.78^{a}	20.65^{b}
	Stromal epithelial	36.84^{a}	18.19^{d}	$19.04^{c,d}$	22.01^{c}	18.17^{d}	25.48^{b}	18.13^{d}	14.42^{e}
	Villus	0.08^{d}	0.08^{d}	2.51^{c}	1.47^{c}	5.43^{b}	0.18^{d}	1.83^{c}	13.61^{a}

Pearson's chi-squared ($p<0.001$), Bonferroni ($p<0.05$). Superscripts that differ indicate significant difference between sex and dietary interventions ($p<0.05$).

Table 5.7 The quantified percentage of SerpinB1 immunopositive and immunonegative BNCs, according to cellular context, in sex and periconception diets

SerpinB1 Stain	Cellular context	Periconception			
		Female		Male	
		High	Low	High	Low
Negative	Stromal	19.63[a]	19.01[a,b]	17.62[b]	14.32[c]
	Stromal villi	32.41[a]	33.55[a]	27.37[b]	24.72[c]
	Stromal epithelial	0.51[b]	0.34[b]	0.33[b]	1.78[a]
	Villus	0.46[d]	3.89[b]	2.48[c]	8.13[a]
Positive	Stromal	0.88[b]	2.07[a]	1.83[a]	2.34[a]
	Stromal villi	20.25[b]	18.66[b]	25.78[a]	25.13[a]
	Stromal epithelial	25.78[a]	20.47[b]	21.66[b]	16.46[c]
	Villus	0.08[d]	2.01[c]	2.93[b]	7.11[a]

Pearson's chi-squared (p<0.001), Bonferroni (p<0.05). Superscripts that differ indicate significant difference between sex and dietary interventions (p<0.05).

Table 5.8 The quantified percentage of SerpinB1 immunopositive and immunonegative BNCs, according to cellular context, in sex and postconception diets

SerpinB1 Stain	Cellular context	Postconception			
		Female		Male	
		High	Low	High	Low
Negative	Stromal	16.13[b]	22.08[a]	16.27[b]	15.40[b]
	Stromal villi	34.44[a]	31.73[b]	27.93[c]	23.68[d]
	Stromal epithelial	0.64[b]	0.23[c]	0.24[c]	2.10[a]
	Villus	2.69[c]	1.79[d]	3.65[b]	7.61[a]
Positive	Stromal	0.86[b]	2.03[a]	1.89[a]	2.34[a]
	Stromal villi	17.08[c]	21.48[b]	28.40[a]	21.98[b]
	Stromal epithelial	26.70[a]	19.94[b]	18.15[c]	19.78[b,c]
	Villus	1.46[d]	0.71[c]	3.47[b]	7.10[a]

Pearson's chi-squared (p<0.001), Bonferroni (p<0.05). Superscripts that differ indicate significant difference between sex and dietary interventions (p<0.05).

5.3.2 GATM Localisation in the 98 Days Postconception Bovine Placentome

In 98 days postconception placentomes, immunohistochemical localisation of GATM was limited to the fetal tissue and was consistently observed in fetal villi and to a lesser extent in fetal stroma within feto-maternal junctions (Figure 5.6). GATM expression was also cytoplasmic, in stroma and connective tissue, but was not nuclear localised (Figure 5.6). Variation in GATM-positive UTCs was observed (Figure 5.6). GATM was consistently expressed in UTCs in contact with the fetal villi (Figure 5.6). However, UTCs within the feto-maternal junctions did not express GATM (Figure 5.6). The BNC nuclei and cytoplasm did not positively express GATM protein versus negative controls (Figure 5.6). BNCs were seen clearly surrounded by stain in connective tissue of the fetal villus, meaning the BNCs were clearly absent of GATM immunopositivity (Figure 5.6). GATM-positive localisation was observed in fetal stroma within feto-maternal junctions proximal to fetal villus. Villus connective tissue or vasculature did not positively stain (Figure 5.6).

GATM localisation in the 98 dpc bovine placenta was present in the fetal villus, fetal stroma, and to a lesser extent in maternal epithelial cell cytoplasm and maternal stroma (Figure 5.6). The percentage of placentomes presenting GATM-positive staining in maternal epithelial cell cytoplasm varied. 50% of placentomes exposed to the LL diet were GATM-positive, compared to 17%, 0%, and 0% in HH, HL and LH diets respectively (Figure 5.6). No placentomes exposed to the HL diet presented GATM-positive localisation in maternal stroma, compared to 17% of samples in HH, LH and LL groups (Figure 5.6 A). 83% of placentomes exposed to HL or LH diets presented GATM-immunopositive localisation in the fetal stroma compared to 100% of samples in HH and LL groups (Figure 5.6 A). Overall, the majority of samples positively stained for GATM in the fetal stroma, however 67% of FLH and MHL samples positively stained compared to 100% in all other groups (Figure 5.6 C D). The greatest percentage of GATM-immunopositivity in the maternal epithelial cell cytoplasm were observed in FLL samples (Figure 5.6 C). Generally, positive staining was not observed in the maternal stroma, however 33% FHH, MLH and MLL samples positively stained for GATM (Figure 5.6 C D).

In 98 days postconception bovine placentome, GATM immunohistochemical localisation was similar between fetal sexes (Figure 5.6 G). Positive staining in the fetal stroma and fetal villus did not differ between female and male samples (Figure 5.6 G). Maternal epithelial cells generally did not stain positively for GATM, however 23% and 8% of female and male placentomes respectively were GATM-immunopositive (Figure 5.6 G). Similarly, the majority of female and male placentomes were not GATM-positive in the maternal stroma (Figure 5.6 G).

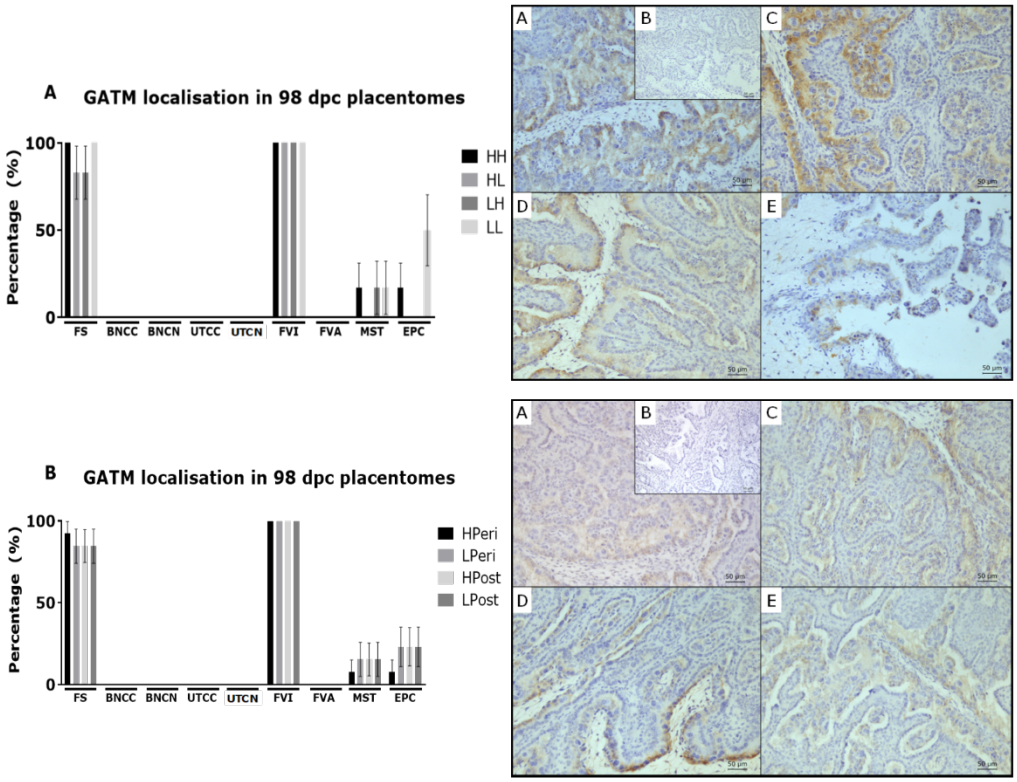


Figure 5.6 The percentage of samples displaying GATM immunopositivity and the cellular localisations in 98 days postconception placentomes. Graphs and complimentary photomicrographs (B=negative control, A, C-E represent each graph key, e.g. A=HH, E=LL) display results between maternal dietary interventions (A), periconception, and postconception diets (B). H=high protein, L=low protein. Letters denote periconception and postconception diet, e.g. HL= high protein periconception, low protein postconception. Peri=periconception, Post=postconception, FS=fetal stroma, BNC=binucleate cell, UTC=uninucleate trophoblast cell, FVI=fetal villus, FVA=fetal vasculature, MST=maternal stroma, EP=epithelial cell, MVA=maternal vasculature, C=cytoplasm, N=nucleus. Scale bars represent 50 µm.

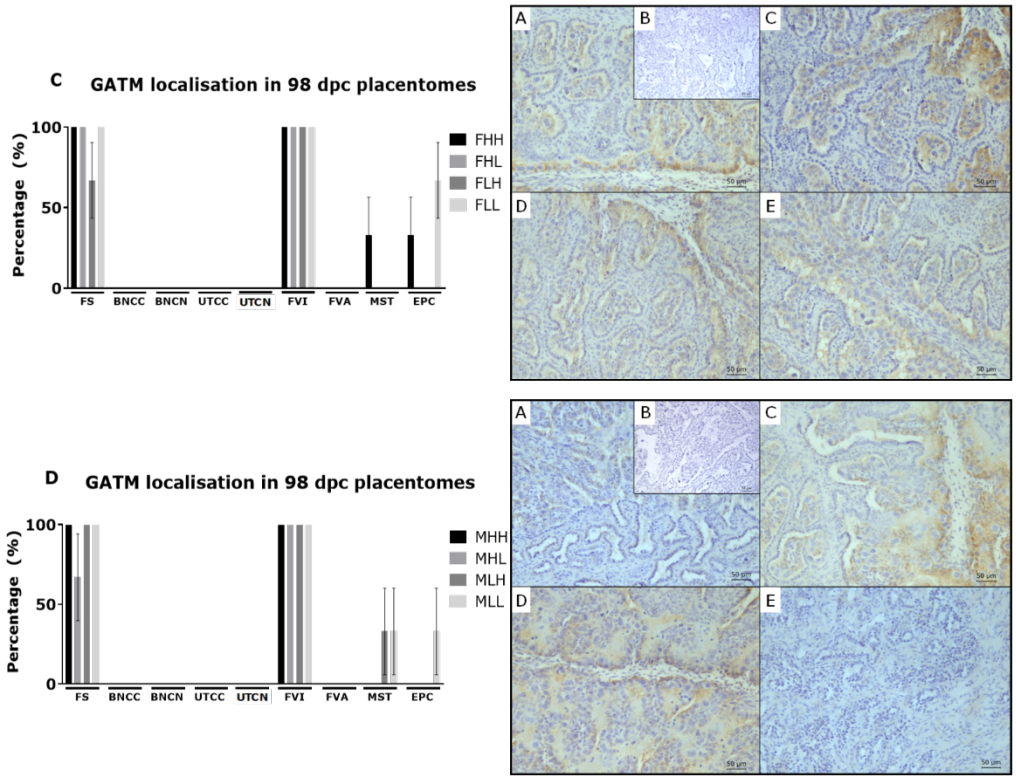


Figure 5.6 The percentage of samples displaying GATM immunopositivity and the cellular localisations in 98 days postconception placentomes continued. Graphs and complimentary photomicrographs (B=negative control, A, C-E represent each graph key, e.g. A=FHH, E=FLL) display results between female (C) and male (D) placentomes exposed to maternal dietary interventions. F=female, M=male, H=high protein, L=low protein. Letters denote periconception and postconception diet, e.g. HL= high protein periconception, low protein postconception. FS=fetal stroma, BNC=binucleate cell, UTC=uninucleate trophoblast cell, FVI=fetal villus, FVA=fetal vasculature, MST=maternal stroma, EP=epithelial cell, MVA=maternal vasculature, C=cytoplasm, N=nucleus. Scale bars represent 50 μm.

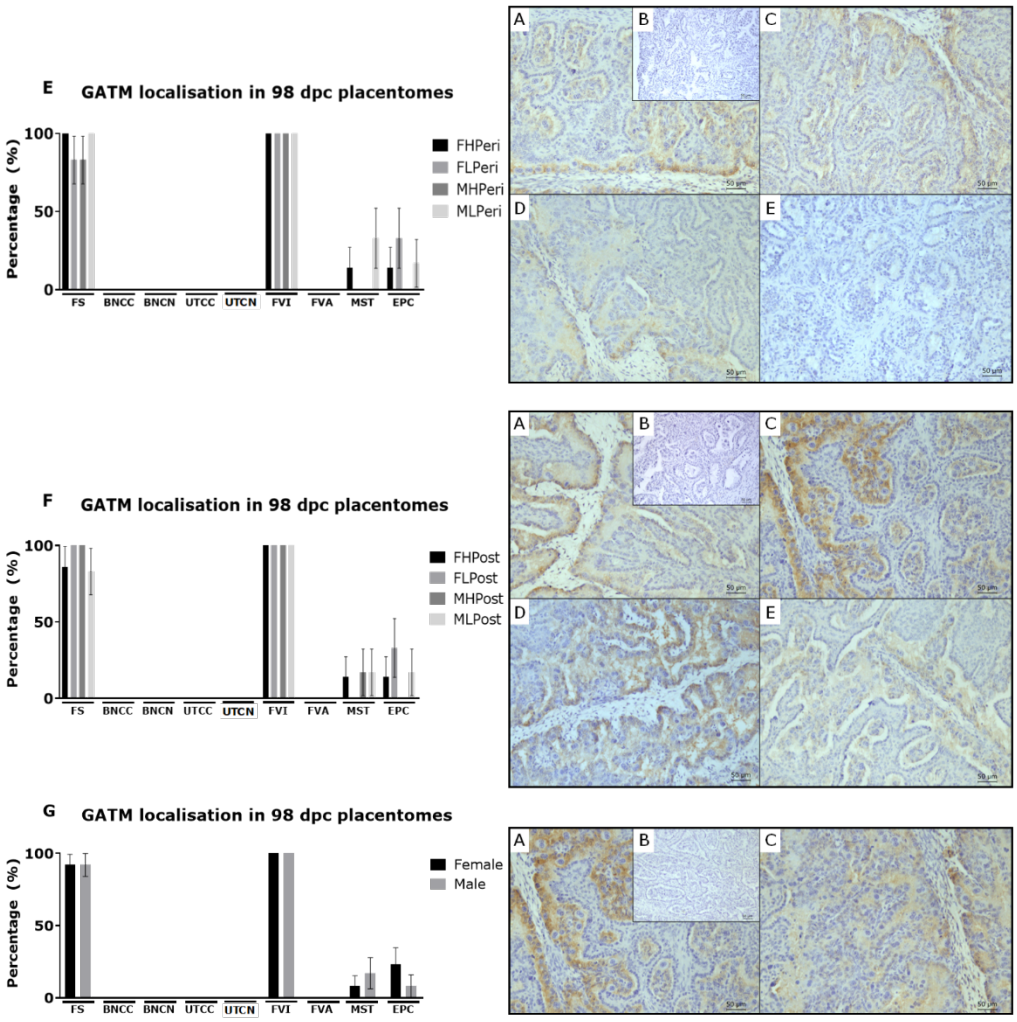


Figure 5.6 The percentage of samples displaying GATM immunopositivity and the cellular localisations in 98 days postconception placentomes continued. Graphs and complimentary photomicrographs (B=negative control, A, C-E represent each graph key, e.g. A=FHPeri, E=MLPeri) display results between female and male placentomes exposed to periconception (E) and postconception diets (F), and female and males (G) independent of dietary intervention. F=female, M=male, H=high protein, L=low protein, Peri=periconception, Post=postconception, FS=fetal stroma, BNC=binucleate cell, UTC=uninucleate trophoblast cell, FVI=fetal villus, FVA=fetal vasculature, MST=maternal stroma, EP=epithelial cell, MVA=maternal vasculature, C=cytoplasm, N=nucleus. Scale bars represent 50 μm.

5.3.3 STRA6 Localisation in the 98 Days Postconception Bovine Placentome

STRA6 immunohistochemical localisation was cytoplasmic and localised in fetal tissue and endometrial epithelium the 98 days postconception bovine placentome. Expression was present in fetal stroma and villi within feto-maternal junctions, epithelial cell cytoplasm, and vasculature. STRA6 was absent in BNC and UTC nuclei (Figure 5.7). Epithelial cytoplasmic immunopositivity was observed in 100% of HH, HL and LH samples, while 67% of MLL and FLL samples were STRA6-immunopositive (Figure 5.7 A C D). In maternal vasculature, FHL was absent of STRA6-immunopositivity, while the percentage of FHH samples staining was 33%, 67%, 50%, and 33% greater than FLH, FLL, MLH and MLL respectively (Figure 5.7 C D).

In certain samples, staining appeared to be localised to the BNC cell membrane at high magnification (63x), particularly the BNC cell membrane adjacent to the maternal endometrial epithelium. This was not widely observed, therefore this was not a parameter which was analysed throughout the sample population. The percentage of male and female samples with STRA6-immunopositivity in fetal villus did not differ (M 100% versus F 100% samples stained; Figure 5.7 G). The percentage of male samples which presented STRA6-immunopositivity in fetal stroma, maternal stromal cell cytoplasm, and maternal vasculature was 8%, 26%, and 10% greater than females, respectively (Figure 5.7 G). In UTC cytoplasm, and fetal vasculature, a 12%, and 19% greater percentage of female samples displayed STRA6-immunopositivity compared with males respectively (Figure 5.7 G).

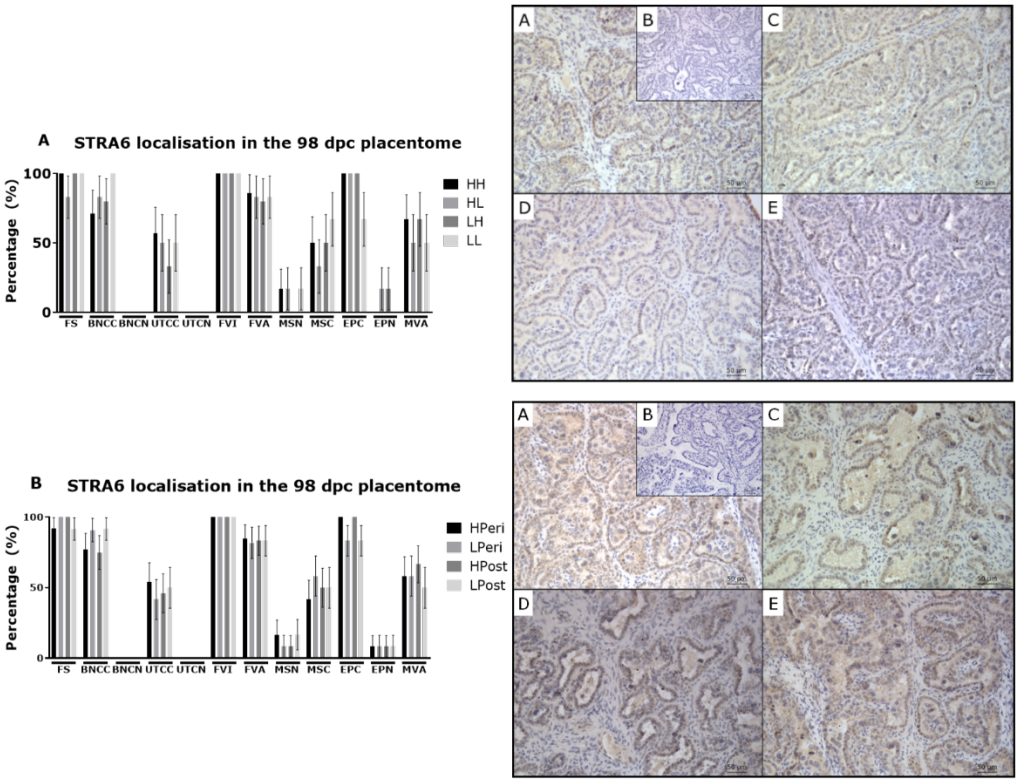


Figure 5.7 The percentage of samples displaying STRA6 immunopositivity and the cellular localisations in 98 days postconception placentomes. Graphs and complimentary photomicrographs (B=negative control, A, C-E represent each graph key, e.g. A=HH, E=LL) display results between maternal dietary interventions (A), periconception, and postconception diets (B). H=high protein, L=low protein. Letters denote periconception and postconception diet, e.g. HL= high protein periconception, low protein postconception. Peri=periconception, Post=postconception, FS=fetal stroma, BNC=binucleate cell, UTC=uninucleate trophoblast cell, FVI=fetal villus, FVA=fetal vasculature, MST=maternal stroma, EP=epithelial cell, MVA=maternal vasculature, C=cytoplasm, N=nucleus. Scale bars represent 50 µm.

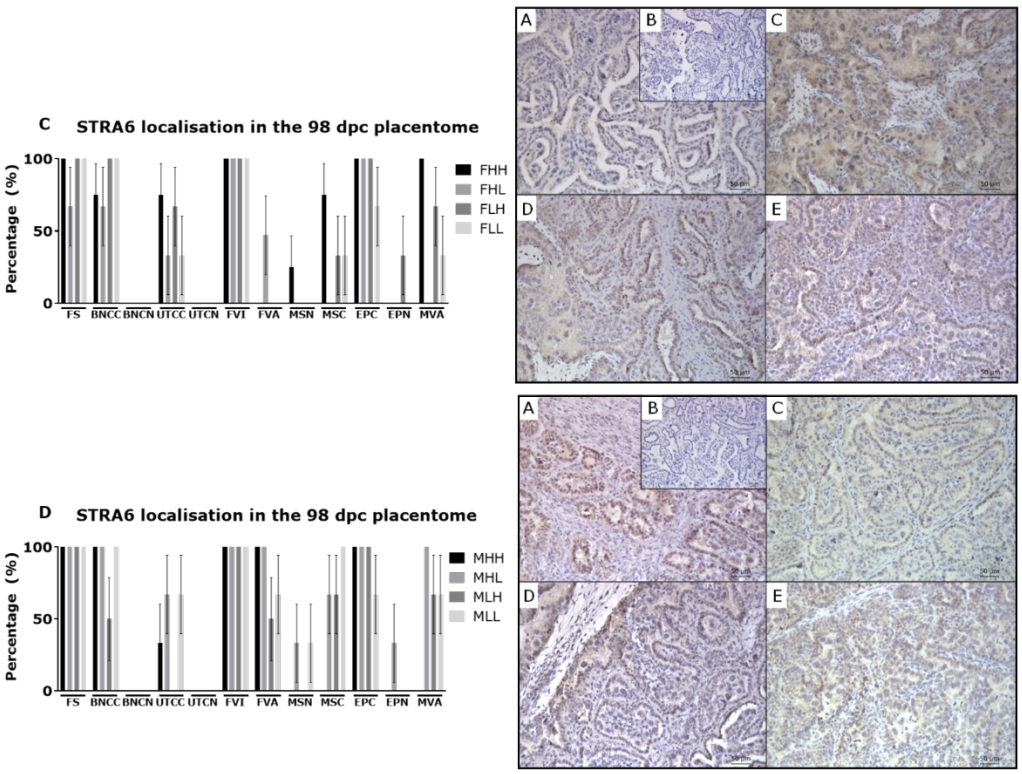


Figure 5.7 The percentage of samples displaying STRA6 immunopositivity and the cellular localisations in 98 days postconception placentomes continued. Graphs and complimentary photomicrographs (B=negative control, A, C-E represent each graph key, e.g. A=FHH, E=FLL) display results between female (C) and male (D) placentomes exposed to maternal dietary interventions. F=female, M=male, H=high protein, L=low protein. Letters denote periconception and postconception diet, e.g. HL= high protein periconception, low protein postconception. FS=fetal stroma, BNC=binucleate cell, UTC=uninucleate trophoblast cell, FVI=fetal villus, FVA=fetal vasculature, MST=maternal stroma, EP=epithelial cell, MVA=maternal vasculature, C=cytoplasm, N=nucleus. Scale bars represent 50 μm.

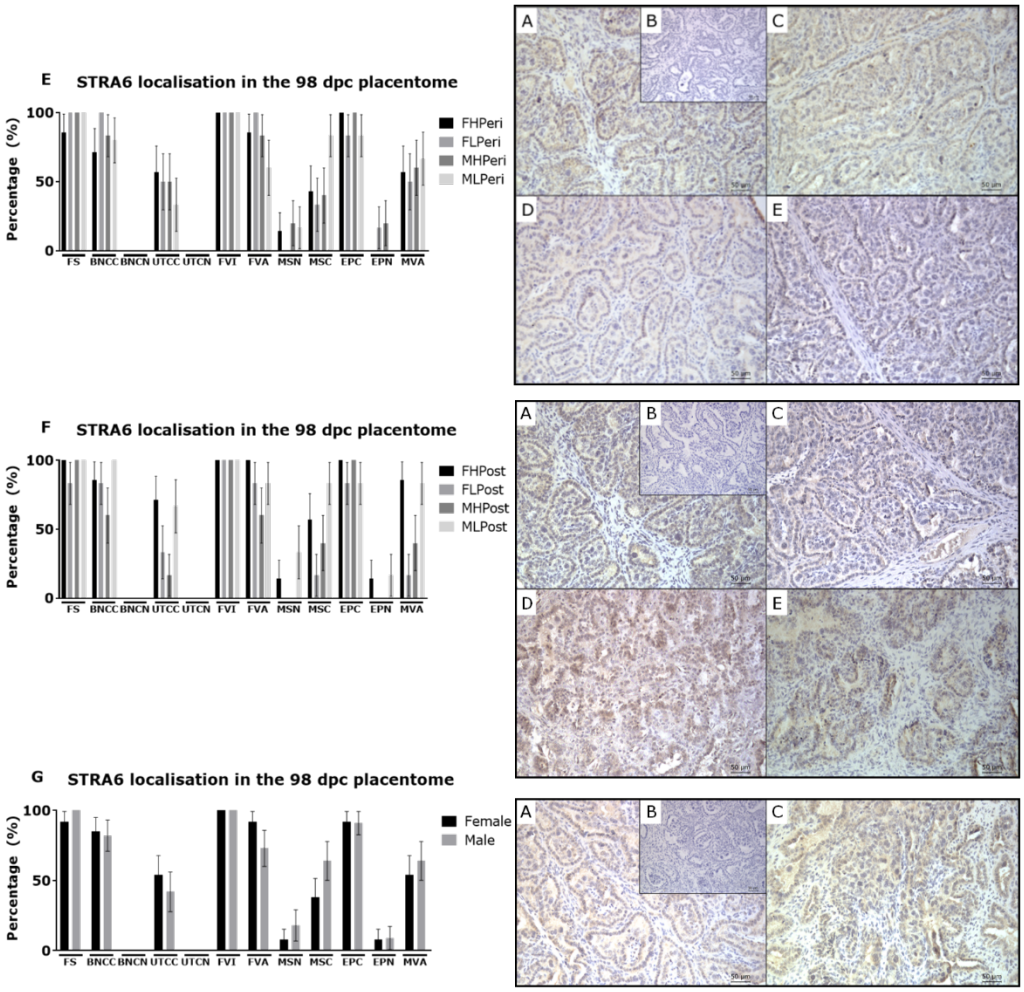


Figure 5.7 The percentage of samples displaying STRA6 immunopositivity and the cellular localisations in 98 days postconception placentomes continued. Graphs and complimentary photomicrographs (B=negative control, A, C-E represent each graph key, e.g. A=FHPeri, E=MLPeri) display results between female and male placentomes exposed to periconception (E) and postconception diets (F), and female and males (G) independent of dietary intervention. F=female, M=male, H=high protein, L=low protein, Peri=periconception, Post=postconception, FS=fetal stroma, BNC=binucleate cell, UTC=uninucleate trophoblast cell, FVI=fetal villus, FVA=fetal vasculature, MST=maternal stroma, EP=epithelial cell, MVA=maternal vasculature, C=cytoplasm, N=nucleus. Scale bars represent 50 μm.

5.3.4 GJA1 Localisation in the 98 Days Postconception Bovine Placentome

GJA1 immunohistochemical localisation was identified in fetal stroma within feto-maternal junctions, and fetal villus. Within these tissues, BNC nuclei, cytoplasm, and UTC cytoplasm were observed to positively stain for GJA1 protein. Positive staining was observed in maternal stroma, epithelial cell cytoplasm and nuclei, and maternal vascular endothelial cells. UTC nuclei were consistently immuno-negative for GJA1 protein.

In the fetal vasculature, the percentage of samples which displayed GJA1 immunopositivity was 16.7% lower in HL compared with HH, LH and LL exposed tissues (Figure 5.8 A). In BNC nuclei, the percentage of GJA1-positive samples was 17%, 17% and 34% lower in LH compared with HH, HL and LL counterparts (Figure 5.8 A). In BNC cytoplasm, the percentage of LH samples positively stained in was 33%, 50% and 50% greater than HH, HL, and LL counterparts (Figure 5.8 A). In UTC cytoplasm, the percentage of GJA1-positive samples was 50%, 33%, and 33% lower in LL versus HH, HL, and LH counterparts (Figure 5.8 A). In fetal villus, the percentage of samples displaying GJA1 immunopositivity was 67% lower in FHL than FLH, FLL, MHH, MHL, MLH and MLL (Figure 5.8 C D). In fetal vasculature, MHPeri displayed 16%, 19%, and 16% fewer GJA1-positive samples than FHPeri, FLPeri and MLPeri respectively (Figure 5.8 E F). FHL had the lowest percentage of positively stained samples overall, with only three parameters (fetal stroma, fetal vasculature, and epithelial cell cytoplasm) displaying greater than 50% of samples with GJA1-positivity (Figure 5.8 C). In maternal vasculature, 19%, 46% and 19% fewer FLPost samples displayed GJA1-immunopositivity than FHPost, MHPost and MLPost respectively (Figure 5.8 F). In UTC cytoplasm, 27% more male samples displayed GJA1-immunopositivity than female samples (Figure 5.8 G).

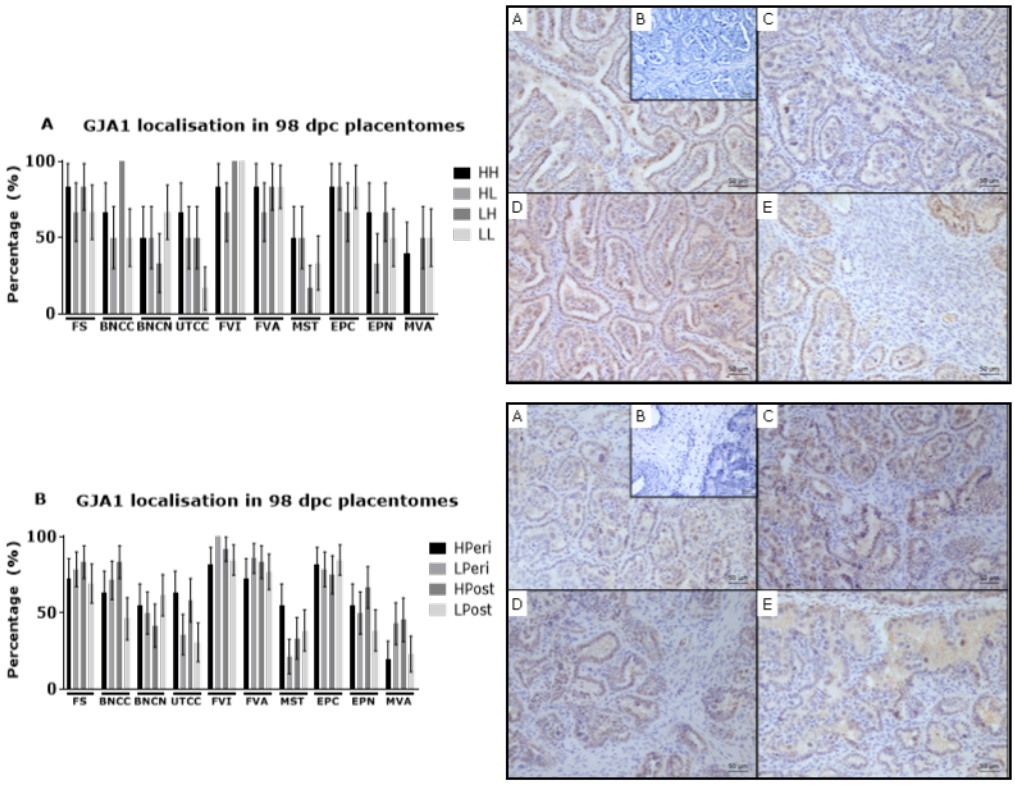


Figure 5.8 The percentage of samples displaying GJA1 immunopositivity and the cellular localisations in 98 days postconception placentomes. Graphs and complimentary photomicrographs (B=negative control, A, C-E represent each graph key, e.g. A=HH, E=LL) display results between maternal dietary interventions (A), periconception, and postconception diets (B). H=high protein, L=low protein. Letters denote periconception and postconception diet, e.g. HL= high protein periconception, low protein postconception. Peri=periconception, Post=postconception, FS=fetal stroma, BNC=binucleate cell, UTC=uninucleate trophoblast cell, FVI=fetal villus, FVA=fetal vasculature, MST=maternal stroma, EP=epithelial cell, MVA=maternal vasculature, C=cytoplasm, N=nucleus. Scale bars represent 50 µm.

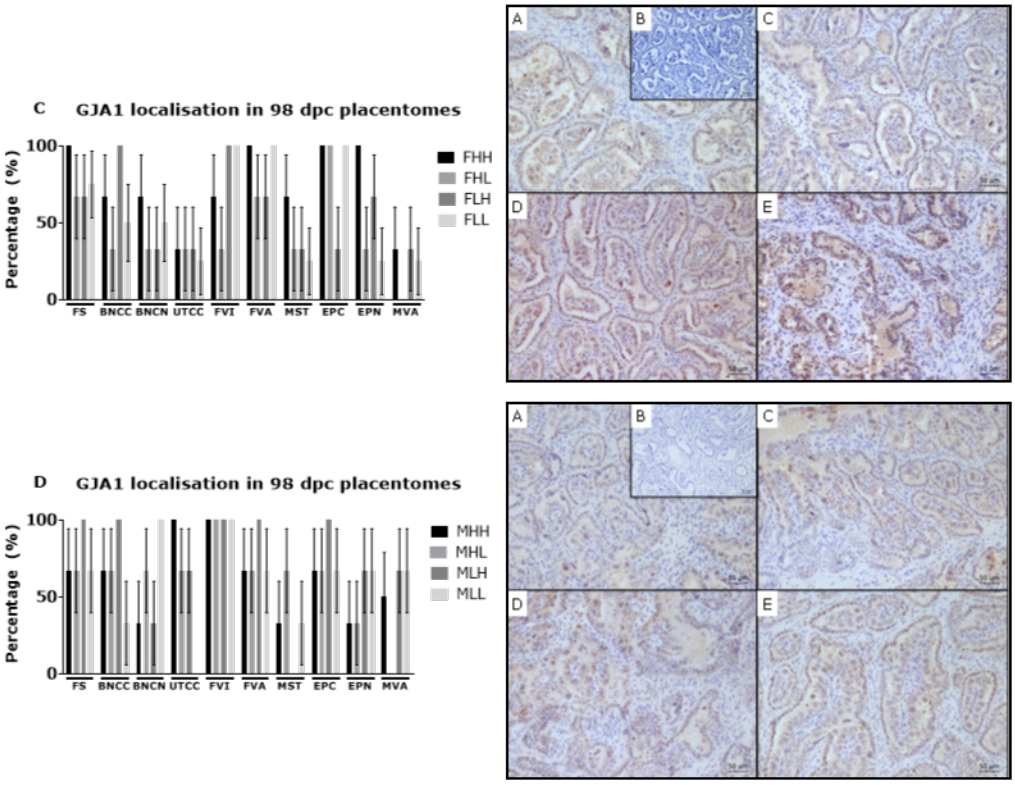


Figure 5.8 The percentage of samples displaying GJA1 immunopositivity and the cellular localisations in 98 days postconception placentomes continued. Graphs and complimentary photomicrographs (B=negative control, A, C-E represent each graph key, e.g. A=FHH, E=FLL) display results between female (C) and male (D) placentomes exposed to maternal dietary interventions. F=female, M=male, H=high protein, L=low protein. Letters denote periconception and postconception diet, e.g. HL= high protein periconception, low protein postconception. FS=fetal stroma, BNC=binucleate cell, UTC=uninucleate trophoblast cell, FVI=fetal villus, FVA=fetal vasculature, MST=maternal stroma, EP=epithelial cell, MVA=maternal vasculature, C=cytoplasm, N=nucleus. Scale bars represent 50 μm.

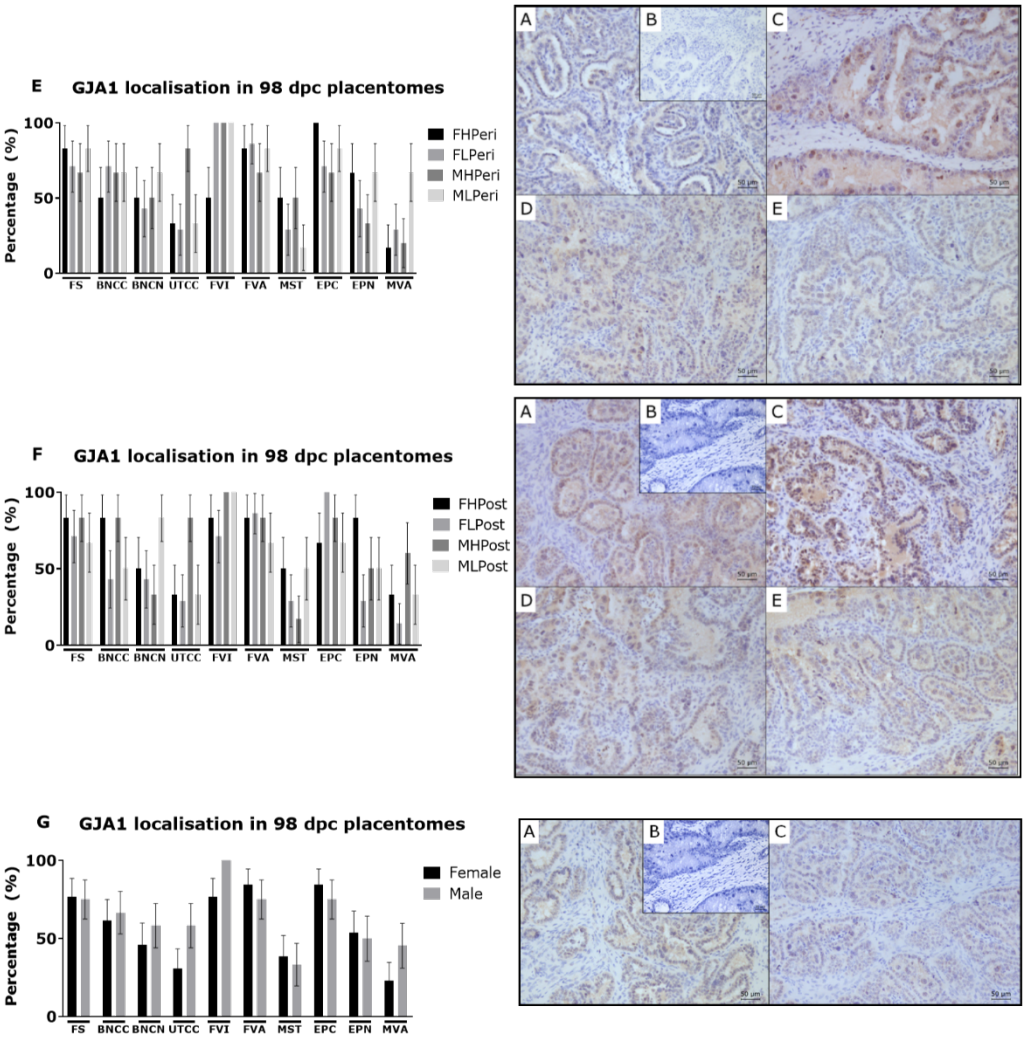


Figure 5.8 The percentage of samples displaying GJA1 immunopositivity and the cellular localisations in 98 days postconception placentomes continued. Graphs and complimentary photomicrographs (B=negative control, A, C-E represent each graph key, e.g. A=FHPeri, E=MLPeri) display results between female and male placentomes exposed to periconception (E) and postconception diets (F), and female and males (G) independent of dietary intervention. F=female, M=male, H=high protein, L=low protein, Peri=periconception, Post=postconception, FS=fetal stroma, BNC=binucleate cell, UTC=uninucleate trophoblast cell, FVI=fetal villus, FVA=fetal vasculature, MST=maternal stroma, EP=epithelial cell, MVA=maternal vasculature, C=cytoplasm, N=nucleus. Scale bars represent 50 μm

5.3.5 FOXO4 Localisation in the 98 Days Postconception Bovine Placentome

FOXO4 immunohistochemical localisation in the 98 dpc bovine placentome was cytoplasmic in BNCs, UTCs and epithelial cells (Figure 5.9). In the fetal stroma, and villus, 100% of HH, HL, LH and LL exposed samples displayed FOXO4-immunopositivity (Figure 5.9 A). Staining was observed in the fetal stroma bordering feto-maternal junctions, and connective tissue bordering the fetal villus (Figure 5.9). FOXO4 nuclear localisation was lowly (0-33%) observed in BNCs and was absent in UTCs and epithelial cell nuclei (Figure 5.9 A). In maternal vasculature, 33% more FHH, FHL, and MHL samples displayed FOXO4-immunopositivity than FLL, MLH and MLL samples (Figure 5.9 C D). In fetal blood vessels, 33% fewer FLL samples positively stained all other sex-diet groups (Figure 5.9 C D). In BNC cytoplasm, 53% and 19% more FLPeri samples displayed FOXO4-immunopositivity than FHPeri and MHPeri (Figure 5.9 E). In UTC cytoplasm, 17% more MHPost and MLPost samples displayed FOXO4-immunopositivity than FHPost and FLPost (Figure 5.9 F). In maternal epithelial cell cytoplasm, 0% of females displayed FOXO4 staining, compared with 25% of male samples (Figure 5.9 G).

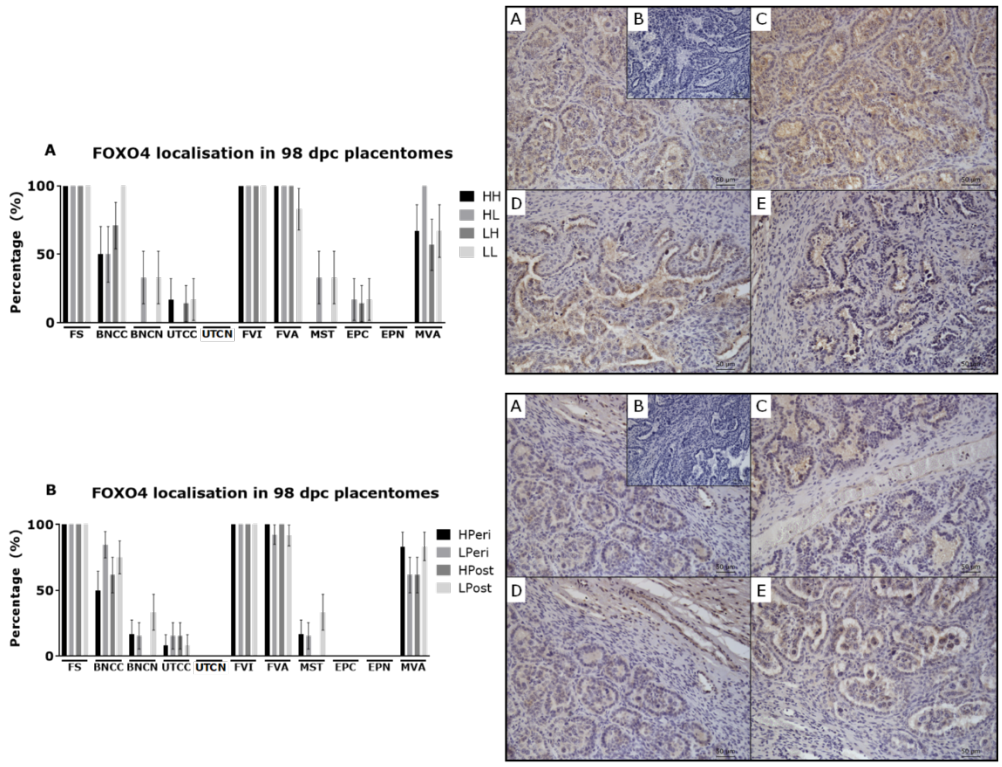


Figure 5.9 The percentage of samples displaying FOXO4 immunopositivity and the cellular localisations in 98 days postconception placentomes. Graphs and complimentary photomicrographs (B=negative control, A, C-E represent each graph key, e.g. A=HH, E=LL) display results between maternal dietary interventions (A), periconception, and postconception diets (B). H=high protein, L=low protein. Letters denote periconception and postconception diet, e.g. HL= high protein periconception, low protein postconception. Peri=periconception, Post=postconception, FS=fetal stroma, BNC=binucleate cell, UTC=uninucleate trophoblast cell, FVI=fetal villus, FVA=fetal vasculature, MST=maternal stroma, EP=epithelial cell, MVA=maternal vasculature, C=cytoplasm, N=nucleus. Scale bars represent 50 μm.

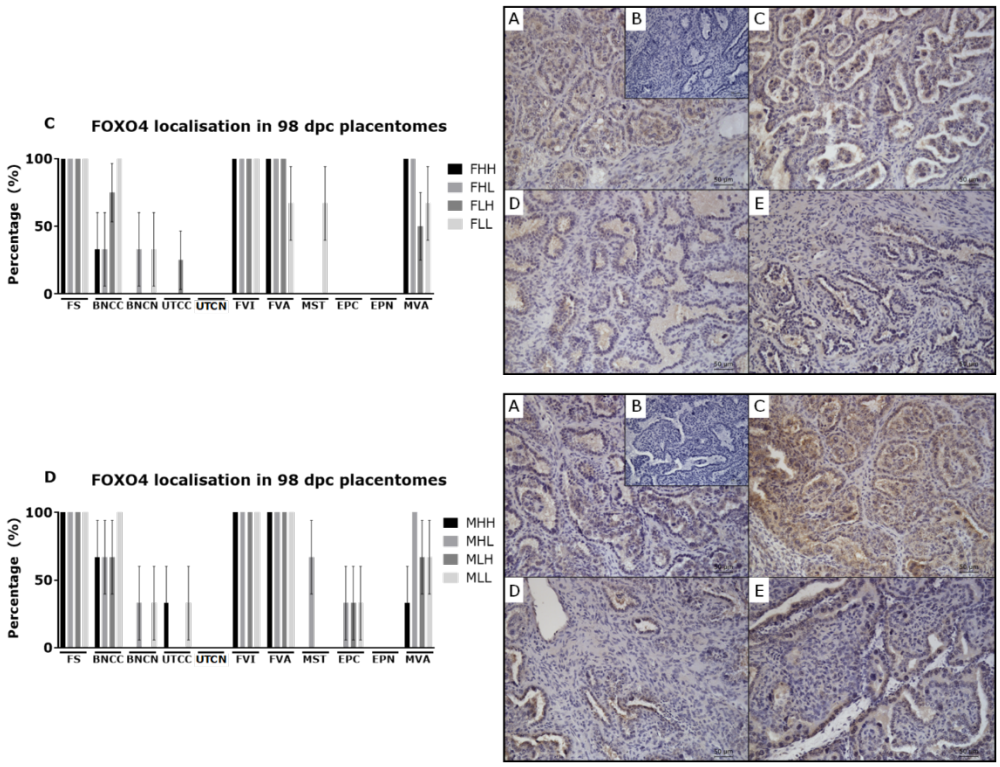


Figure 5.9 The percentage of samples displaying FOXO4 immunopositivity and the cellular localisations in 98 days postconception placentomes continued. Graphs and complimentary photomicrographs (B=negative control, A, C-E represent each graph key, e.g. A=FHH, E=FLL) display results between female (C) and male (D) placentomes exposed to maternal dietary interventions. F=female, M=male, H=high protein, L=low protein. Letters denote periconception and postconception diet, e.g. HL= high protein periconception, low protein postconception. FS=fetal stroma, BNC=binucleate cell, UTC=uninucleate trophoblast cell, FVI=fetal villus, FVA=fetal vasculature, MST=maternal stroma, EP=epithelial cell, MVA=maternal vasculature, C=cytoplasm, N=nucleus. Scale bars represent 50 μm.

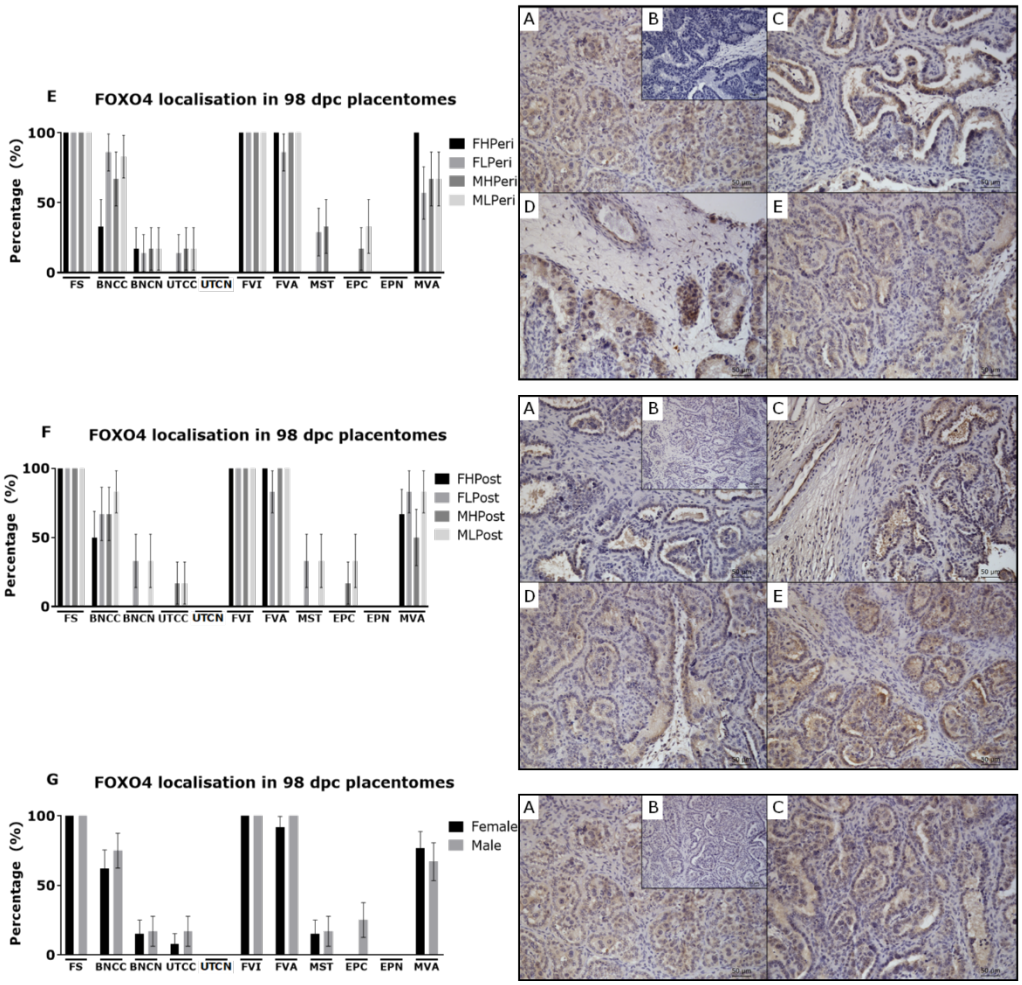


Figure 5.9 The percentage of samples displaying FOXO4 immunopositivity and the cellular localisations in 98 days postconception placentomes continued. Graphs and complimentary photomicrographs (B=negative control, A, C-E represent each graph key, e.g. A=FHPeri, E=MLPeri) display results between female and male placentomes exposed to periconception (E) and postconception diets (F), and female and males (G) independent of dietary intervention. F=female, M=male, H=high protein, L=low protein, Peri=periconception, Post=postconception, FS=fetal stroma, BNC=binucleate cell, UTC=uninucleate trophoblast cell, FVI=fetal villus, FVA=fetal vasculature, MST=maternal stroma, EP=epithelial cell, MVA=maternal vasculature, C=cytoplasm, N=nucleus. Scale bars represent 50 μm.

5.3.6 Notch1 Localisation in the 98 Days Postconception Bovine Placentome

Notch1-immunopositivity was ubiquitously localised in the 98 dpc bovine placentome (Figure 5.10). Notch1 immunopositivity in fetal stroma was present in 80-100% of all samples within maternal dietary interventions (Figure 5.10 A). In BNCs and UTCs, cytoplasmic Notch1 localisation was observed in a lower percentage of HL samples than HH, LH, and LL (67% versus 100%) (Figure 5.10 A). In fetal stroma and villus, 17-20% fewer HL exposed samples displayed Notch1-immunopositivity versus HH, LH and LL (80-83% versus 100%) (Figure 5.10 A). Sex-diet interactions revealed this difference was due to FHL and not MHL, where only 50% of FHL displayed Notch1 fetal stroma localisation, compared with 100% of MHL samples (Figure 5.10 C D). The percentage of samples displaying fetal vascular Notch1-immunopositivity was 17% lower in HL compared to HH, LH and LL samples (83% versus 100%) (Figure 5.10 A). This was attributable to FHL, as all male samples displayed 100% Notch1 localisation in fetal vasculature (Figure 5.10 C D). In BNC and UTC cytoplasm, HPeri samples displayed a lower percentage (83 and 67% respectively) of Notch1-immunopositive samples compared to LHPeri, which did not differ between sexes (Figure 5.10 E). In BNC cytoplasm, 17% fewer HPeri and LPost samples displayed Notch1-immunopositivity than LPeri and HPost samples (Figure 5.10 B). Independent of dietary intervention, the percentage of Notch1-immunopositive samples was unaffected by sex in UTC (75% of male and females) and BNC (92% of male and females) cytoplasm (Figure 5.10 G). The percentage of male samples displaying Notch1-immunopositivity in maternal stroma and epithelial cell cytoplasm were 42 and 16% lower than female counterparts (Figure 5.10 G).

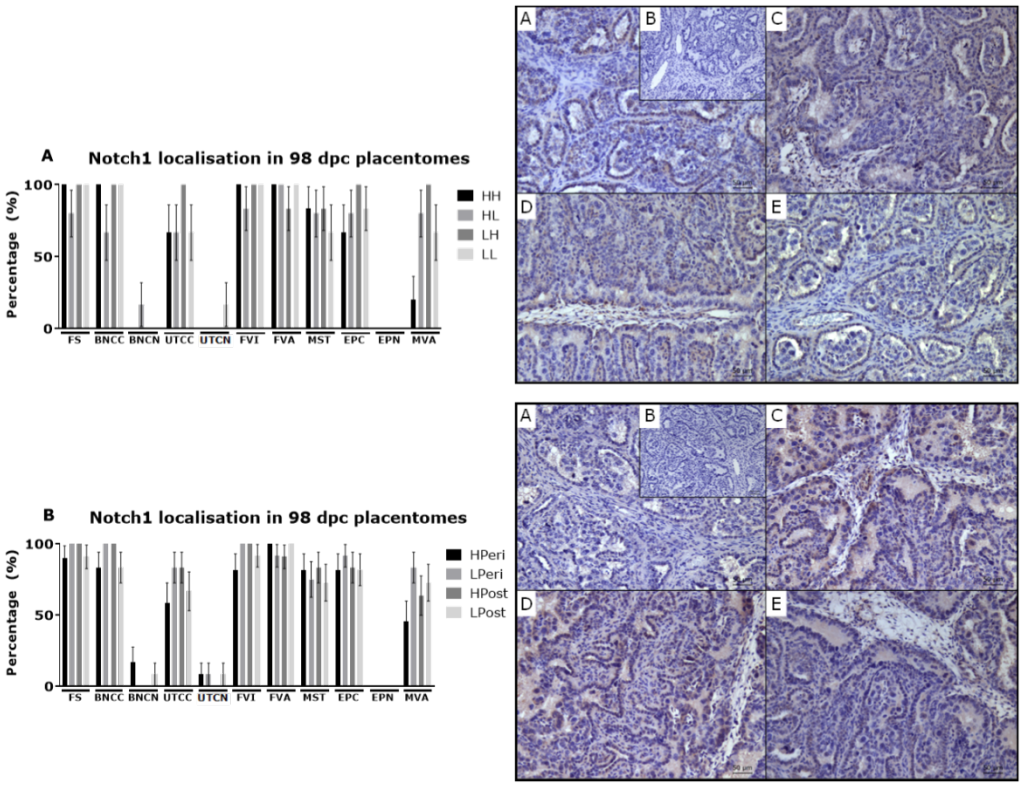


Figure 5.10 The percentage of samples displaying Notch1 immunopositivity and the cellular localisations in 98 days postconception placentomes. Graphs and complimentary photomicrographs (B=negative control, A, C-E represent each graph key, e.g. A=HH, E=LL) display results between maternal dietary interventions (A), periconception, and postconception diets (B). H=high protein, L=low protein. Letters denote periconception and postconception diet, e.g. HL= high protein periconception, low protein postconception. Peri=periconception, Post=postconception, FS=fetal stroma, BNC=binucleate cell, UTC=uninucleate trophoblast cell, FVI=fetal villus, FVA=fetal vasculature, MST=maternal stroma, EP=epithelial cell, MVA=maternal vasculature, C=cytoplasm, N=nucleus. Scale bars represent 50 μm.

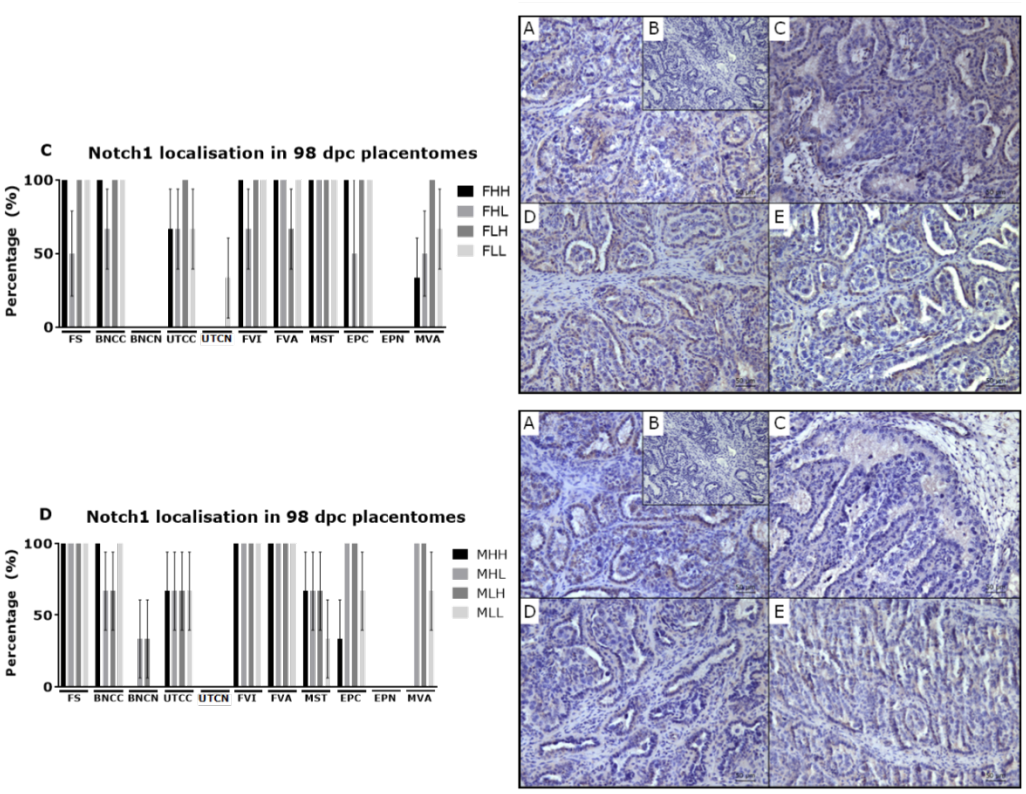


Figure 5.10 The percentage of samples displaying Notch1 immunopositivity and the cellular localisations in 98 days postconception placentomes continued. Graphs and complimentary photomicrographs (B=negative control, A, C-E represent each graph key, e.g. A=FHH, E=FLL) display results between female (C) and male (D) placentomes exposed to maternal dietary interventions. F=female, M=male, H=high protein, L=low protein. Letters denote periconception and postconception diet, e.g. HL= high protein periconception, low protein postconception. FS=fetal stroma, BNC=binucleate cell, UTC=uninucleate trophoblast cell, FVI=fetal villus, FVA=fetal vasculature, MST=maternal stroma, EP=epithelial cell, MVA=maternal vasculature, C=cytoplasm, N=nucleus. Scale bars represent 50 μm.

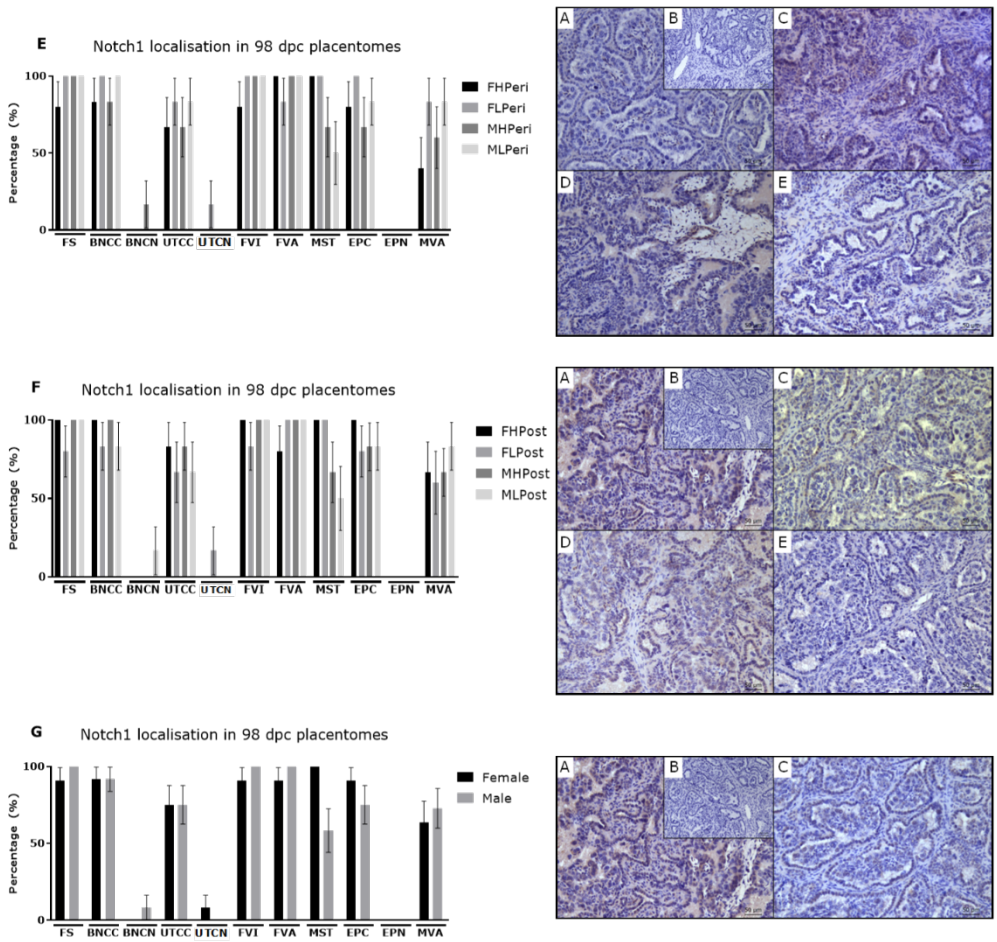


Figure 5.10 The percentage of samples displaying Notch1 immunopositivity and the cellular localisations in 98 days postconception placentomes continued. Graphs and complimentary photomicrographs (B=negative control, A, C-E represent each graph key, e.g. A=FHPeri, E=MLPeri) display results between female and male placentomes exposed to periconception (E) and postconception diets (F), and female and males (G) independent of dietary intervention. F=female, M=male, H=high protein, L=low protein, Peri=periconception, Post=postconception, FS=fetal stroma, BNC=binucleate cell, UTC=uninucleate trophoblast cell, FVI=fetal villus, FVA=fetal vasculature, MST=maternal stroma, EP=epithelial cell, MVA=maternal vasculature, C=cytoplasm, N=nucleus. Scale bars represent 50 μm.

5.4 Discussion

At 98 days postconception, fetal growth and development relies on the placenta. The bovine placentome facilitates nutrient transfer, gaseous exchange and removal of waste products (Hemberger and Cross, 2001, Vonnahme and Lemley, 2011). Additionally, the placenta is a source of pregnancy associated glycoproteins, transferred from BNCs to the endometrial epithelium (Hashizume et al., 2006, Hashizume, 2007, Wallace et al., 2015). The current study investigated the effect of maternal dietary protein on protein expression and localisation in the placenta. It is important to note that immunohistochemistry provides evidence for protein localisation, but not evidence for protein abundance. Qualitative analysis in the current study used a binary scoring of 0 or 1 to represent if staining was present in a certain cellular context or structure within the tissue sections. There was not scope for quantification of protein abundance (Western Blot) in the current study, although this would have ideally been performed. The proteins of interest were SerpinB1, GATM, STRA6, GJA1, FOXO4 and Notch1. Additionally, the sex and diet interaction, and sex alone were studied.

5.4.1 SerpinB1 Localisation in the 98 Days Postconception Bovine Placentome

During initial qualitative analysis, the current study observed a pattern of SerpinB1 protein localisation in BNCs within the feto-maternal junction (Table 5.3). Positive staining of SerpinB1 was initially observed to be dependent on cellular localisation (Figure 5.5, Table 5.3). Further quantitative image analysis confirmed this observation (Table 5.4). Previously, the proportion of BNCs within 98 dpc placentomes was unaffected by dietary intervention (Edwards, 2016). Analysis of BNC cellular contexts in the current study suggests that maternal dietary intervention altered the proportion of BNCs in each cellular context (Table 5.5). The biological significance of this is unclear. Although based on the primary function of BNCs, the proportion of epithelial localised BNCs may suggest a variation in level of BNC-derived glycoprotein transfer to the endometrial epithelium at 98 dpc. This may have been a placental adaptation to maternal diet. One possible reason for this is to regulate fetal growth in cases of maternal nutrient restriction, especially as heifers were nulliparous and therefore still growing (Gluckman and Hanson, 2004). Presence of SerpinB1-

immunopositivity in caruncular epithelial cells (Figure 5.3) may suggest cell activity such as phagocytosis of a BNC remnant, or alternatively, that SerpinB1 protein was present in the BNC remnant. SerpinB1-immunopositivity in vascular endothelial nuclei suggests that SerpinB1 plays an important role in this cell type, as it was ubiquitously expressed in caruncular blood vessels (Figure 5.3). This suggests that SerpinB1 expression in maternal vascular endothelial cells may be independent of fetal sex or maternal diet exposure during the first trimester of gestation. Although further investigation is required to elucidate the exact role of SerpinB1 in maternal vasculature in the bovine placentome.

Furthermore, the molecular and cellular activity of BNCs in each cellular context investigated (Table 5.5) is not fully understood. Differences in BNC granulocyte gene expression have previously been studied between cotyledonary and intercotyledonary locations (Patel et al., 2004, Wooding et al., 1996). Furthermore, gene expression within BNCs has been described as spatiotemporal, and protein expression is influenced by the anatomical localisation of BNCs (Patel et al., 2004). This supports the possibility of differential SerpinB1 protein expression during BNC migration from the fetal stroma, towards the endometrial epithelium. Stromal localised BNCs are thought to be in the early stage of migration or may be preparing to start migration (Hashizume, 2007, Klisch et al., 1999, Patel et al., 2004). Quantification of SerpinB1-immunopositivity in fetal stromal localised BNCs was 9.2% (Table 5.4). Stromal villi localised BNCs are thought to be in a stage before further migration to an epithelial cell, and have migrated from stromal localisation. Quantification of SerpinB1-immunopositivity in stromal villi BNCs was 43.1% (Table 5.4). This suggests stromal villi localised BNCs have different transcriptional activity within this cellular context. There may be a sub-set of cells which cannot be identified via histological image analysis or explained by cellular localisation alone. This indicates that further characterisation may be required further to the four discrete cellular contexts of the current study (Table 5.3). Results suggest that the percentage of stromal villi-localised BNCs did not differ between FHL (60.13%) and FLH (58.87%) (Table 5.5; $p>0.05$). However, within this cellular context, SerpinB1 immunopositivity was greater in FHL (22.51%) than FLH (17.18%) (Table 5.6). Furthermore, this may account for the previous result of greater SerpinB1 gene expression in FHL versus FLH 98 dpc placentomes (Edwards, 2016). Similarly, between the FHH and FLL diets, the percentage of stromal-

villi localised BNCs did not differ (Table 5.5), although SerpinB1-immunopositivity was greater in FLL (20.26%) than FHH (16.95%) (Table 5.6). The biological significance of SerpinB1 expression in BNCs localised in the stromal-villi context is unclear. These results suggest a difference in cellular activity within the same cellular context.

The percentage of stromal-villi, and fetal villus localised BNCs was greater in MLH than MHL, while the percentage of stromal epithelial-localised BNCs in MHL was greater than MLH (Table 5.5). In stromal, stromal villi, and villus localised BNCs, the percentage of SerpinB1-immunopositive BNCs was greater in MLH than MHL (Table 5.5). These localisation results of the current study complimented previous RT-qPCR results of the same placentomes (Edwards, 2016). Relative SerpinB1 expression was increased in MLH compared to MHL and FLH (Edwards, 2016). This may suggest an adaptation of the male placenta in response to changes in maternal dietary protein at 23 dpc. This is consistent with adaptations in response to environmental conditions (Sandovici et al., 2012, Sullivan et al., 2009c, Barker, 1997, Coan et al., 2008). Although the biological significance of this is unclear, it may relate to maternal hormonal regulation of placental BNC glycoprotein production to regulate fetal growth (Bersinger and Ødegård, 2004, Gluckman and Hanson, 2004). Further investigation is required to determine whether BNC cellular context and molecular activity is involved in regulating fetal growth in response to maternal diet.

Stromal epithelial localised BNCs are thought to be in the final stages of cell migration, and are preparing to or have fused with a maternal epithelial cell (Wooding et al., 1996, Wooding et al., 1986). The initial observation that ~100% of stromal epithelial localised BNCs expressed SerpinB1 was quantified to be 96.5% (Table 5.3, Table 5.4). Results suggest that SerpinB1 was expressed during fusion or contact with an epithelial cell (Table 5.4), although the role SerpinB1 has is unclear, interpretation of current literature identified some putative functions (Figure 5.1, Figure 5.11). A possible explanation for stromal epithelial BNC SerpinB1 expression is a role in cell migration and fusion (Figure 5.11).

Finally, the fetal villus (also collectively termed villi) localised BNCs and their role in this location is not as well understood, in comparison to BNCs within the feto-maternal junction. Migration likely occurs as it is a key function of

BNCs to migrate towards the caruncular epithelium, and if it achieves sufficient migration to contact an epithelial cell, to fuse and pass on glycoproteins such as pregnancy associated glycoprotein (PAG) (Wooding et al., 1996, Wooding and Wathes, 1980, Wooding et al., 2005). BNCs in the fetal villus did not have an obvious pattern of SerpinB1 expression (Table 5.4, Figure 5.4). This was consistent with the original qualitative pattern of SerpinB1 immunopositivity within the fetal villus (Table 5.3, Figure 5.5). The lack of a spatial pattern of SerpinB1-immunopositivity in the fetal villus may indicate that expression was cell-specific and not solely based on localisation within the villi. The cell-specific expression may be dependent on individual cellular activity. Alternatively, it is possible that the categorisation level of localisation "fetal villus" was not an adequate enough discreet context and that further subcategorization was required.

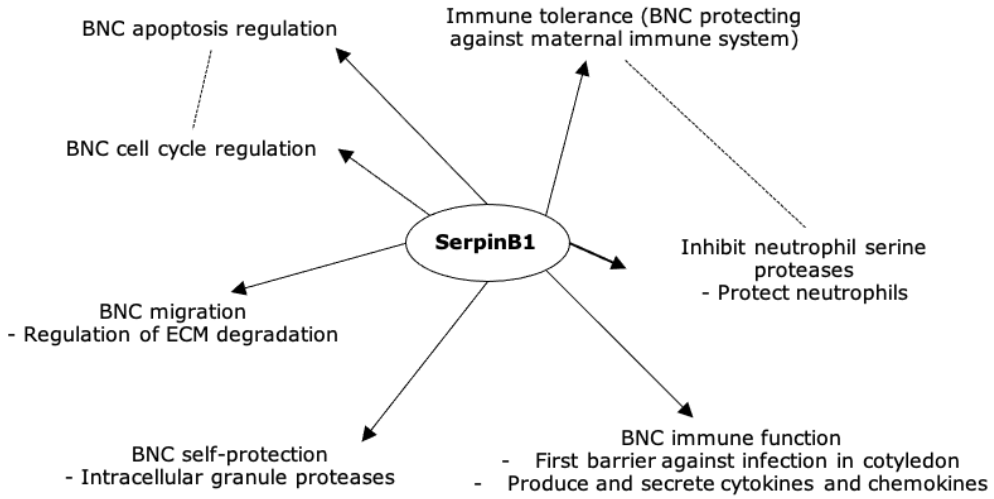


Figure 5.11 Putative roles of SerpinB1 in the bovine placentome. (Riley et al., 2000, Aluvihare et al., 2004, Carroll et al., 2004, Heutinck et al., 2010b). Bold arrow indicates established function, unbold arrows indicate known roles confirmed in other species. Dotted lines indicate putative link between functions.

It is unknown whether SerpinB1 stimulates or inhibits cell migration in bovine BNCs, or in fact whether it is involved at all. However, literature provides evidence to suggest that SerpinB1 may play a role in BNC migration. Matrix-metalloproteinases (MMP) are targets of SerpinB1, which are involved in cell migration and invasion via ECM breakdown (Woessner, 1991). *MMP2* and *MMP9* expression has previously been identified in first trimester human trophoblast cells (Xu et al., 2002, Isaka et al., 2003, Staun-Ram et al., 2004). Studies have reported that *SerpinB1* may inhibit cell migration in hepatocellular carcinoma (Cui et al., 2014), and glioma (Huasong et al.,

2015), however another study contradicted this (Tseng et al., 2009). *SerpinB1* expression was up-regulated in highly mobile oral cancer cell line (Cal-27) compared to a low mobility cell line (SAS) (Tseng et al., 2009). *SerpinB1* gene expression was confirmed and validated via immunoblotting, 2D-electrophoresis, semi-quantitative PCR, and RT-qPCR, and correlated with highly invasive carcinomas (Tseng et al., 2009). This partly compliments the results of the current study, in relation to SerpinB1 localisation within cellular contexts of migrating BNCs. The cellular distribution of human SerpinB1 was both cytoplasmic and nuclear (Silverman et al., 2004). In the current study, SerpinB1 was found to be also cytoplasmic and nuclear in BNCs (Figure 5.3). Further research may involve cell-culture to investigate the role of SerpinB1 in BNCs. For example, a co-culture of the bovine trophoblast cell line, BT-1, and cultured epithelium to direct migration (Haeger et al., 2015).

Another putative function of SerpinB1 in the bovine placentome in apoptosis. Riley and colleagues (2000) suggested that in the ovine placenta, BNC apoptosis was stimulated in response to ECM degradation. Coordinated apoptosis and ECM degradation may aid tissue remodelling during placental adaptation, such as increasing the surface area of feto-maternal junction (Riley et al., 2000). Apoptosis was observed in ovine trophoblast cells located basally in feto-maternal junctions (Riley et al., 2000). These cellular contexts were described as stromal-villi and stromal-epithelial in the current study. This is consistent with SerpinB1-immunopositivity being predominantly identified in these cellular contexts (Table 5.4). Based on previous functions of SerpinB1 and evidence of BNC apoptosis, there is a putative role of SerpinB1 in BNC apoptosis (Hayashi et al., 2011). Although confirmation via histological analysis using an apoptosis detection kit (e.g. Click-iT™ TUNEL Colorimetric IHC Detection Kit, Thermo Fisher Scientific, Waltham, MA, USA) would further understanding of the incidence of BNC apoptosis in the bovine placenta in the current study. Alternatively, immunofluorescence combining apoptosis markers and SerpinB1 to confirm dual expression in individual cells may further elucidate the association between apoptotic cells expressing SerpinB1, and reveal whether SerpinB1 is involved in trophoblast cell apoptosis in the bovine placentome.

Previously, the proportion of vasculature in LPeri exposed cotyledons was greater than HPeri, but was unaffected by postconception diet (Edwards,

2016). Edwards (2016) also reported that SerpinB1 expression was 1.62-fold, 2.33-fold, 1.93-fold, and 1.81-fold greater in MHL, MLL, MLH, and FHL compared with FLH exposed 98 dpc placentomes. As SerpinB1 immunopositivity was consistently localised in fetal and maternal vasculature (100%; Figure 5.3 A), this may suggest that the previously reported SerpinB1 differential gene expression was due to a difference in the proportion of vasculature. However, further in-depth quantitative analysis of SerpinB1 gene and protein expression would further elucidate this.

In summary, SerpinB1 protein was localised in all analysed locations of the 98 dpc placentome (Figure 5.3). SerpinB1 protein expression in BNCs appeared to be spatiotemporal, differing between the discreet cellular contexts analysed within the 98 dpc bovine placentome, and differed between placentome sex, and exposure to maternal diet (Table 5.4, Table 5.5, and Table 5.6). This may suggest adaptations in the placentome at 98 dpc.

5.4.2 GATM Localisation in the 98 Days Postconception Bovine Placentome

GATM was localised to discrete regions of the 98 dpc bovine placentome (Figure 5.6). GATM was consistently expressed in the stroma of the fetal villus (Figure 5.6). This suggests a role of GATM during fetal villi remodelling, and a potential requirement for creatine in the fetal villus. This may be important as this tissue is growing and remodelling, and therefore may have higher energy demands compared to the feto-maternal junctions (Reynolds et al., 2013). Overall, localisation of GATM protein in regions of the 98 dpc placentome suggests the capacity for creatine synthesis. However, as there is more than one enzyme involved in creatine synthesis, GATM localisation alone is no confirmation of this (Bronte and Zanovello, 2005). GATM immunopositivity was not observed in UTCs within feto-maternal junctions (Figure 5.6). Furthermore, GATM expression was not localised in vascular endothelial cells (Figure 5.6). Although GATM human placental gene expression was previously reported, protein localisation specific to vasculature was not reported (Uusküla et al., 2012). Absence in these cellular contexts suggests no creatine synthesis activity.

Previous GATM mRNA expression from RNA-seq was lower in FHL compared to FLH, MLL, MLH and MHL (Edwards, 2016). However, qPCR by the same study contradicted this, suggesting FHL GATM expression was greater than FLH and MLL (Edwards, 2016). Human placental GATM gene expression was low at first trimester, peaked at mid-gestation, and decreased at term (Uusküla, 2013). Placental GATM expression was increased in complicated pregnancies, such as gestational diabetes, and in babies small and large for gestational age, compared to normal placentae (Uusküla, 2013). This suggests increased expression of GATM in nutrient restricted females. This relates to the current study of maternal protein restriction. Additionally, increased expression of GATM may be linked with maternal under-nutrition at critical periods during gestation. However, the study by Uusküla (2013) did not report fetal sex, which was shown to affect GATM expression (Edwards, 2016). Similarly, creatine was implicated in enhanced mouse pup survival following hypoxia (Ireland et al., 2008). Maternal protein restriction may illicit a similar stress as hypoxia, and initiate a placental adaptation, in this case altered GATM expression, to support fetal survival. In summary, the current study positively identified GATM protein immunopositivity in the 98 dpc bovine placentome, suggesting the required machinery for creatine biosynthesis is present. This may be altered by maternal diet and fetal sex.

5.4.3 STRA6 Localisation in the 98 Days Postconception Bovine Placentome

Another protein of interest which was localised in the 98 dpc bovine placentome was STRA6 (Figure 5.7). STRA6 localisation in maternal and fetal blood vessels (Figure 5.7) suggests a biological requirement and capacity for retinoic acid transport. Previously, in E12.5 mouse placentae, STRA6 was localised in the labyrinth, which is the region of feto-maternal exchange (Bouillet et al., 1997). This agrees with the localisations of STRA6-immunopositivity observed in the feto-maternal junctions of 98 dpc bovine placentomes in the current study (Figure 5.7). Previously, STRA6 was absent in mouse trophoblast giant cells throughout gestation (Bouillet et al., 1997). This is contrary to BNC expression of STRA6 in the current study (Figure 5.7), which may highlight differences in cell activity and localisation between the two placentae. In the ovine placenta, retinoid transport was observed as both fetal transfer and return to the maternal system, which indicated both influx and efflux of retinoid across the placentome (Donoghue et al., 1982).

STRA6-immunopositivity in trophoblasts, epithelial cells and vasculature (Figure 5.7) supports retinoid influx and efflux across the placenta previously observed in ewes (Donoghue et al., 1982). The current study provides novel evidence for STRA6 protein localisation in the 98 dpc bovine placenta (Figure 5.7), which agrees with previous research in mice and sheep (Donoghue et al., 1982, Bouillet et al., 1997). The biological implications of STRA6 expression in the bovine placentome remain unclear, however existing research provides some indication of the possible functions of STRA6 in the bovine placentome, and potential future effects on the calf (Figure 5.12).

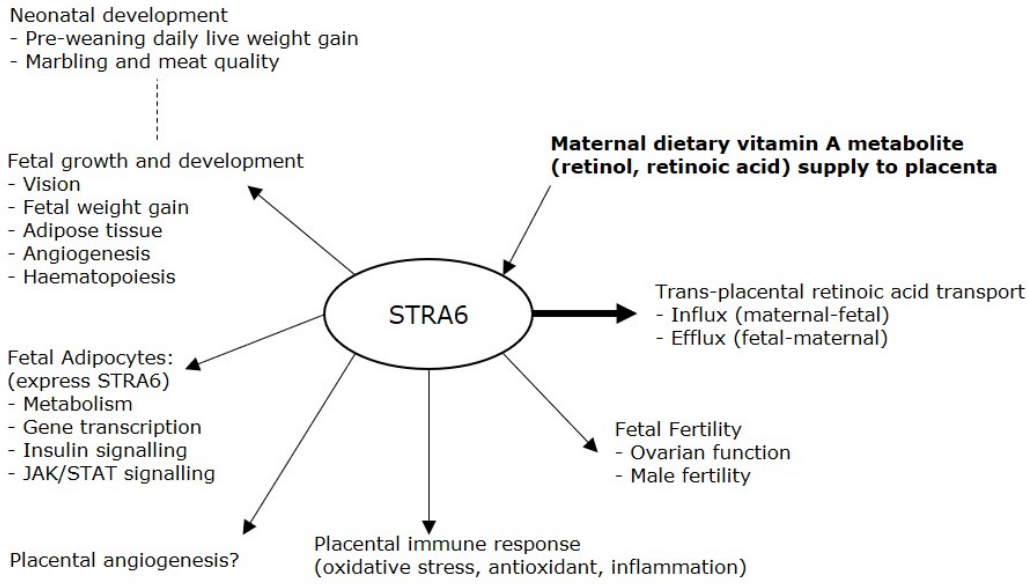


Figure 5.12 Putative direct and indirect roles of STRA6 in the bovine placentome and fetus. (Kawaguchi et al., 2007, Kawaguchi et al., 2011, Kawaguchi et al., 2012, Kawaguchi et al., 2015, Nozière et al., 2006, Donoghue et al., 1982). Bold arrow indicates key function, dashed line indicates indirect effects, and inwards pointing arrow indicates putative effect on placental STRA6.

5.4.4 GJA1 Localisation in the 98 Days Postconception Bovine Placentome

GJA1 protein localisation in the 98 dpc bovine placentome (Figure 5.8) supports the previous finding of GJA1 expression in the 120 dpc bovine placentome (Pfarrer et al., 2006). In human extravillus trophoblasts, GJA1 expression was down-regulated by *SPARC (secreted protein acidic and cysteine rich*), which is thought to promote trophoblast invasion of the endometrial epithelium (Jiang et al., 2013). Considering this, GJA1 expression may be spatio-temporal (relates to both space or location and

time, or timing) within BNCs and UTCs dependent on cellular activity. Down-regulation of *GJA1* mRNA was also thought to favour receptivity of the bovine endometrium to implantation (Salilew-Wondim et al., 2010). The commonality between these studies and structures is a reduction in cell-cell gap junctions. This may favour BNC migration through loss of BNC-BNC or UTC-BNC gap junctions, the equivalent of which was previously been demonstrated in humans (Cronier et al., 2002). In bovine endometria, reduced *GJA1* expression was beneficial to successful implantation and pregnancy, with fewer gap junction connections, increasing the surface area and thus receptivity to implantation (Bauersachs et al., 2009). Similarly in human placentae, *GJA1* down-regulation was proposed to aid implantation (Jiang et al., 2013). This is further evidence to suggest GJA1 may function in BNC migration, and cell-cell communication within trophoblasts.

Connexons can be homomeric or heteromeric (Kumar and Gilula, 1996, Winterhager and Kidder, 2015, Wang and Daniel, 2001). The expression of other connexins within the placentome in the current study is unknown. Therefore, it is possible that multiple types of connexons were present in the placentome. One explanation for varying protein localisation may be the presence of different connexin proteins with varying proportions of GJA1 protein. In the fetal villus, GJA1 localisation was observed in over 50% of samples in all dietary interventions and was observed in 100% of LH and LL exposed placentomes (Figure 5.8, A). However, these differences were largely accounted for by sex and diet interactions (Figure 5.8, C, D). Interactions between sex and diet were observed for GJA1 localisation in the fetal villus. 100% of male samples presented GJA1-immunopositivity whereas localisation in female placentomes was affected by diet (Figure 5.8, C, D). This suggests that GJA1 protein expression in the fetal villus was ubiquitous across male but not female placentomes. This may be important, as the villus is remodelling, communication between cells likely aids tissue growth and development (Malassine and Cronier, 2005). This result indicates a possible difference in fetal villus remodelling between placentome sexes. Further investigation into connexin gene expression and immunohistochemical investigation of the connexin family within the bovine placentome would further the understanding of connexons in the placentome.

GJA1 protein localisation in maternal stroma was less than 50% in all samples, particularly in LH samples (Figure 5.8, A). This was largely due to 0% of MLH samples displaying GJA1 protein (Figure 5.8, D). Between male and female placentomes, the percentage of samples displaying GAJ1-immunopositivity was similar (Figure 5.8, G). However, sex-diet interactions were observed (Figure 5.8, C, D). This suggests that the differences in GJA1 expression between maternal diets in the maternal stroma was sex specific. Results suggest that males were more sensitive to nutritional intervention (LH) compared to female placentomes. It is thought that *GJA1* is involved in supporting stromal differentiation.

GJA1 protein localisation in maternal vasculature was absent in 100% of HL samples. This suggests a reduction in cell-cell communication between maternal endothelial cells. In this location, no difference between placentome sexes (FHL versus MHL) was observed (Figure 5.8, C, D). It is thought that GJA1 plays an important role in neovascularization and support of angiogenesis in early gestation (Winterhager et al., 2013). Previous investigation of the same samples in the current study reported that the proportion of placentome area covered by blood vessels was greater in HPeri exposed 98 dpc placentomes compared with LPeri counterparts (Edwards, 2016). However, Edwards (2016) reported no differences in blood vessel area, total area, number or perimeter. 50% of LH and LL exposed placentomes expressed *GJA1* in maternal vasculature. A possible implication of altered GJA1 protein expression is altered placental transfer of metabolites such as ATP and glucose which are known to be transported through gap junctions, particularly GJA1 (Cx43) (Goldberg et al., 2002). However, the extent of GJA1 and gap-junction transport of glucose and other small molecules and ions in the placenta is unknown. In a previous mouse study, *GJA1* gene expression was deleted in murine uterine epithelial cells expressing the prolactin receptor (Laws et al., 2008). Previously, 50% of *GJA1*-ablated mice were infertile despite mating normally (Laws et al., 2008). It is possible that other connexins are involved in maintaining the function of gap junctions in the placentome. The study by Laws and colleagues (2008) did not investigate uterine expression of other connexins. Additionally, gap junctions are known to facilitate communication between cells (Dunk et al., 2012, Winterhager et al., 2000). It is possible that differential expression of *GJA1* may affect the number of or properties of connexons between maternal and fetal cells. Finally, GJA1 was previously

suggested to influence trophoblast proliferation (Nishimura et al., 2004, Winterhager et al., 2000). The proportions of UTCs and BNCs in 98 dpc placentomes of the current study were previously analysed by Edwards (2016), who reported no differences in proportion between maternal dietary interventions. This conflicts with previous suggestions of GJA1, although it is possible that the level of differential expression did not produce a phenotypic response in UTC and BNC proportions.

5.4.5 FOXO4 Localisation in the 98 Days Postconception Bovine Placentome

FOXO4 protein was consistently expressed in the fetal stroma, within feto-maternal junctions, and fetal villus (Figure 5.9). Previous understanding of the role of the transcription factor is predominantly in oxidative stress/hypoxia (Araujo et al., 2011, Ho et al., 2010, Nakayoshi et al., 2014, Tang and Lasky, 2003). Previous human immunohistochemical localisation of FOXO4 indicates presence in trophoblast cells, and identified the role of FOXO4 in ECM remodelling (Lappas et al., 2010). The consistent localisation, and thus spatial regulation of FOXO4 protein expression in the fetal stroma of feto-maternal junctions and villus supports this, as these regions are expected to be remodelling. There is limited evidence in the literature for the presence of FOXO4 in the bovine placentome. The majority of previous studies were in humans, however a genome wide study of the human placenta indicated that FOXO4 may play an important role in placental physiology, being highly expressed compared with other tissues (Saben et al., 2014b). Transcriptomic analysis of the bovine conceptus at 16 dpc reported *FOXO4* expression (Weldenegodguad, 2013). In the current study, FOXO4 was localised in trophoblast cells (Figure 5.9). This is in agreement with Lappas and colleagues (2010). FOXO4 localisation has previously been shown to be nuclear and cytoplasmic in the placenta (Saben et al., 2014b). This is in agreement with results of the current study, although findings from the current study show a higher percentage of samples expressed cytoplasmic FOXO4 compared with nuclear expression (Figure 5.9, A). Interestingly, differences in expression between diets for nuclear FOXO4 expression were observed. Placentomes exposed to low protein in the postconception period presented FOXO4 expression in the BNC nuclei, whereas no such expression was observed for placentomes exposed to high protein in postconception (Figure 5.9, A, B). The possible impact of FOXO4

nuclear localisation in LPost exposed placentomes, may be increased transcription of genes, which are mediated by FOXO4 expression in BNCs. Results indicate that FOXO4 expression is not sexually dimorphic in the 98 dpc bovine placentome (Figure 5.9, G). This suggests that FOXO4 plays an important role in placentome physiology and is not involved in structural differences between female and male placentomes. Although no bovine literature supports this finding, the human study by Saben and colleagues (2014b) is in agreement.

5.4.6 Notch1 Localisation in the 98 Days Postconception Bovine Placentome

Notch1 protein immunohistochemical localisation in the bovine placenta is absent from the literature. Comparisons were therefore guided by mouse and human placental localisation, which raises many molecular and cellular discrepancies. Further to structural differences, a recent study reported that bovine and mouse trophoblast transcriptomes differed greatly (Pfeffer et al., 2017). Therefore, caution was taken when discussing the putative functions and biological implications of Notch1 localisation in the bovine placentome.

It is well established that Notch signalling is vital for embryonic development and cell fate in many tissues, since *Notch1* homozygous-null mice did not survive to term (Hellström et al., 2007). Notch1 may be involved in aspects of the bovine placenta such as vascular development, and cell-cell signalling, potentially indicating a role in branching of the fetal villus. Previously, *Notch1* inhibition decreased trophoblast migration and invasion (Yu et al., 2014). In the current study no pattern of Notch1-immunopositivity was observed in BNCs between the discrete cellular contexts in feto-maternal junctions, as was observed in SerpinB1 localisation (Table 5.4). The lower percentages of MHL and MLH exposed placentomes expressing Notch1 in BNC cytoplasm may suggest a difference in BNC migration activity in MHL and MLH versus MHH and MLL samples, which all presented Notch1 immunopositivity in BNC cytoplasm (Figure 5.10, D). This may compliment SerpinB1 results, and its putative role in BNC migration (Chapter 5.3.1, Figure 5.11). Furthermore, irrespective of SerpinB1 expression, MHL and MLH had an equal percentage of stromal localised BNCs (Table 5.5). Although MHL exposed placentomes had a 5.56% lower percentage of BNCs localised in stromal villi and a 7.52% greater percentage of stromal epithelial localised BNCs (Table 5.5). In male

98 dpc placentomes, there were no differences in the percentage of epithelial localised BNCs between dietary interventions (MHH, MHL, MLH, ML) (Table 5.5). Therefore, it is unlikely that the qualitative variation in Notch1 protein localisation in BNC cytoplasm was linked to BNC migration.

In vascular development, *Notch1* is essential for normal vascular development, as *Notch1* knockdown leads to embryonic lethality. However, it is possible that *Notch1* works in part with *Notch4,* as double deletion of both *Notch -1* and *-4* is more detrimental to vascular development than *Notch1* deletion alone. The consequences of insufficient vascularisation are reduced nutrient, growth factor, hormone and oxygen delivery to the tissues (Limbourg et al., 2005). Eifert and colleagues (2015) observed that ovine maternal nutrient restriction decreased placental vasculature. As nutrient and gaseous transfer of the placenta is via the circulation, normal vascular development is important for fetal development. Kofler and colleagues (2015) reported that *Notch1* heterozygous null mice displayed reduced pericyte and endothelial cell interactions. Endothelial cell quiescence was reduced as a result of reduced pericyte coverage, and overall abnormalities in angiogenesis. Notch signalling plays a role in correct vasculature maturation (Kofler et al., 2015). Considering previous findings , localisation of Notch1 protein in the current study may also indicate improper vascular development if localisation is low in maternal and fetal angiogenesis, however, the study by Kofler and colleagues (2015) is not directly comparable to the current study. Firstly, Kofler and colleagues (2015) studied notch in *in vitro* murine eyes, while the current study was *in vivo,* in the bovine placenta. In the current study, the reduced percentage of samples exhibiting Notch1 localisation, indicates that vascular development of placentomes may be reduced. Subsequent effects of reduced *Notch1* localisation may suggest that placentome transfer of nutrients and gaseous exchange is impaired. Furthermore, Notch1 protein was localised in human adult epithelia in various organs, including the mammary gland, and arterial vascular endothelial cells (Baldi et al., 2004). This is supporting evidence for Notch1 expression in maternal epithelial cells in the bovine placenta at 98 dpc. Notch1 signalling may also be involved in placental angiogenesis.

The function of *Notch1*-Dll4 signalling in branching morphogenesis is to control tip cell formation (Hellström et al., 2007). This signalling regulates vascular sprouting and branching morphogenesis (Hellström et al., 2007).

The specific role of *Notch1* is dependent on ligand interactions. However, *Notch1* ligand expression in the current study was not identified as differentially expressed between maternal dietary interventions, therefore the focus was on the effect of *Notch1* localisation in specific placental cell types. *Notch1* deletion in endothelial cells increased branching morphogenesis (Hellström et al., 2007). This suggests that absence of *Notch1* in vascular endothelial cells may increase branching morphogenesis. Reduced *Notch1* expression in vascular endothelial cells may coincide with increased vascularisation. It was previously reported that *Notch1* expression in the placenta decreases throughout gestation (Limbourg et al., 2005). Bovine placentome localisation of Notch1 in literature is limited. Although the biological significance of *Notch1* expression is partially dependent on which ligands bind, the presence or absence of Notch1 protein would also affect angiogenesis. Immunofluorescence or colourimetric analysis may elucidate further the interactions between Notch1 localisation and vessel structure. Notch1 protein localisation was identified above the basement membrane and in human umbilical-cord epithelial cells (Ng et al., 2008). This is consistent with epithelial localisation in the current study. In areas requiring oxygen and nutrients, signals including VEGFs induce angiogenesis. Levin and colleagues (2017) reported *Notch1* localisation in maternal and fetal vasculature at E12.5. This stage of gestation is mid first trimester, thus is not directly comparable to the end of the first trimester. However, these findings support results of the current study as *Notch1* was found to be present in vasculature. It also supports findings in the literature that *Notch1* is present throughout gestation. Maternal capillary endothelial cells expressed Notch1 protein (Levin et al., 2017). This agrees with the current study, which identified Notch1-immunopositivity in maternal vascular endothelial (Figure 5.10). In early human gestation, Notch1 was expressed at the feto-maternal interface (Sarikaya and Jerome-Majewska, 2011). This agrees with Notch1 localisation in all cellular contexts of the feto-maternal junction.

5.5 Conclusions

Each protein of interest was localised within the 98 dpc bovine placentome. Cellular localisation of each protein of interest was identified, offering new insights into protein expressions within the first trimester bovine placentome. These protein localisations therefore further validate previous work which identified expression of the genes encoding these proteins. Further to the aims of this chapter, the putative functions of each protein were postulated.

Irrespective of protein localisation, results suggest that the proportion of BNCs in each cellular context differed between fetal sex and maternal diet. SerpinB1 was localised in all analysed cellular contexts in qualitative analysis. SerpinB1 immunopositivity was found to be spatio-temporal within BNCs, where the majority of BNCs fused with the epithelium expressed SerpinB1. Furthermore, SerpinB1 protein expression was consistently observed in vasculature. GATM localisation indicated creatine synthesis capability, particularly in the fetal villus. While STRA6 localisation suggested retinoid transport across the feto-maternal junction and through both maternal and fetal vasculature, agreeing with previous rudimentary investigation of retinoid transport in the ovine placenta.

GJA1 immunopositivity was ubiquitously localised in the 98 dpc bovine placentome, which although this is a novel finding within this organ and species, agrees with existing understanding in other mammalian organs. FOXO4 was localised in the fetal stroma, within feto-maternal junctions, and fetal villus. Further investigation is required to elucidate the exact functions of FOXO4 in each placental location. Notch1 immunopositivity was localised to BNC and UTC cytoplasm, which suggests possible Notch1 signalling occurred between adjacent trophoblast cells. The implication is unclear, although based on previous knowledge, Notch1 is likely involved in vascular development and cell-cell communication events. Overall, this chapter achieved the original aims, and although it was beyond the scope of this study, explored possible functions of each protein and postulated potential implications in the bovine placenta.

6 The Effects of Maternal Dietary Intervention on Term Cotyledon Protein Localisation

6.1 Introduction

The placenta is a vital transient organ during gestation and has a vital function at parturition. It is essential that the fetal and maternal tissues formed over gestation separate at parturition. At parturition, the maternal caruncles are not expelled, while the fetal membranes, the chorioallantois and cotyledons, are normally expelled (Schlafer et al., 2000). Retained placenta is an abnormality with reported incidences ranging between 3-39% of dairy calvings (Kimura et al., 2002, Laven and Peters, 1996, Schlafer et al., 2000). Immunosuppression and insufficient caruncle-cotyledon separation are some known causes of retained placenta (Laven and Peters, 1996). Retained placenta is more common in cows of aborted or premature calving, twins, and induced parturition (Schlafer et al., 2000). Specific nutrient deficiencies such as Vitamin E, selenium and have also been associated with retained fetal membranes (Beagley et al., 2010).

Before parturition, the BNC population normally decreases greatly (Schlafer et al., 2000). Immune activity is thought to activate the uterus from a quiescent pregnancy maintaining state to prepare for parturition (Christiaens et al., 2008, Attupuram et al., 2016). Immune action is also thought to stimulate the degradation of the ECM and subsequently release fetal membranes (Schlafer et al., 2000, Attupuram et al., 2016). It is unknown whether BNCs play a role in parturition or separation of maternal and fetal membranes (Schlafer et al., 2000). Bovine parturition is initiated by fetal maturation and rising fetal cortisol levels (Attupuram et al., 2016). These fetal signals activate the heifer's or cow's HPA-axis (Attupuram et al., 2016). Down-stream effects are increased oestrogen, PGF2α, relaxin, and oxytocin, and decreased progesterone (Attupuram et al., 2016).

In the current study, term placentome consisted of cotyledonary (fetal) tissue only. Immunohistochemical protein expression of genes differentially affected by maternal dietary intervention in 98 days postconception (dpc) placentomes (caruncular and cotyledonary) was investigated (Chapter 5). Further analysis of protein expression and localisation in term cotyledons was performed. This analysis determined protein expression at term and

allowed immunohistochemical comparison between the two time-points within cotyledonary tissue. Structural analysis of term cotyledons was previously performed (Edwards, 2016).

6.1.1 Term Cotyledon Structure and Composition

A summary of research performed by Edwards (2016) on samples also included in the current study was necessary in order to consider protein expression and localisation, with tissue structure and composition. Cotyledon volume (ml) was unaffected by maternal dietary intervention, when separated by placentome sex. In LH exposed cotyledons, blood vessel perimeter was greater, versus HL exposed counterparts (Edwards, 2016). There was no effect of maternal dietary intervention on blood vessel size (individual blood vessel area), perimeter, number, or proportion of cotyledon area containing blood vessels (Edwards, 2016).

Periconception low protein (LPeri) exposed term cotyledons had a greater total vascular area versus high counterparts (HPeri) (Edwards, 2016). Overall, cotyledons exposed to LPeri had a greater proportional area of vasculature than HPeri counterparts (Edwards, 2016). Postconception low protein (LPost) exposed term cotyledons had a smaller blood vessel size versus HPost counterparts (Edwards, 2016). However, unlike periconception diet, there was no effect of postconception diet on the total vascular area in term cotyledons (Edwards, 2016). The proportion of collagen in term cotyledons was unaffected by dietary intervention (Edwards, 2016). The percentage of BNCs decreased to around 5% in term cotyledons compared with 98 dpc placentomes, and was unaffected by dietary intervention at term (Edwards, 2016). The above information aids comprehension of the results and rationale behind undertaking the protein expression and localisation study. Literature reviews of the proteins of interest were performed (Chapter 5). Additional literature reviews relevant to parturition are discussed below for SerpinB1, GATM, STRA6, GJA1, FOXO4 and Notch1.

6.1.2 The Role of SerpinB1 at Parturition

Matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPS) play a role in the onset of bovine parturition, through two mechanisms (Attupuram et al., 2016). One mechanism is via neutrophil

secretion of MMP2 and MMP9 (Xu et al., 2002). MMPs perform proteolysis of the ECM and consequent expulsion of bovine fetal membranes (Attupuram et al., 2016). In this case, proteolysis is beneficial to normal parturition, therefore inhibitors of neutrophil proteases such as SerpinB1 should not be required in this mechanism. Increased SerpinB1 expression in bovine postpartum uteri has also been associated with bacterial uterine infection (Ledgard et al., 2015).

6.1.3 GATM in Placentae

In human 196 dpc placentae, mRNA expression of *GATM* was reportedly decreased in *in utero* growth-restricted placentae compared with non *in utero* growth-restricted placentae (McMinn et al., 2006). The biological implication of this is currently unknown. However, it is thought that GATM plays a role in maternal energy preservation (Sandell et al., 2003). Therefore, in humans, when the intra uterine environment is nutrient restricted, the result is a decrease in *GATM* expression in the placenta. If this is also the case in cows, GATM protein expression may be reduced in low protein exposed bovine placentomes and is worth studying as a protein of interest. Additionally, one study reported *GATM* relative mRNA expression was on average lowest in early gestation, peaked at mid-gestation and moderate in late gestation (Uusküla et al., 2012). Furthermore, *GATM* mRNA expression was increased in all human placentae with a complicated pregnancy compared with control placentae (Uusküla et al., 2012). This suggests that complicated pregnancies were not nutrient restricted. Complicated pregnancies included gestational diabetes mellitus, preeclampsia, and small or large for gestational age new-borns, (n=12 per group) (Uusküla et al., 2012).

6.1.4 The Function of STRA6 in Placentomes

The role of STRA6 in the bovine placenta is largely unknown, therefore there is a lack of existing evidence for STRA6 in bovine placentomes, at any gestational stage. Vitamin A is vital for embryogenesis (Rothman et al., 1995, Zile, 2001). Retinol transfer to the fetus may be altered by a switch in maternal diet at 23 dpc. Sex-specific *STRA6* expression was reported in term human placentae, in a dietary intervention study where the intervention group consumed a reduced omega-6:omega-3 ratio (Sedlmeier et al., 2014).

STRA6 expression was down-regulated in the intervention group compared with the control in both male and female placentae (Sedlmeier et al., 2014). This is evidence for maternal dietary intervention influence on placental STRA6 expression. *Negative* energy balance and consequent ketosis may occur as a result of nutrient restriction in cattle (Loor et al., 2007, Grummer, 1995).

Ketosis is a metabolic disorder which is associated with hepatic lipidosis and negative energy balance, where the diet does not meet the energy requirements of the animal (Loor et al., 2007). In peripartal dairy cows, a recent study reported 40% higher serum retinol concentrations in non-ketotic cows compared with ketotic cows (Rodriguez-Jimenez et al., 2018). This suggested higher inflammation in ketotic cows, as low retinol is an indication of inflammation (Rodriguez-Jimenez et al., 2018, Goff et al., 2002). In relation to the current study of STRA6 in term cotyledons, STRA6 expression is of interest as it may be a putative placental adaptation to regulate retinol transport in response to maternal serum retinol levels. Vitamin A is lipid-soluble and can be toxic at high levels (Fanali et al., 2017). Therefore the placenta may regulate transfer to the fetus to avoid toxicity, as previously reported in sheep (Donoghue et al., 1982). STRA6 expression in the placenta may therefore modulate maternal-fetal transfer of retinol. Considering the maternal dietary interventions in the current study, there may be a link between maternal diet-induced alterations of maternal serum retinol and STRA6 expression. The placental expression of STRA6 may be both a protective mechanism, to avoid fetal retinol toxicity, but also may be retinol 'scavenging' to ensure fetal requirements. As retinol is transported via the circulation, *STRA6* gene expression and subsequent STRA6 protein expression may be altered in the placental vasculature, but also within the trophoblast layer in placentomes. However, maternal serum was not analysed in the current study.

In term cotyledons, STRA6 protein expression may be altered according to maternal diet in the following ways. Firstly, in heifers fed the periconception low protein, the diet met 85% of the energy requirements (Table 2.2). There is potential for a negative energy balance, however no data on the heifers was available in terms of body composition or blood samples. In the case where dietary energy is lower than requirements, maternal plasma retinol concentrations could be expected to be lower than that of non-ketotic cows.

Heifers fed HH are likely to have been non-ketotic with subsequently higher plasma retinol levels compared with periconception low protein fed heifers. Placental STRA6 expression may increase in heifers fed the periconception low protein diet inducing ketosis (low retinol), in order to ensure adequate retinol transfer to the fetus. Secondly, placental STRA6 expression may decrease in heifers fed a diet (HH) which does not induce ketosis (high retinol), in order to protect against fetal retinol toxicity.

6.1.5 The Role of GJA1 at Parturition

Literature searches for GJA1 (Cx43) specific to the bovine term placentome yielded few results. Therefore, human and mouse studies were the focus of comparative discussion. Simultaneously being aware that the trophoblast transcriptome between mouse and bovine were shown to differ (Pfeffer et al., 2017). Bovine placentome *GJA1* expression was greater at 220 dpc compared with 120 dpc (Pfarrer et al., 2006). *GJA1* expression was increased in the maternal stroma in response to trophoblast invasion. In bovine 240 and 270 dpc placentomes, *GJA1* mRNA was present in caruncular connective tissue and to a lesser amount in the fetal mesenchyme (Pfarrer et al., 2006). 270 dpc is close to term and comparable with term samples in the current study which ranged between 270-293 dpc. In humans and rodents, GJA1 expression and subsequent gap-junction connections were shown to be important in the initiation of parturition (Dong et al., 2009, Cluff et al., 2006, Bernal, 2007, Tong et al., 2009, Shynlova et al., 2013). However, considering the large differences in placental structure between bovine synepitheliochorial and hemochorial placentae, it is unclear whether GJA1 is of equal importance for bovine parturition.

6.1.6 The Role of FOXO4 in Term Placentomes

FOXO4 was not previously reported in bovine term placentomes. In human placentae, cytoplasmic FOXO4-immunopositivity was localised in syncytiotrophoblasts, but not cytotrophoblasts or endothelial cells (Lappas et al., 2010). Lappas and colleagues (2010) obtained tissue from elective caesareans prior to onset of natural labour in women. In other human studies, *FOXO4* mRNA was present in human term placentae samples and was highly expressed (Lappas et al., 2010, Saben et al., 2014b). The high

expression of *FOXO4* in the placenta in comparison to other tissues may suggest a specific role of FOXO4 in placental function (Saben et al., 2014b).

At human parturition, placental *FOXO4* reportedly regulated *COX2* and prostaglandin expression (Lim et al., 2012). Human parturition reportedly down-regulated cytoplasmic FOXO4 mRNA expression in the chorion compared with preparturient chorions (Lim et al., 2012). FOXO4 protein was immunohistochemically localised in the chorionic trophoblast (Lim et al., 2012).

6.1.7 Notch1 in Placentae

At term, *Notch1* expression in human trophoblast cell cytoplasm was reported to decrease compared with the first trimester (De Falco et al., 2007). *Notch1* was localised in endothelial cell cytoplasm. *Notch1* was localised in fetal villi stroma (De Falco et al., 2007). At term, strong positive staining was present in the trophoblast membrane (De Falco et al., 2007). Furthermore, De Falco and colleagues (2007) reported cytoplasmic localisation of *Notch1* in trophoblast cells invading the endometrium, suggesting that trophoblast binucleate cells in the bovine may express Notch1 protein. Evidence for SerpinB1, GATM, STRA6, FOXO4, and Notch1 localisation in the bovine placenta is lacking, therefore the current study will increase existing knowledge.

The aims of this chapter were to 1) determine and characterise the localisation of the proteins of interest detailed above in the term fetal cotyledonary tissue, and 2) investigate the effect of maternal dietary intervention, and fetal sex, on protein localisation.

6.2 Materials and Methods

SerpinB1, *GATM*, *STRA6*, *GJA1*, *FOXO4* and *Notch1* genes were differentially expressed in the 98 dpc bovine placentome (Edwards, 2016). Further validation of previous molecular results, via immunohistochemical protein localisation was performed for the proteins encoded by these genes.

Immunohistochemistry protocols were performed according to the general method (Chapter 2.3) and optimised specifically for each antibody (Table

5.1). Antigen retrieval was optimised to 20 minutes at medium-low microwave settings for STRA6, GJA1, FOXO4, and Notch1. GATM was optimised to 1 minute high, 4 minutes medium. Microscopy and image analysis were also carried out as described (Chapter 2.4). One slide (per sample) containing multiple tissue sections was used in each immunohistochemistry. n=3 samples per sex (Female and Male) and dietary intervention were analysed (FHH n=3, FHL n=3, FLH n=3, FLL n=3; MHH n=3, MHL n=3, MLH n=3, MLL n=3) equalling n=24 samples per antibody. Antibodies used for immunohistochemical analysis of term cotyledons are detailed in Table 5.2.1. Qualitative analysis was performed using approximately 40 fields per slide.

6.3 Results

Fetal membrane expulsion time was unaffected by dietary intervention, sex or sex-diet interactions (Appendix 14). Proteins encoded by differentially expressed genes in 98 dpc placentomes were localised in term cotyledonary tissues (Table 6.1).

Table 6.1 Summary of protein localisation in term cotyledons

Cotyledonary localisation	Protein of interest					
	SerpinB1	GATM	STRA6	GJA1	FOXO4	Notch1
N	*24*	*24*	*24*	*24*	*24*	*24*
Fetal stroma / connective tissue	+	+	+	-	+	+
BNC nucleus	+	-	+	+	+	-
BNC cytoplasm	+	-	+	+	+	+
UTC nucleus	+	-	-	+	+	-
UTC cytoplasm	+	+	+	+	+	+
Vascular endothelial cell	+	-	+	+	+	+

6.3.1 SerpinB1 Localisation in Term Bovine Cotyledonary Tissues

SerpinB1 immunopositivity in term cotyledons was identified in fetal stroma, trophoblasts, and vasculature (Figure 6.1). 100% of samples presented SerpinB1 immunopositivity in stroma and BNCs (Figure 6.1 A). UTC nuclear SerpinB1 immunopositivity varied, and was absent in LL exposed tissues (0-67%) (Figure 6.1 A). Variation in the percentage of samples displaying SerpinB1 immunopositivity was further explained by sex and diet interactions (Figure 6.1 C D). In UTC cytoplasm, a 30% greater percentage

of female samples displayed SerpinB1 immunopositivity than male counterparts (Figure 6.1 C D). Nuclear SerpinB1 immunopositivity in UTCs was absent in FLL, MHH, and MLL samples, and was observed in FHH, MLH (n=1/3, 33%) and FLH and MHL (n=2/3, 67%) samples (Figure 6.1 C D). In vasculature, 30% more LPost exposed samples displayed SerpinB1 immunopositivity than HPost counterparts (Figure 6.1 B). Independent of dietary intervention, the percentage of females displaying SerpinB1 in UTC cytoplasm and vasculature were 24% and 18% greater than males respectively (Figure 6.1 G).

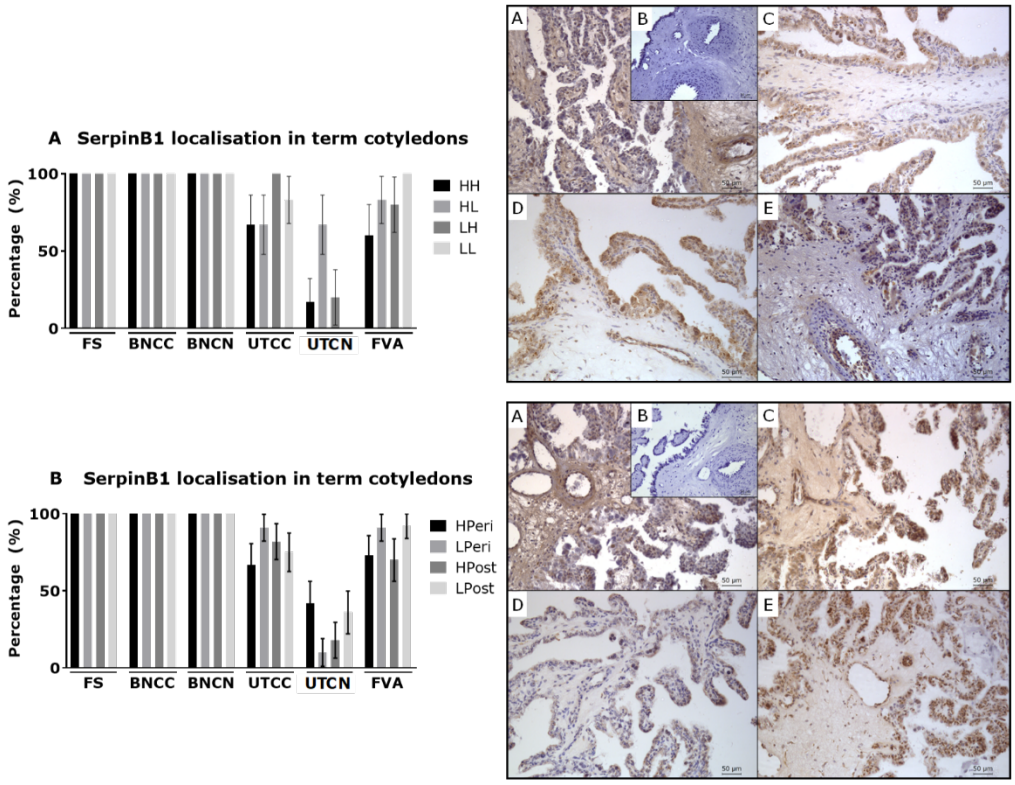


Figure 6.1 The percentage of samples displaying SerpinB1 immunopositivity and the cellular localisations in term cotyledons. Graphs and complimentary photomicrographs (B=negative control, A, C-E represent each graph key, e.g. A=HH, E=LL) display results between maternal dietary interventions (A), periconception, and postconception diets (B). H=high protein, L=low protein. Letters denote periconception and postconception diet, e.g. HL= high protein periconception, low protein postconception. Peri=periconception, Post=postconception, FS=fetal stroma, BNC=binucleate cell, UTC=uninucleate trophoblast cell, FVA=fetal vasculature, C=cytoplasm, N=nucleus. Scale bars represent 50 μm.

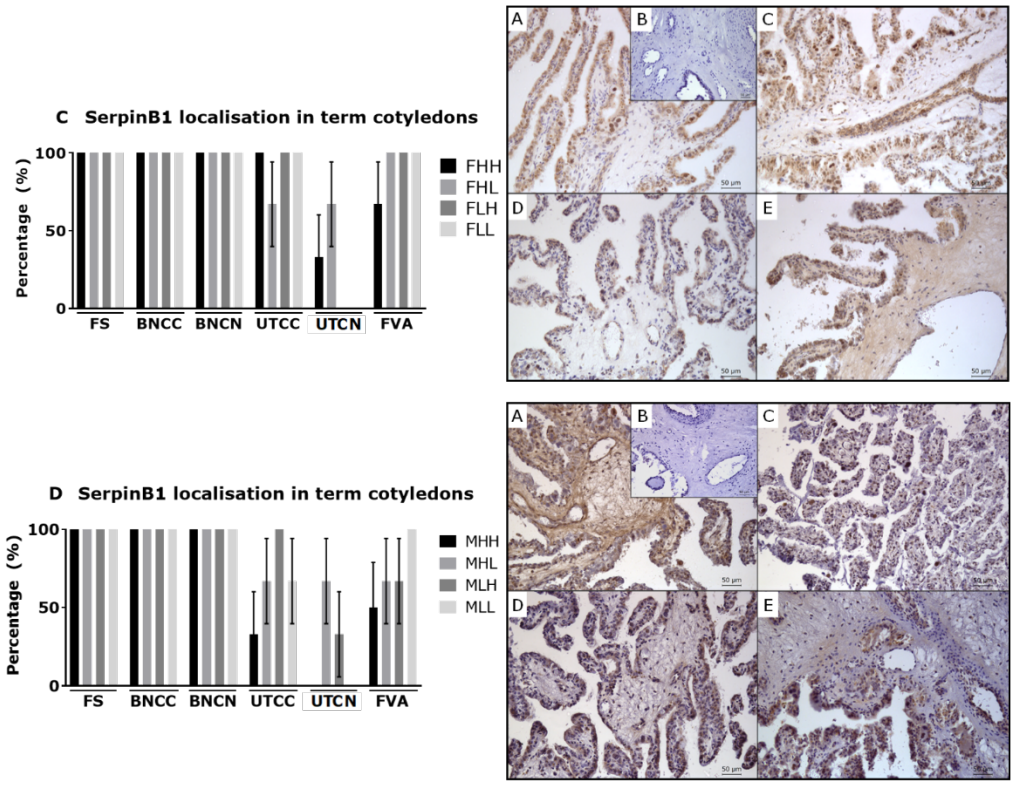


Figure 6.1 The percentage of samples displaying SerpinB1 immunopositivity and the cellular localisations in term cotyledons continued. Graphs and complimentary photomicrographs (B=negative control, A, C-E represent each graph key, e.g. A=FHH, E=FLL) display results between female (C) and male (D) cotyledons exposed to maternal dietary interventions. F=female, M=male, H=high protein, L=low protein. Letters denote periconception and postconception diet, e.g. HL= high protein periconception, low protein postconception. FS=fetal stroma, BNC=binucleate cell, UTC=uninucleate trophoblast cell, FVA=fetal vasculature, C=cytoplasm, N=nucleus. Scale bars represent 50 μm.

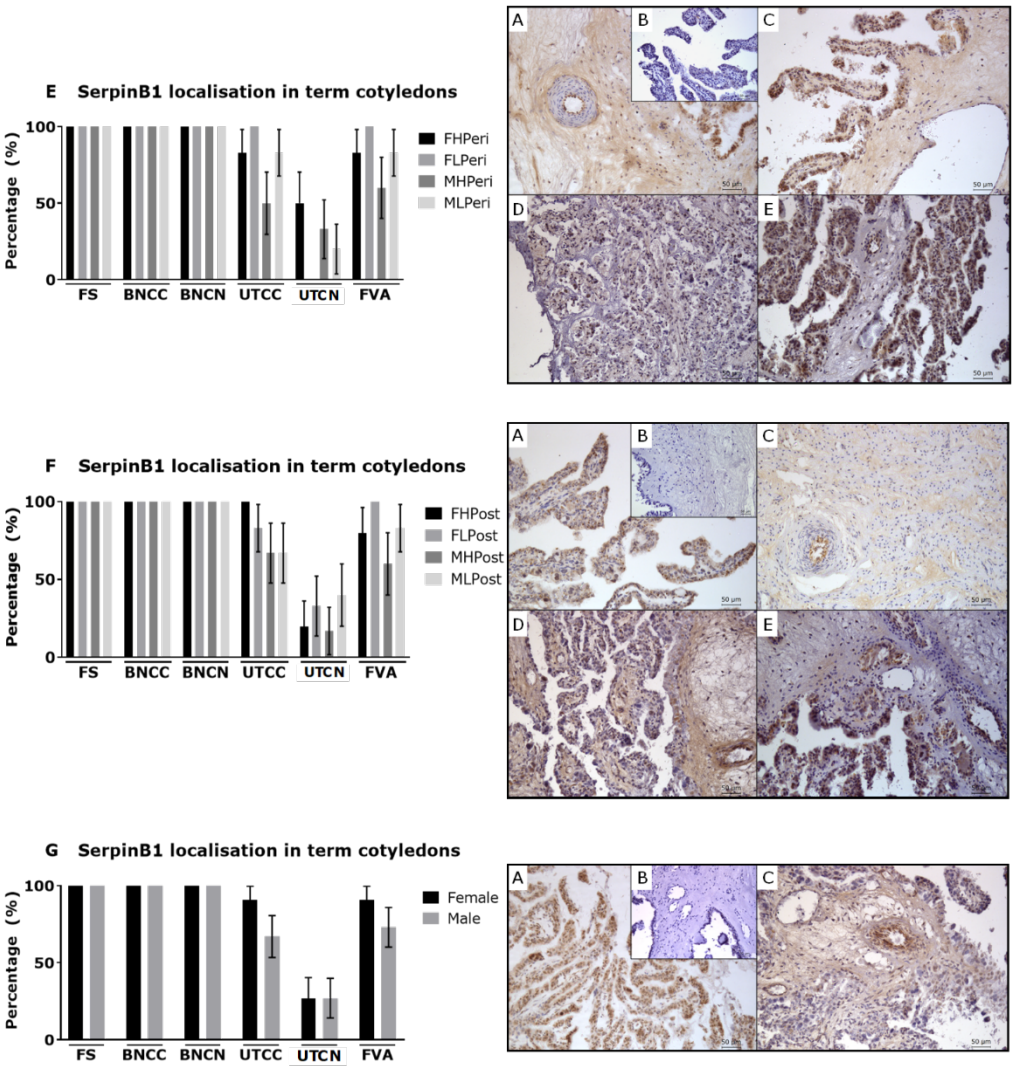


Figure 6.1 The percentage of samples displaying SerpinB1 immunopositivity and the cellular localisations in term cotyledons continued. Graphs and complimentary photomicrographs (B=negative control, A, C-E represent each graph key, e.g. A=FHPeri, E=MLPeri) display results between female and male cotyledons exposed to periconception (E) and postconception diets (F), and female and males (G) independent of dietary intervention. F=female, M=male, H=high protein, L=low protein, Peri=periconception, Post=postconception, FS=fetal stroma, BNC=binucleate cell, UTC=uninucleate trophoblast cell, FVA=fetal vasculature, C=cytoplasm, N=nucleus. Scale bars represent 50 μm.

6.3.2 GATM Localisation in Term Bovine Cotyledonary Tissues

In term bovine cotyledonary tissues, immunohistochemical GATM protein localisation was restricted to the peripheral fetal stroma (0-67%), and UTC cytoplasm (15-83%) in maternal dietary interventions (Figure 6.2 A). In stroma, GATM-immunopositivity did not differ between fetal sexes (33%) (Figure 6.2 G). In stroma, the percentage of GATM-immunopositive samples was 67% greater in HL and LL exposed tissues, compared with HH and LH counterparts (Figure 6.2 A). GATM immunopositivity in stroma was absent (0%) in HPost exposed tissues compared with 67% of LPost, and 33% of HPeri and LPeri exposed tissues (Figure 6.2 B). The percentage of samples displaying GATM-immunopositivity in stroma localisation was 100% in FHL and MLL, whereas the percentage varied in all other groups (0-33%) (Figure 6.2 C D). In UTC cytoplasm, the percentage of GATM-immunopositive samples was high (67-100%) in all maternal dietary interventions (Figure 6.2 A). The percentage of samples presenting GATM-positive UTC localisation was unaffected by postconception diet (83% HPeri versus 83% LPeri) (Figure 6.2 B). In UTC cytoplasm, the percentage of samples displaying GATM immunopositivity was 67% lower in female FHL than FLH, FLL, MHH, MHL, and MLL exposed tissues (Figure 6.2 C D). Overall, GATM-immunopositivity in UTC cytoplasm was 17% greater in males than female tissues (n=11/12, 92% and n=9/12, 75% respectively) (Figure 6.2 G).

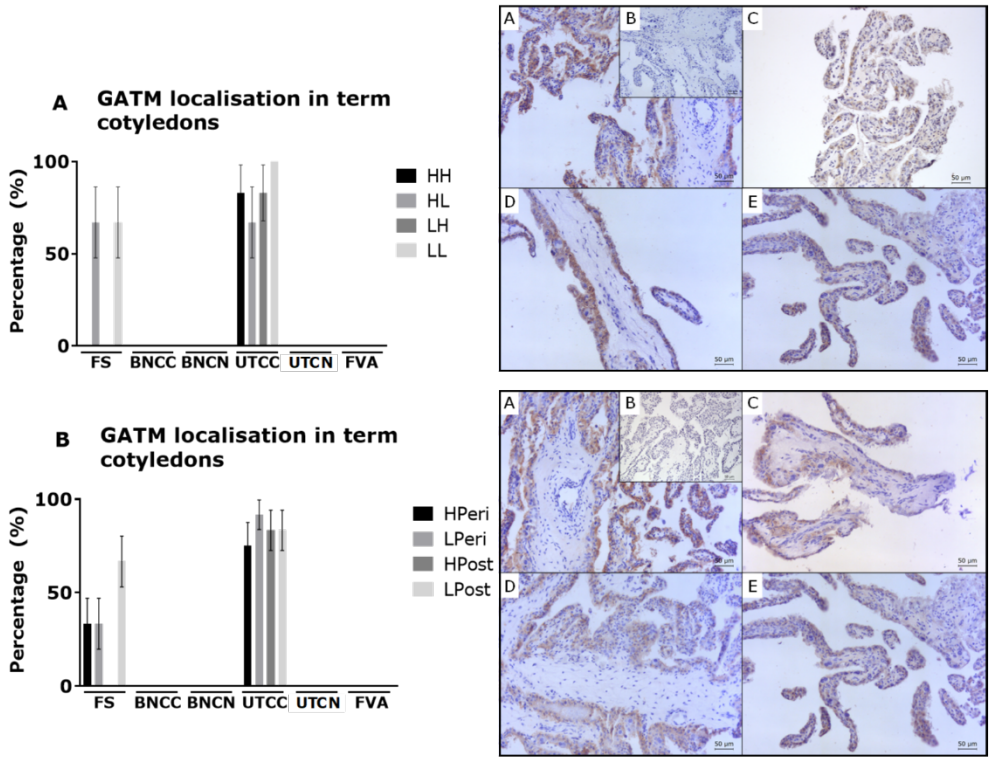


Figure 6.2 The percentage of samples displaying GATM immunopositivity and the cellular localisations in term cotyledons. Graphs and complimentary photomicrographs (B=negative control, A, C-E represent each graph key, e.g. A=HH, E=LL) display results between maternal dietary interventions (A), periconception, and postconception diets (B). H=high protein, L=low protein. Letters denote periconception and postconception diet, e.g. HL= high protein periconception, low protein postconception. Peri=periconception, Post=postconception, FS=fetal stroma, BNC=binucleate cell, UTC=uninucleate trophoblast cell, FVA=fetal vasculature, C=cytoplasm, N=nucleus. Scale bars represent 50 μm.

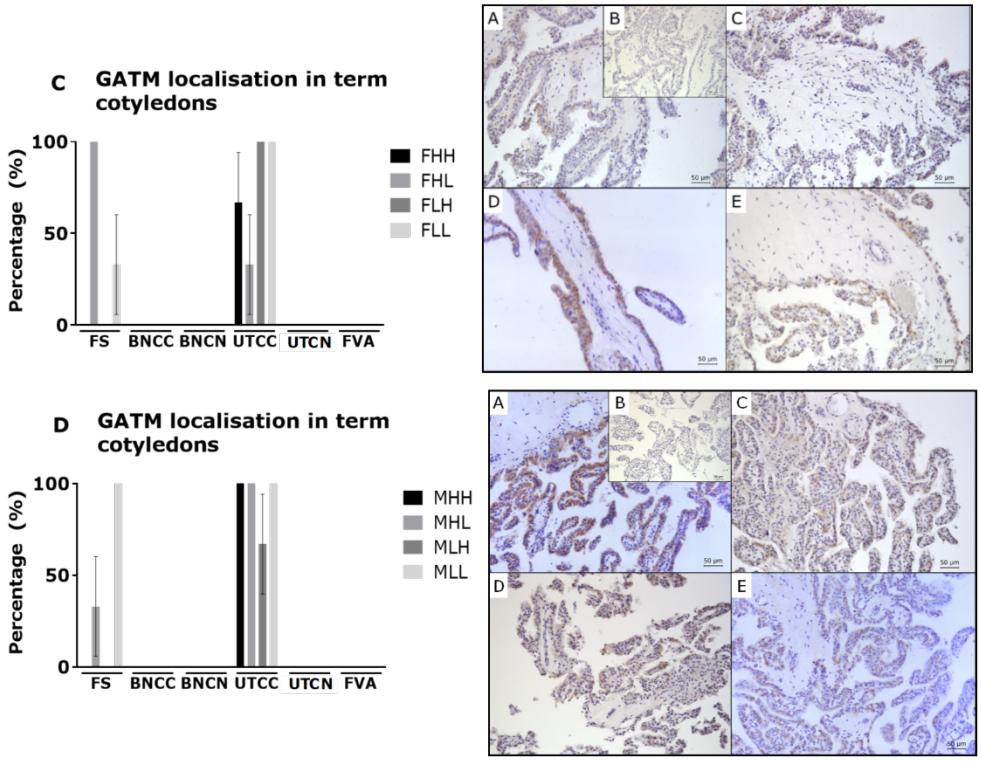


Figure 6.2 The percentage of samples displaying GATM immunopositivity and the cellular localisations in term cotyledons continued. Graphs and complimentary photomicrographs (B=negative control, A, C-E represent each graph key, e.g. A=FHH, E=FLL) display results between female (C) and male (D) cotyledons exposed to maternal dietary interventions. F=female, M=male, H=high protein, L=low protein. Letters denote periconception and postconception diet, e.g. HL= high protein periconception, low protein postconception. FS=fetal stroma, BNC=binucleate cell, UTC=uninucleate trophoblast cell, FVA=fetal vasculature, C=cytoplasm, N=nucleus. Scale bars represent 50 μm.

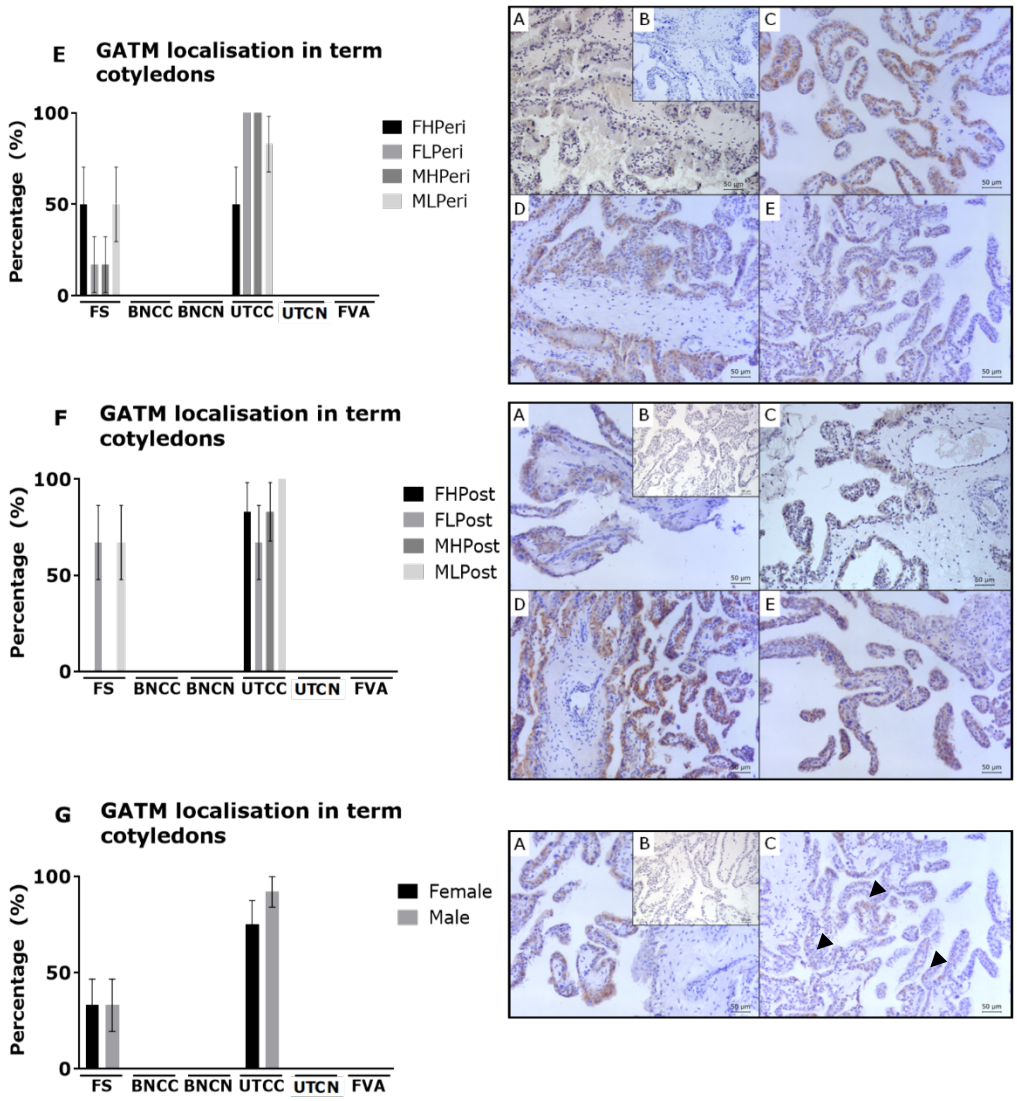


Figure 6.2 The percentage of samples displaying GATM immunopositivity and the cellular localisations in term cotyledons continued. Graphs and complimentary photomicrographs (B=negative control, A, C-E represent each graph key, e.g. A=FHPeri, E=MLPeri) display results between female and male cotyledons exposed to periconception (E) and postconception diets (F), and female and males (G) independent of dietary intervention. F=female, M=male, H=high protein, L=low protein, Peri=periconception, Post=postconception, FS=fetal stroma, BNC=binucleate cell, UTC=uninucleate trophoblast cell, FVA=fetal vasculature, C=cytoplasm, N=nucleus. Arrowheads indicate examples of immunopositivity (brown staining). Scale bars represent 50 μm.

6.3.3 STRA6 Localisation in Term Bovine Cotyledonary Tissues

In term bovine cotyledonary tissues, immunohistochemical *STRA6* protein localisation was observed in BNC nuclei, BNC and UTC cytoplasm, vasculature, and stroma (Figure 6.3). Within stroma, STRA6 immunopositivity was restricted to the periphery of the stroma (Figure 6.3). The percentage differences in stromal localisation were due to FHL sample STRA6-immunopositivity, which was 33% versus 100% of all other sex-diet groups (Figure 6.3 C D). Cytoplasmic BNC STRA6-immunopositivity was 45% lower in males versus females (Figure 6.3 G). In males, BNC STRA6-immunopositivity was restricted to MLH (Figure 6.3, D). BNCs were present in all samples, except one MHH and one FHL sample. In vasculature, STRA6 localisation was absent (0%) in FHH, FHL, and MLH (Figure 6.3 C D). In vasculature, the percentage of LL samples displaying STRA6-immunopositivity was 17%, 50% and 47% greater than HH, HL and LH samples respectively (Figure 6.3 A). The percentage of samples presenting vascular STRA6 localisation was 67%, 100%, and 33% greater in MHH compared with MHL, MLH and MLL exposed cotyledons respectively (Figure 6.3 D). Vascular STRA6-immunopositivity was absent in FHPeri (0%), and was observed in 67% of FLL samples (Figure 6.3 C). The percentage of samples which displayed UTC cytoplasm STRA6-immunopositivity was 33% greater in males than females (Figure 6.3 G). In vasculature, 23% more male samples displayed STRA6 immunopositivity than females (Figure 6.3 G).

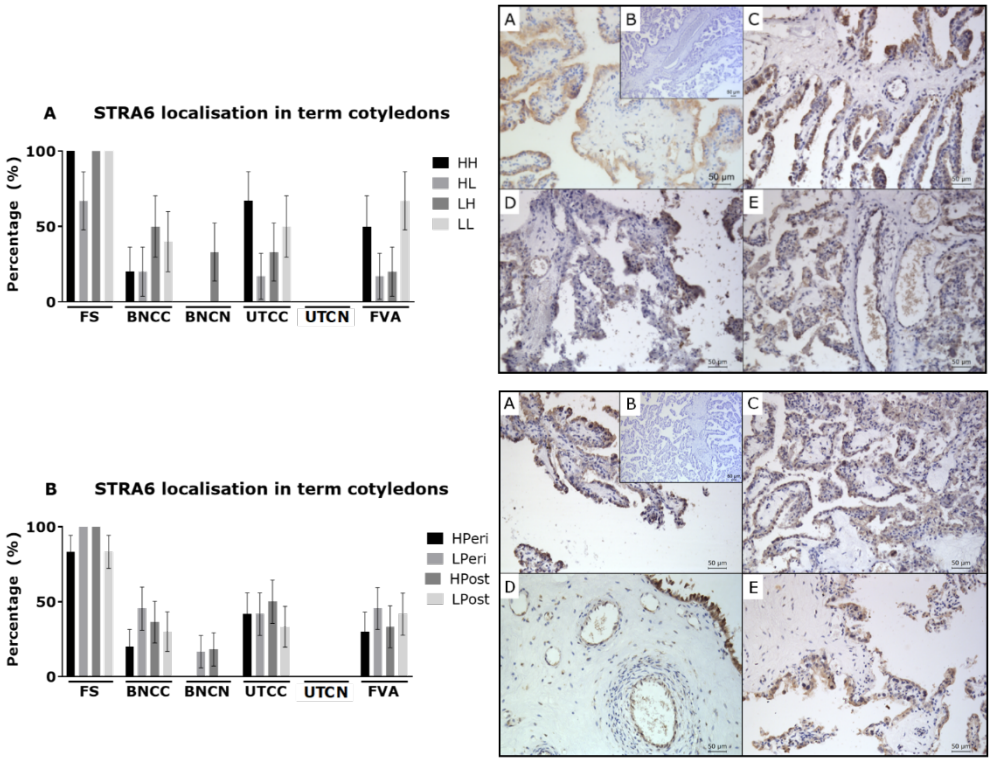


Figure 6.3 The percentage of samples displaying STRA6 immunopositivity and the cellular localisations in term cotyledons. Graphs and complimentary photomicrographs (B=negative control, A, C-E represent each graph key, e.g. A=HH, E=LL) display results between maternal dietary interventions (A), periconception, and postconception diets (B). H=high protein, L=low protein. Letters denote periconception and postconception diet, e.g. HL= high protein periconception, low protein postconception. Peri=periconception, Post=postconception, FS=fetal stroma, BNC=binucleate cell, UTC=uninucleate trophoblast cell, FVA=fetal vasculature, C=cytoplasm, N=nucleus. Scale bars represent 50 μm.

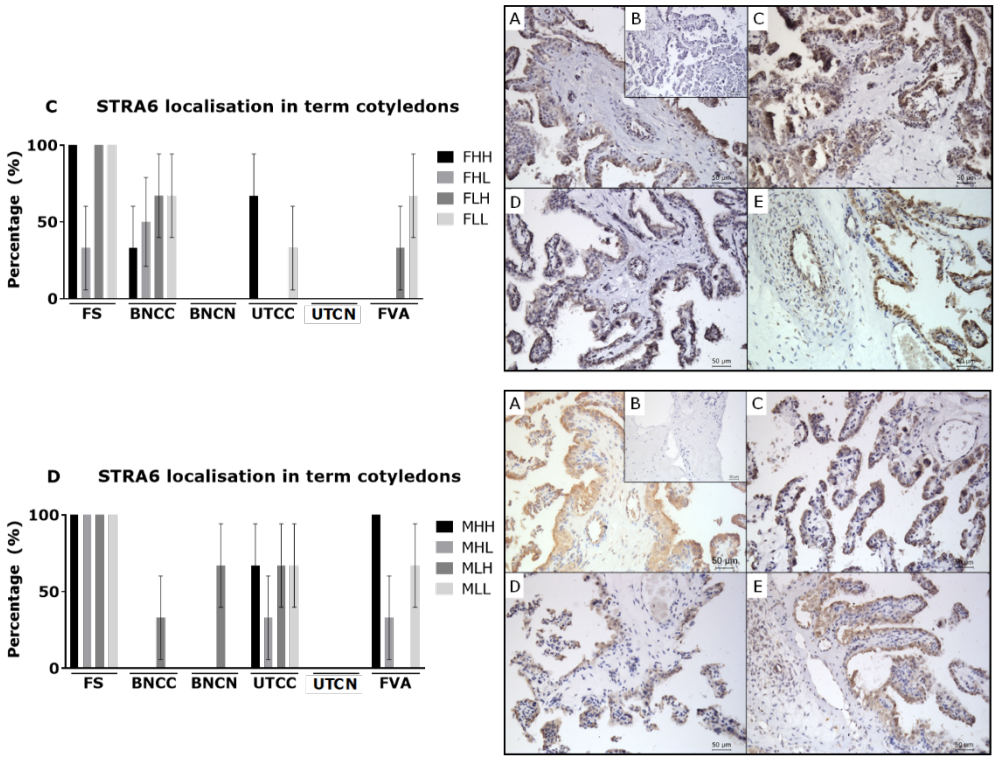


Figure 6.3 The percentage of samples displaying STRA6 immunopositivity and the cellular localisations in term cotyledons continued. Graphs and complimentary photomicrographs (B=negative control, A, C-E represent each graph key, e.g. A=FHH, E=FLL) display results between female (C) and male (D) cotyledons exposed to maternal dietary interventions. F=female, M=male, H=high protein, L=low protein. Letters denote periconception and postconception diet, e.g. HL= high protein periconception, low protein postconception. FS=fetal stroma, BNC=binucleate cell, UTC=uninucleate trophoblast cell, FVA=fetal vasculature, C=cytoplasm, N=nucleus. Scale bars represent 50 μm.

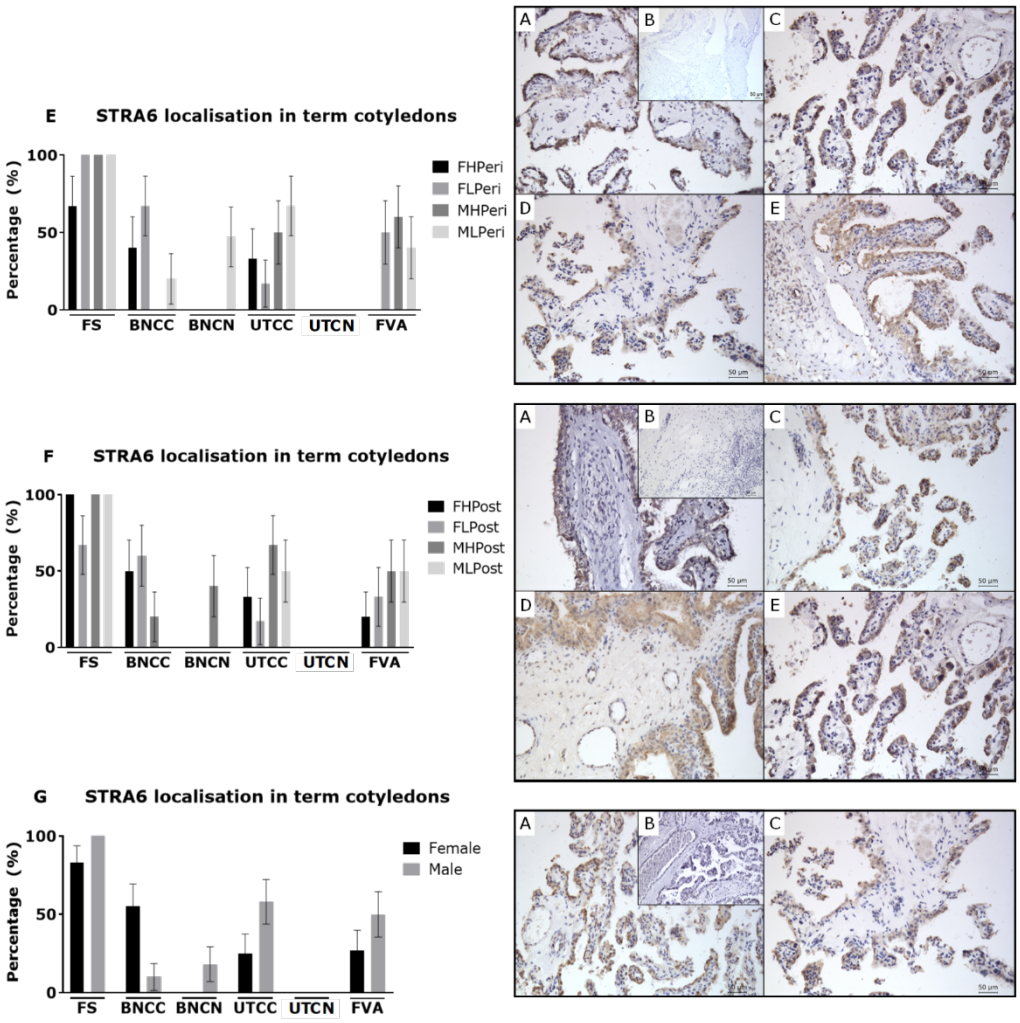


Figure 6.3 The percentage of samples displaying STRA6 immunopositivity and the cellular localisations in term cotyledons continued. Graphs and complimentary photomicrographs (B=negative control, A, C-E represent each graph key, e.g. A=FHPeri, E=MLPeri) display results between female and male cotyledons exposed to periconception (E) and postconception diets (F), and female and males (G) independent of dietary intervention. F=female, M=male, H=high protein, L=low protein, Peri=periconception, Post=postconception, FS=fetal stroma, BNC=binucleate cell, UTC=uninucleate trophoblast cell, FVA=fetal vasculature, C=cytoplasm, N=nucleus. Scale bars represent 50 μm.

6.3.4 GJA1 Localisation in Term Bovine Cotyledonary Tissues

In term cotyledonary tissues, GJA1 protein localisation was ubiquitous in BNC and UTC nuclei and cytoplasm but was absent in stroma (Figure 6.4). The percentage of samples presenting cytoplasmic BNC localisation was 8%, 25% and 16% greater in LPeri than LPost exposed tissues than HPeri and HPost respectively (Figure 6.4 B). The percentage of samples presenting nuclear BNC localisation was 17% greater in LPeri than HPeri and HPost samples (Figure 6.4 B). The percentage of samples which displayed vascular GJA1-immunopositivity was 25% greater in LPeri than HPeri (Figure 6.4 B). The percentage of samples displaying vascular GJA1 localisation was similar (33-41%) between postconception diets (Figure 6.4 B). GJA1-immunopositivity was ubiquitously localised throughout MLL samples (100%), except for stroma (0%) (Figure 6.4 D). BNC nuclear GJA1 localisation was absent (0%) in female cotyledons and ranged from 33-100% in males (Figure 6.4 C D). The cellular location with the highest variability of GJA1-immunopositivity was the vasculature (0-100%) (Figure 6.4). In MHPeri, GJA1 was absent (0%) from vasculature (Figure 6.4 E). In vasculature, FLPeri and FLPost presented 34% lower GJA1-immunopositivity compared to FHPeri and FHPost (Figure 6.4 E F). A greater percentage of males (41-66%) presented GJA1-immunopositivity than females (33%) in UTCs and vasculature, while a similar percentage of females and males (50-58%) displayed GJA1-immunopositivity in BNC cytoplasm (Figure 6.4 G).

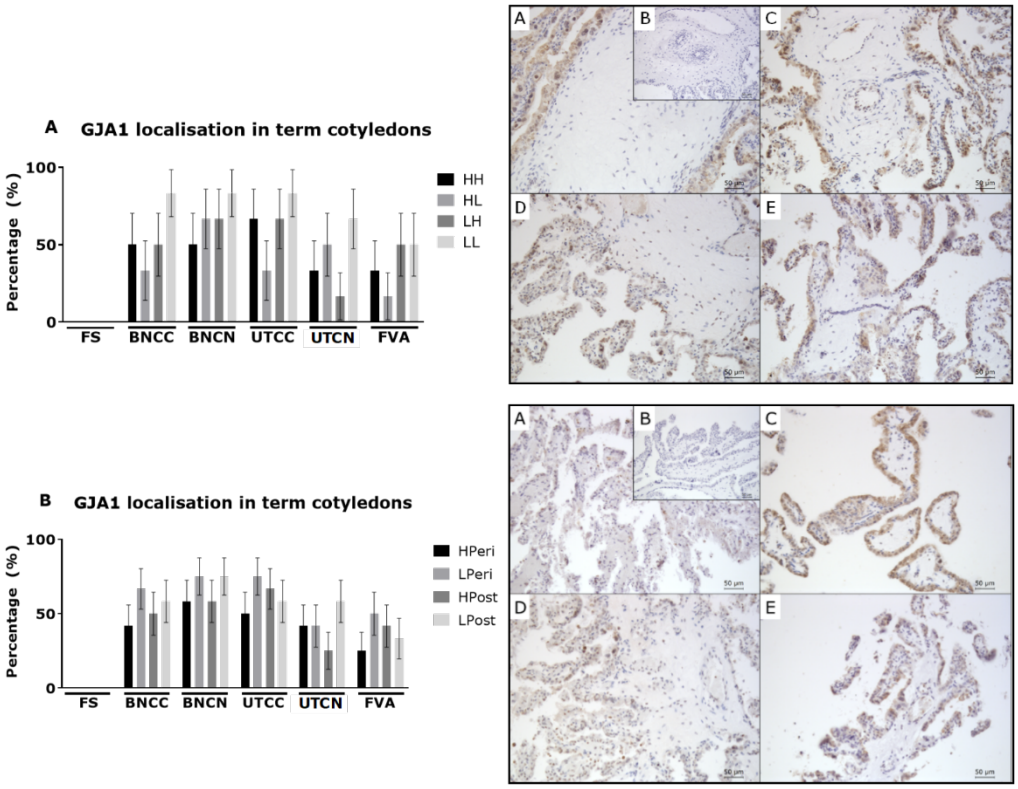


Figure 6.4 The percentage of samples displaying GJA1 immunopositivity and the cellular localisations in term cotyledons. Graphs and complimentary photomicrographs (B=negative control, A, C-E represent each graph key, e.g. A=HH, E=LL) display results between maternal dietary interventions (A), periconception, and postconception diets (B). H=high protein, L=low protein. Letters denote periconception and postconception diet, e.g. HL= high protein periconception, low protein postconception. Peri=periconception, Post=postconception, FS=fetal stroma, BNC=binucleate cell, UTC=uninucleate trophoblast cell, FVA=fetal vasculature, C=cytoplasm, N=nucleus. Scale bars represent 50 μm.

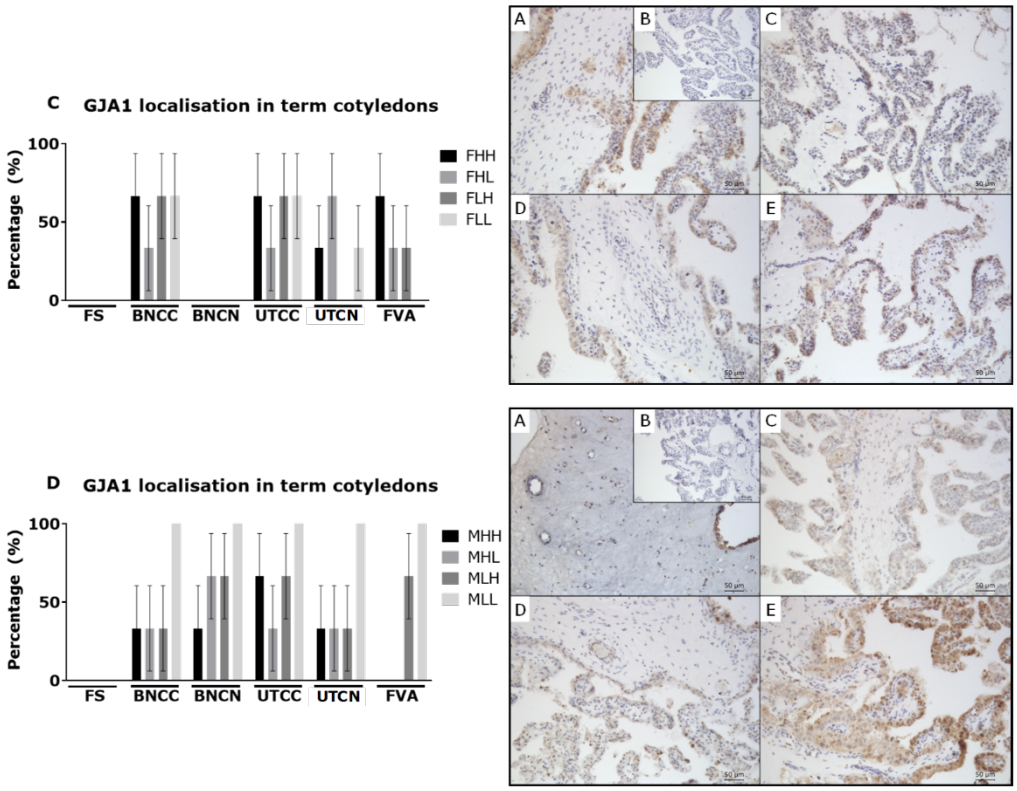


Figure 6.4 The percentage of samples displaying GJA1 immunopositivity and the cellular localisations in term cotyledons continued. Graphs and complimentary photomicrographs (B=negative control, A, C-E represent each graph key, e.g. A=FHH, E=FLL) display results between female (C) and male (D) cotyledons exposed to maternal dietary interventions. F=female, M=male, H=high protein, L=low protein. Letters denote periconception and postconception diet, e.g. HL= high protein periconception, low protein postconception. FS=fetal stroma, BNC=binucleate cell, UTC=uninucleate trophoblast cell, FVA=fetal vasculature, C=cytoplasm, N=nucleus. Scale bars represent 50 μm.

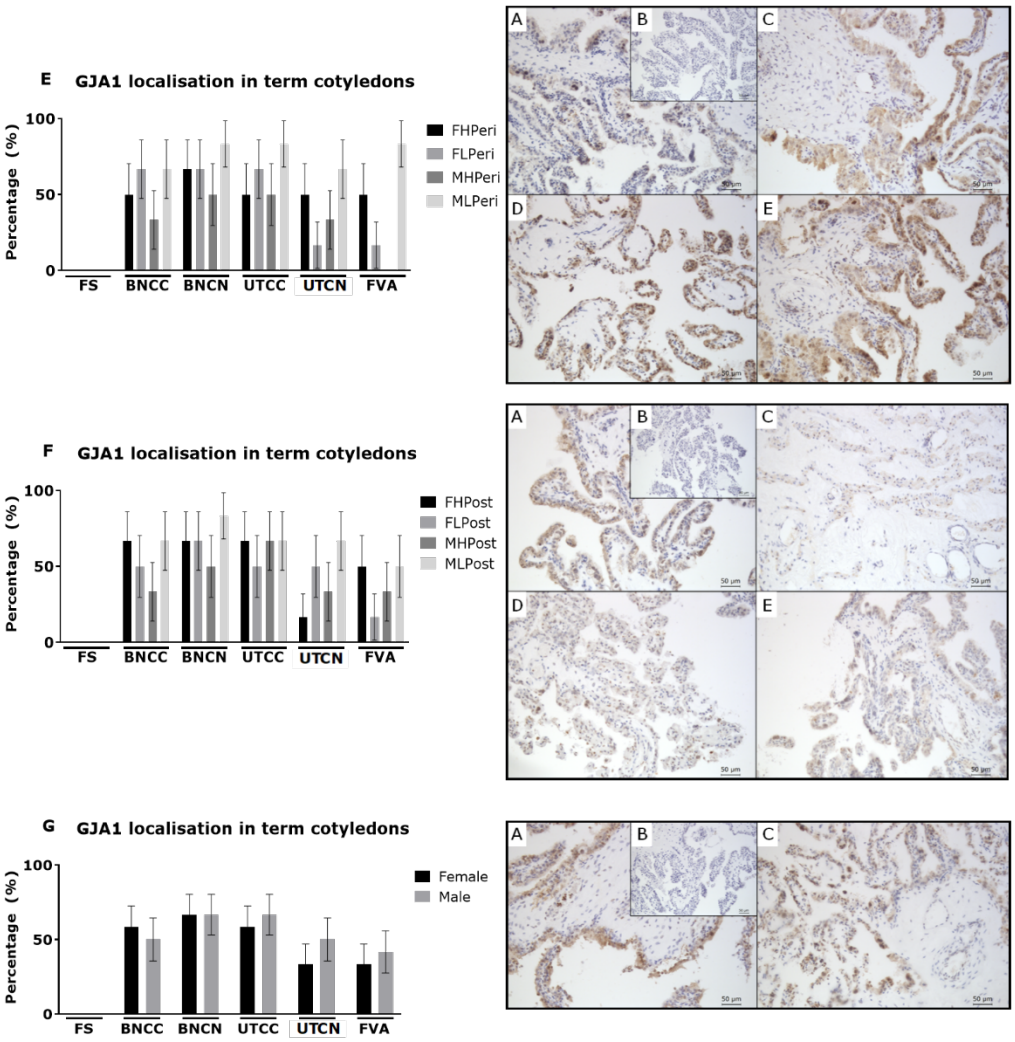


Figure 6.4 The percentage of samples displaying GJA1 immunopositivity and the cellular localisations in term cotyledons continued. Graphs and complimentary photomicrographs (B=negative control, A, C-E represent each graph key, e.g. A=FHPeri, E=MLPeri) display results between female and male cotyledons exposed to periconception (E) and postconception diets (F), and female and males (G) independent of dietary intervention. F=female, M=male, H=high protein, L=low protein, Peri=periconception, Post=postconception, FS=fetal stroma, BNC=binucleate cell, UTC=uninucleate trophoblast cell, FVA=fetal vasculature, C=cytoplasm, N=nucleus. Scale bars represent 50 μm.

6.3.5 FOXO4 Localisation in Term Bovine Cotyledonary Tissues

In term cotyledons, FOXO4 localisation was lowly observed in the stroma, BNC and UTC nuclei, and vascular endothelial cells (Figure 6.5). FOXO4-immunopositivity in trophoblasts was predominantly cytoplasmic (Figure 6.5). 83% of HL and LH exposed tissues displayed UTC cytoplasmic FOXO4-immunopositivity compared with 100% of HH and LL samples (Figure 6.5 A). UTC cytoplasmic FOXO4 localisation was unaffected by peri- and post-conception diets (91% versus 91% respectively) (Figure 6.5 B). This pattern of UTC cytoplasmic localisation was not consistent in BNC cytoplasm. Vascular FOXO4-immunopositivity was not observed in LPeri exposed tissues (0%) and was unaffected by postconception diet (8% for both HPost and LPost) (Figure 6.5 B). FHL was the only group to present FOXO4-positive staining in stroma (33%, n=1/3) (Figure 6.5 C). 100% of FHL exposed tissues displayed FOXO4-immunopositivity in BNC nuclei, with only 33% of MHH and FHH samples displaying FOXO4-immunopositivity (Figure 6.5 C D). BNC cytoplasmic FOXO4 localisation was lowest in LPeri exposed tissues (58%) (Figure 6.5 B). 100% of MHH, FHH, and FLL samples presented FOXO4-immunopositivity in BNC cytoplasm (Figure 6.5 C D). Vascular FOXO4 localisation was only observed in FHPeri exposed cotyledons (33%; Figure 6.5, E, F).

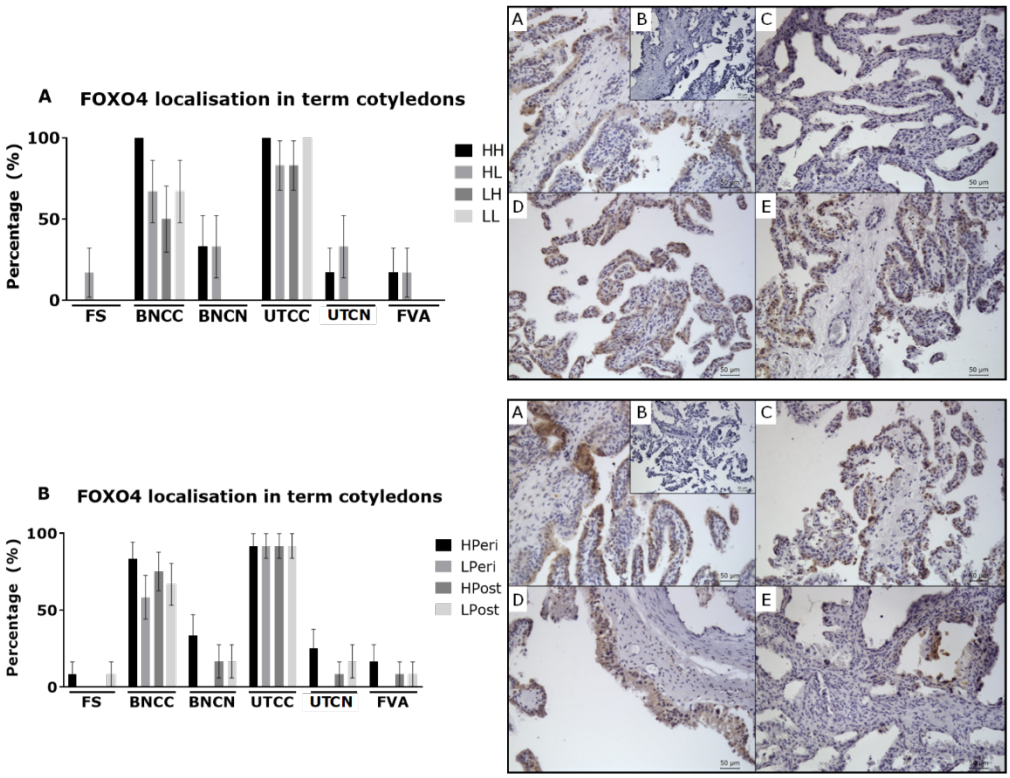


Figure 6.5 The percentage of samples displaying FOXO4 immunopositivity and the cellular localisations in term cotyledons. Graphs and complimentary photomicrographs (B=negative control, A, C-E represent each graph key, e.g. A=HH, E=LL) display results between maternal dietary interventions (A), periconception, and postconception diets (B). H=high protein, L=low protein. Letters denote periconception and postconception diet, e.g. HL= high protein periconception, low protein postconception. Peri=periconception, Post=postconception, FS=fetal stroma, BNC=binucleate cell, UTC=uninucleate trophoblast cell, FVA=fetal vasculature, C=cytoplasm, N=nucleus. Scale bars represent 50 μm.

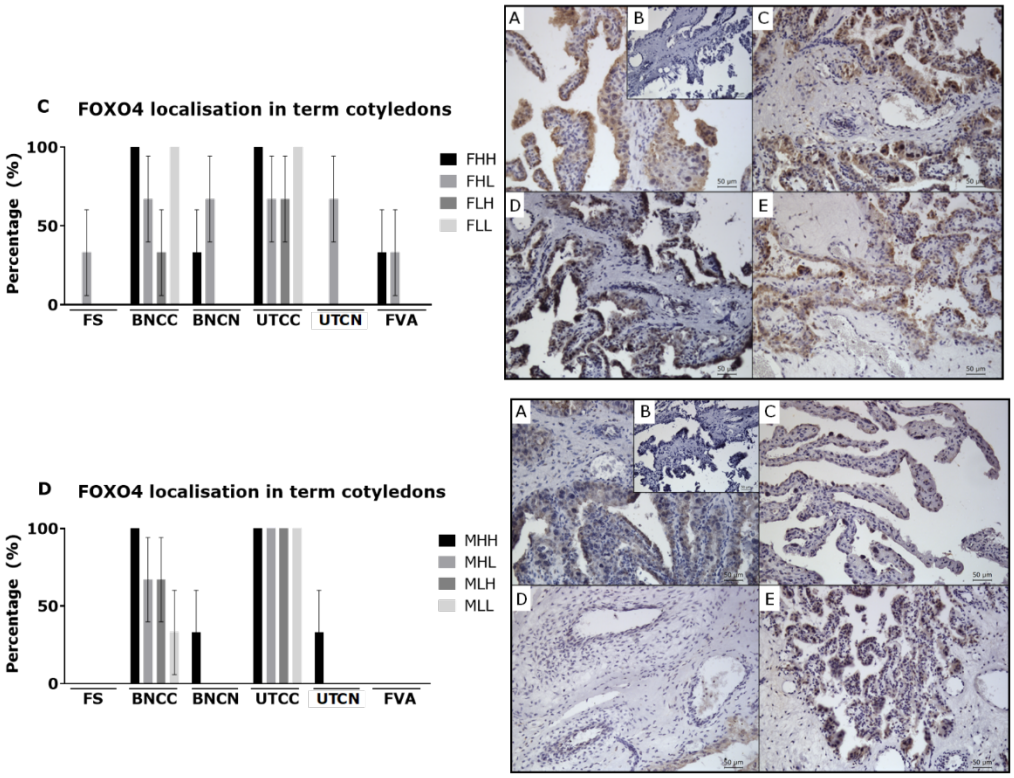


Figure 6.5 The percentage of samples displaying FOXO4 immunopositivity and the cellular localisations in term cotyledons continued. Graphs and complimentary photomicrographs (B=negative control, A, C-E represent each graph key, e.g. A=FHH, E=FLL) display results between female (C) and male (D) cotyledons exposed to maternal dietary interventions. F=female, M=male, H=high protein, L=low protein. Letters denote periconception and postconception diet, e.g. HL= high protein periconception, low protein postconception. FS=fetal stroma, BNC=binucleate cell, UTC=uninucleate trophoblast cell, FVA=fetal vasculature, C=cytoplasm, N=nucleus. Scale bars represent 50 μm.

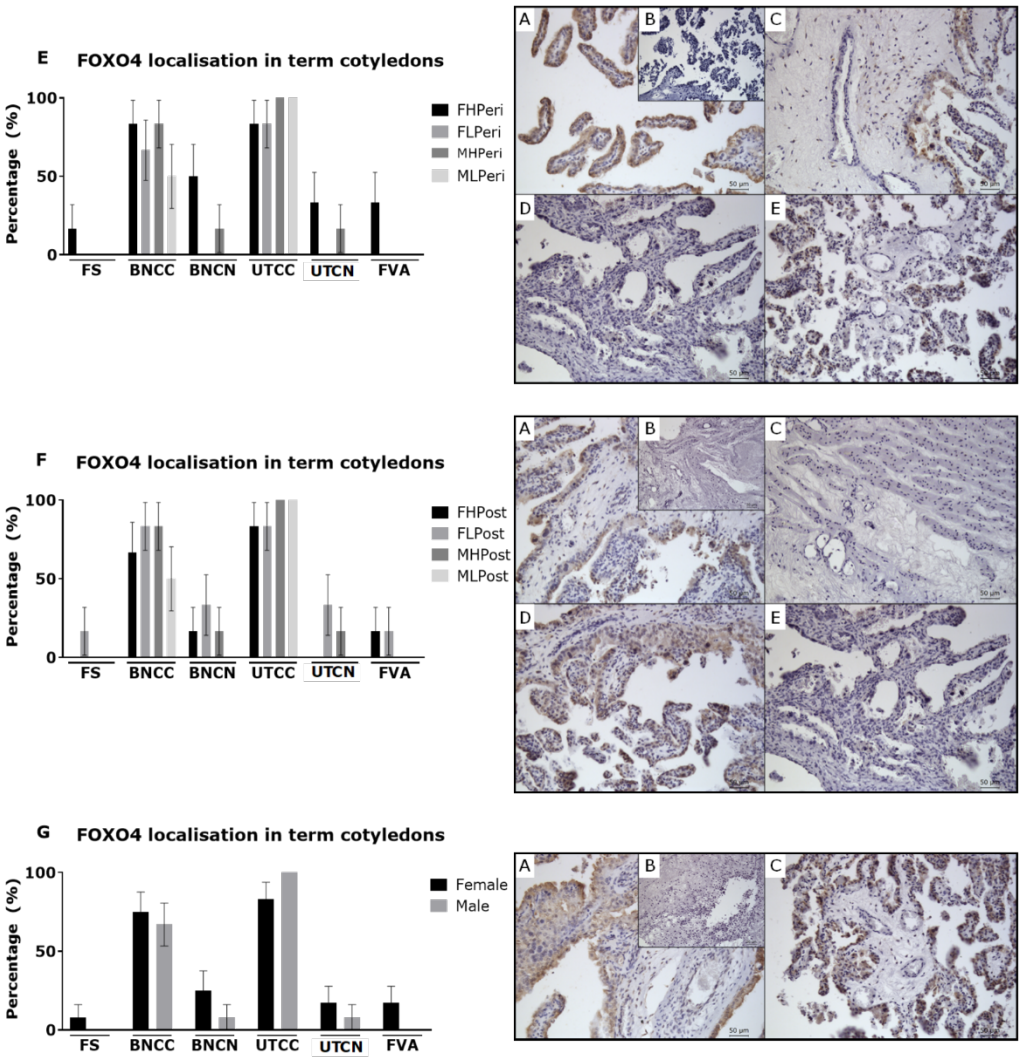


Figure 6.5 The percentage of samples displaying FOXO4 immunopositivity and the cellular localisations in term cotyledons continued. Graphs and complimentary photomicrographs (B=negative control, A, C-E represent each graph key, e.g. A=FHPeri, E=MLPeri) display results between female and male cotyledons exposed to periconception (E) and postconception diets (F), and female and males (G) independent of dietary intervention. F=female, M=male, H=high protein, L=low protein, Peri=periconception, Post=postconception, FS=fetal stroma, BNC=binucleate cell, UTC=uninucleate trophoblast cell, FVA=fetal vasculature, C=cytoplasm, N=nucleus. Scale bars represent 50 μm.

6.3.6 Notch1 Localisation in Term Bovine Cotyledonary Tissues

Notch1 protein localisation in term cotyledonary tissue was restricted to stroma, BNC and UTC cytoplasm, and vasculature (Figure 6.6). No nuclear Notch1 localisation was observed (Figure 6.6). In stroma, Notch1 immunopositivity was restricted to MHH and MLL exposed tissues (Figure 6.6 A D). Stromal localisation of Notch1 was restricted to MHH and MLL samples (33% of MHH and MLL samples) (Figure 6.6 D). UTC cytoplasmic Notch1-immunopositivity was lower in samples exposed to different protein levels between peri- and post-conception versus those exposed to constant protein levels (60-83% in HL and LH versus 100% in HH and LL) (Figure 6.6 A). Vascular Notch1-localisation was restricted to FHH and FLH samples (25-33%) (Figure 6.6 C). BNC cytoplasmic Notch1-localisation was lowest in FHL (50%), MHH and MLH (66%) compared with 100% of MHH, MLH, MLL, FHH and FLL exposed cotyledons (Figure 6.6 C D). BNC cytoplasmic localisation was 77-91% across periconception and postconception groups (Figure 6.6 B). Stromal and vascular localisation was only observed in 16% and 15% of male and female tissues respectively (Figure 6.6 G). In the vasculature, Notch1-immunopositivity was 0% in LL and HL, compared with 16% (n=1/6) and 14% (n=1/7) of HH and LH samples respectively (Figure 6.6 A).

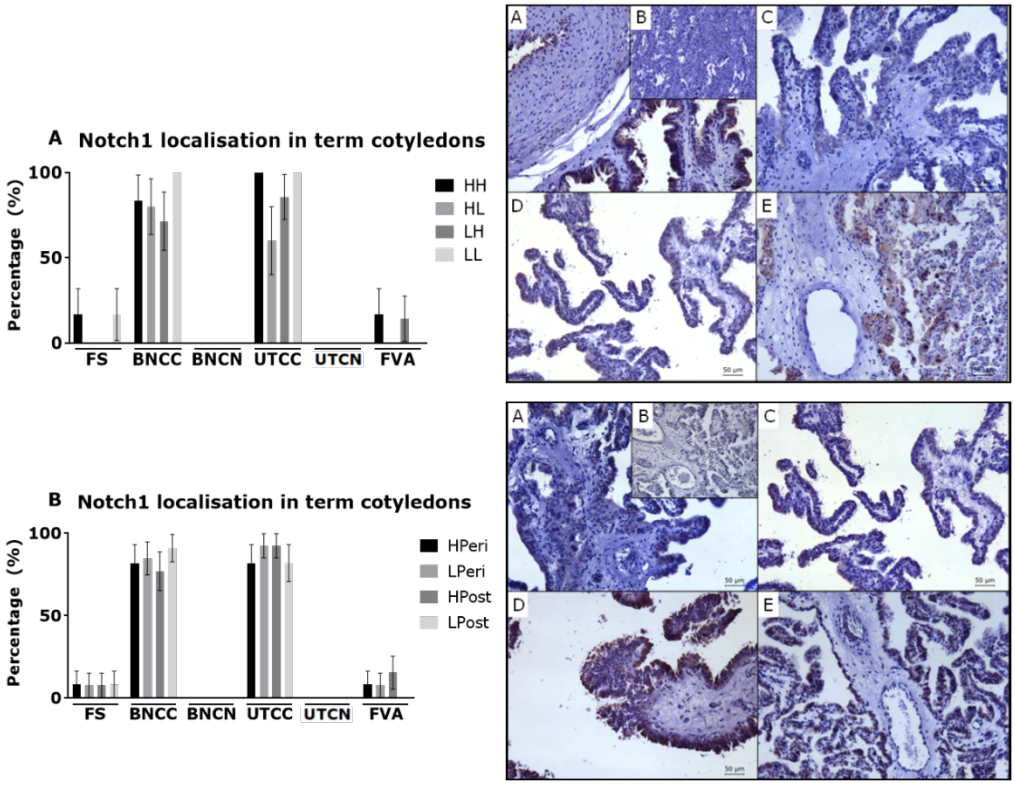


Figure 6.6 The percentage of samples displaying Notch1 immunopositivity and the cellular localisations in term cotyledons. Graphs and complimentary photomicrographs (B=negative control, A, C-E represent each graph key, e.g. A=HH, E=LL) display results between maternal dietary interventions (A), periconception, and postconception diets (B). H=high protein, L=low protein. Letters denote periconception and postconception diet, e.g. HL= high protein periconception, low protein postconception. Peri=periconception, Post=postconception, FS=fetal stroma, BNC=binucleate cell, UTC=uninucleate trophoblast cell, FVA=fetal vasculature, C=cytoplasm, N=nucleus. Scale bars represent 50 μm.

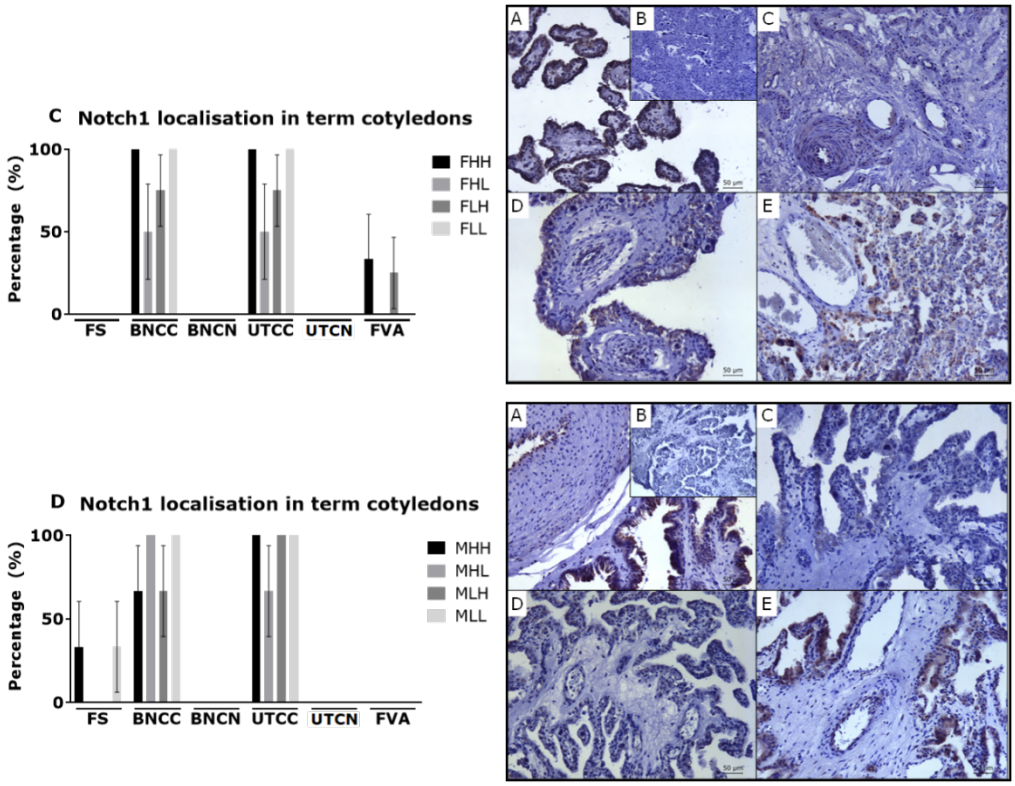


Figure 6.6 The percentage of samples displaying Notch1 immunopositivity and the cellular localisations in term cotyledons continued. Graphs and complimentary photomicrographs (B=negative control, A, C-E represent each graph key, e.g. A=FHH, E=FLL) display results between female (C) and male (D) cotyledons exposed to maternal dietary interventions. F=female, M=male, H=high protein, L=low protein. Letters denote periconception and postconception diet, e.g. HL= high protein periconception, low protein postconception. FS=fetal stroma, BNC=binucleate cell, UTC=uninucleate trophoblast cell, FVA=fetal vasculature, C=cytoplasm, N=nucleus. Scale bars represent 50 μm.

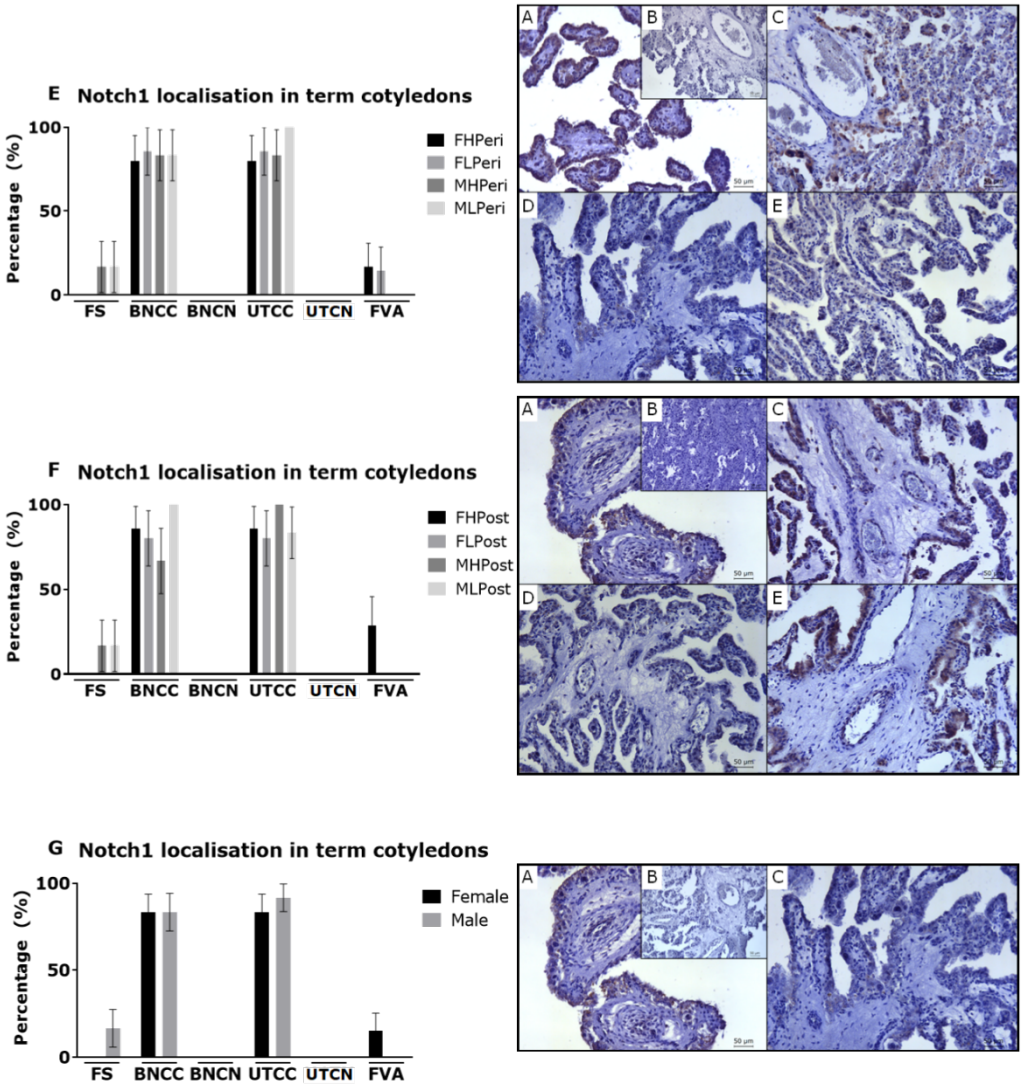


Figure 6.6 The percentage of samples displaying Notch1 immunopositivity and the cellular localisations in term cotyledons continued. Graphs and complimentary photomicrographs (B=negative control, A, C-E represent each graph key, e.g. A=FHPeri, E=MLPeri) display results between female and male cotyledons exposed to periconception (E) and postconception diets (F), and female and males (G) independent of dietary intervention. F=female, M=male, H=high protein, L=low protein, Peri=periconception, Post=postconception, FS=fetal stroma, BNC=binucleate cell, UTC=uninucleate trophoblast cell, FVA=fetal vasculature, C=cytoplasm, N=nucleus. Scale bars represent 50 μm.

6.4 Discussion

The aims of this chapter were to identify whether the proteins of interest, encoded by the previously identified genes of interest (Edwards, 2016), were present in term cotyledons, and to characterise the immunohistochemical localisation of the proteins of interest.

6.4.1 SerpinB1 Localisation in Term Bovine Cotyledons

In the current study, SerpinB1 was ubiquitously localised in term bovine cotyledons (Figure 6.1). This suggests SerpinB1 is important at term and may have multiple cell-specific roles. Extracellular matrix remodelling, apoptosis, inflammatory and immune responses are all involved in initiating bovine parturition (Georgiou et al., 2015, Attupuram et al., 2016). The role of SerpinB1 at bovine parturition is currently unknown, although literature suggests (Chapter 5.1.1) links to the processes involved in the initiation of bovine parturition.

Decreased neutrophil function was associated with retained placenta at calving, although SerpinB1 was not discussed (Kimura et al., 2002). Decreased neutrophil function may result in decreased neutrophil protease secretion, degradation of ECM and subsequent expulsion of fetal membranes. Neutrophils and their proteolytic enzymes appear to be required for normal membrane expulsion (Beagley et al., 2010). Fetal macrophages are present within the bovine placentome, and produce pro-inflammatory cytokines (Schlafer et al., 2000). Macrophages gradually increased with gestation length, and parturition (Schlafer et al., 2000). Previously, SerpinB1 was present in secretory granules of sheep polymorphonuclear neutrophils (Subramaniam et al., 2010). In the current study, immune cells were not positively identified in the term cotyledons, therefore no inference of SerpinB1 immune cell localisation was possible. At 15 days postpartum a strong-positive correlation between PMN percentage and SerpinB1 was reported in dairy cows (Ledgard et al., 2015). This supports neutrophil expression of SerpinB1 but does not infer the role of SerpinB1 in parturition.

The immune response is a known mechanism of expulsion of fetal membranes, via promoting proteolytic enzymes, collagenases and elastases (Attupuram et al., 2016). SerpinB1 may therefore be involved in regulating

ECM degradation and the subsequent expulsion of fetal membranes. While overexpression may contribute to retained placenta, as SerpinB1 is thought to be anti-protease in activity, some expression may be required for regulation of normal membrane expulsion. SerpinB1 may be regulated by bovine progesterone and PGF2α, as evidenced by human progesterone and prostaglandin E2 (Denison et al., 1999, King et al., 2003). Secretory leukocyte protease inhibitor, similar to SerpinB1, has a putative progesterone response element on the promoter region, where progesterone was shown to increase expression (Denison et al., 1999). Progesterone has a role during gestation to maintain quiescence and maintenance of pregnancy. A reduction in progesterone may reduce promotion of anti-protease expression, increasing protease activity and assisting in parturition.

Further research into the role of SerpinB1 in the immune response at parturition is required, to elucidate its exact role and optimal expression levels at bovine parturition. Whether BNC migration, epithelial-fusion and degranulation is involved in parturition is unknown (Schlafer et al., 2000). In the current study, not all BNCs presented SerpinB1 localisation at term, although 100% of samples presented SerpinB1 immunopositivity (Figure 6.1). This suggests variation in molecular activity within the BNC population. Certain proteins expressed in the ruminant placenta were described as spatio-temporal, particularly those expressed by BNCs (Takahashi et al., 2013).

Similarly to 98 dpc placentome localisation, in term cotyledons SerpinB1 localisation did not differ between dietary interventions in the BNC cytoplasm, BNC nuclei (overall, not considering discreet cellular context), and the fetal vasculature (Figure 5.3 A, Figure 6.1 A). This suggests that the putative function of SerpinB1 is required at both 98 dpc and term.

In term cotyledons, the pattern of SerpinB1 localisation was similar between male and females across all locations assessed (Figure 6.1 G). This may be because parturition is a conserved mechanism which is not dependent on offspring sex. These similarities between fetal sexes in term cotyledons were also seen in 98 dpc placentomes, with the exception of UTCs (Figure 5.3 G). The current study characterised the cellular localisation of SerpinB1 within term cotyledons (Figure 6.1). In summary, this provided a basis for further investigation of the role of SerpinB1 in term bovine placentomes within

identified cellular contexts. Further investigation may include RNA-seq analysis and validation via RT-qPCR to confirm SerpinB1 expression, and further understanding of the level of expression required for normal parturition in term bovine cotyledons.

6.4.2 GATM Localisation in Term Bovine Cotyledons

Another protein of interest in the bovine placenta was GATM (Figure 6.2). Localisation of GATM protein in the term bovine placentome was not previously reported in literature. Overall, GATM protein expression is thought to be related to fetal growth, although results suggest no differences in localisation between sexes (Figure 6.2, G). Previously, placental expression of creatine synthesising enzymes was lacking (Dickinson et al., 2014). Recently, term human placentae expressed both GATM and GAMT, and subsequent synthesis of the creatine-precursor guanidinoacetate, and creatine was identified (Ellery et al., 2017). The current study provided evidence of GATM protein localisation in both 98 dpc placentomes (Figure 5.6) and term (Figure 6.2) bovine cotyledons. This is evidence that the bovine placenta may synthesise and release creatine. Although GAMT and creatine synthesis are lacking in the current study, the existing findings agree with human placentae findings (Ellery et al., 2017). Term bovine placentome creatine requirement is unknown. Previously it was suggested that placental creatine synthesis may be a compensatory adaptation in cases of intrauterine growth restriction (Dickinson et al., 2014). Cotyledons exposed to LPost presented the greatest percentage of samples with stromal GATM localisation (Figure 6.2, B). This indicates that postconception protein restriction may have led to placental adaptation, to increase creatine synthesis to meet placenta energy requirements. Localisation was specific to UTC cytoplasm at term (Figure 6.2). The biological significance of this may be sustained creatine synthesis requirement in UTCs throughout gestation, as UTCs are present throughout gestation, and are less differentiated than BNCs. Human placental GATM gene expression peaked at mid-gestation and was reduced at term (Uusküla, 2013). Human term placental GATM expression was higher in pregnancy complications versus normal pregnancies (Uusküla, 2013). This agrees with Dickinson and colleagues (2014). Overall, future analysis of mRNA expression in term placentomes would elucidate the effect of maternal diet on term GATM expression.

6.4.3 STRA6 Localisation in Term Bovine Cotyledons

The cellular localisation of another protein of interest, STRA6, was also characterised in bovine term cotyledons (Figure 6.3). STRA6 protein localisation suggests vitamin A uptake is possible in trophoblasts and the fetal vasculature, which is in agreement with previous evidence for retinol transfer across the ovine placentome (Donoghue et al., 1982). Whilst the precise vitamin A requirement in term bovine placenta is unknown (Amengual et al., 2014), retinoic acid is vital for epithelial differentiation (Rothman et al., 1995, De Luca et al., 1972, Rogers et al., 1991). As trophoblasts are specialised epithelial cells, vitamin A transport into trophoblasts may be involved in supporting trophoblast differentiation until term (Cross et al., 1994). A greater percentage of STRA6-postive BNC cytoplasm was observed in females than males, independent of diet (Figure 6.3 C, D). Furthermore, results may indicate placental transfer of Vitamin A to the fetal circulation. This is supported by STRA6 localisation in both trophoblasts and the vasculature (Figure 6.3). The lower percentages of samples with STRA6-positive vasculature in HL and LH (17-20%) groups compared with HH and LL (50-67%) exposed tissues suggests that expression was affected by a switch in maternal dietary protein, rather than continuous high or low protein throughout periconception and postconception (Figure 6.3, A). This putative switch in STRA6 protein expression around 23 dpc appears to have persisted to term fetal membranes, and was limited to the vasculature and UTC cytoplasm (Figure 6.3). This may impact fetal growth if STRA6 expression and subsequent retinol transfer to the fetus did not meet fetal requirements for growth (Rothman et al., 1995). In fetal vasculature, the greater percentage of STRA6-localisation in males than females (Figure 6.3 G) indicates sex-specific STRA6 protein localisation. This may indicate a greater influx of retinol through the male cotyledons at term than females. Within male cotyledons, STRA6-immunopositivity in the vasculature was observed in MHH, MLL, and MHL but was absent in MLH. Whereas 67% of MLL cotyledons displayed vascular STRA6 localisation (Figure 6.3), which supported the hypothesis that placental STRA6 expression may increase in response to LL (potential negative energy balance, and low serum retinol). In female cotyledons, vascular STRA6-immunopositivity supported the hypothesis, as FHH exposed cotyledons displayed 0% immunopositivity, while FLL presented 67% (Figure 6.3). This may suggest increased capacity in FLL

cotyledons for retinol transport to the fetus. Results may suggest that FLPeri exposed cotyledons displayed 50% vascular STRA6-immunopositivity, while this localisation was absent in FHPeri samples (Figure 6.3). Between postconception diets, vascular STRA6-immunopositivity was similar, suggesting that postconception maternal diet did not affect vascular STRA6 expression.

STRA6 localisation in bovine placental vasculature was characterised, although the influence of maternal diet on expression in cellular contexts remains unclear. Although putative functions were discussed, further research is required. Further characterisation of STRA6 expression via spatial transcriptomics profiling would benefit the understanding of the effect of maternal diet on gene expression within the bovine placentome (Salmén et al., 2018). Equally, term cotyledon RNA-seq analysis and RT-qPCR of STRA6 would provide further evidence for the effect of maternal diet on term cotyledon *STRA6* expression. Thereby elucidating the putative homeostatic function of STRA6 in placental retinoid transport at this stage of gestation. Further investigation in the current study may include analysis of serum retinol concentrations from cord blood samples (LeBlanc et al., 2004).

6.4.4 GJA1 Localisation in Term Bovine Cotyledons

In term fetal membranes, existing research of GJA1 protein localisation is limited. *GJA1* RNA was present in 120 and 220 dpc bovine placentomes (Pfarrer et al., 2006). In human trophoblasts, *GJA1* down-regulation was favourable to trophoblast epithelial invasion (Jiang et al., 2013). This agrees with an earlier finding that *GJA1* expression was high in normal human parturition, and expression was reduced in prolonged parturition (Cluff et al., 2006). Moreover, loss of GJA1 impaired murine parturition (Tong et al., 2009). These studies suggest that relatively high GJA1 expression is beneficial to human parturition. In bovine parturition, based on the hormonal regulation of *GJA1* expression, GJA1 may be up-regulated at parturition via increased oestrogen (Bernal, 2007, Bauersachs et al., 2005, Attupuram et al., 2016). Additionally, increased $PGF_{2\alpha}$ through fetal cortisol reduces progesterone, and subsequently may increase GJA1 expression (Attupuram et al., 2016). This increase in GJA1 agrees with human and mouse studies, nevertheless the functions of GJA1 at parturition may differ between bovine, mouse and human.

In human placentae, gap junctions between cytotrophoblast-cytotrophoblast, and syncytiotrophoblast-syncytiotrophoblast were demonstrated, which is comparable to UTC-UTC and BNC-BNC (Cronier et al., 2002). GJA1-immunopositivity was observed in both UTC and BNCs in the current study (Figure 6.4). This agrees with human placentae findings by Cronier and colleagues (2002). Overall, the percentage of GJA1-positive samples was greater in LL exposed cotyledons (Figure 6.4). Within bovine UTC-UTC and BNC-BNC connections, greater numbers of gap junctions may be required for the crucial signalling events involved in parturition. Furthermore, *GJA1* expression may be important for immune response involved in parturition (Chen et al., 2017, Seo et al., 2013). Although RNA-Sequencing of term cotyledons was beyond the scope of the current study, further analysis of term cotyledon gene expression via RNA-seq would elucidate *GJA1* expression levels. This would allow comparison between 98 dpc placentome RNA-seq results, and further understanding of the role of GJA1 during bovine gestation. However, disparity exists between 98 dpc placentome and term cotyledon, as only 98 dpc placentome contained maternal caruncles. Cell-specific transcriptome analysis would allow molecular comparison between cell populations and at different time-points (Liu et al., 2018, Pavličev et al., 2017).

Bovine peripartum polymorphonuclear neutrophil mRNA expression was studied (Seo et al., 2013). Though not directly related to fetal membranes, the dam's immune system is involved in parturition (Chen et al., 2017). Polymorphonuclear neutrophil GJA1 expression was constant through -10 to 21 days post-partum (Seo et al., 2013). Parturition is associated with an increased inflammatory response of the cow (Chen et al., 2017). This includes increased amniotic fluid proinflammatory cytokines, neutrophil and macrophage infiltration of the fetal membranes (Chen et al., 2017). Genemania revealed genetic interactions between *GJA1* and *CASP8 (proteolytic)*, *IL1A (inflammatory cytokine)*, and *BCL2 (apoptosis inhibitor)*, which are genes involved in separation and expulsion of bovine fetal membranes (Warde-Farley et al., 2010, Attupuram et al., 2016). *GJA1* was reportedly co-expressed with *MMP2*, *MMP9*, and *IL8* which are involved in bovine feto-maternal unit separation and fetal membrane expulsion (Attupuram et al., 2016, Warde-Farley et al., 2010). The immunohistochemical localisation of *GJA1* protein in term cotyledons

provides a basis for further investigation. Immunopositive localisation supports a possible role of GJA1 in bovine separation of fetal membranes, though the exact role of gap junctions in fetal membranes is unclear.

6.4.5 FOXO4 Localisation in Term Bovine Cotyledons

The function of another protein of interest, FOXO4, is also yet to be elucidated in the term bovine placenta. FOXO4 was previously identified in human coronary artery endothelial cells, and is thought to suppress vascular endothelial cell and vascular smooth muscle cell growth (Paik et al., 2007, Ding et al., 2011). Therefore, localisation of FOXO4 in endothelial cells suggests negative regulation of cell growth. However, there is no supporting evidence in cows. Results suggest that FOXO4 protein was absent in term cotyledon endothelial cells exposed to periconception low protein (Figure 6.5). This indicates a lack of endothelial cell growth suppression. Absence of FOXO4 endothelial localisation was maintained in term cotyledons exposed to the LH diet (Figure 6.5), suggesting that periconception diet had a lasting effect which was not altered by postconception period, and persisted to term. The biological significance of this is unclear, however one explanation is compensatory vascular growth in protein restricted placentomes. Periconception nutrient restriction (50% of requirements) increased ovine placentome vascularisation (Zhu et al., 2007). Average placentome weight was lower in nutrient restricted compared to control fed ewes, however vascular density was increased approximately 2-fold and 3-fold, in 78 dpc caruncles and cotyledons respectively (Zhu et al., 2007). Furthermore, in the current study, male placentome endothelial cells appeared to lack FOXO4 localisation (Figure 6.5). This may be a mechanism which allows increased growth in males compared to females (Copping et al., 2014, Sullivan et al., 2009c). The targets of FOXO4 within bovine vascular endothelial cells are currently unknown. FOXO4 was not the predominant FOXO- transcription factor in human vascular endothelial cells (Potente et al., 2005). Overexpression of FOXO4 did not affect vascular endothelial cell migration or tube formation (Potente et al., 2005). However, in endothelial progenitor cells (EPCs), FOXO4 was the predominant FOXO (Urbich et al., 2005). FOXO4 phosphorylation and subsequent inactivation induced apoptosis of EPCs (Urbich and Dimmeler, 2004). EPCs are considered crucial for neovascularisation and postnatal vascular regeneration (Urbich et al., 2005). Adiponectin was demonstrated to maintain FOXO4 phosphorylation, and

thereby promoted human vascular smooth muscle cell differentiation (Ding et al., 2011). Human trophoblast cells contained adiponectin receptor-2 (Aye et al., 2013). This suggests the potential for adiponectin to influence FOXO4 expression in trophoblasts. Maternal adiposity and FOXO4 expression was previously reported in obese pregnant women (Saben et al., 2014a). FOXO4 nuclear localisation was associated with maternal lipid accumulation (Saben et al., 2014a). In the current study, FOXO4 nuclear localisation was lowly observed in term cotyledons (Figure 6.5). This may indicate a diet which did not promote increased BCS. There is however no data on heifer BCS in the current study. Adiponectin was negatively correlated with cow body condition score (BCS) (De Koster et al., 2017, Locher et al., 2015). Furthermore, in non-pregnant non-lactating dairy cows, subcutaneous adipocyte adiponectin concentration decreased with increasing BCS, however serum levels were unaffected (Locher et al., 2015). In pregnant women, adiponectin was high and low in lean and obese women respectively (Aye et al., 2013). This agreed with bovine studies. However in pregnant women, serum adiponectin concentrations were negatively correlated with maternal body fat, and fetal birth weight (Aye et al., 2013). Adiponectin inhibited amino acid uptake in trophoblasts (Aye et al., 2013). This suggests a mechanism of the maternal adipokine to control fetal growth during maternal low energy reserves, and potentially in nutrient restricted states. Literature also suggests maternal adiposity reduced placental angiogenic factors via FOXO4 signalling (Saben et al., 2014a). Transcriptomic analysis of bovine term cotyledons, with interest in adiponectin expression, would further elucidate the effects of maternal dietary protein during periconception and postconception on placentome gene expression at term.

Fetal sex suggests that overall, FOXO4 protein was absent in endothelial cells of all males independent of diet (Figure 6.5). Whereas in females exposed to HH and HL, FOXO4 protein was localised in endothelial cells (Figure 6.5). This suggests that vascular endothelial cell growth in male term cotyledons was not suppressed. FOXO4 localisation in female vascular endothelial cells suggests that cell growth was open to growth suppression. . The biological significance is potentially that females exposed to periconception high protein developed normal vascularisation.

Previous human immunohistochemical localisation of FOXO4 indicates presence in trophoblast cells in the peri-parturition period (Lappas et al.,

2010, Lim et al., 2012). Immunohistochemical localisation in term placentae were observed in the syncytiotrophoblast layer. The syncytiotrophoblast is comparable to the feto-maternal junctions, as the point of contact between the fetal and maternal tissues where trophoblast cells invade the endometrial epithelium. There are fundamental differences such as the invasiveness of the human syncytiotrophoblast, which acts as a barrier to maternal immune cells to avoid an immune response to foreign fetal proteins (Thellin and Heinen, 2003).

Compared to previous findings, immunohistochemical localisation of FOXO4 in the current study is in agreement with Lappas and colleagues (2010), as FOXO4 was also identified in term human trophoblast cells. Results of the current study suggest reduced percentages of samples presenting FOXO4 protein localisation in term fetal tissues exposed to a change in maternal dietary protein (HL or LH), compared to consistent protein levels (HH and LL) (Figure 6.5).

6.4.6 Notch1 Localisation in Term Bovine Cotyledons

The final protein of interest, Notch1, was also previously unstudied in the bovine placenta. There is no existing evidence for Notch1 protein localisation in the bovine placenta. In human tissues, Notch1 was suggested to have varying roles in numerous cell types, including epithelial and endothelial cells (Baldi et al., 2004, De Falco et al., 2007). In the current study, Notch1 protein was localised in cytoplasm but absent in nuclei of BNCs and UTCs (Figure 6.6). This suggests that the Notch1 intracellular domain was not activated in term trophoblasts, as only the activated NICD is able to translocate to the nucleus (Chapter 5.1.6) (Sarikaya and Jerome-Majewska, 2011). In the human placenta, De Falco and colleagues (2007) reported Notch1 expression in extravillus trophoblast, in the third trimester of gestation in healthy placentae. Cytotrophoblasts in the human placenta are most comparable to UTCs in the bovine placentome, although it should be noted that the trophoblast transcriptome likely differs between these species (Pfeffer et al., 2017). Between diets, cytoplasmic localisation was similar between BNCs and UTCs (Figure 6.6 A). This suggests no clear effect of diet on Notch1 localisation. Qualitatively, HPeri did not affect the percentage of Notch1-positive samples within BNC and UTC cytoplasm (BNC cytoplasm 82%, UTC cytoplasm 82%; Figure 6.6 B). While LPeri and HPost displayed

lower Notch1-positive staining in BNC than UTC cytoplasm (Figure 6.6). In summary, Notch1 was present within trophoblasts in the majority of samples (Figure 6.6 C D). In female cotyledons, results appear to show fewer Notch1 immunopositive samples exposed to HL and LH, than HH and LL. This suggests that a switch in maternal dietary protein at 23 dpc may have altered Notch1 protein localisation. This pattern was not seen in male cotyledons, suggesting a sexually dimorphic response to maternal dietary changes in periconception and postconception. Notch1 gene was 1.3-fold upregulated in MHL versus MLH tissues in 98 dpc placentomes (Edwards, 2016). At term, the percentage of samples with Notch1-immunopositive BNC cytoplasmic localisation was greater in MHL than MLH, although the inverse was observed in UTC cytoplasm (Figure 6.6). Furthermore, all MLL samples displayed Notch1 protein in trophoblast cytoplasm (Figure 6.6). Whereas Notch1 immunopositivity in trophoblast cytoplasm of females was similar between diets (Figure 6.6). This suggests sexually dimorphic protein localisation, and differential response to diet.

Notch and Notch1 signalling was reportedly involved in promoting trophoblast cell migration and invasion in human choriocarcinoma cells (Massimiani et al., 2015, Pang et al., 2010). This suggests a potential role of Notch in bovine trophoblasts, although further research is required, such as utilising bovine trophoblast cell line BT-1 (Ushizawa et al., 2005, Hirata et al., 2003). A co-culture of BT-1 and cultured epithelium to direct migration may be of interest for future investigation (Haeger et al., 2015). Knockdown studies of Notch1 and all other genes of interest would be valuable to further understanding of their functions in the bovine placentome. Furthermore, Notch1 was localised in human fetal villi stroma and trophoblast membranes (De Falco et al., 2007). In the current study, Notch1 localisation in fetal stroma was only observed in HH and LL exposed tissues (Figure 6.6). This may suggest that the switch in maternal dietary protein at 23 dpc reduced Notch1 protein expression which persisted to term in cotyledons.

Human Notch1 was also localised in vascular endothelial cell cytoplasm, particularly in arteries (Baldi et al., 2004, De Falco et al., 2007). This was also observed in mice by E13.5 (Villa et al., 2001). This is supporting evidence for Notch1 localisation in large arteries but not veins, in term cotyledonary tissue. In the current study, term endothelial Notch1 protein localisation was only observed in female cotyledons. The biological

significance of this is unclear based on the data of the current study. Notch1 is essential for normal vascular development, as Notch1 functional loss in endothelial cells was associated with murine embryonic lethality (Limbourg et al., 2005). The consequences of insufficient vascularisation are reduced nutrient, growth factor, hormone and oxygen delivery to the tissues (Limbourg et al., 2005). In the current study, reduced Notch1 localisation may have indicated that vascular development of placentomes was reduced at term. This is potentially not detrimental as by the end of gestation, the vasculature is likely fully developed. Notch1-Dll4 signalling regulated branching morphogenesis through control of tip cell formation, thereby regulating sprouting and branching in mouse retina (Hellström et al., 2007). The specific role of Notch1 is dependent on ligand interactions, however, Notch1 ligand expression was not investigated in the current study. Therefore, although the biological significance of Notch1 expression is partially dependent on which ligands bind, the presence or absence of Notch1 protein could affect angiogenesis. In the current study, term Notch1 localisation in vascular endothelial cells was uncommon, with 15 % of female placentomes positively expressing Notch1, compared with 0 % of males (Figure 6.6 G). This may indicate that at term, the notch signalling pathway is not upregulated in vascular endothelial cells in males. This suggests sexually dimorphic expression of Notch1 within endothelial cells, which may have favoured increased branching angiogenesis in female tissues. However, down-stream effects of Notch1 activation was reportedly ligand-specific in mouse endothelial cells (Hellström et al., 2007). As ligand binding was unknown in the current study, it was not possible to infer a biological implication of Notch1 expression. Further investigation via term cotyledon RNA-seq analysis may reveal which Notch1 ligands were present, and further immunofluorescence analysis may be of use to determine receptor-ligand interactions in tissue, which would further elucidate Notch1 function in term bovine cotyledons. Moreover, validation via RT-qPCR would provide more robust evidence. Immunofluorescence may elucidate further the interactions between Notch1 localisation and its ligands.

Bovine placentome localisation of Notch1 does not currently exist in literature, although Notch1 expression has been reported in bovine muscle and blastocysts (Liu et al., 2017, Goossens et al., 2013). The current study therefore presents novel findings of Notch1 protein in UTC and BNCs, and endothelial cells at term (Figure 6.6). In summary, Notch1 localisation in

term cotyledons is evidence to suggest Notch1 signalling persists to term. Notch1 signalling may be involved in vascular remodelling, angiogenesis, and trophoblast migration in term bovine cotyledons (Massimiani et al., 2015, Hunkapiller et al., 2011).

6.5 Conclusions

Overall, SerpinB1, GATM, STRA6, GJA1, FOXO4 and Notch1 proteins were positively identified in term bovine cotyledons via immunohistochemistry. Immunopositivity indicates each protein expression occurred at term, although it does not infer that each protein expression persisted from 98 dpc until term. Specific cellular localisations may indicate putative individual roles based on existing knowledge.

SerpinB1 may be involved in expulsion of fetal membranes at parturition. Although evidence is lacking, there may be an optimal expression level at term which allows normal membrane expulsion and avoids preterm labour and retained placenta. Increased incidence of GATM protein localisation in LPost exposed tissues may suggest GATM is involved in intrauterine growth restriction. Localisation was restricted to stroma and UTC cytoplasm. The functions in the bovine placenta are not currently understood. Future analysis of mRNA expression in term placentomes would elucidate the effect of maternal diet on term GATM expression. STRA6 may be involved in supporting trophoblast differentiation until term, and facilitating transport of maternal dietary retinol to the calf. This was supported by trophoblast and vascular endothelial localisation in term cotyledons. Retinol transfer to the fetus may be altered by a switch in maternal diet at 23 dpc. Results suggest sexually-dimorphic STRA6 localisation in blood vessels, where more female samples displayed STRA6 localisation than male counterparts regardless of diet. GJA1 expression and localisation is thought to be beneficial for normal separation and expulsion of fetal membranes. GJA1 localisation at term supports this putative role. FOXO4 localisation was rarely nuclear. FOXO4 may be involved in placental compensation of maternal diet via altered angiogenesis. Notch1 localisation in trophoblast and endothelial cells suggests a role in trophoblast migration and vascular remodelling. Endothelial localisation was sexually dimorphic, restricted to female cotyledons. Trophoblast cytoplasm localisation indicated that male

cotyledons may be more susceptible to altered protein localisation than females.

Edwards (2016) did not conduct RNA-seq of term cotyledons which were investigated via immunohistochemistry in the current study. Further analysis of term cotyledon gene expression via RNA-seq would elucidate gene expression levels of each protein of interest. This would allow comparison between previous 98 dpc placentome RNA-seq results (Edwards, 2016), and further understanding of the effect of maternal dietary intervention during periconception and postconception on gene expression in the 98 dpc and term placenta. However, disparity exists between 98 dpc placentome and term cotyledon, as only 98 dpc placentome contained maternal caruncles. Further analysis of mRNA expression in the term cotyledonary tissues via RNA-seq is required to validate the expression of genes encoding the proteins investigated in the term cotyledons. RNA-seq analysis would also potentially reveal differentially expressed genes between fetal sex and maternal dietary interventions in term cotyledons. Additionally, RNA-seq analysis would reveal the expressions of related genes of the proteins of interest. This may allow further understanding of genes affected by maternal dietary interventions. Knockdown studies of genes of interest in co-culture of bovine trophoblast cell line, BT-1 and bovine epithelial cells would further elucidate protein functions in the bovine placentome.

7 General Discussion, Conclusions, and Future Directions

The current study provided qualitative analysis of ducts, postulating the degree of development and putative presence of terminal ductal units at 98 dpc (Chapter 3). To our knowledge, this study was the first to present quantitative analysis of ducts within the fetal mammary gland, vasculature, and total collagen in the bovine fetal mammary gland. Additionally, the cellular contexts of total collagen were described in relation to ducts (Chapter 3). Moreover, adipocytes were immunopositively identified within the fetal mammary gland (Chapter 3) which agrees with recent findings (Hara et al., 2018). This study presented novel findings into the effects of maternal diet on the fetal mammary transcriptome at 98 days postconception (dpc) (Chapter 4). The current study identified differences in the proportion of BNCs in discrete cellular contexts within the feto-maternal junction, between fetal sex and maternal diets (Chapter 4). The current study provided in-depth qualitative analysis of SerpinB1, GATM, STRA6, GJA1, FOXO4 and Notch1 protein localisation in the bovine 98 dpc placentome (Chapter 5) and term cotyledons (Chapter 6). A summary of key findings is presented (Tables 7.1 and 7.2).

Table 7.1 Summary of findings in the bovine placenta

98 dpc	Term
SerpinB1, GATM, STRA6, GJA1, FOXO4 and Notch1 were immunopositively identified and qualitatively localised in the 98 dpc bovine placentome.	SerpinB1, GATM, STRA6, GJA1, FOXO4 and Notch1 were immunopositively identified and qualitatively localised in term cotyledons.
SerpinB1 was localised to BNCs, and expression was dependent on cellular context in relation to migration.	
The proportion of BNCs in discrete cellular contexts within the feto-maternal junction differed between maternal diet and fetal sex.	

Table 7.2 Summary of findings in the bovine fetal mammary gland

Cellular effects:
Duct lumen size (micro, patent) was greater in HPost versus LPost.
Stromal blood vessels were affected by periconception diet.
Total collagen area was unaffected by maternal dietary intervention.
Adipocytes were immunopositively identified within ducts.
Molecular effects:
45 and 46 characterised genes were up- and down-regulated in LH versus HH exposed tissues respectively.
Up-regulated gene ontologies indicated immune system activation. Down-regulated gene ontologies indicated roles in structural development.
Adipocyte related genes were differentially (log2 fold-change) expressed (+1.3 *UCP2*, -2.7 *APCDD1*, -2.6 *KLF5*).

7.1 Ruminant Dietary Protein: Beyond Crude Protein

The present study focused on crude protein (CP), although effective rumen degradable protein (ERDP) and rumen undegradable protein (UDP) were estimated (Table 2.2). It is important to consider ERDP and UDP as levels determine what is absorbed as ammonia, peptides and amino acids. The level of ERDP is important to ensure optimum fibre digestibility and subsequent feed efficiency (Köster et al., 1996, Arroquy et al., 2004). Barley straw was the main fibre component of the current study (Table 2.1). Beef cows were fed diets varying in ERDP from 0, 180, 360, 540, or 720g/day (Köster et al., 1996). Neutral detergent fibre (NDF) digestion was improved by 180 g/day versus 0 g/day, while no benefit was observed from feeding greater ERDP between 360-720 g/day (Köster et al., 1996). The estimated ERDP for the LPeri and LPost diets were 405g/day and 562g/day respectively (Table 2.2). HPeri and HPost ERDP was estimated at 714g/day and 901g/day respectively (Table 2.2). This suggests that fibre digestion was adequate in all maternal dietary interventions.

Furthermore, soybean meal was the major dietary protein source in the current study (Table 2.1). Future environmental and economic pressure may lead to increased commercial feeding of alternative protein sources in livestock production, particularly non-human edible protein (Wilkinson and Lee, 2017, Wilkinson and Garnsworthy, 2017, Broderick, 2017, Abbasi et al., 2018). In future research, a more detailed characterisation of dietary protein, including amino acid levels, ERDP, and DUP would allow increased

depth in understanding, negating the requirement to investigate the effects of multiple protein sources on fetal programming.

The effects of excess dietary protein on rumen function include increased ruminal proteolysis and deamination by rumen microbiota, of which there are around 45 microbial species involved in protein, peptide, amino acid and urea degradation (Hartinger et al., 2018). This leads to increased levels of ammonia in the rumen, which is absorbed through the rumen and converted to urea (Hartinger et al., 2018). Oocyte development to the blastocyst stage was impaired in oocytes exposed to high maternal plasma ammonia and urea in an *in vivo* study in beef heifers (Sinclair et al., 2000). Rees and colleagues (2006) reported that both an excess and a deficiency in methionine can be detrimental to the developing human fetus. Excess methionine is catabolised, and this process requires the use of glycine and serine, which reduces their availability and can lead to glycine and serine deficiency (Rees et al., 2006). Therefore, future investigation into the effects of maternal dietary protein on fetal programming should further investigate amino acid levels in the diet, with attention to methionine, considering also dietary amino acid balance.

The effects of dietary protein restriction in ruminants can alter the rumen microbiota population, as dietary protein is one of the two key nutrients rumen microbes use for microbial growth (Latham et al., 2018), and can reduce the microbial protein source available to the ruminant and developing fetus (Figure 1.1). Restricted dietary protein can also increase rumen pH, and reduce rumen fermentation (Ma et al., 2017). Reduced microbial fermentation can subsequently lead to a reduction in the metabolites of rumen fermentation, ammonia and total volatile fatty acid (VFA) concentration, which are the main energy source of the ruminant (Ma et al., 2017).

Methionine is of particular interest due to its requirement for S-adenosyl-methionine (SAM) production (Rees et al., 2006). For example, there may be a putative link between maternal dietary methionine level, and placental creatine synthesis, as SAM is a substrate involved in endogenous creatine synthesis (Brosnan et al., 2009). There may be a link between maternal dietary methionine and placental expression of amidinotransferases such as GATM investigated in the current study. The amino acids arginine and glycine

are the substrates used by GATM in the first step of creatine synthesis (Humm et al., 1997, Ellery et al., 2017). Further investigation of methionine, arginine and glycine levels in relation to creatine synthesis woudlfurther elucidate the effect of maternal dietary protein on placental creatine synthesis.

Secondly, it is possible that the low CP diet was lower in methionine, which is a methyl group donor and can alter DNA methylation in offspring, leading to permanent changes to gene expression (Sinclair et al., 2007). Therefore, maternal levels of these amino acids, particularly methionine, are also of interest in future investigation of the effects of maternal protein level and placental gene expression, and the subsequent molecular and cellular effects on fetal development.

The underlying mechanisms involved in the effects of maternal dietary protein on placentome protein expression, cellular localisation, and fetal programming in the bovine fetal mammary gland remain unknown. It is postulated that specific amino acids such as methionine may be the key factor in the maternal diet influencing placental gene expression and fetal programming (Sinclair et al., 2007). A Human study of intra uterine growth restriction have suggested that the rapamycin (mTOR) pathway may be involved in nutrient sensing in the placenta (Jansson and Powell, 2006). Studies in dairy cows (Liang et al., 2019) and sheep (Arroyo et al., 2009) also support the possibility of the mTOR pathway as a putative mechanism (Jansson et al., 2012).

Alternatively, future work may consider the molecular and cellular effects of maternal dietary protein on the placenta and the influence on fetal nutrient demands on placental gene expression (Gaccioli et al., 2013). The current study observed fetal sex-specific differences in the proportional localisation of BNCs within the feto-maternal junction. The current study of SerpinB1 protein localisation also suggested fetal sex-specific differences in protein expression, which stemmed from previous molecular findings by Edwards (2016). Current understanding points towards methionine as a key amino acid in the maternal diet which can influence fetal development, however other amino acids such as glycine, arginine, and serine should not be disregarded from further study.

7.2 The Implications of Maternal Diet on Fetal Mammary Epithelial Development

The current study investigated the effects of maternal diet on fetal mammary epithelial cell structures, and differential gene expression in fetal mammary tissues. Differential IGF1 expression was 0.99-fold greater in LH exposed tissues compared with HH ($p<0.05$), (Appendix 14). *IGF1* is known to promote epithelial development in mammary tissue (Macias and Hinck, 2012, Sternlicht, 2005, Hinck and Silberstein, 2005). *IGF1* is known to promote ductal elongation and branching morphogenesis in pubertal mouse mammary glands (Hinck and Silberstein, 2005, Richards et al., 2004). If fetal mammary *IGF1* expression during periconception is permanently altered, this may affect later pubertal development in the bovine mammary gland. Mouse mammary end-bud branching was reduced in *IGF1* null pubertal mice (Richards et al., 2004). *IGF1* expression has previously been reported in a number of fetal bovine tissues however the mammary gland was not investigated (Ghanipoor-Samami et al., 2018). Equally the study by Ghanipoor-Samami and colleagues (2018) reported 45 dpc and 153 dpc fetal expression, which is not comparable to the present study (98 dpc). In the current study, *IGF1* expression was greater in LH than HH 98 dpc mammary tissues, however *TGF-β* expression was unaffected (Appendix 14). Cellular protein localisation of *IGF1* and *TGF-β*, and further validation via RT-qPCR would elucidate whether these proteins appear to be involved in epithelial ductal morphogenesis at 98 dpc. In relation to lumen size, it is unclear whether an association between postconception diet on lumen size and periconception diet on *IGF1* expression exists. In the current study, lumen size was 54.5% greater in HPost exposed mammary tissues than LPost counterparts (Figure 3.8). This difference was potentially related to expression of cell adherins and integrins. Adherins and integrins are responsible for epithelial cell-cell contact (Knudsen and Wheelock, 2005). However, RT-qPCR suggested that relative expression of E-cadherin and integrin subunit beta 2 (ITGB2) did not differ between maternal dietary interventions. The 6.3-fold greater E-cadherin expression in LPost versus HPost mammary tissues (Figure 4.4) may be related to the differences in lumen size. E-cadherin is present in both luminal and myoepithelial cells within mammary ducts (Glukhora et al., 1995). Since total duct area, or duct size did not differ between dietary interventions (Appendix 1), this suggests that the differential expression may be due to differences in cell expression and not cell number.

In the current study, only a proportion (n=48, of which n=20 were female) of fetuses were culled at 98 dpc. Female fetuses which were carried to term entered a commercial farm (Personal Communication, Dr Viv Perry). With protocols such as RNA isolation of mammary epithelial cells derived from milk-fat globules from expressed milk, it is possible to analyse mRNA expression of lactating cows (Lemay et al., 2013, Chen et al., 2016, Choudhary et al., 2017, Irmak et al., 2012). Future investigation could include analysis of mRNA expression from the offspring carried to term. This would provide a more longitudinal study of the effects of maternal diet on fetal mammary gene expression, development, and additionally mature offspring epithelial gene expression. This would not allow for a direct comparison between the RNA-seq data of the current study and mRNA expression of mature offspring mammary epithelial cells, as the former is mammary fat pad whereas the latter would be luminal epithelial derived. However, a more longitudinal study may further the understanding of whether the effects of maternal diet during gestation are linked to permanent changes in offspring gene expression in the mammary gland.

Transcriptomic data suggests down-regulation of genes related to branching morphogenesis, such as *TFAP2A* and *TFAP2C* (Table 4.11). Investigation of the cellular effects of maternal dietary protein on fetal mammary development indicated no effect on ductal area or duct size (Appendices 1 and 2). While changes in gene expression at 98 dpc did not appear to alter mammary physiology at this stage of development, discrete contexts such as extent of branching development were not studied in the current study. Further analysis may involve comparison of epithelial-specific, branching morphogenesis genes between the 98 dpc placentome and fetal mammary gland. This would provide in-depth evidence for organ-specific effects of maternal dietary protein. This may provide a basis for further validation of organ-specific effects of maternal diet on fetal and placental gene expression.

One gene also expressed in epithelial cells is *KRT18*. *KRT18*, a cytokeratin and human epithelial cell marker (Wilson and Dering, 2004, Charafe-Jauffret et al., 2006, Bièche et al., 2002), was lowly expressed and unaffected by diet (Appendix 14). Mammary epithelial cell number was not investigated in the current study, although results indicated no significant differences in duct

size or area. Ducts are comprised of epithelial cells which are known to express *KRT18*. Gene expression of KRT18, though not directly comparable to duct area, was in agreement with cellular findings.

The balance between cell death and renewal in the lactating mammary gland has been linked with milk yield (Capuco et al., 2001). This involves cell proliferation and apoptosis (Sorensen et al., 2006). Furthermore, Marker of proliferation Ki-67 (*MKI67*) gene was expressed and unaffected by diet (LH 15.5 versus HH 18.8 FPKM, 0.27 Log^2FC) in the current study (Appendix 14). This compliments recent findings, which reported immunohistochemical localisation in epithelial cells distributed throughout bovine mammary ducts (n=3) at ~90 dpc (Hara et al., 2018). No markers of proliferation were used in immunohistochemistry in the current study. However, transcriptomic analysis of *MKI67* gene expression in 98 dpc fetal mammary tissues in the current study may have indicated no differences in proliferation (Appendix 14). If this persists to adult offspring, this may suggest no effects of maternal diet on offspring mammary cell renewal during lactation (Capuco et al., 2001).

Furthermore, immunohistochemistry and transcriptomics analysis combined are valuable tools to determine the cellular localisation and quantified mRNA expression. Particularly with advances such as Spatial RNA-seq (Salmén et al., 2018).

7.3 Adipocytes in the Fetal Mammary Gland

Further to epithelial cells, the current study investigated the presence of adipocytes in the fetal mammary fat pad. Although adipocytes were observed, no cellular quantitative analysis was performed following the immunohistochemical identification of adipocytes (Figure 3.12, Figure 3.13). Transcriptomic analysis of genes expressed by adipocytes was performed between the LH and HH exposed tissues (Appendix 14). Firstly, brown adipocyte specific UCP1 expression (LH, 0 versus HH, 0.27 FPKM) supports brown adipose tissue presence in HH exposed but not LH exposed tissues (Appendix 14). UCP1 immunopositive adipocytes were observed in n=3/6 and n=1/4 mammary samples exposed to HH and LH respectively (Figure 3.12). Although transcriptomics data in the current study was not cell-specific, these agree with histological results for UCP1. Gene expression of

UCP1 was low, which is representative of the (unquantified) low number of adipocytes observed throughout the tissue sections. Further RT-qPCR analysis of *UCP1* expression in all samples would further elucidate expression and indicate whether there was a significant difference in *UCP1* gene expression between treatment groups. Secondly, *UCP2* expression was 1.3-fold greater in LH compared with HH ($p<0.01$). However, *UCP2* is not specific to adipocytes. *UCP2* is known to be expressed in immune cells (Nègre-Salvayre et al., 1997), therefore this difference does not strictly support evidence for differential adipocyte cell populations. It may indicate differences in cell activity, rather than cell numbers. If further investigation occurred, both molecular and cellular analysis is recommended, to determine whether maternal dietary intervention affects adipocyte cell activity, cellular development (number, size) or a combination of both. Further analysis of adipocytes in 98 dpc is of interest as adipocytes are known to promote postnatal ductal development (Gouon-Evans et al., 2000). Adipocyte adiponectin expression is well-established to negatively correlate with level of adiposity (Locher et al., 2015, De Koster et al., 2017, Berg et al., 2002, Aye et al., 2013).

ADIPOQ gene expression in 98 dpc mammary tissue was not significantly affected by LH and HH diets (Appendix 14). This suggests that fetal mammary triglyceride stores were unaffected by maternal periconception diet followed by postconception high protein (LH versus HH). Mammary gland *ADIPOQ* expression was previously up-regulated in lactating versus non-lactating dairy cows (Dai et al., 2018). This may suggest that adiponectin expression may only be altered in a state of high metabolic stress such as lactation. Further investigation of *ADIPOQ* expression via RT-qPCR in all maternal dietary interventions, particularly expression in LL exposed tissues, may further elucidate this.

The current study confirmed gene expression of o*estrogen receptor alpha (ESR1*), which was not significantly different between LH and HH exposed 98 dpc mammary tissues (Appendix 14). *ESR1* was previously immunohistochemically identified in bovine fetal mammary adipocytes and epithelial cells (Hara et al., 2018). Although Hara and colleagues (2018) estimated the age of fetal mammary tissues by crown rump length, this provided evidence for fetal adipocytes which were comparable to those identified in the current study (Figure 3.12, Figure 3.13). Future work in the

current study may include ESR1 immunohistochemistry to identify the protein localisation in fetal mammary tissues of known age.
In summary, a combination of immunohistochemistry, and RT-qPCR for adipocyte related genes such as *Adiponectin*, UCP1, UCP2, *Adenomatosis polyposis coli down-regulated 1* (*APCDD1), and Krüppel-like factor 5 (KLF5)* would further confirm the molecular effects of maternal dietary protein on fetal adipocyte gene expression. Further study may involve Western Blotting to quantify protein expression of UCP1, UCP2, APCDD1 and KLF5. The current study provided the basis for gene expression in the fetal bovine mammary gland. Single-cell RNA-seq would provide transcriptomics data for specific cell-types (Liu et al., 2018, Negrón-Pérez et al., 2017, Pavličev et al., 2017). Although useful, this technique may not be applicable to the current study. Similarly, the use of spatially resolved single-cell RNA-seq may provide cell-specific data, whereas a more meaningful and useful technique for future investigation is within tissue spatial transcriptomics, which would provide more meaningful data in relation to gene expression within whole organ tissue sections (Achim et al., 2015, Salmén et al., 2018).

7.4 The Implications of an Up-regulated Mammary Immune System

In the current study of fetal mammary transcriptomics data, leukocytes overall were suggested to be affected by maternal dietary intervention as indicated by enriched pathway and gene ontology analysis of candidate genes (Appendix 11, Appendix 12). If these DEGs persist to adult and lactating offspring, this may influence the ability of the mammary gland immune defence against mastitis bacteria (Burvenich et al., 2003, Park et al., 2004).

In addition to adipocytes, it was found that *UCP2* is also expressed by macrophages and monocytes (Nègre-Salvayre et al., 1997, Kim et al., 2007). Although transcriptomics data analysis suggested a predominant effect of maternal dietary intervention on lymphocyte cell activation (Table 4.9, Appendix 10), UCP2 expression is evidence to suggest other immune cells such as monocytes were affected. Although RNA-seq data was no indication of cell-specific expression, this provides a basis for known presence of *UCP*2 expression, which may be further investigated via spatial transcriptomics (Salmén et al., 2018). In the current study, certain immune

cells, neutrophils and lymphocytes, were morphologically identified, though no specific staining was performed. Molecular findings in the current study support further investigation of the effects of maternal dietary intervention on immune cells. A novel role of macrophages in postnatal end-bud growth was presented (Gouon-Evans et al., 2000). This suggests a potential for the fetal immune system to affect future duct development in offspring, although further investigation of immune cells is required to provide evidence of a cellular response to up-regulation of immune related genes. The standard stain to assess microscopic anatomy (cells, nuclei and connective tissue) in pathology would be Haematoxylin and Eosin stain, however the low incidence of these cells within the tissue warrants the use of a marker for immunopositive identification.

Furthermore, there may be a link between placental STRA6 expression and fetal mammary immune function related gene expression (Rezamand et al., 2007). Increased dietary retinol was associated with improved lymphocyte function in peripartal dairy cows (Tjoelker et al., 1990). Similar findings have been reported, suggesting that retinol is beneficial to mammary immune function (Sordillo et al., 2009, LeBlanc et al., 2004). Altered placental transfer of retinol may have resulted in differential expression of immune related genes in the fetal mammary gland (Appendix 8, 9 and 10). Transcriptomics data analysis of term cotyledon expression of *STRA6* and retinol binding proteins are of interest to identify retinol transfer and transport at term.

7.5 Cellular and Molecular Effects of Maternal Dietary Intervention on Mammary Vasculature

The cellular and molecular effects of maternal dietary intervention on fetal mammary vasculature are partly complimentary. Periconception effects on stromal mammary blood vessel number are comparable to molecular results as only HH and LH exposed tissues were analysed via RNA-Seq. However, based on quality control criteria, differential expression of known specific angiogenesis related genes such as *VEGF* isomers were not observed (Appendix 14). Periconception diet had organ-specific effects on vasculature, as the proportion of placental vasculature was greater in periconception high protein compared with low protein (Edwards, 2016). This was converse to the effects of periconception maternal diet on mammary vasculature

(Chapter 3.4.3). This may be due to organ differences, such as the placenta being a transitory organ, thereby development and gene expression is confined to its specific role until term. Whereas the fetal mammary gland may be subjected to different DNA modifications to prepare for the future expected environment, based on *in utero* conditions.

FGFR2 was significantly downregulated in LH versus HH exposed 98 dpc mammary tissues (Appendix 14). As previously described (Chapter 4.1.6), FGFR2 has a role in mammary morphogenesis. Cellular analysis of 98 dpc mammary tissues indicated overall no significant differences in mammary duct size (Appendix 1). Previously, lower *FGFR2* expression was linked to decreased mammary size in neonatal mice, versus controls (Parsa et al., 2008). This suggested that the differences in FGFR2 gene expression in the current study did not affect mammary development.

Notch1 expression in the 98 dpc mammary gland was unaffected by diet between LH and HH exposed samples (Appendix 14). This agrees with cellular results in the current study, which indicated that mammary vasculature was largely unaffected by maternal dietary interventions (Appendix 5, Appendix 6).

7.6 Industrial Implications

The aims of the current study were to determine the cellular and molecular effects of maternal dietary intervention on fetal development in Santa Gertrudis cows. This beef breed is farmed in Australia, which often has limited rainfall and subsequent low protein content in grass (Chapter 1.1.2). In beef production, herd health and production performance are intrinsically linked to sustainability, efficiency and economic outcomes. It is therefore essential to evaluate the implications of the current findings. By 98 dpc, the effects of maternal intervention on rudimentary duct development indicates no effect on total duct tissue concerning epithelial cells. Although the area of ductal tissue is not equivalent to epithelial cell number. Differences in epithelial cell density were observed between ducts of differing developmental stages (Table 3.4). Despite the indication that lumen expansion was greater in HPost exposed tissues than LPost counterparts, this is not known to be related to future milk production performance. The difference in lumen size may indicate differences in the development of the

network of glandular tissue, however more research is required to confirm this, as three-dimensional analysis was not conducted. Overall, duct parameters were unaffected by dietary intervention by 98 dpc. This suggests no cellular effect and thus potentially no detrimental effect of low protein on future mammary development and subsequent milk production performance. However, molecular expression, particularly of FGFR2 may have permanently altered cellular molecular activity. Further research is required to evaluate the effects of periconception and postconception dietary intervention on offspring mammary development and molecular activity.

Mammary stem cell marker *LGR5* was unaffected by diet (Appendix 14), which suggests cell number was unaffected at 98 dpc (Trejo et al., 2017). However this does not indicate cell activity (Trejo et al., 2017). Regarding mammary vasculature, the increase in stromal blood vessel number in LPeri exposed mammary tissues, suggests the potential for increased blood supply to the developing mammary tissue. This may benefit future ductal branching morphogenesis compared with periconception high protein counterparts. However, molecular analysis suggests the structural integrity of the mammary tissue may have been altered. Particularly by cell adhesion molecules E-cadherin and integrins. Future research into the structural integrity of stromal blood vessels in 98 dpc mammary tissue would elucidate whether gene expression was related to vascular structural integrity. Molecular findings support further investigation of specific staining for markers of cell adhesion proteins within 98 dpc mammary tissue. Decreased structural integrity may open the organ to future infection (Anders et al., 2003, Petruzzelli et al., 1999).

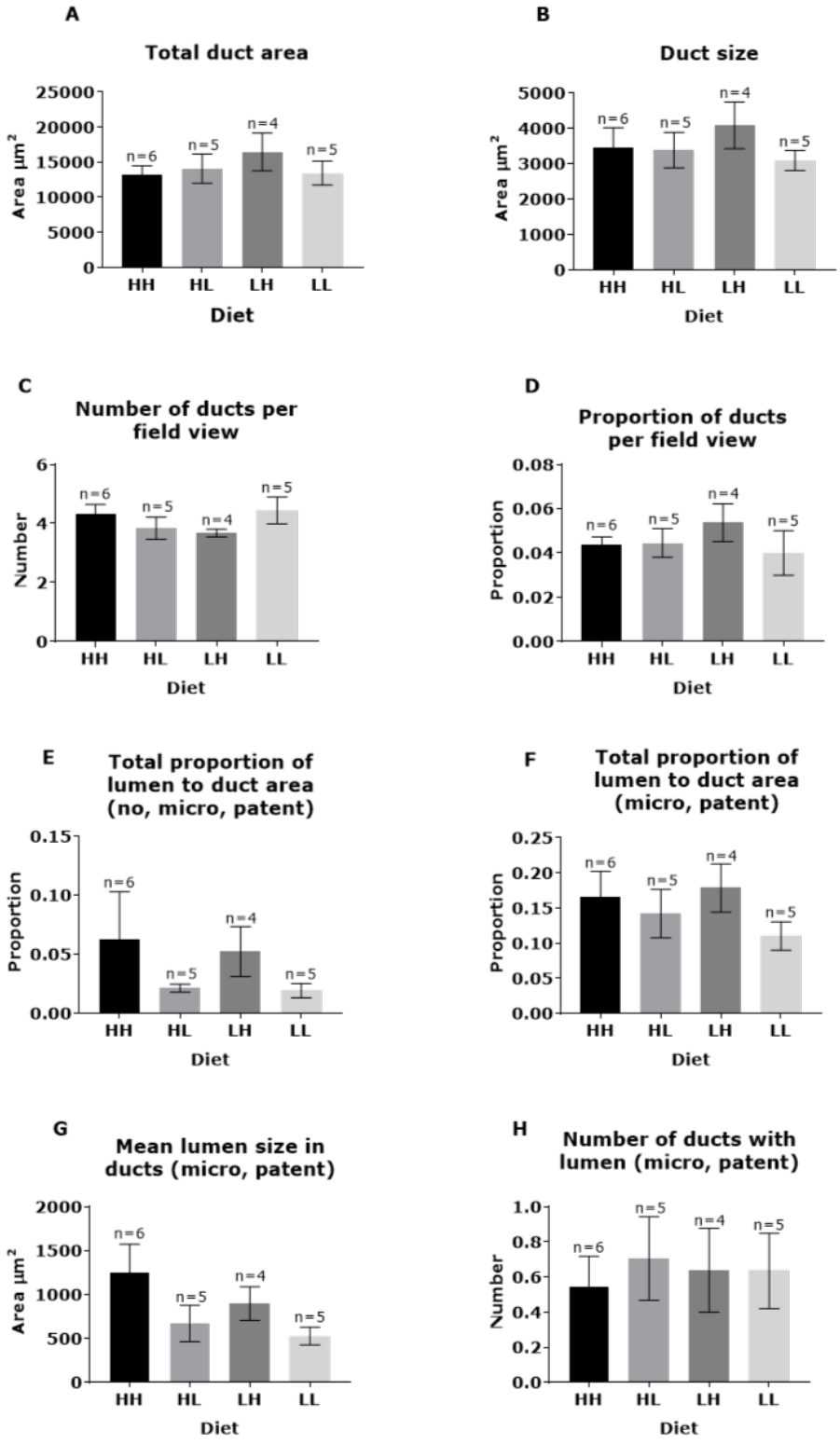
Molecular findings indicate periconception diet affected immune function (Chapter 5). Mammary infection in cows is detrimental to health and milk production. Mastitis accounts for a huge economic cost in dairy production and beef production to an extent. Further to production performance, mastitis also reportedly was detrimental to ovarian function and reproductive competence (Roth et al., 2016). Mastitis-causing bacteria, *S. aureus* and *E. coli* both reduced early embryo development to 2-, 4-cell, and blastocyst stage (Roth et al., 2016). Previously, mRNA expression of genes associated with embryonic development were reduced in oocytes of infected cows (Roth et al., 2016). Supporting evidence was published to suggest that even sub-clinical levels of mastitis negatively affected oocyte viability (Santos et al.,

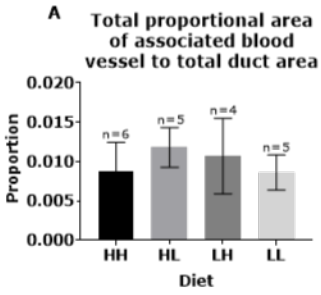
2018, Mellado et al., 2018). Regarding the present study, although future implications are unknown, fetal programming of the mammary gland altered genes associated with immune response. This programming may alter the adult cow's immune function against mastitis-causing bacteria, and subsequently affect ovarian function as previously reported (Santos et al., 2018, Roth et al., 2016, Mellado et al., 2018). Heifer phenotype should also be considered when making comparisons, particularly between beef crossbreeds, and beef and dairy breeds. The effect of high level of maternal diet during the first trimester on placenta weight was dependent on heifer genotype (Sullivan et al., 2009d). These results indicate that different genotypes are differentially affected by nutrition level. This should be taken into account when applying results of the current study to cattle breeds which were not used in the current study.

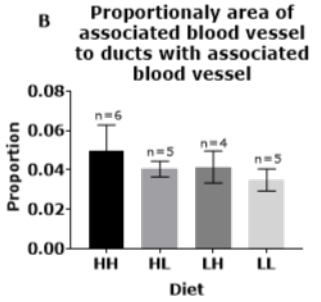
The overarching theme of this study was global food security in relation to beef production. The impact climate change will have on future grasses is unpredictable and was beyond the scope of the current study (Connor and Hawkes, 2018, Polley et al., 2017, Dellar et al., 2018, Dumont et al., 2015). However, irrespective of climate change, the aim was to further the current understanding of maternal low protein diet on fetal gene expression and development. By furthering the understanding of effects, future nutritional and reproductive decision making in cattle production may be made to ensure optimal health and development of the future generations of cattle. The areas investigated in this study, particularly the fetal mammary gland indicate novel findings of the effects of maternal diet on fetal mammary development and gene expression which suggests numerous implications to the offspring in later life. This is valuable as the mammary gland is an organ of economic importance to both the dairy and beef industry. Future research based on these findings may influence policy within agricultural productivity, and impact food security and sustainability, and climate change mitigation policy (Obersteiner et al., 2016). Indeed, further questions were raised from the results of the current study which warrants further research in order to understand the long-term effects of molecular and cellular differences in the bovine fetal mammary gland and placentome.

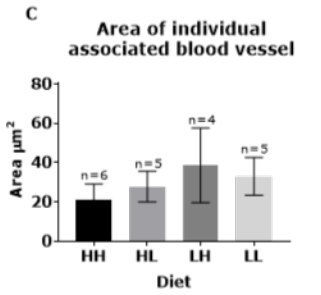
Appendix

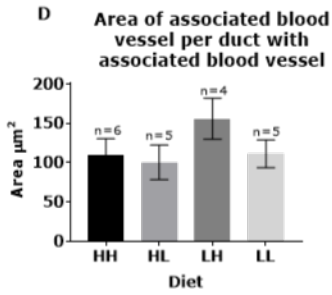
Appendix 1 Total fetal mammary duct analysis (includes ducts with no lumen, micro lumina, and patent lumen) between maternal dietary interventions

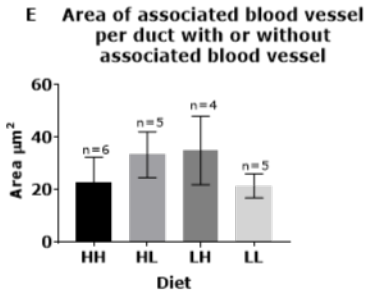


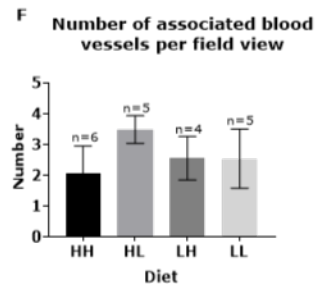

A
Total proportional area of associated blood vessel to total duct area
Proportion
0.020
0.015
0.010
0.005
0.000
n=6
n=5
n=4
n=5
HH
HL
LH
LL
Diet

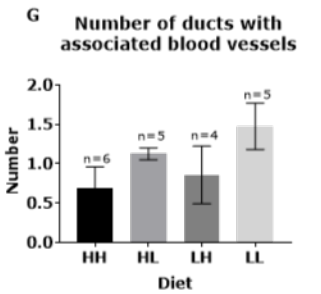

B
Proportionaly area of associated blood vessel to ducts with associated blood vessel
Proportion
0.08
0.06
0.04
0.02
0.00
n=6
n=5
n=4
n=5
HH
HL
LH
LL
Diet

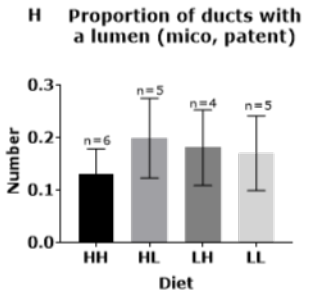

C
Area of individual associated blood vessel
Area µm²
80
60
40
20
0
n=6
n=5
n=4
n=5
HH
HL
LH
LL
Diet

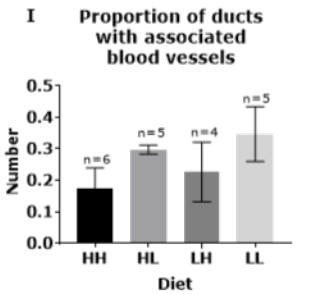

D
Area of associated blood vessel per duct with associated blood vessel
Area µm²
200
150
100
50
0
n=6
n=5
n=4
n=5
HH
HL
LH
LL
Diet


E
Area of associated blood vessel per duct with or without associated blood vessel
Area µm²
60
40
20
0
n=6
n=5
n=4
n=5
HH
HL
LH
LL
Diet

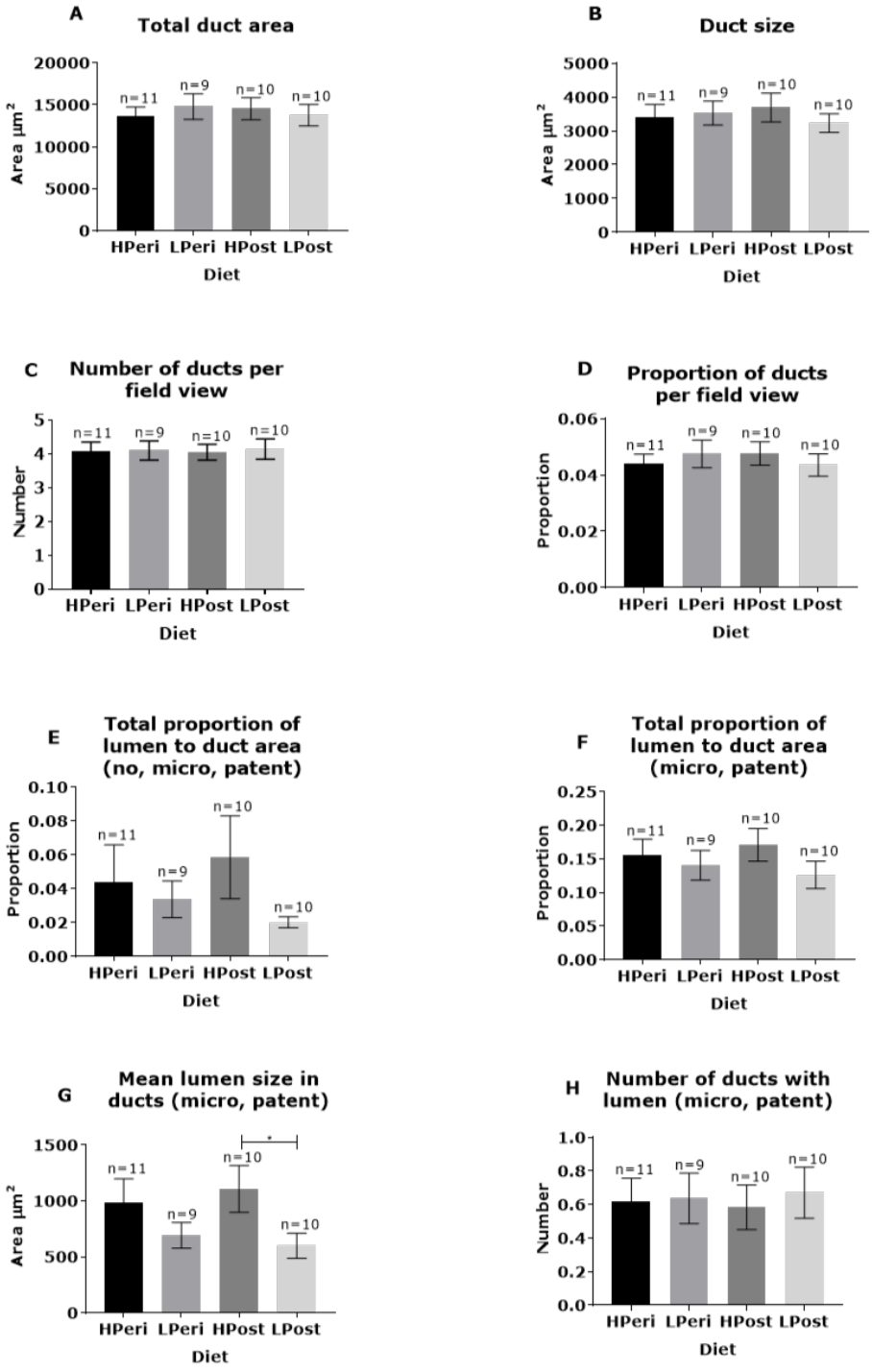

F
Number of associated blood vessels per field view
Number
5
4
3
2
1
0
n=6
n=5
n=4
n=5
HH
HL
LH
LL
Diet

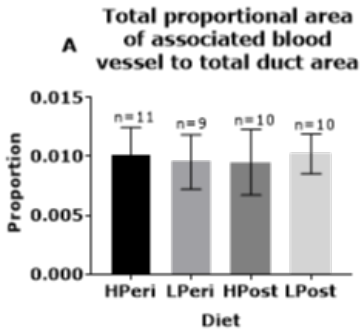

G
Number of ducts with associated blood vessels
Number
2.0
1.5
1.0
0.5
0.0
n=6
n=5
n=4
n=5
HH
HL
LH
LL
Diet

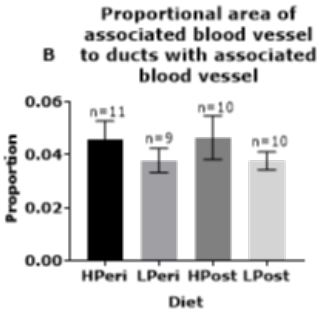

H
Proportion of ducts with a lumen (mico, patent)
Number
0.3
0.2
0.1
0.0
n=6
n=5
n=4
n=5
HH
HL
LH
LL
Diet

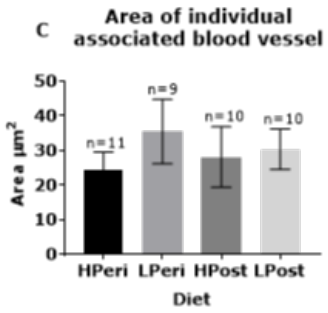

I
Proportion of ducts with associated blood vessels
Number
0.5
0.4
0.3
0.2
0.1
0.0
n=6
n=5
n=4
n=5
HH
HL
LH
LL
Diet

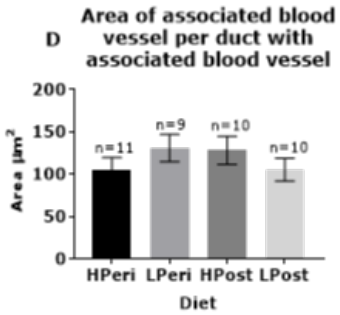
Appendix 2 Total fetal mammary duct analysis (includes ducts with no lumen, micro lumina, and patent lumen) between periconception and postconception diets

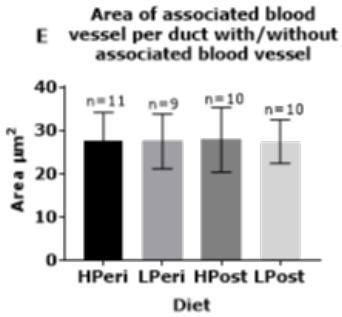


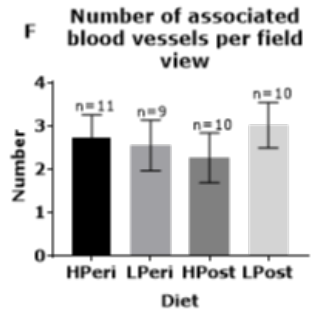

A
Total proportional area of associated blood vessel to total duct area
Proportion
0.015
0.010
0.005
0.000
n=11
n=9
n=10
n=10
HPeri LPeri HPost LPost
Diet

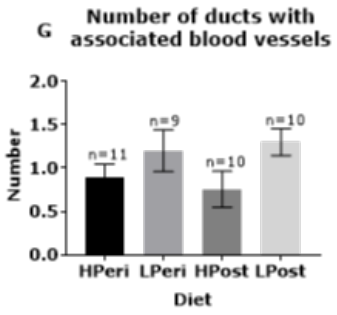

B
Proportional area of associated blood vessel to ducts with associated blood vessel
Proportion
0.06
0.04
0.02
0.00
n=11
n=9
n=10
n=10
HPeri LPeri HPost LPost
Diet

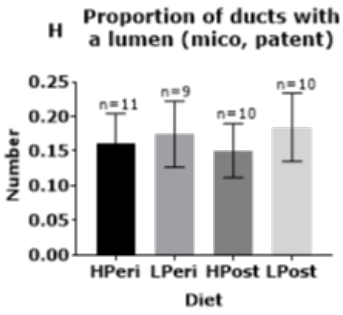

C
Area of individual associated blood vessel
Area µm²
50
40
30
20
10
0
n=11
n=9
n=10
n=10
HPeri LPeri HPost LPost
Diet

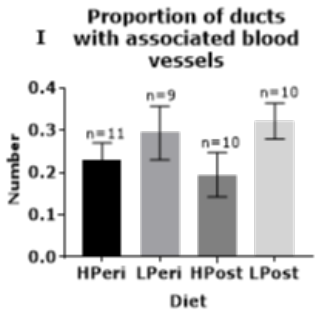

D
Area of associated blood vessel per duct with associated blood vessel
Area µm²
200
150
100
50
0
n=11
n=9
n=10
n=10
HPeri LPeri HPost LPost
Diet


E
Area of associated blood vessel per duct with/without associated blood vessel
Area µm²
40
30
20
10
0
n=11
n=9
n=10
n=10
HPeri LPeri HPost LPost
Diet

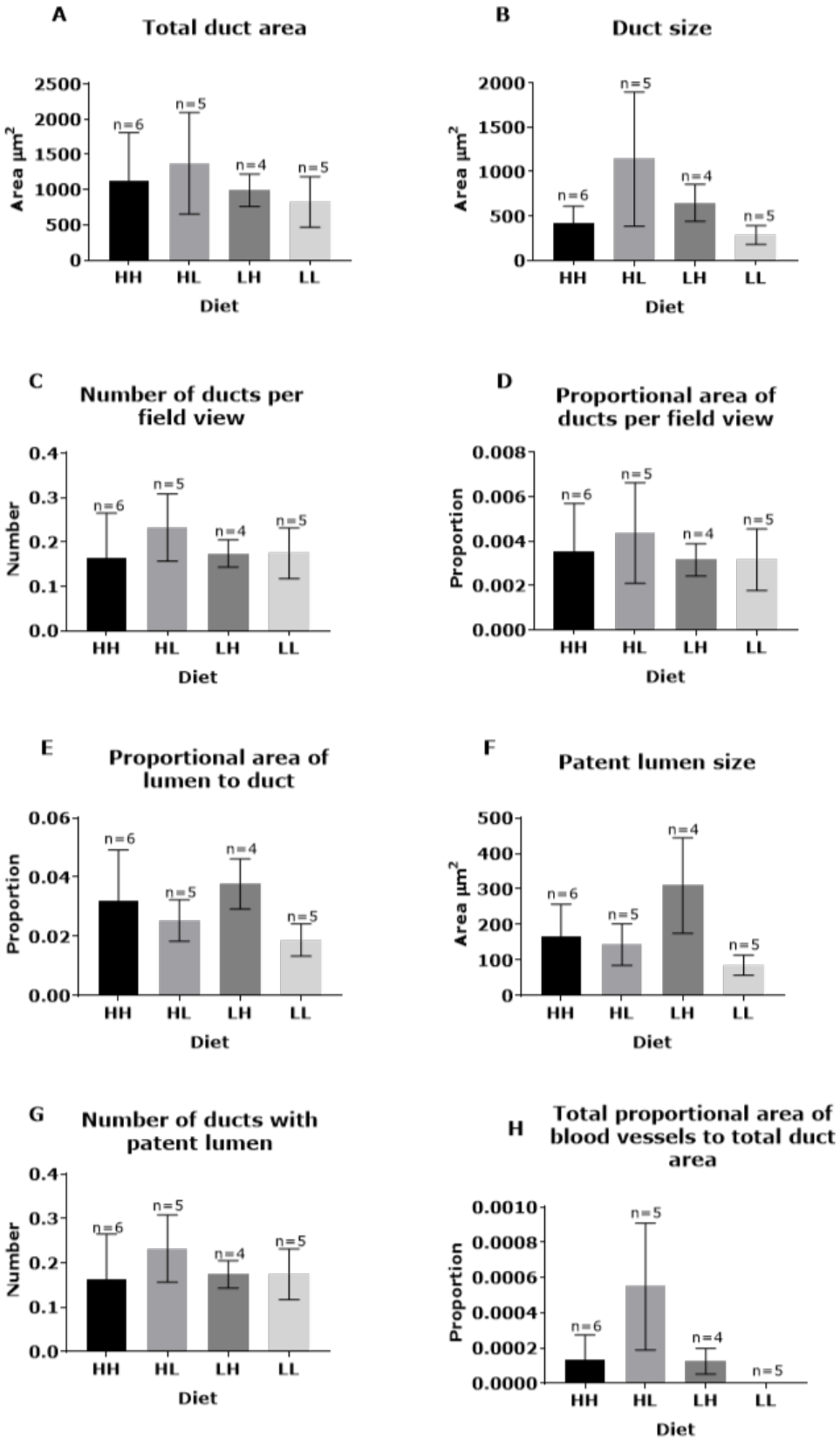

F
Number of associated blood vessels per field view
Number
4
3
2
1
0
n=11
n=9
n=10
n=10
HPeri LPeri HPost LPost
Diet

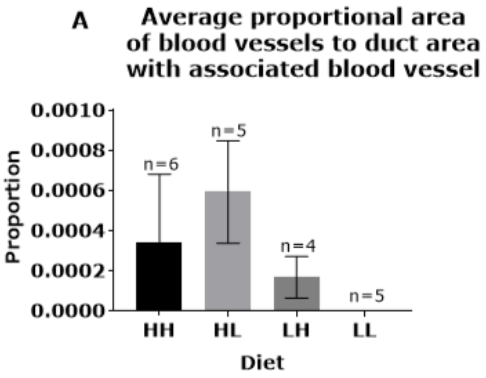

G
Number of ducts with associated blood vessels
Number
2.0
1.5
1.0
0.5
0.0
n=11
n=9
n=10
n=10
HPeri LPeri HPost LPost
Diet

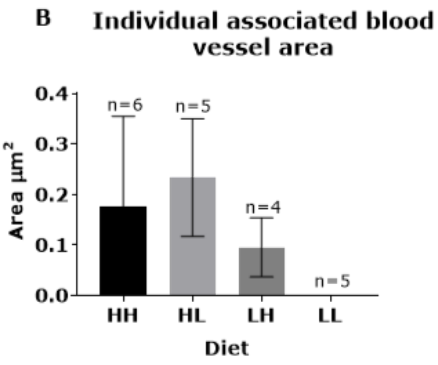

H
Proportion of ducts with a lumen (mico, patent)
Number
0.25
0.20
0.15
0.10
0.05
0.00
n=11
n=9
n=10
n=10
HPeri LPeri HPost LPost
Diet

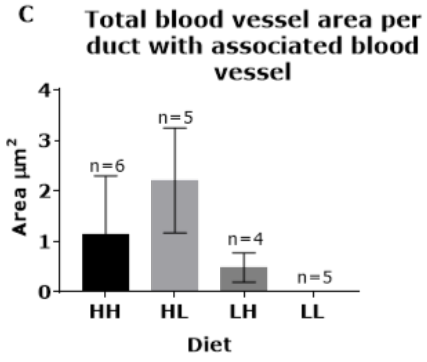

I
Proportion of ducts with associated blood vessels
Number
0.4
0.3
0.2
0.1
0.0
n=11
n=9
n=10
n=10
HPeri LPeri HPost LPost
Diet

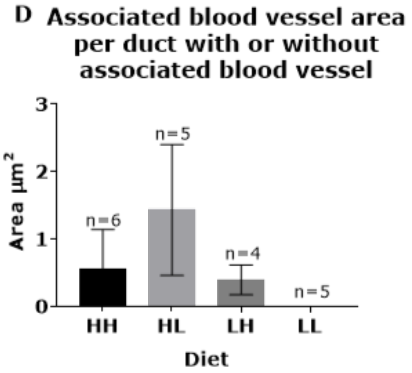
Appendix 3 Patent mammary duct analysis (patent lumen only) between maternal dietary interventions

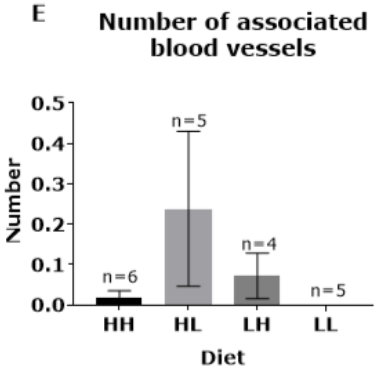


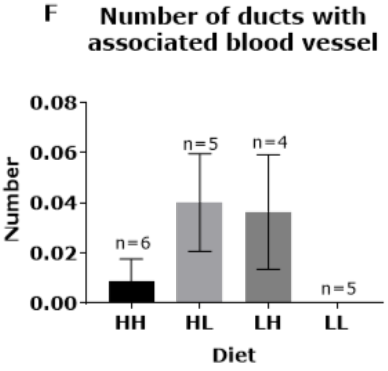

A
Average proportional area of blood vessels to duct area with associated blood vessel
Proportion
0.0010
0.0008
0.0006
0.0004
0.0002
0.0000
n=6
n=5
n=4
n=5
HH
HL
LH
LL
Diet

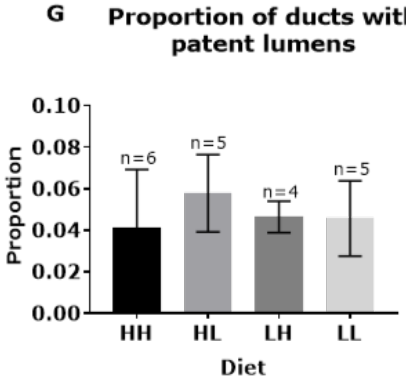

B
Individual associated blood vessel area
Area µm²
0.4
0.3
0.2
0.1
0.0
n=6
n=5
n=4
n=5
HH
HL
LH
LL
Diet

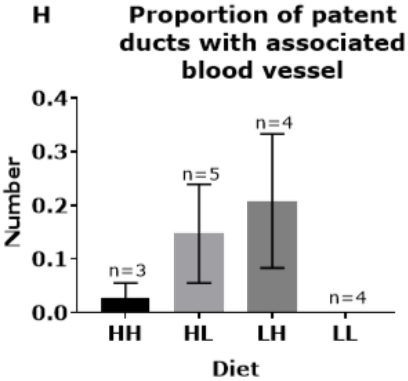

C
Total blood vessel area per duct with associated blood vessel
Area µm²
4
3
2
1
0
n=6
n=5
n=4
n=5
HH
HL
LH
LL
Diet


D
Associated blood vessel area per duct with or without associated blood vessel
Area µm²
3
2
1
0
n=6
n=5
n=4
n=5
HH
HL
LH
LL
Diet

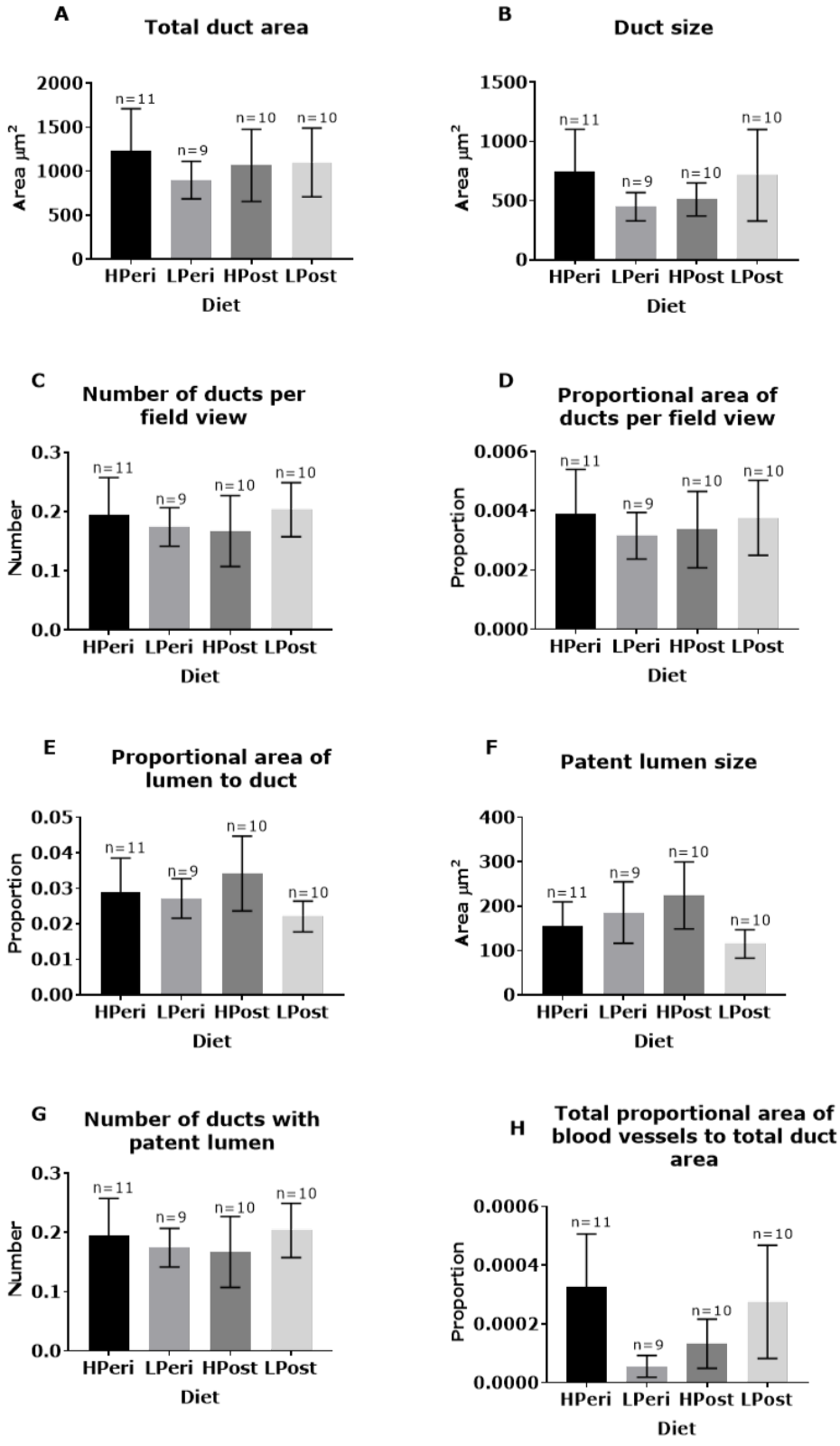

E
Number of associated blood vessels
Number
0.5
0.4
0.3
0.2
0.1
0.0
n=6
n=5
n=4
n=5
HH
HL
LH
LL
Diet

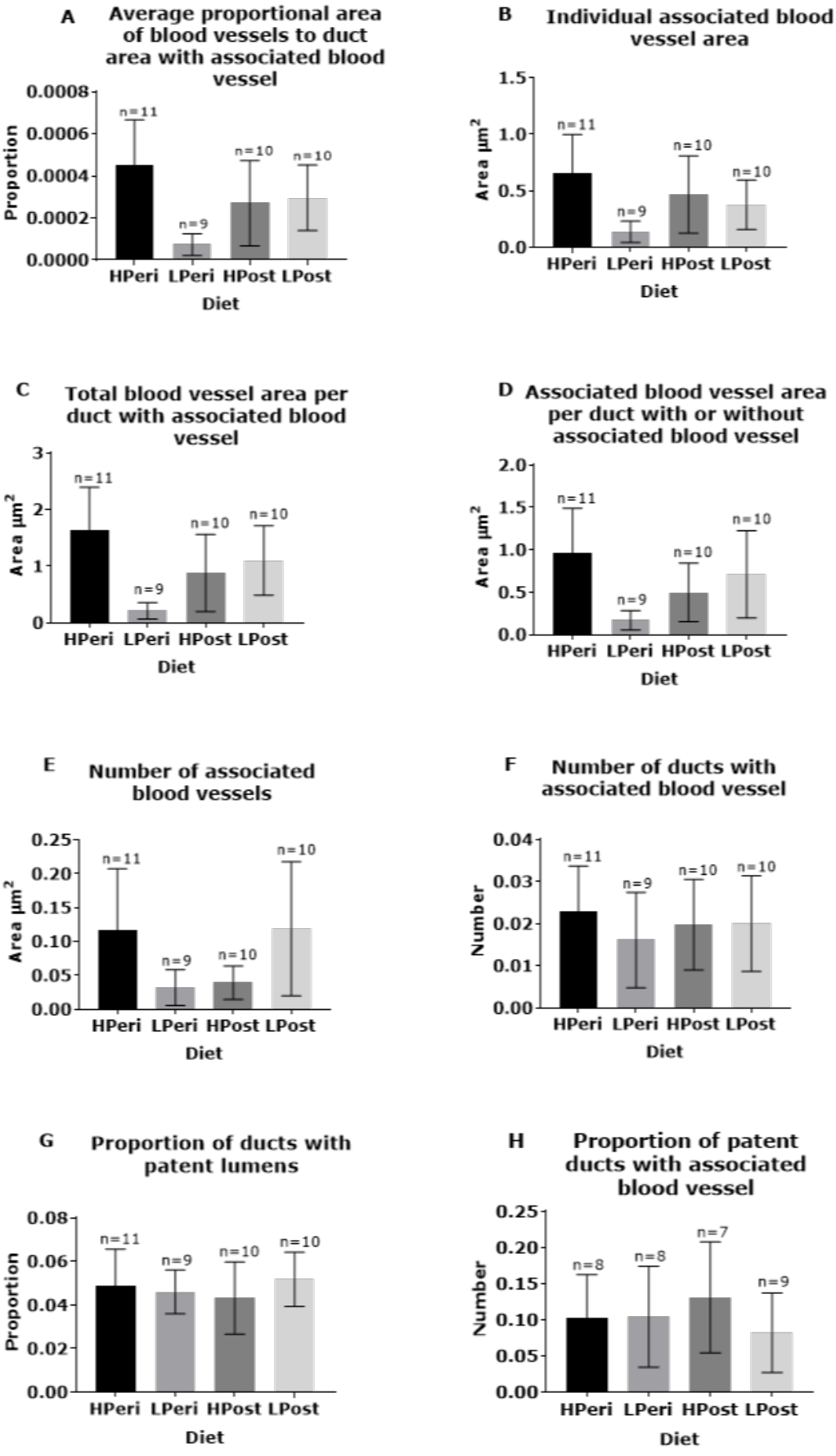

F
Number of ducts with associated blood vessel
Number
0.08
0.06
0.04
0.02
0.00
n=6
n=5
n=4
n=5
HH
HL
LH
LL
Diet


G
Proportion of ducts with patent lumens
Proportion
0.10
0.08
0.06
0.04
0.02
0.00
n=6
n=5
n=4
n=5
HH
HL
LH
LL
Diet

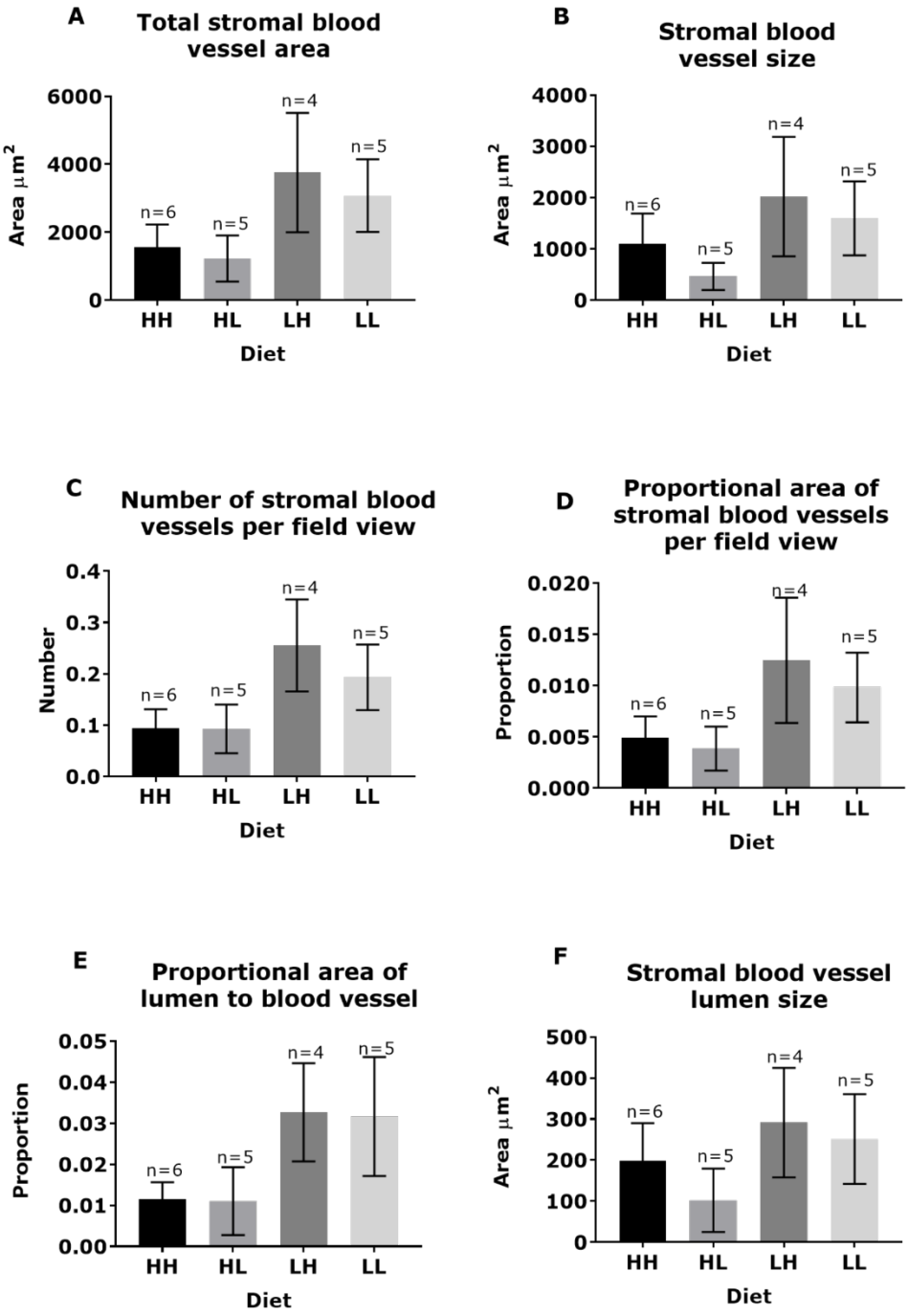

H
Proportion of patent ducts with associated blood vessel
Number
0.4
0.3
0.2
0.1
0.0
n=3
n=5
n=4
n=4
HH
HL
LH
LL
Diet

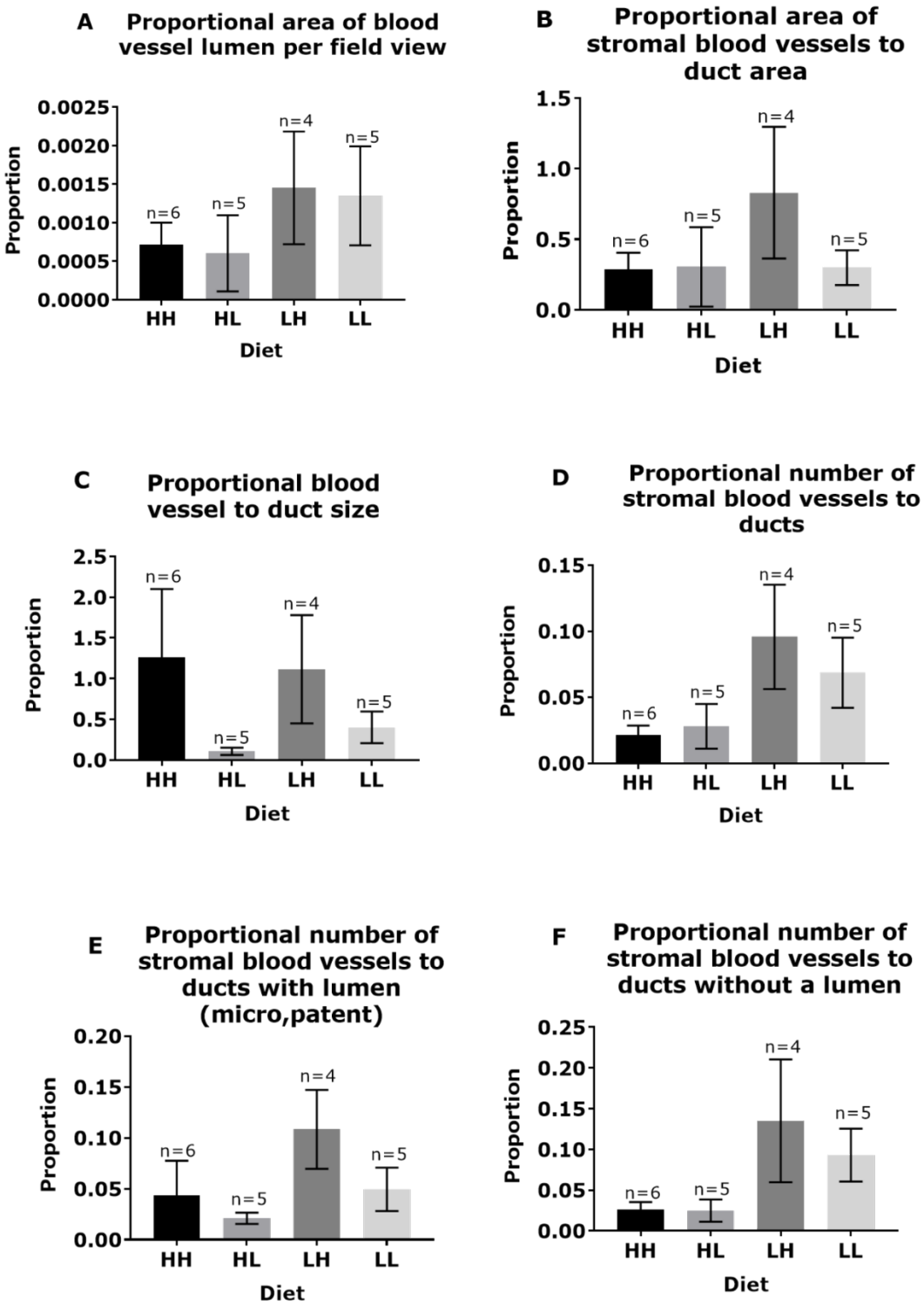
Appendix 4 Patent mammary duct analysis (patent lumen only) between periconception and postconception diets



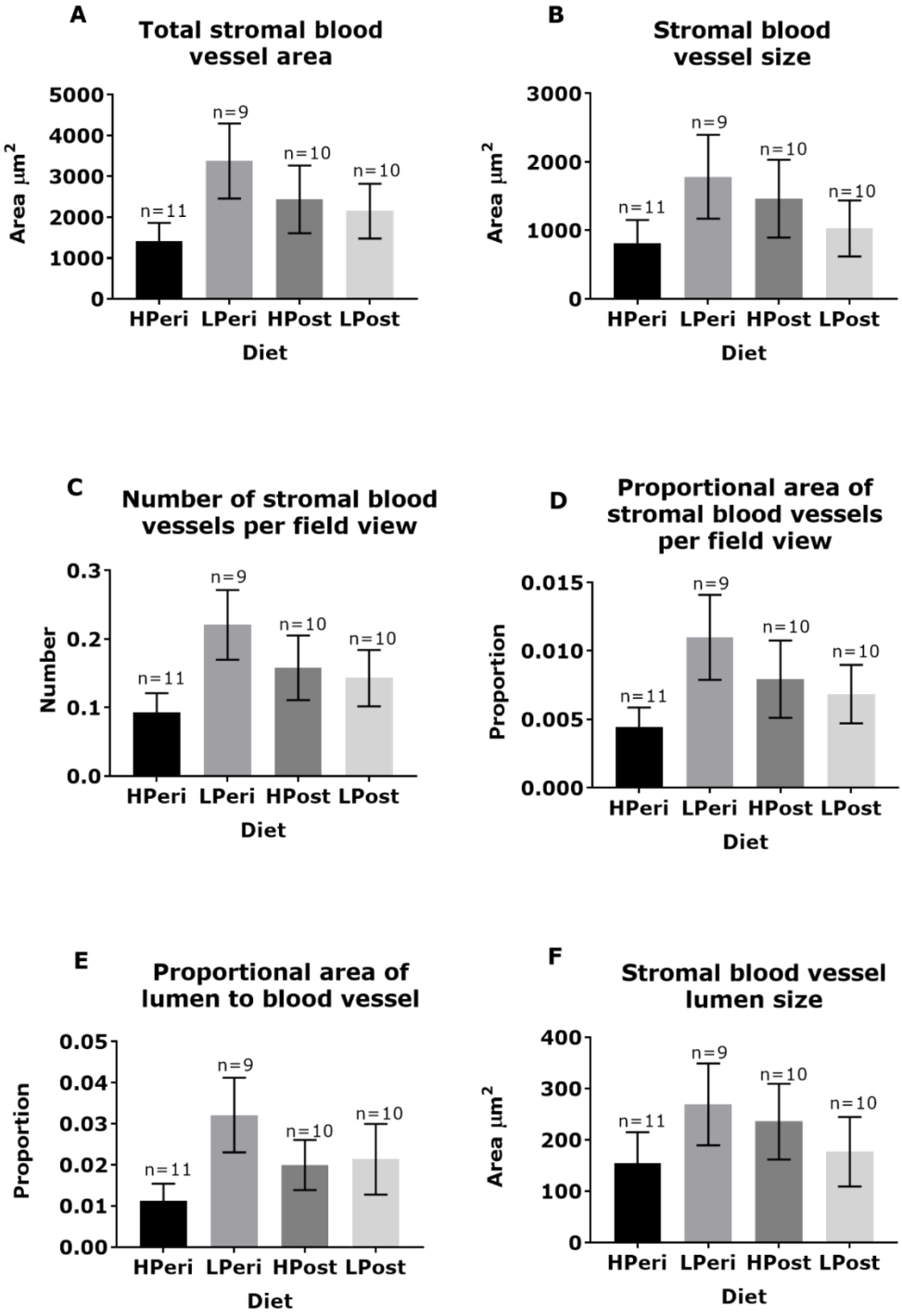

A
Average proportional area of blood vessels to duct area with associated blood vessel
Proportion
0.0008
0.0006
0.0004
0.0002
0.0000
n=11
n=9
n=10
n=10
HPeri LPeri HPost LPost
Diet
B
Individual associated blood vessel area
Area µm²
1.5
1.0
0.5
0.0
n=11
n=9
n=10
n=10
HPeri LPeri HPost LPost
Diet
C
Total blood vessel area per duct with associated blood vessel
Area µm²
3
2
1
0
n=11
n=9
n=10
n=10
HPeri LPeri HPost LPost
Diet
D
Associated blood vessel area per duct with or without associated blood vessel
Area µm²
2.0
1.5
1.0
0.5
0.0
n=11
n=9
n=10
n=10
HPeri LPeri HPost LPost
Diet
E
Number of associated blood vessels
Area µm²
0.25
0.20
0.15
0.10
0.05
0.00
n=11
n=9
n=10
n=10
HPeri LPeri HPost LPost
Diet
F
Number of ducts with associated blood vessel
Number
0.04
0.03
0.02
0.01
0.00
n=11
n=9
n=10
n=10
HPeri LPeri HPost LPost
Diet
G
Proportion of ducts with patent lumens
Proportion
0.08
0.06
0.04
0.02
0.00
n=11
n=9
n=10
n=10
HPeri LPeri HPost LPost
Diet
H
Proportion of patent ducts with associated blood vessel
Number
0.25
0.20
0.15
0.10
0.05
0.00
n=8
n=8
n=7
n=9
HPeri LPeri HPost LPost
Diet

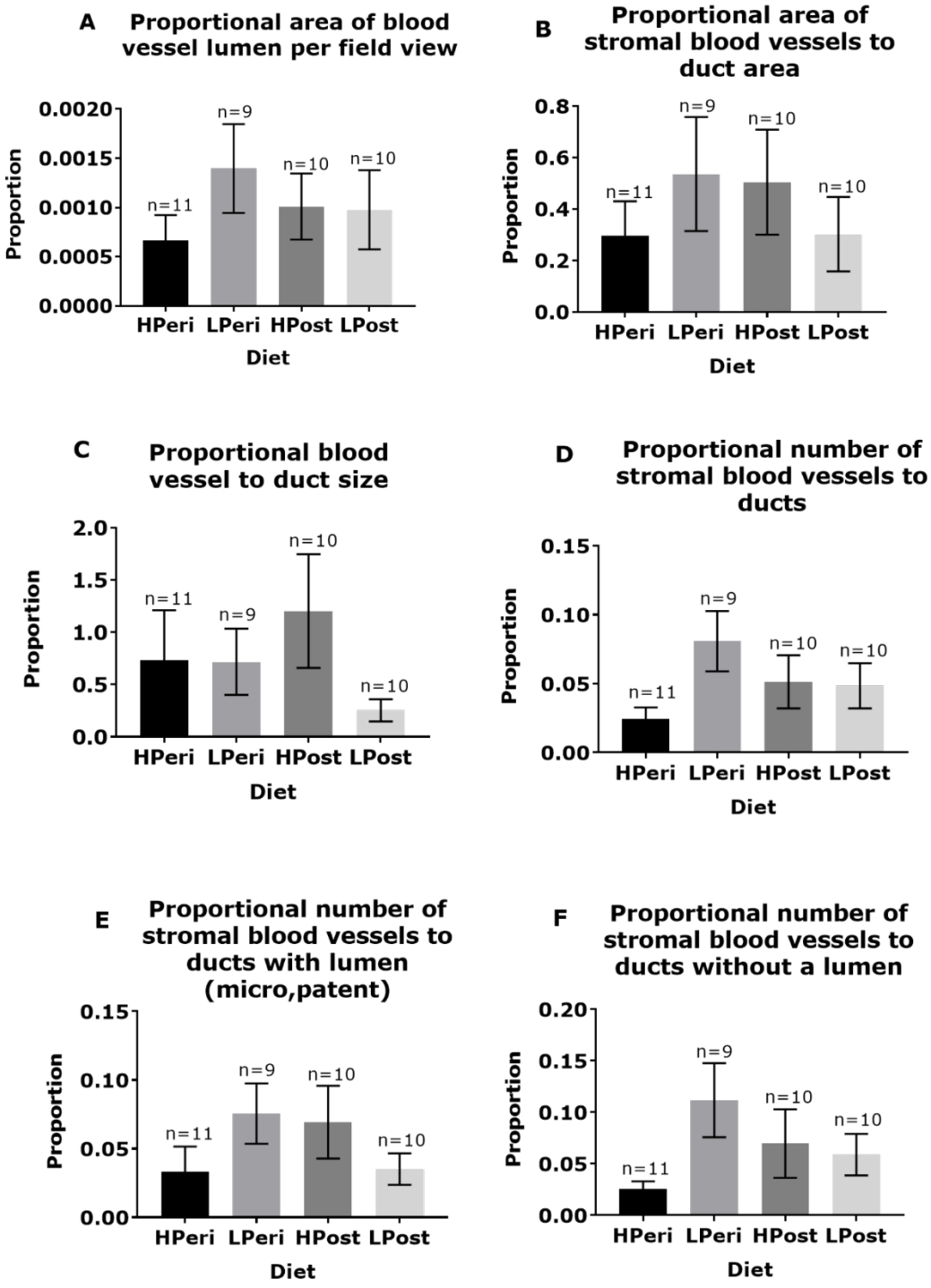
Appendix 5 Stromal blood vessel analysis in 98 days postconception bovine mammary glands between maternal dietary interventions



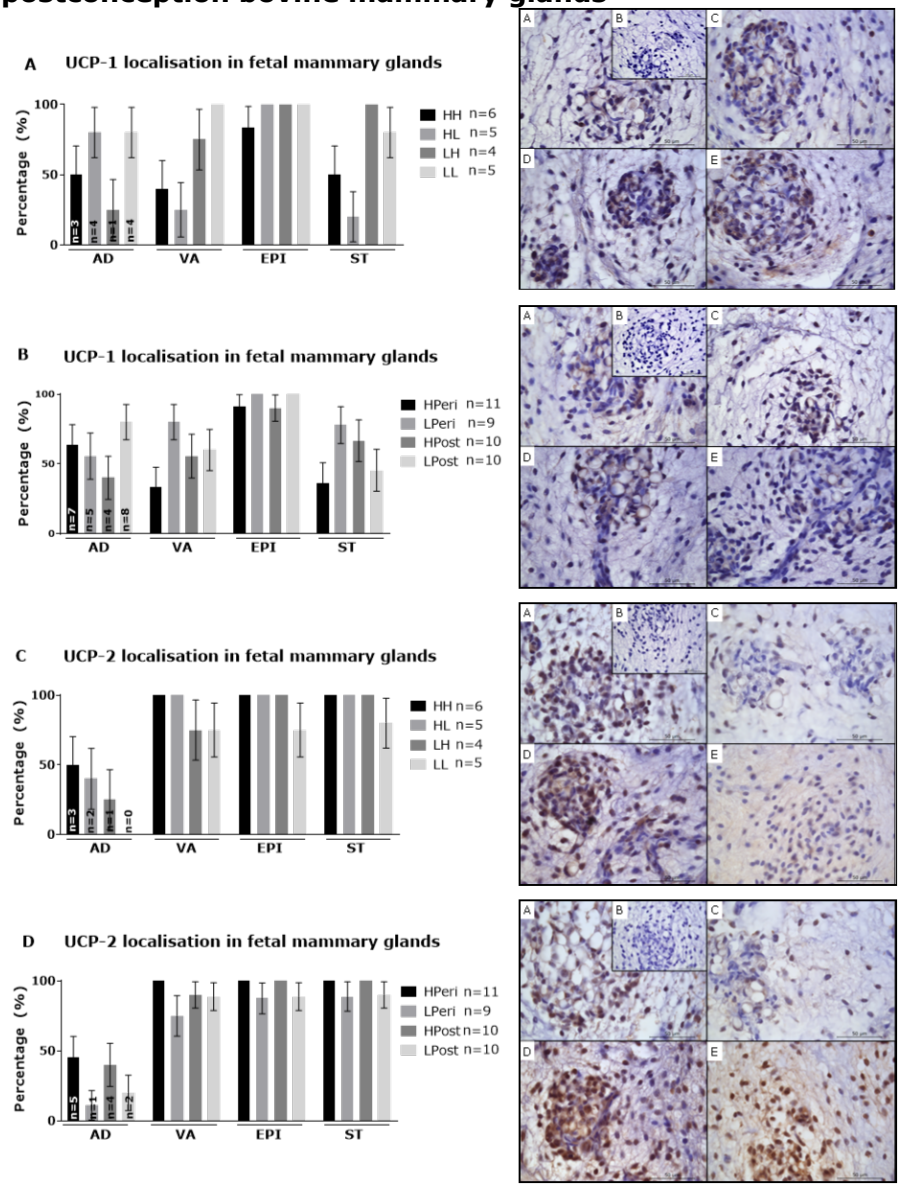

A
Proportional area of blood vessel lumen per field view
Proportion
0.0025
0.0020
0.0015
0.0010
0.0005
0.0000
n=6
n=5
n=4
n=5
HH
HL
LH
LL
Diet
B
Proportional area of stromal blood vessels to duct area
Proportion
1.5
1.0
0.5
0.0
n=6
n=5
n=4
n=5
HH
HL
LH
LL
Diet
C
Proportional blood vessel to duct size
Proportion
2.5
2.0
1.5
1.0
0.5
0.0
n=6
n=5
n=4
n=5
HH
HL
LH
LL
Diet
D
Proportional number of stromal blood vessels to ducts
Proportion
0.15
0.10
0.05
0.00
n=6
n=5
n=4
n=5
HH
HL
LH
LL
Diet
E
Proportional number of stromal blood vessels to ducts with lumen (micro,patent)
Proportion
0.20
0.15
0.10
0.05
0.00
n=6
n=5
n=4
n=5
HH
HL
LH
LL
Diet
F
Proportional number of stromal blood vessels to ducts without a lumen
Proportion
0.25
0.20
0.15
0.10
0.05
0.00
n=6
n=5
n=4
n=5
HH
HL
LH
LL
Diet

Appendix 6 Stromal blood vessel analysis in 98 days postconception bovine mammary glands between periconception and postconception diets




A
Proportional area of blood vessel lumen per field view
Proportion
0.0020
0.0015
0.0010
0.0005
0.0000
n=11
n=9
n=10
n=10
HPeri LPeri HPost LPost
Diet
B
Proportional area of stromal blood vessels to duct area
Proportion
0.8
0.6
0.4
0.2
0.0
n=11
n=9
n=10
n=10
HPeri LPeri HPost LPost
Diet
C
Proportional blood vessel to duct size
Proportion
2.0
1.5
1.0
0.5
0.0
n=11
n=9
n=10
n=10
HPeri LPeri HPost LPost
Diet
D
Proportional number of stromal blood vessels to ducts
Proportion
0.15
0.10
0.05
0.00
n=11
n=9
n=10
n=10
HPeri LPeri HPost LPost
Diet
E
Proportional number of stromal blood vessels to ducts with lumen (micro,patent)
Proportion
0.15
0.10
0.05
0.00
n=11
n=9
n=10
n=10
HPeri LPeri HPost LPost
Diet
F
Proportional number of stromal blood vessels to ducts without a lumen
Proportion
0.20
0.15
0.10
0.05
0.00
n=11
n=9
n=10
n=10
HPeri LPeri HPost LPost
Diet

Appendix 7 UCP1 and UCP2 immunohistochemistry in the 98 days postconception bovine mammary glands



The percentage ±SEM of samples displaying UCP1 and UCP2 immunopositivity and the cellular localisation in 98 days postconception fetal bovine mammary glands exposed to maternal dietary interventions. Graphs and complimentary photomicrographs (B=negative control, A, C-E represent each graph key e.g. A=HH, C=HL, D=LH, E=LL) display UCP1 (A) and UCP2 (C) results between maternal dietary interventions, and periconception and postconception diets (B, D respectively). H=high protein, L=low protein Letters denote periconception (Peri) and postconception (Post) diets, e.g. HL= high protein periconception, low protein postconception. AD=adipocyte, VA=vasculature, EPI=epithelial cell, ST=stroma. Total samples per group: HH n=6, HL n=5, LH n=4. Samples with observed adipocytes (UCP1): HH n=3, HL n=4, LH n=1, LL n=4. Samples with observed adipocytes (UCP2): HH n=3, HL n=2, LH n=1, LL n=0. Scale bar represents 50 μm.

Appendix 8 Up-regulated genes in LH versus HH exposed 98 days postconception bovine mammary tissues

Ensembl ID	Gene symbol	Log2 FC	p value
ENSBTAG00000011238	CD48	4.40059	7.60E-03
ENSBTAG00000038496	CR2	4.33353	5.05E-03
ENSBTAG00000020116	JSP.1	4.29758	1.46E-02
ENSBTAG00000019588	BOLA-DQB	4.18912	9.55E-03
ENSBTAG00000006466	CD53	3.76645	1.09E-02
ENSBTAG00000007103	ITGAL	3.72411	6.15E-03
ENSBTAG00000013185	TIMD4	3.71059	1.37E-02
ENSBTAG00000012684	CCL19	3.68023	1.79E-02
ENSBTAG00000014725	CD27	3.64875	4.82E-02
ENSBTAG00000023144	PTPRC	3.54174	1.65E-03
ENSBTAG00000021145	MAP4K1	3.51786	8.05E-03
ENSBTAG00000006730	SUSD2	3.50521	9.70E-03
ENSBTAG00000005628	CD52	3.34637	1.93E-02
ENSBTAG00000046257	GIMAP4	3.16301	2.50E-02
ENSBTAG00000007626	IL2RG	3.11497	5.50E-04
ENSBTAG00000021141	CD8A	3.02412	3.38E-02
ENSBTAG00000008953	TAP1	3.01824	2.19E-02
ENSBTAG00000007079	LCP1	2.98873	3.30E-03
ENSBTAG00000002705	REC8	2.8488	2.77E-02
ENSBTAG00000012695	LCK	2.8366	4.08E-02
ENSBTAG00000033334	SPATA2L	2.78065	3.65E-02
ENSBTAG00000021906	COL6A5	2.60107	4.85E-02
ENSBTAG00000011766	C7	2.57789	5.00E-05
ENSBTAG00000037605	BOLA-DQA1	2.57487	2.40E-03
ENSBTAG00000001001	ANKRD55	2.56134	4.35E-02
ENSBTAG00000005404	MSC	2.54793	3.01E-02
ENSBTAG00000022590	BOLA	2.53375	1.07E-02
ENSBTAG00000031701	TAGAP	2.49893	4.66E-02
ENSBTAG00000020080	MYBPC2	2.49393	4.36E-02
ENSBTAG00000020674	LTB	2.41647	5.00E-04
ENSBTAG00000019711	LRMP	2.35905	4.87E-02
ENSBTAG00000001882	CD79A	2.27269	4.66E-02
ENSBTAG00000017060	ITGB2	2.26868	4.25E-03
ENSBTAG00000023028	WDFY4	2.21916	6.35E-03
ENSBTAG00000002041	SASH3	2.17549	2.13E-02
ENSBTAG00000002846	TRAF3IP3	2.16875	1.77E-02
ENSBTAG00000005305	NTS	2.1654	2.52E-02
ENSBTAG00000001492	FYB	2.15925	6.95E-03
ENSBTAG00000001009	HCLS1	2.14494	2.05E-03
ENSBTAG00000008631	CORO1A	2.12612	1.00E-04
ENSBTAG00000014612	DOCK2	2.09075	1.57E-02
ENSBTAG00000010645	BOLA-DRA	2.07538	5.00E-05

ENSBTAG00000021144	PSTPIP1	2.06905	2.01E-02
ENSBTAG00000003016	PTPN7	2.00218	2.31E-02
ENSBTAG00000002450	FAM111A	2.44837	4.95E-02
ENSBTAG00000001219	ENSBTAG00000001219	2.55721	1.09E-02
ENSBTAG00000030845	ENSBTAG00000030845	3.00432	3.95E-03
ENSBTAG00000010522	ENSBTAG00000010522	3.19211	2.14E-02
ENSBTAG00000048049	ENSBTAG00000048049	3.32661	2.46E-02
ENSBTAG00000020684	ENSBTAG00000020684	2.68993	3.71E-02
ENSBTAG00000034138	ENSBTAG00000034138	2.08073	4.11E-02
ENSBTAG00000038938	ENSBTAG00000038938	2.67243	4.21E-02
ENSBTAG00000004043	ENSBTAG00000004043	2.62232	4.33E-02
ENSBTAG00000001627	ENSBTAG00000001627	2.04854	4.95E-02

Appendix 9 Enriched pathways for up-regulated genes in LH versus HH exposed 98 days postconception bovine mammary tissues

Pathway name	Set size	Candidates contained (%)	p-value	q-value
lck and fyn tyrosine kinases in initiation of T cell receptor activation	13	3 (23.1%)	3.74E-06	3.37E-05
T cell receptor signalling pathway	55	4 (7.3%)	9.21E-06	4.14E-05
Role of mef2d in t-cell apoptosis	31	3 (9.7%)	5.71E-05	1.71E-04
Activation of csk by camp-dependent protein kinase inhibits signalling through the t cell receptor	45	3 (6.7%)	1.76E-04	3.96E-04
Antigen processing and presentation	12	2 (16.7%)	3.77E-04	6.79E-04
IL2 signalling pathway	14	2 (14.3%)	5.19E-04	7.67E-04
IL4 signalling pathway	15	2 (13.3%)	5.98E-04	7.67E-04
IL7 signal transduction	16	2 (12.5%)	6.82E-04	7.67E-04
The co-stimulatory signal during t-cell activation	21	2 (10.0%)	1.07E-03	1.07E-03

Appendix 10 Gene ontology terms for up-regulated genes in LH versus HH exposed 98 days postconception bovine mammary tissues

Gene ontology term		Category	Set size	Candidates contained (%)	p-value	q-value
GO:0006955	immune response	BP	1578	22 (1.4%)	4.01E-14	2.28E-12
GO:0046649	lymphocyte activation	BP	631	16 (2.5%)	6.05E-14	7.74E-12
GO:0045321	leukocyte activation	BP	733	16 (2.2%)	6.04E-13	1.72E-11
GO:0007159	leukocyte cell-cell adhesion	BP	475	13 (2.7%)	8.91E-12	1.51E-09
GO:0001775	cell activation	BP	895	16 (1.8%)	1.25E-11	7.98E-10

GO:0070489	T cell aggregation	BP	434	12 (2.8%)	5.83E-11	2.49E-09
GO:0042110	T cell activation	BP	434	12 (2.8%)	5.83E-11	4.93E-09
GO:0002682	regulation of immune system process	BP	1462	18 (1.2%)	1.98E-10	6.33E-09
GO:0002684 process	positive regulation of immune system	BP	963	15 (1.6%)	4.54E-10	2.56E-08
GO:0002252	immune effector process	BP	841	14 (1.7%)	8.67E-10	1.65E-08
GO:0016337	single organismal cell-cell adhesion	BP	708	13 (1.8%)	1.24E-09	5.23E-08
GO:0050776	regulation of immune response	BP	906	14 (1.5%)	2.31E-09	7.80E-08
GO:0098602	single organism cell adhesion	BP	758	13 (1.7%)	2.83E-09	4.04E-08
GO:0006952	defense response	BP	1591	17 (1.1%)	6.33E-09	1.62E-07
GO:0030098	lymphocyte differentiation	BP	305	9 (3.0%)	1.20E-08	3.37E-07
GO:0007155	cell adhesion	BP	1439	16 (1.1%)	1.31E-08	1.49E-07
GO:0098609	cell-cell adhesion	BP	885	13 (1.5%)	1.81E-08	3.87E-07
GO:0002521	leukocyte differentiation	BP	466	10 (2.2%)	3.26E-08	3.49E-06
GO:0050778	positive regulation of immune response	BP	687	11 (1.6%)	1.23E-07	6.60E-06
GO:0002449	lymphocyte mediated immunity	BP	286	8 (2.8%)	1.29E-07	3.12E-06
GO:0002250	adaptive immune response	BP	416	9 (2.2%)	1.61E-07	2.94E-06
GO:0002696 activation	positive regulation of leukocyte	BP	310	8 (2.6%)	2.33E-07	7.58E-06
GO:0050867	positive regulation of cell activation	BP	318	8 (2.5%)	2.83E-07	7.58E-06
GO:0048534 development	hematopoietic or lymphoid organ	BP	772	11 (1.4%)	3.87E-07	6.18E-06
GO:0002443	leukocyte mediated immunity	BP	356	8 (2.2%)	6.82E-07	9.70E-06
GO:0002520	immune system development	BP	822	11 (1.3%)	7.12E-07	6.77E-06
GO:0022409	positive regulation of cell-cell adhesion	BP	246	7 (2.9%)	7.47E-07	1.60E-05
GO:0046651	lymphocyte proliferation	BP	259	7 (2.7%)	1.06E-06	2.14E-05

GO:0032943	mononuclear cell proliferation	BP	262	7 (2.7%)	1.14E-06	2.14E-05
GO:0070661	leukocyte proliferation	BP	276	7 (2.5%)	1.62E-06	2.07E-05
GO:0051249	regulation of lymphocyte activation	BP	402	8 (2.0%)	1.66E-06	2.97E-05
GO:0002253	activation of immune response	BP	557	9 (1.6%)	1.95E-06	1.59E-05
GO:0030097	hemopoiesis	BP	729	10 (1.4%)	2.04E-06	3.30E-05
GO:0002460	adaptive immune response based on somatic recombination of immune receptors built from immunoglobulin superfamily domains	BP	287	7 (2.4%)	2.15E-06	3.30E-05
GO:0001771	immunological synapse formation	BP	13	3 (23.1%)	2.50E-06	3.82E-05
GO:1903037	regulation of leukocyte cell-cell adhesion	BP	304	7 (2.3%)	3.07E-06	4.11E-05
GO:0006950	response to stress	BP	3759	21 (0.6%)	4.38E-06	3.12E-05
GO:0002694	regulation of leukocyte activation	BP	459	8 (1.7%)	4.46E-06	6.28E-05
GO:0045087	innate immune response	BP	835	10 (1.2%)	6.57E-06	7.64E-05
GO:0048584	positive regulation of response to stimulus	BP	1992	15 (0.8%)	6.65E-06	8.64E-05
GO:0050865	regulation of cell activation	BP	492	8 (1.6%)	7.42E-06	8.96E-05
GO:0050851	antigen receptor-mediated signalling pathway	BP	228	6 (2.6%)	8.01E-06	9.52E-05
GO:0002757	immune response-activating signal transduction	BP	500	8 (1.6%)	8.48E-06	9.04E-05
GO:0002429	immune response-activating cell surface receptor signalling pathway	BP	368	7 (1.9%)	1.10E-05	0.000124
GO:0071345	cellular response to cytokine stimulus	BP	692	9 (1.3%)	1.14E-05	0.000122
GO:0022407	regulation of cell-cell adhesion	BP	379	7 (1.9%)	1.30E-05	0.000137
GO:0002764	immune response-regulating signalling pathway	BP	533	8 (1.5%)	1.35E-05	0.000133
GO:0045785	positive regulation of cell adhesion	BP	382	7 (1.8%)	1.37E-05	0.000137
GO:0002768	immune response-regulating cell surface receptor signalling pathway	BP	400	7 (1.8%)	1.88E-05	0.000176

GO:0031295	T cell costimulation	BP	80	4 (5.1%)	2.33E-05	0.00021
GO:0031294	lymphocyte costimulation	BP	81	4 (5.0%)	2.45E-05	0.00015
GO:0002697	regulation of immune effector process	BP	426	7 (1.6%)	2.81E-05	0.00025
GO:0034097	response to cytokine	BP	799	9 (1.1%)	3.55E-05	0.0003
GO:0007166	cell surface receptor signalling pathway	BP	2697	16 (0.6%)	5.88E-05	0.00047
GO:0048583	regulation of response to stimulus	BP	3683	19 (0.5%)	5.97E-05	0.00047
GO:0051283	negative regulation of sequestering of calcium ion	BP	106	4 (3.8%)	7.40E-05	0.00072
GO:0051282	regulation of sequestering of calcium ion	BP	107	4 (3.7%)	7.68E-05	0.00060
GO:0002366	leukocyte activation involved in immune response	BP	210	5 (2.4%)	7.86E-05	0.00060
GO:0002263	cell activation involved in immune response	BP	212	5 (2.4%)	8.22E-05	0.00061
GO:0051208	sequestering of calcium ion	BP	110	4 (3.6%)	8.55E-05	0.00062
GO:0007165	signal transduction	BP	5761	24 (0.4%)	0.00010	0.00051
GO:0050900	leukocyte migration	BP	363	6 (1.7%)	0.00010	0.00051
GO:0019882	antigen processing and presentation	BP	225	5 (2.2%)	0.00010	0.00051
GO:0019221	cytokine-mediated signalling pathway	BP	546	7 (1.3%)	0.00013	0.00093
GO:0051238	sequestering of metal ion	BP	124	4 (3.2%)	0.00013	0.00096
GO:0042113	B cell activation	BP	238	5 (2.1%)	0.00013	0.00093
GO:0001767	establishment of lymphocyte polarity	BP	9	2 (22.2%)	0.00015	0.0014

GO:0060402	calcium ion transport into cytosol	BP	130	4 (3.1%)	0.000163	0.001106
GO:0009615	response to virus	BP	421	6 (1.4%)	0.000239	0.001197
GO:0002285	lymphocyte activation involved in immune response	BP	147	4 (2.7%)	0.000261	0.001164
GO:0009988	cell-cell recognition	BP	64	3 (4.7%)	0.000338	0.002204
GO:0030155	regulation of cell adhesion	BP	636	7 (1.1%)	0.000338	0.002228
GO:0044700	single organism signalling	BP	6201	24 (0.4%)	0.000368	0.001151
GO:0051235	maintenance of location	BP	293	5 (1.7%)	0.000037	0.001151
GO:1903708	positive regulation of hemopoiesis	BP	164	4 (2.4%)	0.000396	0.003303
GO:0007154	cell communication	BP	6232	24 (0.4%)	0.00004	0.001152
GO:0051668	localization within membrane	BP	69	3 (4.3%)	0.000422	0.0027
GO:0050857	positive regulation of antigen receptor-mediated signalling pathway	BP	15	2 (13.3%)	0.000454	0.003324
GO:0001911	negative regulation of leukocyte mediated cytotoxicity	BP	16	2 (12.5%)	0.000518	0.003326
GO:0002579	positive regulation of antigen processing and presentation	BP	16	2 (12.5%)	0.000518	0.003326
GO:0032845	negative regulation of homeostatic process	BP	178	4 (2.2%)	0.000054	0.003315
GO:0002456	T cell mediated immunity	BP	79	3 (3.8%)	0.000627	0.003373
GO:0031342	negative regulation of cell killing	BP	18	2 (11.1%)	0.000658	0.003371
GO:0051607	defense response to virus	BP	337	5 (1.5%)	0.000069	0.000405
GO:0048518	positive regulation of biological process	BP	5198	21 (0.4%)	0.000703	0.000405
GO:0016477	cell migration	BP	1193	9 (0.8%)	0.000	0.000405

					728	
GO:0001909	leukocyte mediated cytotoxicity	BP	85	3 (3.5%)	0.000776	0.00277
GO:2000021	regulation of ion homeostasis	BP	201	4 (2.0%)	0.000851	0.00464
GO:0051716	cellular response to stimulus	BP	6988	25 (0.4%)	0.000907	0.00304
GO:0070663	regulation of leukocyte proliferation	BP	207	4 (1.9%)	0.000932	0.00525
GO:0002577	regulation of antigen processing and presentation	BP	23	2 (8.7%)	0.00108	0.00571
GO:0051641	cellular localization	BP	2451	13 (0.5%)	0.00115	0.00363
GO:0048513	animal organ development	BP	3149	15 (0.5%)	0.00128	0.00683
GO:0048870	cell motility	BP	1309	9 (0.7%)	0.00141	0.00401
GO:0051674	localization of cell	BP	1309	9 (0.7%)	0.00141	0.00401
GO:1902105	regulation of leukocyte differentiation	BP	238	4 (1.7%)	0.00154	0.00824
GO:1902580	single-organism cellular localization	BP	1101	8 (0.7%)	0.00188	0.00962
GO:0008283	cell proliferation	BP	1967	11 (0.6%)	0.00196	0.00532
GO:0048873	homeostasis of number of cells within a tissue	BP	31	2 (6.5%)	0.00197	0.01
GO:0070670	response to interleukin-4	BP	32	2 (6.2%)	0.00209	0.0102
GO:0002819	regulation of adaptive immune response	BP	124	3 (2.4%)	0.0023	0.0107
GO:0006954	inflammatory response	BP	654	6 (0.9%)	0.0024	0.0123
GO:1990090	cellular response to nerve growth factor stimulus	BP	36	2 (5.7%)	0.0025	0.0124
GO:0006928	movement of cell or subcellular component	BP	1739	10 (0.6%)	0.00281	0.0138
GO:0006897	endocytosis	BP	678	6 (0.9%)	0.00281	0.0125
GO:1990089	response to nerve growth factor	BP	39	2 (5.3%)	0.00294	0.014
GO:0043207	response to external biotic stimulus	BP	947	7 (0.7%)	0.00329	0.0151

GO:0051707	response to other organism	BP	947	7 (0.7%)	0.003329	0.0159
GO:0050854	regulation of antigen receptor-mediated signalling pathway	BP	42	2 (4.8%)	0.003559	0.0154
GO:0043113	receptor clustering	BP	44	2 (4.5%)	0.003793	0.0185
GO:0009607	response to biotic stimulus	BP	984	7 (0.7%)	0.004008	0.0106
GO:0008037	cell recognition	BP	153	3 (2.0%)	0.004116	0.0184
GO:0002703	regulation of leukocyte mediated immunity	BP	153	3 (2.0%)	0.004116	0.0171
GO:0071310	cellular response to organic substance	BP	2177	11 (0.5%)	0.004449	0.0205
GO:0016032	viral process	BP	748	6 (0.8%)	0.004559	0.0196
GO:1903706	regulation of hemopoiesis	BP	322	4 (1.2%)	0.004661	0.0205
GO:0042348	NF-kappaB import into nucleus	BP	48	2 (4.2%)	0.004666	0.018
GO:0044764	multi-organism cellular process	BP	752	6 (0.8%)	0.004711	0.0117
GO:0034341	response to interferon-gamma	BP	160	3 (1.9%)	0.004772	0.018
GO:0017038	protein import	BP	324	4 (1.2%)	0.004882	0.0206
GO:0002478	antigen processing and presentation of exogenous peptide antigen	BP	162	3 (1.9%)	0.004888	0.0206
GO:0032655	regulation of interleukin-12 production	BP	52	2 (3.8%)	0.005445	0.0201
GO:0019884	antigen processing and presentation of exogenous antigen	BP	169	3 (1.8%)	0.005549	0.0227
GO:0032615	interleukin-12 production	BP	53	2 (3.8%)	0.005666	0.0228
GO:0001910	regulation of leukocyte mediated cytotoxicity	BP	53	2 (3.8%)	0.005666	0.0228
GO:0006873	cellular ion homeostasis	BP	547	5 (0.9%)	0.005776	0.0202
GO:0044403	symbiosis, encompassing mutualism through parasitism	BP	784	6 (0.8%)	0.005777	0.0231
GO:0019724	B cell mediated immunity	BP	173	3 (1.7%)	0.005886	0.0202
GO:0044419	interspecies interaction between organisms	BP	787	6 (0.8%)	0.005887	0.014
GO:0042267	natural killer cell mediated cytotoxicity	BP	57	2 (3.5%)	0.006652	0.0149

GO:0048002	antigen processing and presentation of peptide antigen	BP	181	3 (1.7%)	0.006064	0.02577
GO:0080134	regulation of response to stress	BP	1359	8 (0.6%)	0.006093	0.0272
GO:0030595	leukocyte chemotaxis	BP	184	3 (1.6%)	0.006095	0.0258
GO:0051649	establishment of localization in cell	BP	1982	10 (0.5%)	0.007707	0.0258
GO:0002228	natural killer cell mediated immunity	BP	60	2 (3.3%)	0.00772	0.0276
GO:0098542	defense response to other organism	BP	592	5 (0.9%)	0.007756	0.0284
GO:0031341	regulation of cell killing	BP	63	2 (3.2%)	0.007791	0.0281
GO:0044802	single-organism membrane organization	BP	874	6 (0.7%)	0.009966	0.0334
GO:0098552	side of membrane	CC	439	10 (2.3%)	1.93E-08	6.57E-07
GO:0009986	cell surface	CC	771	11 (1.4%)	3.77E-07	6.40E-06
GO:0044459	plasma membrane part	CC	2583	18 (0.7%)	1.47E-06	1.67E-05
GO:0005887	integral component of plasma membrane	CC	1599	14 (0.9%)	2.55E-06	6.15E-05
GO:0031226	intrinsic component of plasma membrane	CC	1661	14 (0.8%)	4.01E-06	0.000164
GO:0034687	integrin alphaL-beta2 complex	CC	2	2 (100.0%)	4.40E-06	6.15E-05
GO:0098797	plasma membrane protein complex	CC	223	6 (2.7%)	7.05E-06	6.58E-05
GO:0009897	external side of plasma membrane	CC	241	6 (2.5%)	1.10E-05	0.000225
GO:0005886	plasma membrane	CC	5069	23 (0.5%)	4.09E-05	0.000348
GO:0001772	immunological synapse	CC	34	3 (8.8%)	5.07E-05	0.000693
GO:0071944	cell periphery	CC	5176	23 (0.4%)	5.87E-05	0.000399
GO:0098796	membrane protein complex	CC	625	7 (1.1%)	0.000307	0.00174
GO:0098802	plasma membrane receptor complex	CC	176	4 (2.3%)	0.000517	0.0053

GO:0042613	MHC class II protein complex	CC	16	2 (12.5%)	0.000518	0.006673
GO:0043235	receptor complex	CC	328	5 (1.5%)	0.000619	0.003101
GO:0001891	phagocytic cup	CC	18	2 (11.1%)	0.000658	0.0054
GO:0070062	extracellular exosome	CC	2813	14 (0.5%)	0.001128	0.008335
GO:1903561	extracellular vesicle	CC	2826	14 (0.5%)	0.001134	0.008473
GO:0043230	extracellular organelle	CC	2827	14 (0.5%)	0.001135	0.005573
GO:0042611	MHC protein complex	CC	27	2 (7.4%)	0.001149	0.008335
GO:0098553	lumenal side of endoplasmic reticulum membrane	CC	29	2 (6.9%)	0.001172	0.008473
GO:0071556	integral component of lumenal side of endoplasmic reticulum membrane	CC	29	2 (6.9%)	0.001172	0.01112
GO:0043234	protein complex	CC	2898	14 (0.5%)	0.001175	0.006626
GO:0098636	protein complex involved in cell adhesion	CC	30	2 (6.7%)	0.001184	0.006626
GO:0008305	integrin complex	CC	30	2 (6.7%)	0.001184	0.008473
GO:0031982	vesicle	CC	4005	17 (0.4%)	0.001192	0.008473
GO:0016021	integral component of membrane	CC	5705	21 (0.4%)	0.002249	0.0102
GO:0030176	integral component of endoplasmic reticulum membrane	CC	132	3 (2.3%)	0.002275	0.01228
GO:0031227	intrinsic component of endoplasmic reticulum membrane	CC	137	3 (2.2%)	0.003005	0.01111
GO:0045121	membrane raft	CC	289	4 (1.4%)	0.003021	0.01228
GO:0098857	membrane microdomain	CC	290	4 (1.4%)	0.003025	0.01111
GO:0031224	intrinsic component of membrane	CC	5819	21 (0.4%)	0.003026	0.0101
GO:0030666	endocytic vesicle membrane	CC	153	3 (2.0%)	0.004116	0.01311
GO:0030669	clathrin-coated endocytic vesicle membrane	CC	48	2 (4.2%)	0.004466	0.0163
GO:0012507	ER to Golgi transport vesicle membrane	CC	56	2 (3.6%)	0.00633	0.0196

GO:0098589	membrane region	CC	364	4 (1.1%)	0.007724	0.0205
GO:0030369	ICAM-3 receptor activity	MF	3	2 (66.7%)	1.32E-05	0.00029
GO:0042605	peptide antigen binding	MF	31	3 (9.7%)	3.82E-05	0.00107
GO:0032403	protein complex binding	MF	724	8 (1.1%)	0.000117	0.00164
GO:0032395	MHC class II receptor activity	MF	11	2 (18.2%)	0.000239	0.00263
GO:0003823	antigen binding	MF	156	4 (2.6%)	0.000328	0.00283
GO:0004872	receptor activity	MF	1663	11 (0.7%)	0.000486	0.00283
GO:0038023	signalling receptor activity	MF	1406	10 (0.7%)	0.000053	0.00283
GO:0042288	MHC class I protein binding	MF	19	2 (10.5%)	0.000735	0.00441
GO:0004888	transmembrane signalling receptor activity	MF	1300	9 (0.7%)	0.00128	0.0103
GO:0099600	transmembrane receptor activity	MF	1352	9 (0.7%)	0.00168	0.0103
GO:0043548	phosphatidylinositol 3-kinase binding	MF	30	2 (6.7%)	0.00184	0.0103
GO:0042287	MHC protein binding	MF	31	2 (6.5%)	0.00197	0.0144
GO:0044877	macromolecular complex binding	MF	1252	8 (0.6%)	0.0042	0.0168
GO:0019901	protein kinase binding	MF	549	5 (0.9%)	0.00581	0.0174
GO:0019900	kinase binding	MF	616	5 (0.8%)	0.00934	0.0429
GO:0003779	actin binding	MF	397	4 (1.0%)	0.00976	0.0429

BP, biological process. CC, cellular component. MF, molecular function.

Appendix 11 Down-regulated genes in LH versus HH exposed 98 days postconception bovine mammary tissues

Ensembl ID	Gene symbol	Log2 FC	p value
ENSBTAG00000018237	MYO16	-5.40233	0.0039
ENSBTAG00000046350	PKP1	-4.76989	0.00215
ENSBTAG00000013732	GPR37	-4.59468	0.005
ENSBTAG00000015106	DSP	-4.43748	5.00E-05
ENSBTAG00000020824	KRT10	-4.2663	5.00E-05
ENSBTAG00000001250	TFAP2A	-4.26473	0.00835
ENSBTAG00000018019	BCL11B	-4.16662	0.00815
ENSBTAG00000002065	TFAP2C	-3.79148	0.0137
ENSBTAG00000046295	CCER2	-3.58522	5.00E-05
ENSBTAG00000038682	FAM83H	-3.57853	0.01445
ENSBTAG00000015991	E-CADHERIN	-3.49339	0.01735
ENSBTAG00000009076	ADD2	-3.42348	0.0182
ENSBTAG00000020837	ARHGEF16	-3.34967	0.0263
ENSBTAG00000017188	C20ORF82	-3.28889	0.00015
ENSBTAG00000037775	ENSBTAG00000037775	-3.24271	0.02055
ENSBTAG00000026278	CLDN4	-3.19604	0.0184
ENSBTAG00000019125	*SLC1A1*	-3.15089	0.02855
ENSBTAG00000012992	CDH6	-3.13123	5.00E-05
ENSBTAG00000005288	GFRA3	-3.01416	0.026
ENSBTAG00000004924	PENK	-2.93082	0.0267
ENSBTAG00000017877	PVRL4	-2.91375	0.0369
ENSBTAG00000011173	FAM189A2	-2.86247	0.0283
ENSBTAG00000007485	GRHL1	-2.8036	0.0072
ENSBTAG00000017032	FREM2	-2.7677	0.04495
ENSBTAG00000005154	APCDD1	-2.71297	5.00E-05
ENSBTAG00000027832	CNTNAP2	-2.62156	0.04175
ENSBTAG00000002129	KLF5	-2.5714	5.00E-05
ENSBTAG00000007164	FGFR3	-2.54132	0.0461
ENSBTAG00000006694	CXCL14	-2.53833	5.00E-05
ENSBTAG00000000601	COL11A2	-2.53593	0.0171
ENSBTAG00000043568	MT-ND3	-2.51919	5.00E-05
ENSBTAG00000004948	FAM84A	-2.4483	0.00925
ENSBTAG00000008138	HOXA2	-2.43278	0.00745

ENSBTAG00000047547	ENSBTAG00000047547	-2.38912	5.00E-05
ENSBTAG00000039664	ENSBTAG00000039664	-2.3621	0.0478
ENSBTAG00000008493	AQP3	-2.33729	0.0422
ENSBTAG00000020205	PDE8B	-2.31974	0.0008
ENSBTAG00000001764	NCAN	-2.31799	0.00455
ENSBTAG00000046256	TMEM132C	-2.30763	5.00E-05
ENSBTAG00000007204	KRT80	-2.25581	0.0138
ENSBTAG00000021059	TNMD	-2.24376	0.0016
ENSBTAG00000012541	CHP2	-2.23162	0.0259
ENSBTAG00000009059	PITX2	-2.20999	0.0004
ENSBTAG00000015232	RAPGEFL1	-2.19994	0.0167
ENSBTAG00000010007	MAPK13	-2.15475	0.01655
ENSBTAG00000012119	PTPRZ1	-2.1454	0.00715
ENSBTAG00000011741	COL13A1	-2.07844	0.03095
ENSBTAG00000032548	SYBU	-2.07627	0.0389
ENSBTAG00000047499	IRX2	-2.03564	0.00065

Appendix 12 Gene ontology terms for down-regulated genes in LH versus HH exposed 98 days postconception bovine mammary tissues

	Gene ontology term	Category	Set size	Candidates contained (%)	p-value	q-value
GO:0007275	multicellular organism development	BP	4862	30 (0.6%)	1.77E-09	2.56E-07
GO:0048731	system development	BP	4290	28 (0.7%)	3.37E-09	2.56E-07
GO:0044707	single-multicellular organism process	BP	5925	32 (0.5%)	7.92E-09	3.56E-07
GO:0048856	anatomical structure development	BP	5313	30 (0.6%)	1.70E-08	3.83E-07
GO:0044767	single-organism developmental process	BP	5610	30 (0.5%)	6.68E-08	1.00E-06
GO:0048513	animal organ development	BP	3149	23 (0.7%)	3.69E-08	1.87E-06
GO:0009888	tissue development	BP	1765	15 (0.9%)	4.17E-06	1.58E-04
GO:0007155	cell adhesion	BP	1439	12 (0.8%)	6.13E-05	6.90E-04
GO:0008544	epidermis development	BP	300	7 (2.3%)	4.67E-06	9.11E-04

GO:0009653	anatomical structure morphogenesis	BP	2366	15 (0.6%)	1.37E-04	1.23E-03
GO:0043588	skin development	BP	242	6 (2.5%)	1.73E-05	1.26E-03
GO:0060429	epithelium development	BP	1065	11 (1.0%)	1.93E-05	1.26E-03
GO:0009887	animal organ morphogenesis	BP	977	10 (1.0%)	5.29E-05	1.61E-03
GO:0045103	intermediate filament-based process	BP	43	3 (7.0%)	1.28E-04	3.25E-03
GO:0001501	skeletal system development	BP	489	7 (1.4%)	1.06E-04	3.50E-03
GO:0048705	skeletal system morphogenesis	BP	210	5 (2.4%)	1.09E-04	3.50E-03
GO:0045104	intermediate filament cytoskeleton organization	BP	42	3 (7.1%)	1.20E-04	3.50E-03
GO:0042476	odontogenesis	BP	115	4 (3.5%)	1.35E-04	3.50E-03
GO:0030154	cell differentiation	BP	3603	19 (0.5%)	1.44E-04	3.50E-03
GO:0007417	central nervous system development	BP	912	9 (1.0%)	1.77E-04	3.85E-03
GO:0044763	single-organism cellular process	BP	10315	34 (0.3%)	7.44E-04	5.58E-03
GO:0048869	cellular developmental process	BP	3779	19 (0.5%)	2.77E-04	6.01E-03
GO:0021756	striatum development	BP	16	2 (12.5%)	5.98E-04	1.09E-02
GO:0060348	bone development	BP	176	4 (2.3%)	6.69E-04	1.09E-02
GO:0098609	cell-cell adhesion	BP	885	8 (0.9%)	7.65E-04	1.09E-02
GO:0021536	diencephalon development	BP	80	3 (3.8%)	8.05E-04	1.09E-02
GO:0007420	brain development	BP	689	7 (1.0%)	8.61E-04	1.09E-02
GO:0007423	sensory organ development	BP	509	6 (1.2%)	9.85E-04	1.13E-02
GO:0010035	response to inorganic substance	BP	515	6 (1.2%)	1.04E-03	1.13E-02
GO:0021544	subpallium development	BP	22	2 (9.1%)	1.14E-03	1.13E-02
GO:0060322	head development	BP	728	7 (1.0%)	1.19E-03	1.13E-02
GO:0030900	forebrain development	BP	366	5 (1.4%)	1.41E-03	1.21E-02

GO:0001503	ossification	BP	368	5 (1.4%)	1.43E-03	1.21E-02
GO:0007399	nervous system development	BP	2157	13 (0.6%)	7.28E-04	1.35E-02
GO:0007379	segment specification	BP	18	2 (11.1%)	7.61E-04	1.35E-02
GO:0060349	bone morphogenesis	BP	83	3 (3.6%)	8.96E-04	1.41E-02
GO:0045109	intermediate filament organization	BP	20	2 (10.0%)	9.42E-04	1.41E-02
GO:0034332	adherens junction organization	BP	116	4 (3.4%)	1.40E-04	1.46E-02
GO:0030855	epithelial cell differentiation	BP	567	7 (1.2%)	2.71E-04	1.46E-02
GO:0010038	response to metal ion	BP	345	5 (1.5%)	1.07E-03	1.48E-02
GO:0043583	ear development	BP	202	4 (2.0%)	1.14E-03	1.48E-02
GO:0045216	cell-cell junction organization	BP	217	4 (1.8%)	1.49E-03	1.72E-02
GO:0098742	cell-cell adhesion via plasma-membrane adhesion molecules	BP	221	4 (1.8%)	1.59E-03	1.72E-02
GO:0036075	replacement ossification	BP	26	2 (7.7%)	1.60E-03	1.72E-02
GO:0021761	limbic system development	BP	103	3 (2.9%)	1.67E-03	1.72E-02
GO:0034330	cell junction organization	BP	246	4 (1.6%)	2.35E-03	1.88E-02
GO:0033273	response to vitamin	BP	109	3 (2.8%)	1.96E-03	1.92E-02
GO:0008038	neuron recognition	BP	33	2 (6.1%)	2.57E-03	2.39E-02
GO:0031076	embryonic camera-type eye development	BP	36	2 (5.6%)	3.05E-03	2.71E-02
GO:0031214	biomineral tissue development	BP	133	3 (2.3%)	3.38E-03	2.87E-02
GO:0033280	response to vitamin D	BP	39	2 (5.1%)	3.57E-03	2.90E-02
GO:0001764	neuron migration	BP	139	3 (2.2%)	3.91E-03	3.05E-02
GO:0048483	autonomic nervous system development	BP	42	2 (4.8%)	4.13E-03	3.05E-02
GO:0033993	response to lipid	BP	921	7 (0.8%)	4.50E-03	3.05E-02
GO:0021983	pituitary gland development	BP	44	2 (4.5%)	4.53E-03	3.05E-02

GO:0008347	glial cell migration	BP	44	2 (4.5%)	4.53E-03	3.05E-02
GO:0071241	cellular response to inorganic substance	BP	161	3 (1.9%)	5.79E-03	3.64E-02
GO:0030198	extracellular matrix organization	BP	317	4 (1.3%)	5.79E-03	3.64E-02
GO:0006928	movement of cell or subcellular component	BP	1739	10 (0.6%)	4.93E-03	3.70E-02
GO:0008037	cell recognition	BP	153	3 (2.0%)	5.11E-03	3.70E-02
GO:0042221	response to chemical	BP	4129	17 (0.4%)	6.64E-03	3.90E-02
GO:0042330	taxis	BP	547	5 (0.9%)	7.85E-03	3.90E-02
GO:0009605	response to external stimulus	BP	2175	11 (0.5%)	7.90E-03	3.90E-02
GO:0051705	multi-organism behavior	BP	62	2 (3.2%)	8.82E-03	3.90E-02
GO:0042127	regulation of cell proliferation	BP	1532	9 (0.6%)	6.57E-03	4.00E-02
GO:0043062	extracellular structure organization	BP	318	4 (1.3%)	5.86E-03	4.05E-02
GO:0022008	neurogenesis	BP	1433	10 (0.7%)	1.16E-03	4.19E-02
GO:0007267	cell-cell signalling	BP	1524	9 (0.6%)	6.49E-03	4.29E-02
GO:0001958	endochondral ossification	BP	26	2 (7.7%)	1.60E-03	4.31E-02
GO:1904888	cranial skeletal system development	BP	57	2 (3.5%)	7.50E-03	4.39E-02
GO:0048839	inner ear development	BP	176	3 (1.7%)	7.52E-03	4.39E-02
GO:0048468	cell development	BP	1859	10 (0.5%)	7.77E-03	4.39E-02
GO:0006935	chemotaxis	BP	546	5 (0.9%)	7.79E-03	4.39E-02
GO:0050891	multicellular organismal water homeostasis	BP	59	2 (3.4%)	8.02E-03	4.74E-02
GO:0060415	muscle tissue morphogenesis	BP	64	2 (3.1%)	9.38E-03	5.38E-02
GO:0051592	response to calcium ion	BP	120	3 (2.5%)	2.52E-03	5.45E-02
GO:0002064	epithelial cell development	BP	195	3 (1.5%)	9.94E-03	5.54E-02
GO:0071248	cellular response to metal ion	BP	140	3 (2.2%)	3.91E-03	6.47E-02

GO:0051173	positive regulation of nitrogen compound metabolic process	BP	1721	10 (0.6%)	4.41E-03	6.47E-02
GO:0060350	endochondral bone morphogenesis	BP	47	2 (4.3%)	5.15E-03	6.47E-02
GO:0007156	homophilic cell adhesion via plasma membrane adhesion molecules	BP	156	3 (1.9%)	5.39E-03	6.47E-02
GO:0055008	cardiac muscle tissue morphogenesis	BP	54	2 (3.7%)	6.75E-03	7.29E-02
GO:0005911	cell-cell junction	CC	386	8 (2.1%)	2.24E-06	7.83E-05
GO:0030057	desmosome	CC	26	3 (11.5%)	2.78E-05	9.16E-04
GO:0005882	intermediate filament	CC	202	5 (2.5%)	8.90E-05	4.45E-04
GO:0045111	intermediate filament cytoskeleton	CC	245	5 (2.1%)	2.21E-04	5.53E-04
GO:0005913	cell-cell adherens junction	CC	55	3 (5.5%)	2.68E-04	4.41E-03
GO:0070161	anchoring junction	CC	491	6 (1.2%)	8.18E-04	1.43E-02
GO:0005886	plasma membrane	CC	5069	21 (0.4%)	1.65E-03	1.81E-02
GO:0044430	cytoskeletal part	CC	1503	10 (0.7%)	1.65E-03	1.82E-02
GO:0071944	cell periphery	CC	5176	21 (0.4%)	2.19E-03	1.81E-02
GO:0044420	extracellular matrix component	CC	120	3 (2.5%)	2.58E-03	1.81E-02
GO:0005887	integral component of plasma membrane	CC	1599	10 (0.6%)	2.61E-03	3.04E-02
GO:0031226	intrinsic component of plasma membrane	CC	1661	10 (0.6%)	3.46E-03	2.54E-02
GO:0044459	plasma membrane part	CC	2583	13 (0.5%)	3.80E-03	2.01E-02
GO:0099512	supramolecular fiber	CC	898	7 (0.8%)	3.85E-03	2.54E-02
GO:0099081	supramolecular polymer	CC	905	7 (0.8%)	4.02E-03	2.01E-02
GO:0099513	polymeric cytoskeletal fiber	CC	684	6 (0.9%)	4.29E-03	3.04E-02
GO:0005794	Golgi apparatus	CC	1469	9 (0.6%)	5.06E-03	3.04E-02
GO:0098590	plasma membrane region	CC	943	7 (0.7%)	5.06E-03	2.21E-02
GO:0016328	lateral plasma membrane	CC	50	2 (4.1%)	5.59E-03	3.08E-02

GO:0005578	proteinaceous extracellular matrix	CC	350	4 (1.1%)	8.09E-03	3.81E-02
GO:0000982	transcription factor activity, RNA polymerase II core promoter proximal region sequence-specific binding	MF	331	7 (2.1%)	9.08E-06	2.09E-04
GO:0001077	transcriptional activator activity, RNA polymerase II core promoter proximal region sequence-specific binding	MF	229	6 (2.6%)	1.26E-05	1.39E-04
GO:0001228	transcriptional activator activity, RNA polymerase II transcription regulatory region sequence-specific binding	MF	319	6 (1.9%)	8.09E-05	9.30E-04
GO:0001078	transcriptional repressor activity, RNA polymerase II core promoter proximal region sequence-specific binding	MF	105	4 (3.8%)	9.51E-05	5.23E-04
GO:0030280	structural constituent of epidermis	MF	8	2 (25.0%)	1.41E-04	2.83E-03
GO:0019215	intermediate filament binding	MF	13	2 (15.4%)	3.91E-04	2.99E-03
GO:0000981	RNA polymerase II transcription factor activity, sequence-specific DNA binding	MF	631	7 (1.1%)	5.16E-04	1.39E-02
GO:0001227	transcriptional repressor activity, RNA polymerase II transcription regulatory region sequence-specific binding	MF	168	4 (2.4%)	5.74E-04	3.30E-03
GO:0017134	fibroblast growth factor binding	MF	25	2 (8.0%)	1.48E-03	6.79E-03
GO:0003690	double-stranded DNA binding	MF	784	7 (0.9%)	1.72E-03	5.38E-03
GO:0043565	sequence-specific DNA binding	MF	1054	8 (0.8%)	2.25E-03	5.38E-03
GO:0000975	regulatory region DNA binding	MF	833	7 (0.8%)	2.45E-03	5.38E-03
GO:0001067	regulatory region nucleic acid binding	MF	834	7 (0.8%)	2.46E-03	9.44E-03
GO:0005085	guanyl-nucleotide exchange factor activity	MF	303	4 (1.3%)	4.94E-03	4.94E-02
GO:0050839	cell adhesion molecule binding	MF	190	3 (1.6%)	9.27E-03	1.25E-01

BP, biological process. CC, cellular component. MF, molecular function.

Appendix 13 Enriched pathways analysis of down-regulated genes in LH versus HH exposed 98 days postconception bovine mammary tissues

Pathway name	Set size	Candidates contained (%)	p-value	q-value
TFAP2 (AP-2) family regulates transcription of other transcription factors	4	3 (75.0%)	6.13E-08	7.17E-06
TFAP2 (AP-2) family regulates transcription of cell cycle factors	5	2 (40.0%)	6.34E-05	1.05E-03
Apoptotic cleavage of cell adhesion proteins	11	3 (27.3%)	2.50E-06	1.46E-04
Negative regulation of activity of TFAP2 (AP-2) family transcription factors	10	2 (20.0%)	2.83E-04	2.60E-03
Activation of the TFAP2 (AP-2) family of transcription factors	12	2 (16.7%)	4.14E-04	3.23E-03
TFAP2 (AP-2) family regulates transcription of growth factors and their receptors	15	2 (13.3%)	6.55E-04	4.79E-03
SUMOylation of transcription factors	20	2 (10.0%)	1.18E-03	7.49E-03
Adherens junctions interactions	31	3 (9.7%)	6.57E-05	1.05E-03
Apoptotic cleavage of cellular proteins	38	3 (7.9%)	1.22E-04	1.58E-03
Syndecan-3-mediated signalling events	26	2 (7.7%)	1.99E-03	7.49E-03
Transcriptional regulation by the AP-2 (TFAP2) family of transcription factors	40	3 (7.5%)	1.42E-04	1.66E-03
Cell-cell junction organization	64	4 (6.3%)	1.92E-05	5.61E-04
Apoptotic execution phase	52	3 (5.8%)	3.11E-04	2.60E-03
Neural Crest Differentiation	101	5 (5.0%)	5.37E-06	2.09E-04
Stabilization and expansion of the E-cadherin adherens junction	42	2 (4.8%)	5.15E-03	1.12E-02
Syndecan-1-mediated signalling events	43	2 (4.7%)	5.39E-03	1.15E-02
Cell junction organization	89	4 (4.5%)	7.17E-05	1.05E-03
Collagen chain trimerization	44	2 (4.5%)	5.64E-03	1.16E-02
Posttranslational regulation of adherens junction stability and disassembly	53	2 (3.8%)	8.09E-03	1.58E-02
FGF signalling pathway	56	2 (3.6%)	9.00E-03	1.73E-02
Preimplantation Embryo	59	2 (3.4%)	9.95E-03	1.88E-02
Cell-Cell communication	124	4 (3.3%)	2.62E-04	2.60E-03
Protein digestion and absorption - Homo sapiens (human)	90	3 (3.3%)	1.55E-03	7.49E-03
Keratinization	128	4 (3.1%)	3.05E-04	2.60E-03
Apoptosis	118	3 (2.5%)	3.35E-03	7.69E-03
Programmed Cell Death	121	3 (2.5%)	3.60E-03	8.10E-03
Ectoderm Differentiation	142	3 (2.1%)	5.63E-03	1.16E-02
Cell adhesion molecules (CAMs) - Homo sapiens (human)	145	3 (2.1%)	5.74E-03	1.16E-02
Extracellular matrix organization	293	4 (1.4%)	6.40E-03	1.27E-02
Developmental Biology	618	7 (1.1%)	8.06E-04	5.55E-03

Appendix 14 Miscellaneous genes in the 98 days post conception bovine mammary gland

Ensembl ID	Gene symbol	Log2FC	p value
ENSBTAG00000014614	ACTA2	0.26593	0.3332
ENSBTAG00000019813	ADIPOQ	-0.547	0.11485
ENSBTAG00000012210	C5	0	1
ENSBTAG00000014177	C6	-0.29891	1
ENSBTAG00000014177	C6	-0.29891	1
ENSBTAG00000011766	C7	2.57789	5.00E-05
ENSBTAG00000011766	C7	2.57789	5.00E-05
ENSBTAG00000003774	C8A	0	1
ENSBTAG00000003774	C8A	0	NOTEST
ENSBTAG00000003775	C8B	-inf	1
ENSBTAG00000003775	C8B	-inf	NOTEST
ENSBTAG00000016149	C9	0	1
ENSBTAG00000016149	C9	0	NOTEST
ENSBTAG00000015874	CASP3	-0.6186	0.03915
ENSBTAG00000006615	CASP7	-0.1397	0.78695
ENSBTAG00000015133	CCR7	5.2477	0.2475
ENSBTAG00000032122	CD19	-0.2644	0.7284
ENSBTAG00000017256	CD2	inf	5.00E-05
ENSBTAG00000011887	CD22	2.09974	0.07345
ENSBTAG00000002951	CD244	0	NOTEST
ENSBTAG00000021466	COL3A1	0	1
ENSBTAG00000038496	CR2 (CD21)	4.33353	0.00505
ENSBTAG00000007159	ESR1	0.00197	0.9935
ENSBTAG00000014064	FGFR2	-1.00706	0.0015
ENSBTAG00000005198	FGF1	0.186681	0.8951
ENSBTAG00000008623	FGF3	0	NOTEST
ENSBTAG00000004013	FGF7	0.183222	0.4968
ENSBTAG00000008300	FN1	-0.0981	0.8008
ENSBTAG00000004879	FOXO4	0.01456	0.97065
ENSBTAG00000005586	GATM	-0.9173	0.00985
ENSBTAG00000001835	GJA1	0.195	0.47815
ENSBTAG00000011082	IGF1	0.99831	0.0304
ENSBTAG00000015953	IL13	0	NOTEST
ENSBTAG00000018571	IL1RL1	1.01522	0.255
ENSBTAG00000015957	IL4	0	NOTEST
ENSBTAG00000031235	IL5	0	NOTEST
ENSBTAG00000047238	ITGAM	2.30684	0.1384
ENSBTAG00000003176	JAM3	0.18484	0.4876
ENSBTAG00000017685	JUP	-0.182	0.5021
ENSBTAG00000001517	KRT18	-5.0235	0.21025
ENSBTAG00000007108	LGALS12	0.17989	0.8601
ENSBTAG00000013256	LGR5	-0.4163	0.2881

ENSBTAG00000002444	MKI67	0.27752	0.2906
ENSBTAG00000002075	MME	0.14388	0.59665
ENSBTAG00000004580	MS4A1 (CD20)	inf	0.00035
ENSBTAG00000022799	NOTCH1	-0.0467	0.86975
ENSBTAG00000006909	PIK3CB	0.19552	0.677
ENSBTAG00000011975	SERPINB1	0.74087	0.1105
ENSBTAG00000007348	STRA6	0.99139	0.18115
ENSBTAG00000020457	TGFB1	0.08151	0.8343
ENSBTAG00000004647	UCP1	-inf	NOTEST
ENSBTAG00000003692	UCP2	1.29847	0.00295
ENSBTAG00000047561	VEGFA	-0.1073	0.87305
ENSBTAG00000047567	VEGFB	-0.1139	0.7085
ENSBTAG00000014737	VEGFC	-0.1923	0.54675
ENSBTAG00000018463	VIM	-0.326	0.408

Log2 FC (fold change) in LH exposed tissues versus HH counterparts.

Appendix 15 Collagen genes differentially expressed between LH and HH diets

COL6A5 was up-regulated in LH mammary samples versus HH. *COL13A1,* and *COL11A1 and COL11A2* were down-regulated in LH versus HH samples (Appendix 15.1). These genes were displayed as they complemented histological total collagen analysis.

Appendix 15.1 Collagen genes differentially expressed in HH and LH 98 days postconception mammary fat pad samples

Gene	Official name	HH Diet (FPKM)	LH Diet (FPKM)	Log^2 fold-change	*p* value
COL6A5	collagen type VI alpha 5	0.36	2.18	2.60	0.05
COL11A1	collagen type XI alpha 2	78.63	52.70	-0.58	0.04*
COL11A2	collagen type XI alpha 2	3.17	0.55	-2.54	0.02*
COL13A1	collagen type XIII alpha 1	3.72	0.88	-2.08	0.03*

*Significant to $p<0.05$. Fragments Per Kilobase Million (FPKM).

Appendix 15.2 Collagen genes discussion

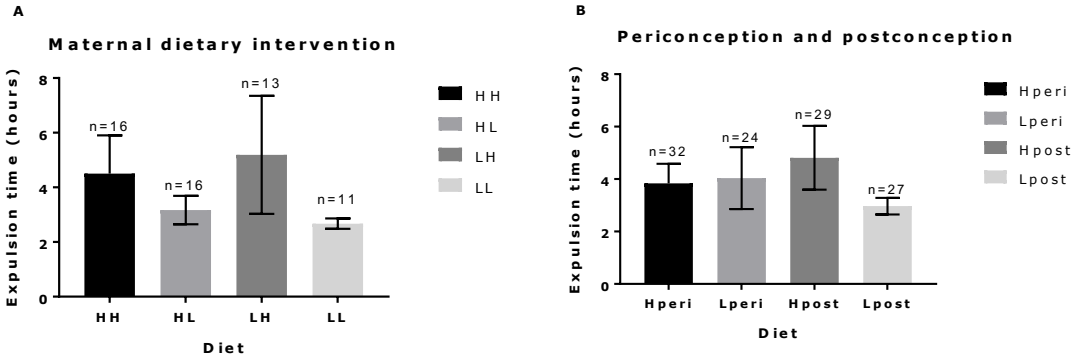
Presence of collagen genes in RNA-seq data supports previous findings that type IV collagen surrounds mammary epithelial cells in ducts (Streuli and Bissell, 1990, Van der Rest and Garrone, 1991, Warburton et al., 1982). This is the first time that the presence of type IV collagen has been reported to be expressed in 98 days postconception bovine mammary fat pad. The predominant collagen thought to surround mammary ducts is collagen IV. Histological investigation of collagen protein in the mammary fat pad, and

quantitative image analysis suggested no significant differences in total collagen area between all four diet groups (See chapter 2). Although there is disparity between total collagen and individual collagen gene and protein expression, both data support each other as overall. There were no significant differences in type IV gene expression between LH and HH (Appendix 15.1). Furthermore, considering the collagen genes with greatest FPKM, there were no significant differences (Appendix 15.1). This supports the histological findings (See Chapter 2).

Specific procollagens were affected by diet. *COL6A5* was upregulated 2.6-fold in LH compared with HH (Appendix 15.1). *COL11A2* and *COL13A1* were down-regulated 2.54 and 2.08-fold respectively in LH compared with HH (Appendix 15.1). All three genes are involved in collagen chain trimerisation. Results show that not all procollagens of Collagen VI, XI and XIII were affected by diet. The biological significance of this is unclear, although likely depends on individual procollagen function rather than the mature collagen molecules. Collagen VI is encoded by six genes (*COL6A 1-6*), but requires only three of these six procollagens to form (Cescon et al., 2015). Collagen VI A1 and A2 appear to be essential, and A3 may be replaced with the recently discovered A4-6 chains (Cescon et al., 2015, Gara et al., 2008, Fitzgerald et al., 2008). This suggests that differential expression of *COL6A5* is not detrimental to overall COL6 production. However, individual procollagens have specific localisation within tissues such as skin and adipose (Cescon et al., 2015, Divoux and Clement, 2011, Sabatelli et al., 2011). In human skin, *COL6A5* was localised in the papillary dermis and surrounding blood vessels (Sabatelli et al., 2011). Collagen VI is predominantly expressed in adipose tissue (Khan et al., 2009). Collagen VI is localised throughout the mammary ECM, and functions to support cells and initiate pathways such as angiogenesis. Collagen expression may be related to adipocyte hypertrophy (Khan et al., 2009, Divoux and Clement, 2011). Decreased expression of procollagens reduces the extracellular structural support of surrounding adipocytes, thus allowing more space for hypertrophy (Divoux and Clement, 2011). The low protein periconception diet may lead to a change in collagen gene expression in favour of energy storage as an adaptation to low nutritive environment. Although the focus is on low protein, the LH diet also only supplied 85% of energy requirements during periconception versus 96% in High protein periconception (Chapter 2.1.6). Therefore, the hypothesis is that a reduced supply of energy during

periconception may lead to altered collagen gene expression to allow greater potential for adipocyte hypertrophy. Further investigation is required to understand a potential link between collagen expression in the mammary fat pad and adipocyte hypertrophy. *COL11A2* encodes the procollagen XI α-2, which forms a heterodimer with XI α -1 and α -3, constituting collagen 11 (XI) (Vázquez-Villa et al., 2015). Literature relating to collagen 11 function is limited in the mammary gland, though Aral and colleagues (1989) indicate collagen 11 may be involved in cell-ECM interactions based on its cell surface localisation. Myoepithelial cells potentially express collagen 11 (Aral et al., 1989).

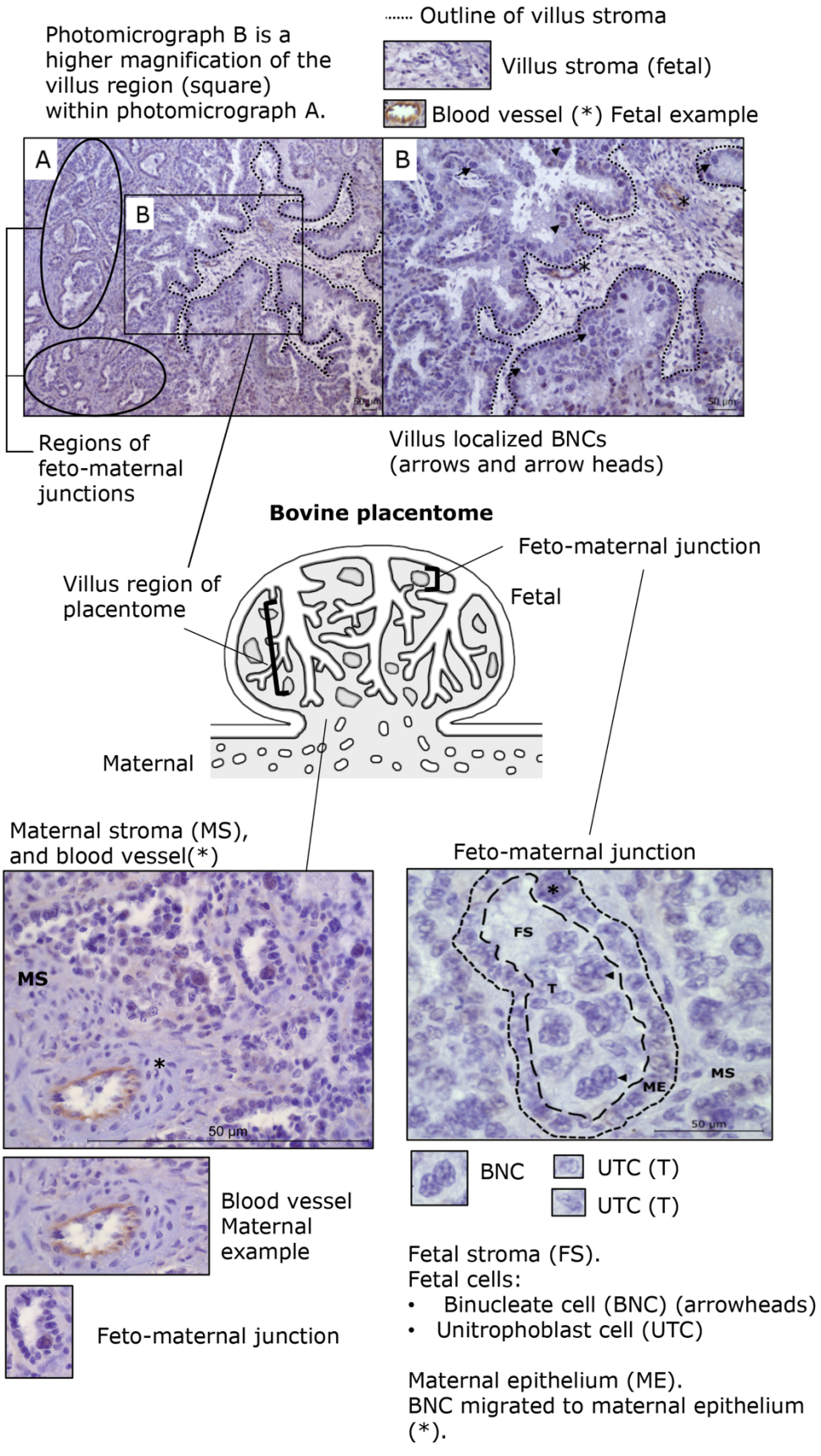
Appendix 16 Fetal membrane expulsion time (hours) was unaffected by maternal dietary intervention, periconception or postconception diet



Appendix 17 Oil-Red-O Stain protocol

Oil-Red-O dye (Acros organics, Geel, Belgium) was prepared by adding 1% powdered dye to 100ml of 1, 2-Propanediol (Acros organics, Geel, Belgium) and heated slowly to 100°C whilst being stirred by a flea. The dye was filtered whilst hot using filter paper. When filtered, the dye was cooled and filtered again overnight. Tissue sections were deparaffinised in xylene for 5 minutes. Following rehydration of sections in a series of graded alcohols (100%, 90%, 70% ethanol respectively), sections were washed in $_dH_2O$ for 5 minutes. Sections were incubated in 100% propylene glycol (200µl per slide) for 2 minutes. Sections were then incubated in Oil-Red-O for 1.5 hours. Sections were then incubated in propylene glycol (85%, then 50% respectively) (200µl per slide; diluted in $_dH_2O$) for 3 minutes, and 2 minutes respectively. Sections were then gently agitated in a water bath with running tap water at room temperature for 5 minutes. Sections were incubated in haematoxylin (200µl per slide) for the shortest time possible. Sections were then washed with $_dH_2O$, then mounted using Hydromount™ (National Diagnostics, Atlanta, Georgia, USA) and cover slips were added. Sections were left over night to dry at room temperature.

Appendix 18 Orientation of the Bovine Placentome



Appendix 19 Professional Internships for PhD Students (PIPS) reflective Statement

The 3-month PIP was a compulsory component of DTP PhD student training, unrelated to the PhD project. This reflective statement describes the skills and experiences gained during the PIP. I was hosted by Anpario Plc, an international company who produce and distribute high performance feed additives for production animals. The product range focuses on addressing issues in animal health, fertility, hygiene and nutrition for aquaculture, swine, poultry, and ruminants with natural solutions. I worked alongside the ruminant technical support team but also joined meetings with the EU sales team and conference calls of technical interest. I performed several tasks which included preparing investment appraisals for R&D, which involved literature reviews and trial design. I assisted with technical presentations to distributors and potential clients. I was responsible for statistics and interpretation of trial data, conducting a rumen batch-culture for fermentation kinetics, and sequential-kill for rumen digestion of two feed additives. I attended technical meetings with distributors, was an active team member in technical – marketing, technical - sales meetings for future work and support. I made a significant contribution to a new product development project, which has now launched, and identified a new product for future development. I furthered my skills in time management, professionalism, team-work and independent working. I gained confidence in assertiveness, presentations, and advising on trial design. I visited the production plant and learned how products were formulated, packaged, and distributed. I gained an insight into the professional experiences of my colleagues.

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